AN EXPERIMENTAL STUDY OF HUMAN MELANOMA CELLS CULTURED *IN VITRO*

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ABSTRACT

This thesis records the results of a series of experiments that were designed to examine the biology of human malignant melanoma cells cultured *in vitro*. The studies were so planned as to document phenotypic differences that exist between melanomas, to define respects in which melanoma cell differentiation could be modulated and to correlate biochemical variability with *in vivo* behaviour as measured in the nude mouse.

Melanoma cell lines were established from biopsy material obtained from 7 patients at Groote Schuur Hospital. Two of these lines synthesized tyrosinase and melanin at a rate that was directly related to cell density. The five remaining lines did not pigment.

All of the lines showed aneuploidy; 5 of the 7 showed anchorage-independent growth; and 6 of the 7 grew as lethal tumours in nude mice.

As has been found with all other melanomas studied, these cells released a plasminogen activator that was chemically and immunologically identical to tissue activator. One of the lines proved to be an exception to this general rule in that it synthesized urokinase-type enzyme.

Unlike most other human cells cultured *in vitro*, melanoma cells proved to be relatively refractory to hormonal stimuli. Addition of estrogen, progesterone, testosterone, dexamethasone or melanocyte-stimulating hormone to the culture medium had very little effect on cellular release of plasminogen activator, upon cell growth, or upon cellular morphology.

Although remarkably resistant to hormonal influences, cellular release of plasminogen activator did appear to be inhibited to a striking degree by cocultivation with normal skin fibroblasts. This observation led to the discovery of a phenomenon in which fibroblasts of many types bound tissue-type plasminogen activator and so removed it from the medium.

This was accompanied by an apparent change in molecular weight of the melanoma cell enzyme from 72K daltons to approximately 115K daltons, suggesting the presence of a 40-50K binding molecule.

In an attempt to influence *in vitro* differentiation, the tumour promoter tetradecanoylphorbol acetate, and the differentiation-inducing retinoid, retinoic acid, were added to the two pigmenting cell lines. The effects of these compounds on induction of tyrosinase activity, morphological change or plasminogen activator release differed. In the one cell line, tetradecanoylphorbol acetate caused morphological maturation with a decrease in the rate of plasminogen activator release and no obvious effect upon pigmentation. This line was relatively resistant to the action of retinoids. The other pigmenting line responded hardly at all to the tumour promoter. Retinoic acid, on the other hand, inhibited the induction of tyrosinase activity, yet caused an inhibition of growth and plasminogen activator release.

A number of interesting observations could be made in experiments in which melanoma cells were inoculated into nude mice. Firstly, the growth rate of the tumours *in vivo* correlated poorly with the doubling times of the corresponding cells cultured *in vitro*. Secondly, despite a marked inhibitory effect of fibroblasts on plasminogen activator *in vitro*, co-injection of fibroblasts and melanoma cells *in vivo* greatly enhanced tumour growth when small tumour cell inocula were used and shortened the latent period for tumour appearance with larger inocula. Thirdly, melanomas growing in nude mice differed strikingly in their ability to elicit a desmoplastic response. Tumours in which large amounts of host connective tissue were deposited tended to be heavily contaminated with murine fibroblasts when re-established *in vitro*. This contamination was not seen with tumours that contained very little connective tissue. These results point to the existence of a melanoma-associated fibrogenic factor.

Finally, by excision of the primary tumour, it was possible to avoid death of the animal from local complications and so allow time for metastases to develop. In three mice, metastatic melanoma deposits could be detected by this device, so establishing a protocol for the use of nude mice as valid models for the experimental study of metastatic spread of human tumours.

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ABBREVIATIONS

Ab	- Antibody
BSA	- Bovine serum albumin
CAM	- Chorioallantoic membrane
cAMP	- cyclic adenosine 3',5'-monophosphate
DB	- Dulbecco's modified Eagle's medium
dBcAMP	- Dibutyryl cAMP
DFP	- Diisopropylfluorophosphate
DMSO	- Dimethyl sulphoxide
DNAse	- Deoxyribonuclease
DOPA	- Dihydroxyphenylalanine
EDTA	- Ethylene-diamine-tetra acetate
FCS	- Foetal calf serum
FC10	- 10% FCS
Hr	- Hour(s)
IgG	- Immunoglobulin G
K dalton	- Kilodalton
LDH	- Lactate dehydrogenase
Min	- Minute(s)
α-MSH	- α-Melanocyte-stimulating hormone
PA	- Plasminogen activator
PBS	- Phosphate buffered saline: 0.14M NaCl; 2.7mM KCl; 8mM Na_2HPO_4; 1.5 mM KH_2PO_4
PIF-BSA	- Protease- and inhibitor-free BSA
PN	- Protease-nexin

RA	- Retinoic acid
RPMI	- Roswell Park Memorial Institute culture medium 1640
s.c	- subcutaneous
SDS	- Sodium lauryl (dodecyl) sulphate
TCA	- Trichloracetic acid
TD	- Tris Dulbecco's Saline 0.14M NaCl; 5mM KCl; 0.7 mM Na_2HPO_4; 24.8 mM Tris.HCl, pH 7.4
TPA(PMA)	- 12-O-tetradecanoylphorbol-13-acetate (Phorbol myristate acetate)
Tris	- Tris (hydroxymethyl)-aminomethane
u	- unit(s) enzyme activity (PA or tyrosinase)
UK	- Urokinase
Versene	- 0.14M NaCl; 2.7 mM KCl; 1.5 mM KH_2PO_4; 6.4 mM Na_2HPO_4; 0.7 mM Na_2EDTA

CHAPTER 1

INTRODUCTION

The work that constitutes the bulk of this thesis developed from my original interests in cell biology as these applied to the study of human cells growing under defined conditions *in vitro*, and culminated in my developing and studying seven human melanoma cell lines.

My choice of malignant melanoma as a subject for study was initially fortuitous, in that one of the first tumours which I developed successfully into a cell line happened to be a pigmenting melanoma which appears in this thesis as UCT-Mel 1. My experience with this cell line and the enquiry that it stimulated soon convinced me of the advisability of accumulating more such cell lines and expanding my interests in this particular neoplasm into a substantive research subject.

My reasons for this decision were manifold. Seen in purely pragmatic terms, melanomas are fairly frequent tumours amongst the patient population of Groote Schuur Hospital. Since I had convenient and effective access to these by virtue of excellent collaborative associations with the surgeons, availability of material presented no problem. Furthermore, melanomas are well known for the relative ease with which they can be used to develop *in vitro* cell lines.

Although not immediately related to the scientific purpose of this study, I was also influenced by the fact that, although relatively rare, the worldwide incidence of this malignancy has risen rapidly since the beginning of cancer registration. The occurrence of melanoma appears to be related primarily to the degree of pigmentation of the skin and to exposure to sunlight, so that the incidence tends to be highest in countries such as Australia and Israel (Davis, 1976) and to a lesser extent, South Africa (Oettle, 1966; Rippey and Rippey, 1979) where light skinned

Caucasians are subject to large amounts of solar radiation. Although I had no clearly defined experimental approach to finding reasons for this increased incidence I was struck by reports indicating that the clinical course of melanoma is frequently influenced by the hormonal status of the patients. The sex of the patient, age in relation to puberty or menopause, and pregnancy have all been implicated in the incidence and prognosis of this disease (Pack and Scharnagel, 1951; Byrd and McGanity, 1954; Allen, 1955; George et al, 1960; Nathanson et al, 1967a, b; Sadoff et al, 1973; Shiu et al, 1976; Lerner et al, 1979b; Rampen and Mulder, 1980). It seemed possible, therefore, that the study of the hormonal responsiveness of these cells *in vitro* might provide information of clinical value.

At a more academic level, melanomas seemed to provide the wherewithal to examine two questions of fundamental biological interest - phenotypic variation and differentiation. With regard to the first of these, it is now well established that melanomas arise in cells derived embryonically from the neural crest. Despite this common origin, and despite the fact that melanomas are so distinctively similar that their clinical and histological diagnosis is seldom in dispute, they are nevertheless known to behave both unpredictably and very differently *in vivo*. I hoped that it would serve some useful purpose to see if fundamental differences or similarities could be documented with cells growing *in vitro*.

As far as differentiation was concerned, melanoma cells seemed eminently suitable for study. Not only does one have melanization as an indisputable and quantifiable manifestation of the differentiated state, but one may also observe morphological changes in the appearance of the cells that resemble very closely those that occur in mature melanocytes *in vivo*.

Finally, I was attracted to the further study of melanomas by a

consideration of the information that was available in the literature. On the one hand, the literature abounds with reports of a great deal of imaginative and extremely interesting work on melanomas of animal origin. This is exemplified by the thoughtful studies of Silagi's group, who used a pigmented clone of the B16 mouse melanoma to study the mechanisms controlling differentiation and expression of the neoplastic phenotype (review by Silagi, 1976). Furthermore, work on the response of Cloudman S-91 melanoma cells to melanocyte stimulating hormone has provided detailed information on the kinetics of this response, the intracellular events that follow, and the interrelationships that exist between the processes of melanization and proliferation (reviews by Pawelek, 1976; Lerner et al, 1979a). In the field of tumour metastasis, Fidler and his co-workers have extensively investigated the metastatic potential and organ preference of clones of the B16 mouse melanoma and the influence of host immunity on metastasis (reviews by Fidler et al, 1978; Fidler and Hart, 1978).

In contrast, work with human melanomas *in vitro* has tended to be largely descriptive, with few attempts to exploit the challenging attributes that recommend these tumours for study. I have attempted, in Tables 1.1 and 1.2, to assemble in summary form a representative cross-section of the essential information that is available in the literature on the subject of human melanomas. Of the reports that I examined, 24 (Table 1.1) dealt more or less exclusively with the characterization of the cell lines according to relatively standard criteria, such as characteristics of growth *in vitro* or in immunosuppressed animals, and karyotype. In some instances, the cells were examined by technically more advanced procedures such as electron microscopy or they were used to examine for the presence of melanoma-specific antigens, recognizable by auto- or allo-antisera.

In 23 reports (Table 1.2) I found that the authors had used melanoma cells in inductive experiments that were designed to examine the

TABLE 1.1

(a) 1. Ponomaryova and Balashova (1964); 2. Oettgen et al (1968);
3. Toshima et al (1968); 4. Maul and Romsdahl (1970);
5. Barranco et al (1971); 6. Romsdahl and Hsu (1967, 1972);
7. Chen and Shaw (1973); 8. Giard et al (1973);
9. Fogh and Trempe (1975); 10. Gerner et al (1975);
11. Liao et al (1975); 12. Aubert et al (1976);
13. Carey et al (1976); 14. Giovanella et al (1976)
15. Fabricant et al (1977); 16. Foa and Aubert (1977);
17. Fogh et al (1977); 18. Creasey et al (1979);
19. Muir and Gunz (1979); 20. Pope et al (1979);
21. Sharkey and Fogh (1979); 22. Aubert et al (1980);
23. Woodbury et al (1980); 24. Wilson et al (1981).

(b) - = not determined

\+ = determined

(c) The figure in parentheses indicates the number of cell lines on which this determination was made.

TABLE 1.1

TABLE 1.1

DESCRIPTIVE REPORTS ON HUMAN MELANOMA CELL LINES

No. cell lines	No. pigmenting	Modal Chromosome no.	Plating Efficiency	Growth in soft agar	Growth in immunodeficient animals	EM	Melanoma Antigens	Reference (a)
2	2	-(b)	-	-	-	-	-	1
1	1	-	-	-	+(b)	+	+	2
2	1	-	-	-	-	+	-	3
2	2	-	-	-	-	+	-	4
3	3	-	-	-	-	-	-	5
8	8	43-78	-	-	-	+	-	6
1	-	45	-	-	-	-	-	7
2	-	67(1)	-	+	+	-	-	8
5	-	aneuploid	-	-	+(1)(c)	+(1)(c)	-	9
14	several	40-300	-	-	+	+	-	10
7	4	46-87	+	-	-	-	-	11
3	3	56-72	-	-	+	+	-	12
35	-	-	-	-	-	-	+	13
46	34	44-108	+	-	+	-	-	14
3	-	-	-	-	-	-	+	15
17	-	-	-	-	-	+	-	16
46	-	-	-	-	+	-	-	17
3	1	46-57	+	+	+	-	-	18
8	-	47-93	-	-	-	-	-	19
10	8	47-93	-	-	-	-	-	20
5	-	-	-	-	+	-	-	21
16	14	35-88	-	-	+	-	-	22
1	-	-	-	-	-	-	+	23
6	-	-	-	-	-	-	+	24

TABLE 1.2

EXPERIMENTAL STUDIES WITH HUMAN MELANOMA CELL LINES

a) Experiments describing attempts to modify the cellular phenotype.

No. Cell Lines	Compound Added	Parameter Studied	Effect	Reference
1	dBcAMP	Morphology	Dendritic	Kanzaki et al, 1977.
	Theophylline	Morphology	Dendritic	
1	Sodium butyrate	Growth	Decrease	Prasad & Sakamoto, 1978
	cAMP stimulating agents	Growth	Decrease	
8	MSH dBcAMP Theophylline Progesterone	Tyrosinase levels & growth	Varied	Fuller & Meyskens, 1979
1	dBcAMP	Morphology Pigmentation Growth	Dendritic Increase Decrease	Nishihira et al, 1979
1	dBcAMP Butyric acid Prednisolone Theophylline	Morphology Growth Tumorigenicity in nude mice	Varied	Kimura et al, 1980
1	Theophylline DMSO RA TPA	Growth Pigmentation	Decrease Increase	Tveit et al, 1980b.
11	Theophylline Prostaglandin E MSH	Tyrosinase levels	Varied	Fuller & Meyskens, 1981
1	MSH	cAMP concentration Tyrosinase levels Growth	Increase Increase Decrease	Legros et al, 1981
1	DMSO TPA	Morphology Pigmentation Growth	Varied Increase Decrease	Huberman et al, 1979
6	RA	Growth	Decrease	Lotan, 1979
1	RA	Pigmentation	Increase	Lotan & Lotan, 1980

TABLE 1.2 CONTINUED

No. Cell Lines	Compound Added	Parameter Studied	Effect	Reference.
1	RA	Tyrosinase levels	Increase	Meyskens & Fuller, 1980
		Growth	Decrease	
2	Estrogen	Tumorigenicity in nude mice	Varied	Beattie et al, 1979
1	DOPA	Growth	Decrease	Wick et al, 1977
2	Cys-DOPA	Growth	Decrease	Fujita et al, 1980
9	Chemotherapeutic agents	Growth	Varied	Tveit et al, 1981

b) Experiments in which specific cellular functions were identified.

No. Cell Lines	Function identified	Reference
1	Metastasis in nude mice	Tseng et al, 1980
4 clones	Secretion of ACTH	Orth, 1973
6	Secretion of NGF	Sherwin et al, 1979.
3	Secretion of PA	Vetterlein et al, 1979, 1980.
6	Secretion of PA	Roblin & Young, 1980.
8	Secretion of PA	Wilson et al, 1980
1	Secretion of TGF	Todaro et al, 1980.

effects of such compounds as cyclic AMP, retinoids or tumour promoters on melanoma cells or to study the unique proteases, hormones or growth factors secreted by these cells. Such reports, however, were few, and appeared only recently.

I felt that this general state of affairs reflected a deficiency in the *in vitro* study of human melanomas, particularly since, in recent years, there have been a number of technical and conceptual advances in tumour cell biology that have been productively applied to the study of other human tumours. The effects, for example, of hormones (Beers et al, 1975; Troll et al, 1975; Lopez et al, 1978; Walker et al, 1978; Lacroix et al, 1979; Ossowski, 1979), glucocorticoids (Vassalli et al, 1976, 1977; Seifert and Gelehter, 1978; Roblin and Young, 1980), cyclic nucleotides (Hsie and Puck, 1971; Masui and Garren, 1971; Shapiro, 1973; Hannon et al, 1976; Leising and Schachtschabel, 1977; review by Pastan and Johnson, 1974), retinoids (Wilson and Dowdle, 1980; review by Schroder and Black, 1980), tumour promoters (Mufson et al, 1979; reviews by Weinstein and Wigler, 1977; Diamond et al, 1980) or compounds thought to be involved in the induction of differentiation of cells in culture, have been investigated in human cells of other types or in cells from other species and have yielded useful and interesting new information regarding cellular control processes and factors that affect expression of the neoplastic phenotype. Similarly, nude mice have increasingly been used to study host responses to tumour cells and in attempts to develop models for the study of metastatic spread or the effects of chemical compounds on tumour cell behaviour in an environment that is perhaps more closely related to the physiological environment of the original host than is the plastic tissue culture dish. I could find surprisingly few accounts, however, of nude mice having been used to quantitate the rate of *in vivo* growth or to study in any systematic way the relationships that exist between growth *in vitro* and *in vivo*.

These, then, were the reasons for my becoming involved with the study of human melanomas. The subsequent chapters of this thesis are a record of this involvement.

In the second chapter, I record the manner in which seven permanent human melanoma cell lines were established *in vitro*. These lines were characterised with respect to their *in vitro* growth characteristics and a number of parameters associated with transformation; such as growth in soft agar, plating efficiency and saturation density. The interests of the department in which I was working centred around regulated proteolysis and its role in tumour promotion and spread. A number of reports had indicated that production of the serine protease, plasminogen activator (PA), could be correlated with the malignant phenotype. I therefore studied the synthesis of this enzyme by melanoma cells, and investigated its modulation by hormones and other compounds of biological interest. Melanoma cells proved to be relatively refractory to these influences.

The third chapter emphasizes the study of PA in this group of cells. The molecular species of PA produced by the melanoma cells was determined by SDS-polyacrylamide gel electrophoresis and immunochemical techniques. The enzyme synthesized and released by 6 of the 7 cell lines was of the tissue-type i.e. a PA with an apparent molecular weight of 72K daltons and was immunochemically distinct from urokinase. The cellular release of PA in two of the cell lines was "density-dependent", i.e. the amount of enzyme produced per cell declined sharply as the cell number increased.

In the fourth chapter, I describe in some detail the effects of a co-carcinogen, tetradecanoyl phorbol acetate, and a retinoid, retinoic acid, on the two pigmenting cell lines. These compounds influenced *in vitro* differentiation in a manner that differed in the two cell lines and that was inconsistent with other reports in the literature. In one case,

retinoic acid caused a pronounced decrease in tyrosinase activity. In the other case, TPA caused a fall in the synthesis and release of PA.

In the fifth chapter, I describe a phenomenon that I observed when melanoma cells were cocultivated with normal adult human skin fibroblasts. Under these circumstances less PA was detected in the medium than was found in the absence of fibroblasts. This was due to a fibroblast component which bound the PA released into the medium by the melanoma cells. The PA-fibroblast component complex could be detected after SDS-polyacrylamide gel electrophoresis as an enzymatically active band with an apparent molecular weight of 115K daltons, from which the molecular weight of the fibroblast component was estimated to be in the region of 40-50K daltons, before binding to the 72K dalton melanoma plasminogen activator.

In the sixth chapter I describe experiments utilising congenitally immunodeficient "nude" mice. The melanoma cell lines were tested for tumorigenicity in this system. Six of the seven cell lines became established as progressively growing tumours after subcutaneous inoculation. No correlation could be found between the *in vivo* growth rates observed in the mice, and the *in vitro* doubling times of the respective cell lines. The co-inoculation of normal skin fibroblasts, which were not tumorigenic when injected alone, was found to increase the tumour take rate of marginally tumorigenic melanoma cell inocula, and to decrease markedly the latency period before the appearance of a measurable tumour mass.

CHAPTER 2

ESTABLISHMENT AND CHARACTERISATION OF SEVEN HUMAN MELANOMA CELL LINES

The research that I report in this thesis was done for the most part with seven human melanoma cell lines that I established from human tumour material obtained at the time of surgery from patients in Groote Schuur Hospital. Although fortuitous, the choice of melanomas as tumours to study proved to be fortunate in a number of respects. Firstly, these neoplasms occur frequently in the South African population with the result that I was able to obtain, in a relatively short space of time, sufficient cell lines for useful comparisons to be drawn. Secondly, melanomas are well known for the ease with which they can be cultured *in vitro* (Giovanella et al, 1976). The large number of melanoma cell lines that have been developed and reported by other workers testify to this fact and have provided a convenient basis in the literature from which my studies could proceed. Finally, melanomas constitute tumours whose similarity is undisputed in terms of their origin in the neural crest, yet whose tendency to differ remarkably in terms of the phenotypic characteristics which they display is equally well documented. These distinctive characteristics and the variability that is manifest in their expression provided the basis for a number of interesting experiments which I was able to undertake. Before proceeding, however, it was necessary to acquire and to characterize the cell lines that formed the substrate for my investigations. In this chapter I describe the way in which the cell lines were established and the procedures that I used to document cellular characteristics that were of interest.

Since much of the work of this thesis is concerned with the synthesis and release of PA's by cultured melanoma cells, I make brief

reference in this chapter to the secretion of these enzymes by the cells and the extent to which this function could be regulated by hormones or other chemical substances of biological interest. At this point my study of PA's and their modulation should be considered only inasmuch as they contribute to the characterization of the cell lines and to the identification of aspects that warranted further study. The justification for my interest in PA synthesis and its control and my further studies with this enzyme are presented in subsequent substantive chapters.

MATERIALS AND METHODS

Primary Culture of Tumour Biopsies.

Samples removed at the time of surgery were placed in bottles containing DB medium supplemented with 2% FCS and were immediately transported to the tissue culture laboratory.

The manner in which the melanoma sample was treated depended firstly on whether it was a primary tumour in skin or whether it was a metastatic deposit, and secondly, on the size and consistency of the specimen. Primary tumours were cultured as explants. Secondary tumours were plated as single-cell suspensions after mechanical or enzymatic dispersion. When adequate amounts of tissue were available, attempts were also made to culture metastases as explants and primary tumours as single-cell suspensions.

Culture of explants: The tissue was cut into small fragments (approximately 1 cu. mm) with a scalpel blade with care not to damage the fragments by squeezing or distorting them in any way. The pieces were then placed as explants on 60 mm tissue culture dishes (Falcon No. 3002), moistened with a thin film of tissue culture medium and left overnight. The following day the adherent explants were covered with medium and the cultures

were examined regularly for evidence of growth. The medium was changed at weekly intervals.

Mechanical dispersion: After mincing finely with sterile scissors, the tumour fragments were suspended in RPMI and pipetted to and fro 4 to 5 times through the orifice of a 5 ml pipette. The suspension was then left to stand for a few minutes during which time larger fragments settled and single cells remained in suspension. The supernate was carefully aspirated into conical tubes and the single cells were sedimented by centrifugation at 350 g for 5 min. The pellet was resuspended in DB or RPMI medium supplemented with 10% FCS. This suspension was placed into tissue culture dishes and observed regularly for evidence of growth.

Enzymatic dispersion: Cells that were retained in fragments that settled after mincing and pipetting were released by treatment with trypsin, followed where necessary by incubation with collagenase.

For trypsinization, the fragments were transferred to a bottle containing 0.25% trypsin in TD and gently agitated at 37°C for a maximum of 20 min. The digest was allowed to stand for 5 min after which the supernatant was removed and added to an equal volume of medium containing 10% FCS to neutralize the proteolytic activity of the trypsin. This cell suspension was centrifuged at 350 g for 5 min. The pellet was resuspended in growth medium and transferred to a tissue culture dish and incubated at 37°C in a humid atmosphere of 5% CO_2 in air.

Fresh 0.25% trypsin was added to any remaining fragments and this procedure was repeated.

After 3-4 sequential trypsin treatments any undigested tumour tissue was incubated overnight at 4°C with collagenase at 1 mg/ml in RPMI-FC5. The cells obtained after this treatment were processed as described above.

Occasionally a gelatinous, viscous suspension was obtained following enzymatic digestion. This was due to DNA released from damaged cells. DNAase, at a final concentration of 10 µg/ml, was added to this gelatinous mixture which was then incubated at 37°C for 5-10 min to give a fluid, easily pipettable solution.

Twenty four hr after cell plating, all cultures were inspected for viable cells. Petri dishes were washed gently with fresh medium to remove non-adherent cells which were pooled, pelleted by centrifugation and reseeded onto new petri dishes.

Maintenance of Early Passage Cultures.

Subsequent treatment of the cultures was determined by the rate of growth of the cells and the need to select for tumour cell growth in the presence of fibroblast contamination. Elimination of fibroblast contamination was achieved by the use of the following strategies either alone or in combination.

If several primary cultures were available, those judged visually to have the best melanoma cell to fibroblast ratio were selected for further passage.

The relative ease with which tumour cells could be detached from the plastic surface was exploited by treating the mixed cultures briefly with 0.02% EDTA in saline. In many cases the fibroblasts remained as an adherent population whereas a population considerably enriched for tumour cells was released by the chelating agent.

Fibroblasts and melanoma cells were found to differ in the rates with which they adhered to plastic surfaces, the former cell type adhering much more rapidly. This fact was used to get rid of fibroblasts by trypsinizing mixed cultures and seeding them into petri dishes which were incubated at 37°C for 10 min. The supernatant containing the non-adherent

population was then removed and reseeded onto a second petri dish, which was also incubated for 10 min. The process could be repeated a number of times.

Selection against fibroblast propagation was continued until any remaining fibroblasts became senescent and were outgrown by melanoma cells.

Long-term Maintenance and Storage of Cell Lines.

Once clearly established as viable and vigorously growing cell lines, the cultures were routinely maintained in DB or RPMI medium supplemented with 10% heat-inactivated FCS, 300 µg penicillin/ml, 200 µg streptomycin sulphate/ml and 10 µg tylocine/ml. The cultures were kept at 37°C in a humid atmosphere containing 5% CO_2 in air.

Cultures were inspected at least twice weekly under phase contrast microscopy and were passaged when they approached confluence. Passaging was performed by aspirating the medium, and covering the cells with 0.25% trypsin in TD at 37°C. After approximately 5 min or less, the detached cells were dispersed by gentle pipetting and the suspensions were added to an equal volume of medium containing FCS to neutralize the protease. The cells were washed by centrifugation at 350 g for 5 min, resuspended in medium, and adjusted to give the desired cell concentration for reseeding of new cultures.

For the long-term preservation of cell stocks, cells were frozen for liquid nitrogen storage as follows. After centrifugation, the cell pellets were resuspended to approximately 2 x 10^6 cells/ml in tissue culture medium containing 10% FCS and 10% DMSO at room temperature. This suspension was distributed in 1 ml volumes into screw-topped 38 x 12.5 mm nylon tubes (Nunc No. 1078) which were kept on chipped ice. These tubes were then frozen by vapor-phase liquid nitrogen freezing in a device built

in the laboratory and designed to give programmed cooling at the rate of $4^{o}C$/min, until a temperature of $-26^{o}C$ was reached. The cells were then held at this temperature for 20 min, after which they were cooled rapidly to $-60^{o}C$ and placed in liquid nitrogen. The freezing protocol chosen was that recommended by Farrant et al (1974) and proved to be extremely satisfactory.

Plating Efficiency

The plating efficiency of the cell lines was determined by two methods:-

Method (i):- Cells were seeded at a density of 250 or 500 cells/60 mm petri dish and left undisturbed for 14 days. The dishes were then washed with PBS, fixed with 70% methanol and stained with haematoxylin and eosin and cell colonies were counted with the aid of a Wild dissecting microscope. Duplicate petri dishes were scored for colonies which consisted of groups of 8 or more cells. In some cases where no colonies were visible after 14 days, the cells were reseeded at a higher density, namely 5×10^{3} cells/60 mm dish. The results include the number of cells that were seeded to give the observed number of colonies.

Method (ii):- Cells removed from dishes in the late log phase of growth were seeded at a density of 5×10^{5} cells/60 mm tissue culture dish in RPMI-FC10. Dishes were washed with PBS 24 hr after seeding at which time adherent cells on triplicate dishes were counted and expressed as a percentage of the number seeded.

Growth in Soft Agar

The detailed method for cloning cells in agar is described in Appendix A.5.

Briefly, cells were seeded at 500, 1000 and 5000 cells/60 mm dish

and scored for colonies after 14 days of incubation. If no colonies were observed the experiment was repeated, seeding cells at higher densities (up to 3 x 10^5 cells/60 mm dish) with or without fibroblast feeder layers. Since certain of the cell lines gave rise to colonies only when seeded at high density, the results include the number of cells that were seeded to give the observed cloning efficiencies.

Growth on the Chorioallantoic Membrane.

The method used was that of Ossowski and Reich (1980a).

Embryonated hen's eggs were obtained from the Golden Grove Poultry Farm, Cape Town and were incubated at 41°C in an egg incubator. Ten-day-old eggs were candled to establish embryo viability and orientation. A shallow punch was used to pierce the shell and shell membrane above the air sac and above the intersection of at least two major blood vessels in the CAM without piercing the CAM itself. The CAM was dropped by applying gentle suction at the air sac. An opening of approximately 1 sq cm was cut in the shell above the dropped CAM. At this stage the egg was moved into a sterile hood. The square of shell and attached shell membrane was removed, taking care that no shell dust contaminated the CAM beneath.

UCT-Mel 1 cells were trypsinized, washed and resuspended in the minimum feasible volume of PBS. Using a fine-tipped pipette, the required number of cells were pipetted gently onto the CAM, as close as possible to the intersection of the major blood vessels. The opening was covered with sterile sellotape and incubated at 37°C in a humidified incubator.

All other tissue culture procedures and reagents were as described in the Appendix.

RESULTS

Biopsies of malignant melanoma tissue were received from 21 patients over a 4 year period. Of these biopsy samples, 4 came from primary cutaneous melanomatous lesions and 17 from metastatic deposits (lymph nodes - 15; liver - 1; brain - 1).

None of the primary biopsies formed cell lines; 7 cell lines were successfully established from metastatic tissue. The origins of these lines are presented in Table 2.1. These lines were characterized and, when established (i.e. at the 10th passage or later) showed features that are summarized in the following brief descriptions and in Tables 2.2 and 2.3 and Figs 2.1 and 2.2.

Cell Line: UCT-Mel 1

History: Tumour tissue was obtained from a 67 year old Caucasian female (EG) who noticed a mole on her right ankle in February, 1976. In May of the same year it began to increase in size and bleed and excision revealed a malignant melanoma of Clark's Level III. In November a superficial inguinal block dissection was performed which yielded the sample obtained for tissue culture. By December the patient presented with an enlarged liver and vague masses in the abdomen and she died in January 1977.

The sample for tissue culture comprised a deeply pigmented lymph node which, on histological examination, showed partial or complete replacement by epithelioid malignant melanoma with melanin production being a prominent feature.

TABLE 2.1

ORIGIN OF MELANOMA CELL LINES.

Cell line	Race[a]	Sex[b]	Age	Site of primary	Site of secondary resulting in line	Morphology on histology	Pigmentation in biopsy
UCT-Mel 1	C	F	67	R.Ankle	R.Inguinal Lymph Node	Epithelioid	+
UCT-Mel 2	C	F	67	L.Shoulder	L.Axillary Lymph Node	Epithelioid	±
UCT-Mel 3	C	F	71	L.Calf	Liver	Epithelioid	-
UCT-Mel 4a & b	C	F	67	L.Foot	L.Inguinal Lymph Node	Epithelioid	+
UCT-Mel 5	C	F	48	L.Calf	Brain	Epithelioid & Spindle	-
UCT-Mel 6	C	F	68	L.Ankle	L.Inguinal Lymph Node	Spindle	-
UCT-Mel 7	B	F	52	L.Heel	L.Femoral Gland	Spindle	-

(a) C = Caucasian B = Black

(b) F = Female

TABLE 2.2

FEATURES OF MELANOMA CELL LINES

Cell Line	Morphology	Pigmentation	Modal Chromosome No.	Plasminogen Activator u/10^6cells/24 hr	Plasminogen Activator Type[a]	Growth in Soft Agar	Growth in Nude Mice
UCT-Mel 1	Triangular Dendritic	+	74	19	Tiss	+	+
UCT-Mel 2	Dendritic	+	61	4	Tiss	+	+
UCT-Mel 3	Triangular Dendritic	-	72	9	Tiss	+	+
UCT-Mel 4a	Cuboid	-	90-158	31	Tiss	-	+
UCT-Mel 4b	Cuboid	-	95-143	50	Tiss	-	+
UCT-Mel 5	Triangular Dendritic	-	72	7	Tiss	+	+
UCT-Mel 6	Epithelioid/Spindle	-	81	67	UK	-	-
UCT-Mel 7	Spindle	-	65	9	Tiss	+	+

(a) Tiss = tissue type PA
UK = urokinase type PA

TABLE 2.3

GROWTH CHARACTERISTICS OF MELANOMA CELL LINES

CELL LINES	GENERATION TIME (hr)		SATURATION DENSITY (cells/sq cm x 10^5)		PLATING EFFICIENCY[c] (i)		(ii)	COLONY FORMATION IN SOFT AGAR	
	DB	RPMI	DB	RPMI	No.cells seeded/ 60mm dish	% colony formation	% adherent cells	No.cells seeded/ 60mm dish	% colony formation
UCT-Mel 1	42	41	1.6	3.6	500	11.7	94.2	$1x10^3$	57.4
" 2	70	50	2.0	3.0	500	n.d[a]	66.8	$1x10^3$	63.2
" 3	50	50	1.6	1.7	500	n.d	91.5	$1x10^3$	53.8
" 4a	n.d[a]	52	1.4	3.8	500	2.5	98.9	$3x10^5$	0
" 4b	83	54	1.5	3.5	500	0	82.6	$3x10^5$	0
" 5	58	58	1.1	3.1	5000	2.0	85.2	$1x10^5$	18.6
" 6	74	33	1.4	2.8	5000	0.8	96.0	$3x10^5$	0
" 7	119	95	1.6	1.6	5000	n.d.	97.5	$1.5x10^5$[b]	1.8
Normal fibroblasts				0.5					

(a) n.d. = not determined (see text)

(b) cells seeded with fibroblast feeder layer.

(c) (i) and (ii) refer to Methods (i) and (ii), page 18.

FIGURE 2.1

FIGURE 2.1

Morphological appearance of melanoma cell lines in culture.

The composite figure shows the morphologies of adherent monolayers of the different melanoma cell lines. The cell lines are as follows: a and b) UCT-Mel 1; c and d) UCT-Mel 2; e and f) UCT-Mel 3; g and h) UCT-Mel 5. In each case, the photograph on the left depicts a sparse culture of the respective melanoma cell line, while that on the right depicts a confluent culture of the same cell line. Note the predominantly triangular/dendritic morphology of UCT-Mel 1, 3 and 5 (a, e and g), and the dendritic morphology of UCT-Mel 2 (c). In all cases, the cells formed giant multilayered clumps on reaching confluence, which were pigmented in the case of UCT-Mel 1 and 2 (b and d) and nonpigmented in the case of UCT-Mel 3 and 5 (f and h). All photographs are at the same magnification. The scale marker in (h) represents 50 μm.

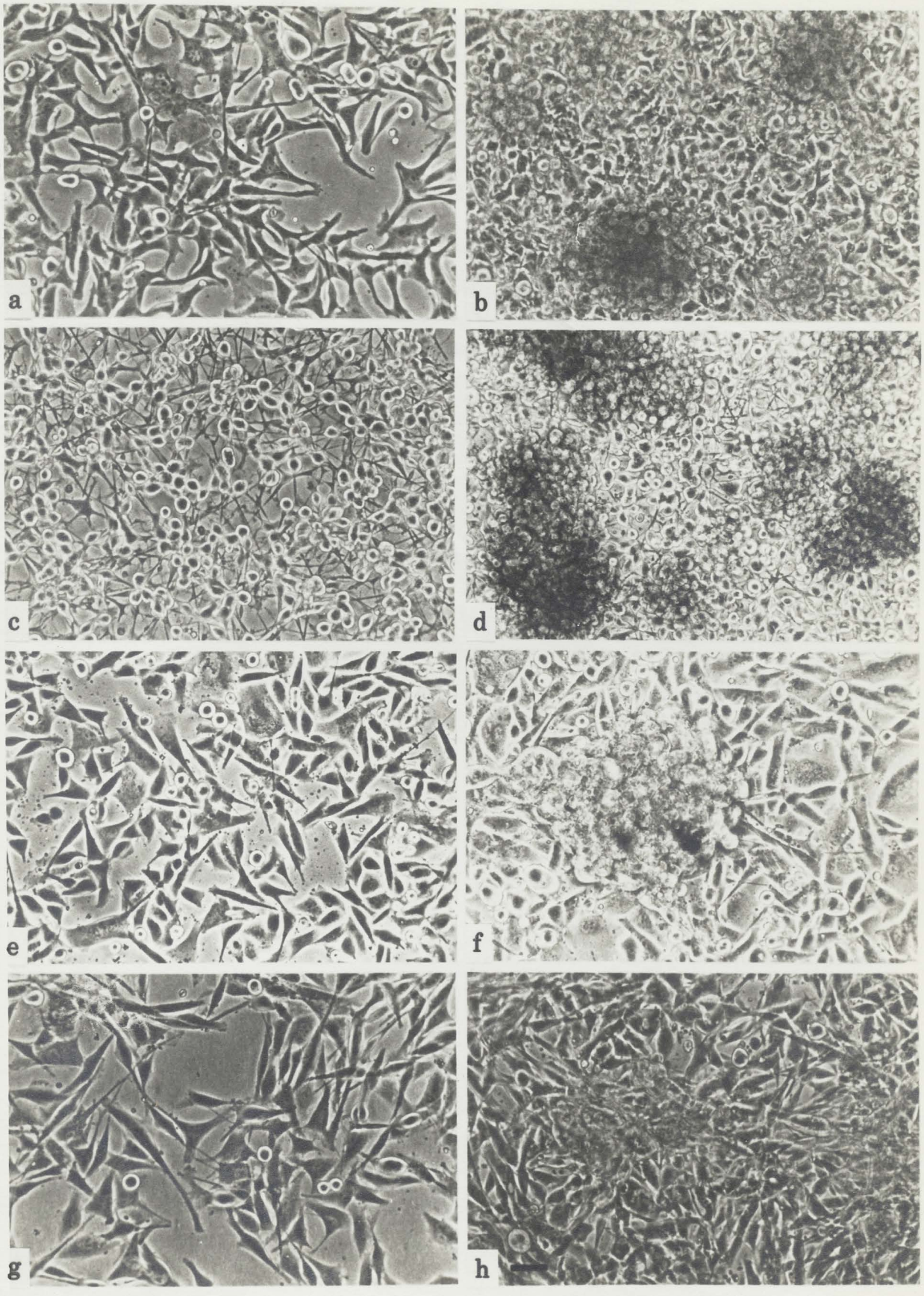


FIGURE 2.1

FIGURE 2.2

FIGURE 2.2

Morphological appearance of melanoma cell lines in culture.

As described in the legend to Fig. 2.1, the figure depicts sparse (left hand photograph) and confluent (right hand photograph) cultures of the melanoma cell lines. The cell lines are as follows: a and b) UCT-Mel 4a; c and d) UCT-Mel 4b; e and f) UCT-Mel 6; g and h) UCT-Mel 7. Note the cuboidal morphology of UCT-Mel 4a and b (a and c), the epithelioid/spindle cell morphology of UCT-Mel 6 (e) and the spindle cell morphology of UCT-Mel 7 (g). UCT-Mel 7 cells formed large multilayered clumps on reaching confluence (h), while UCT-Mel 4a, 4b and 6 cells attained a high density by forming a very dense compact and more crowded layer of cells on the tissue culture dish (b, d and f). All photographs are at the same magnification. The scale marker in (h) represents 50 μm.

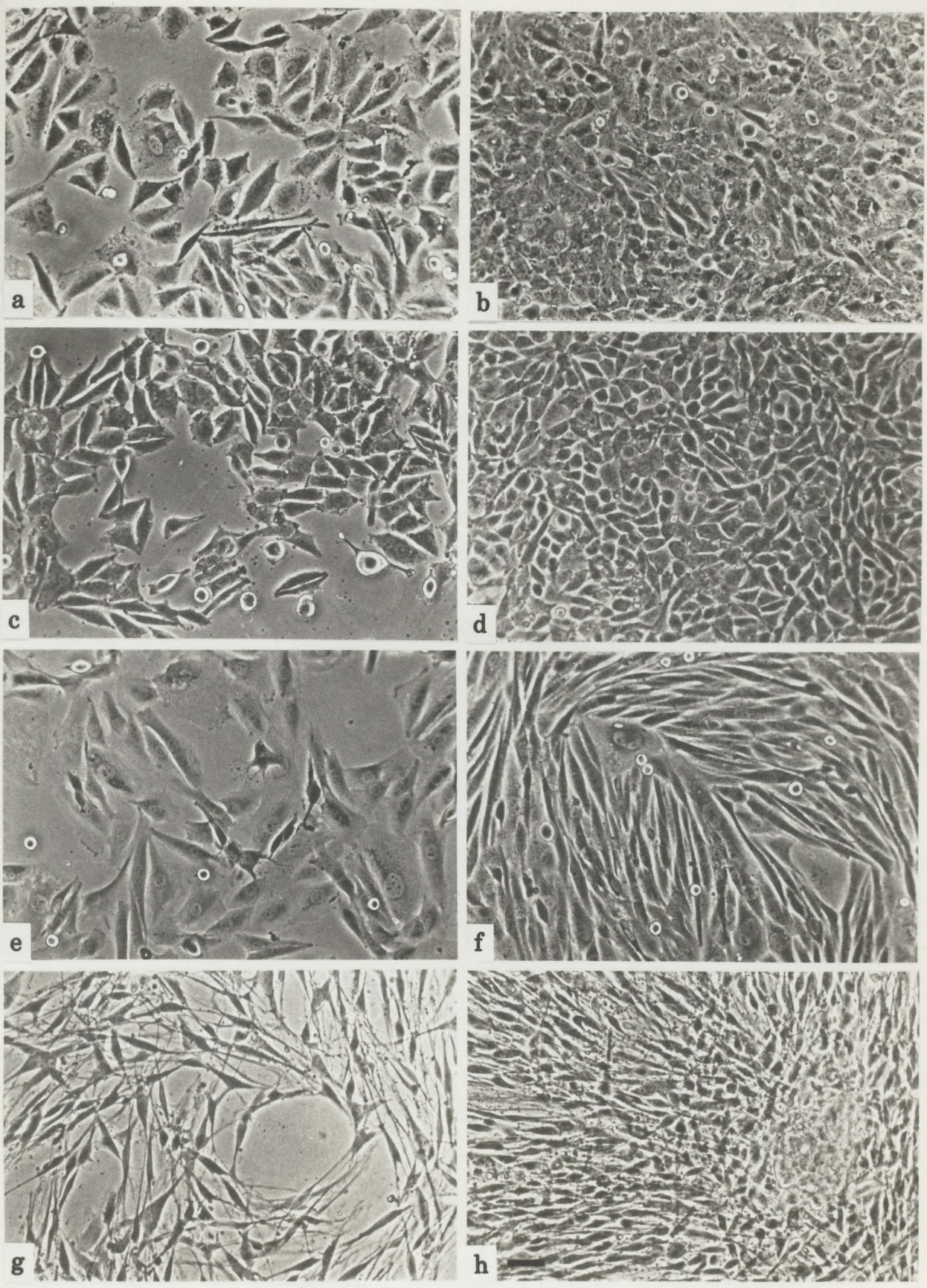


FIGURE 2.2

Initial culture

The biopsy was readily dispersed by mincing with scissors and the tissue fragments were digested with trypsin and with collagenase. Both the trypsin- and collagenase-treated fragments gave viable cell suspensions which readily grew into cell lines. The initial culture dishes were passaged after one week and thereafter at weekly intervals.

The work reported in this thesis was done on the line originating from the trypsin-treated tissue fragments. These cells had the following characteristics after approximately 60 serial passages in culture.

Morphology

The line grew as an adherent monolayer of triangular dendritic cells (Fig. 2.1 a and b). At confluence the cells piled up, forming large multilayered clumps, and therefore failed to show contact inhibition. Giant multinucleated cells were frequently seen.

Pigmentation

The cells were non-pigmented at low density and became deeply pigmented at confluence. This was accompanied by an increase in intracellular tyrosinase (Fig. 2.3).

Growth characteristics

Doubling time:		42 hr (DB-FC10)
		41 hr (RPMI-FC10)
Plating efficiency:	Method (i):	11.7% when 5×10^2 cells/60 mm dish were seeded
	Method (ii):	94.2%

FIGURE 2.3

FIGURE 2.3

Cellular tyrosinase content.

a) UCT-Mel 1 cells were seeded at 1.5 x 10^5 cells/60 mm dish in DB-FC10 and fresh medium was added at 48 hr intervals. At the indicated times, cell lysates of replicate cultures were prepared (Appendix A.9) and assayed for protein content (▲——▲) (Appendix A.12) and tyrosinase activity (■---■) (Appendix A.15).

b) UCT-Mel 2 cells were seeded at 3 x 10^5 cells/60 mm dish in DB-FC10 and treated as in (a).

Note that in both cases tyrosinase activity became apparent while the cells were proliferating, and increased as cell density increased.

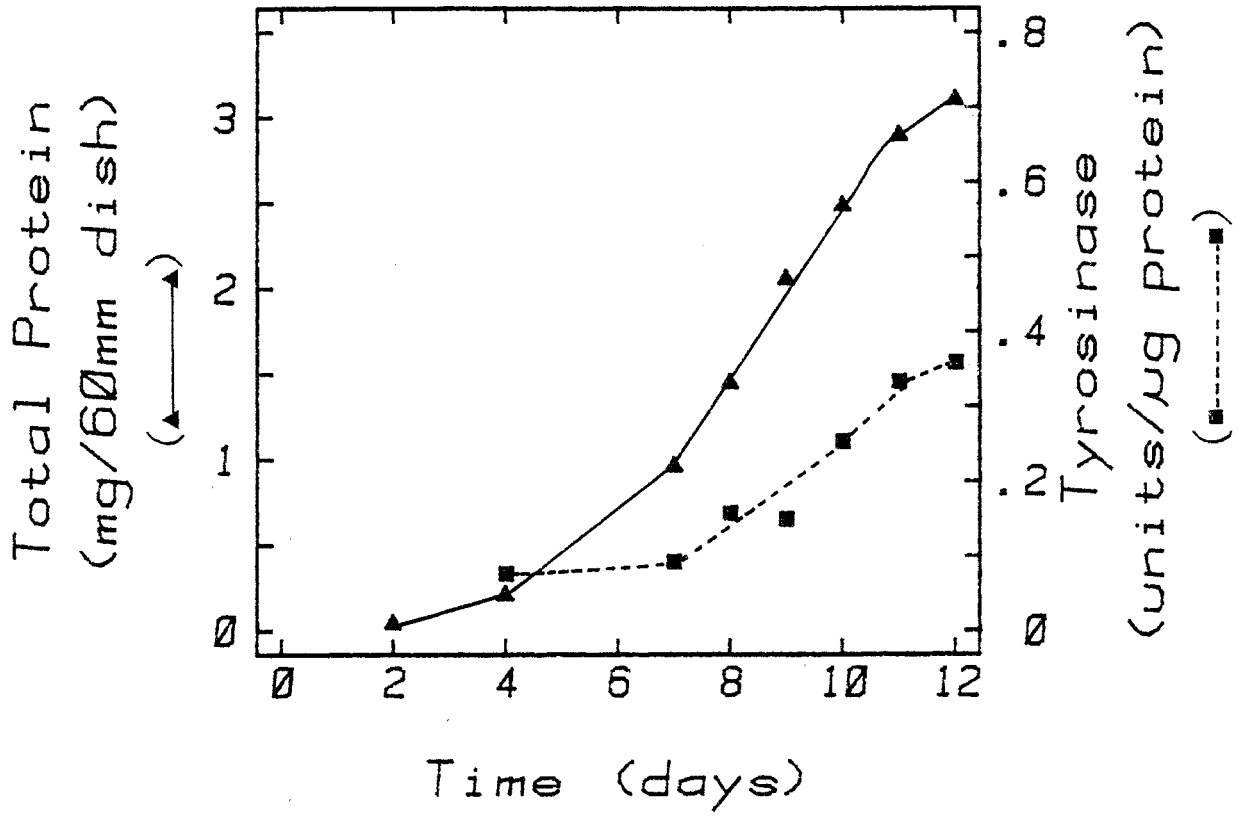


FIGURE 2.3a

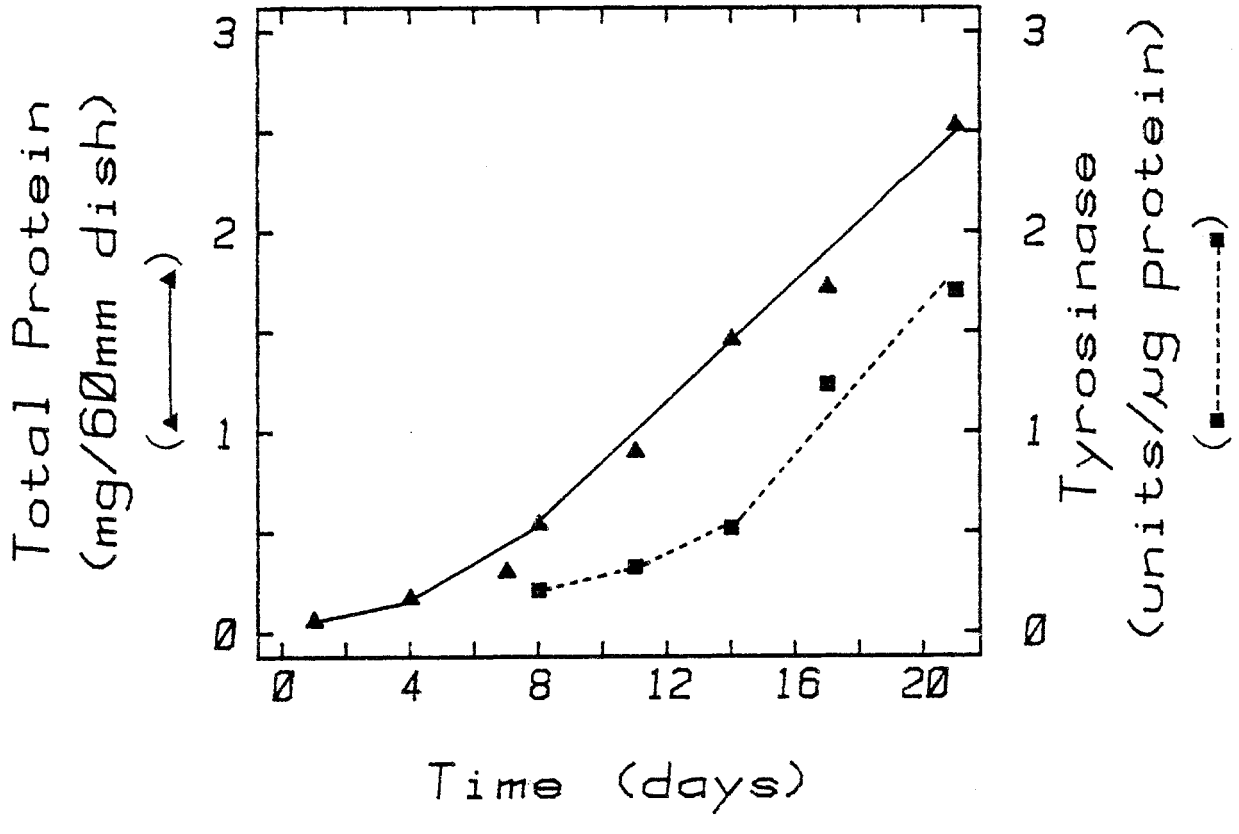


FIGURE 2.3b

Saturation density:	1.6×10^5 cells/sq cm (DB-FC10)
	3.6×10^5 cells/sq cm (RPMI-FC10)
Cloning efficiency in soft agar:	57.4% when 1000 cells/60 mm dish were seeded.
Modal chromosome number:	74
Growth in nude mice:	Positive (see Chapter 6)
Plasminogen activator secretion:	19 u/10^6 cells/24 hr (Tissue-type PA) (see Chapter 3).

Special features

As mentioned previously confluent cultures of UCT-Mel 1 consisted of pigmented aggregates of cells piled in multilayered foci on the surface of the petri dish (Fig. 2.1b). It was of interest to determine whether these densely pigmented cells were capable of growth and adherence or whether the cells, once pigmented, had lost their capacity to divide.

Accordingly cells were seeded on bacteriology dishes on which they could not adhere. Under these circumstances the cells grew in clumps in suspension and once the cell clumps had become large and heavily pigmented they were removed with a wide mouthed pipette and incubated in 0.02% EDTA for 10 min at 37°C to obtain a single cell suspension. This suspension was centrifuged at 350 g for 5 min and the cells were seeded at 5×10^5 cells/60 mm petri dish. The percentage of adherent cells after 24 hr in culture was 71%. Thus even after becoming pigmented these cells were capable of adherence and could still initiate cell division.

Cell Line: UCT-Mel 2

History

Tumour tissue was obtained from a 67 year old Caucasian female (EP).

In December, 1977, a pigmented lesion on the left shoulder of the patient (which had been present for 2 years) increased in size and started to bleed. Excision in January 1978 revealed a nodular malignant melanoma of Clark's Level III. In November 1978 a left axillary clearance was performed from which a sample was obtained for tissue culture. No further recurrence occurred and to date (August 1981) the patient is well and clinically free of melanoma.

The sample received for tissue culture was macroscopically non-pigmented. The histological report confirmed the diagnosis of metastatic malignant melanoma. The cells were epithelioid and pigmentation was visible in certain areas, while other areas were nonpigmented.

Initial culture

Populations of melanoma cells with very little fibroblast contamination were obtained from three sequential trypsin treatments of the original biopsy. These cells were initially passaged on 2 occasions after which time cell division ceased and the cells remained viable but dormant for 4 months. During this period medium was changed at weekly intervals. After 4 months of dormancy, cells on ten 60 mm petri dishes started to divide simultaneously. Thus in this instance it could be said that cell growth was not the result of a random mutation occurring in a cell on one of the plates as cells on all ten plates started to grow at the same time. In this instance it appears that the cells remained dormant for a considerable period of time and after a prolonged latent

period, possibly analagous to the latent period between primary tumour growth and the appearance of metastasis, cell division abruptly started. Cells were subsequently passaged at weekly intervals.

These cells had the following characteristics after approximately 40 serial passages in culture.

Morphology

The line grew as an adherent monolayer of dendritic cells with long spiky projections being a prominent feature of these cultures (Fig. 2.1c and d). At confluence the cells piled up, forming large multi-layered clumps and therefore failed to show contact inhibition.

Pigmentation

The cells were nonpigmented at low density, and became deeply pigmented at confluence. This was accompanied by an increase in intracellular tyrosinase (Fig. 2.3b).

Growth characteristics

Doubling time:		70 hr (DB-FC10)
		50 hr (RPMI-FC10)
Plating efficiency:	Method (i):	not determined as dividing cells detached from existing colonies and reattached, forming new colonies.
	Method (ii):	66.8%
Saturation density:		2.0×10^5 cells/sq cm (DB-FC10)
		3.0×10^5 cells/sq cm (RPMI-FC10)
Cloning efficiency in soft agar:		63.2% when 1000 cells/60 mm dish were seeded.
Modal chromosome number:		61

Growth in nude mice:	Positive
	(see Chapter 6)
Plasminogen activator secretion:	4 u/10^6 cells/24 hr
	(Tissue-type PA)
	(see Chapter 3).

Cell Line: UCT-Mel 3

History

Tumour tissue was obtained from a 71 year old Caucasian female (DH).

A pigmented lesion was removed from the medial aspect of the left calf of this patient in January 1976 and was found to be a malignant melanoma. Recurrence in the periphery of the scar occurred 8 months later. In April 1977, a liver scan indicated possible metastasis and in March 1978 a large melanomatous deposit was removed from the left lobe, from which the cell line in tissue culture was established. Malignant infiltration subsequently occurred in the sternum, lungs, spine and brain and the patient died in December, 1978.

The liver metastasis obtained for tissue culture was very soft and fragile with large pale nodules of tumour visible in the reddish black liver tissue. Histological examination revealed metastatic malignant melanoma of the epithelioid type. Scanty intracellular melanin was demonstrated by a Masson-Fontana silver stain.

Initial culture

The tumour tissue disintegrated readily on mincing with scissors and large numbers of viable cells were obtained from the trypsin digested tissue. The initial culture dishes were passaged after 48 hr. After

5 weekly passages normal fibroblast contamination of the culture had increased markedly and fibroblasts threatened to overgrow the tumour cells. This line, however, shed large numbers of viable cells into the supernatant medium and these were collected from a number of dishes, centrifuged, and reseeded on a separate dish. In this manner a relatively pure culture of melanoma cells was obtained. This procedure was repeated twice before a pure culture of melanoma cells was established. The cells removed from the supernatant adhered readily when plated on a new petri dish.

These cells had the following characteristics after approximately 40 serial passages in culture.

Morphology

The line grew as an adherent monolayer of triangular dendritic cells (Fig. 2.1e and f). At confluence the cells piled up, forming large multilayered clumps and therefore failed to show contact inhibition. Giant multinucleated cells were frequently seen.

Pigmentation

The cells were nonpigmented at low density and remained non-pigmented at confluence.

Growth characteristics

Doubling time:		50 hr (DB-FC10)
		50 hr (RPMI-FC10)
Plating efficiency:	Method (i):	not determined as dividing cells detached from existing colonies and reattached, forming new colonies
	Method (ii):	91.5%

Saturation density:	1.6×10^5 cells/sq cm (DB-FC10)
	1.7×10^5 cells/sq cm (RPMI-FC10)
Cloning efficiency in soft agar:	53.8% when 1000 cells/60 mm dish were seeded.
Modal chromosome number:	72
Growth in nude mice:	Positive (see Chapter 6)
Plasminogen activator secretion:	9 u/10^6 cells/24 hr (Tissue-type PA) (see Chapter 3).

Special features

This line consistently shed viable cells into the medium. At any stage of growth approximately one third of the cell number was found in suspension. The cells in suspension were always viable and approximately 80% of these cells would readily adhere to the surface of a new tissue culture dish if reseeded.

Cell Line: UCT-Mel 4

History

Tumour tissue was obtained from a 67 year old Caucasian female (LH).

A lesion on the left foot of this patient, with the appearance of a scab, was burnt off four times over four years but recurred each time. In November 1978 a wide excision was performed which revealed malignant melanoma of Clark's Level IV. One month later, a left inguinal lymph node dissection was performed which also revealed malignant melanoma which had replaced several lymph nodes. The sample for tissue culture

was obtained at this stage. The melanoma rapidly metastasised to the liver and lungs and the patient died in June, 1979.

The sample for tissue culture comprised a pigmented lymph node. Histological examination showed a metastatic melanoma with strong epithelioid characters and areas of deep pigmentation.

Initial culture

The tumour tissue was minced finely with scissors. Large numbers of cells were readily released from the tumour fragments after brief exposure to trypsin. Twenty four hr later the non-adherent cells from the trypsin treated digests were removed and reseeded on a second petri dish. These cells adhered and had a slightly more spindle-like cell morphology (UCT-Mel 4b) whereas the original adherent cells had a cuboidal, epithelial type morphology (UCT-Mel 4a). Both sublines were passaged 6 times at weekly intervals and representative areas of each subline were removed by cloning to attempt to establish a relatively pure cuboidal (UCT-Mel 4a) and more fibroblastic (UCT-Mel 4b) population. For the cloning procedure, small glass cloning rings which had been coated in high-vacuum grease were carefully placed on a selected area of the dish. Trypsin was added to the centre of the well, the cells were removed and reseeded in a small volume of medium on a new culture dish.

The morphological differences, however, were not retained on cloning and passage and the two sublines thus selected appeared fairly similar, morphologically, but were maintained as separate sublines as they displayed other differences on characterization.

These sublines had the following characteristics after approximately 20 serial passages in culture.

Morphology

Both sublines grew as adherent monolayers of cuboidal cells (Fig. 2.2a, b, c and d). At confluence both UCT-Mel 4a and b did not pile up but formed a very dense tightly packed monolayer, and therefore failed to show contact inhibition.

Pigmentation

Both sublines were nonpigmented, even at high density.

Growth characteristics: (UCT-Mel 4a)

Doubling time:	not determined in DB-FC10 as this medium appeared to be toxic (Fig. 2.5A)
	52 hr (RPMI-FC10)
Plating efficiency: Method (i):	2.5% when 5×10^2 cells/60 mm dish were seeded.
Method (ii):	98.9%
Saturation density:	1.4×10^5 cells/sq cm (DB-FC10)
	3.8×10^5 cells/sq cm (RPMI-FC10)
Cloning efficiency in soft agar:	0% when 3×10^5 cells/60 mm dish were seeded, with or without a fibroblast feeder layer.
Modal chromosome number:	90 - 158, no mode.
Growth in nude mice:	Positive (see Chapter 6)
Plasminogen activator secretion:	31 u/10^6 cells/24 hr. (Tissue-type PA) (see Chapter 3)

Growth characteristics: (UCT-Mel 4b)

Doubling time:	83 hr (DB-FC10)
	54 hr (RPMI-FC10)
Plating efficiency: Method (i):	0% when 5 x 10^2 cells/60 mm dish were seeded.
Method (ii):	82.6%
Saturation density:	1.5 x 10^5 cells/sq cm (DB-FC10)
	3.5 x 10^5 cells/sq cm (RPMI-FC10)
Cloning efficiency in soft agar:	0% when 3 x 10^5 cells/60 mm dish were seeded, with or without a fibroblast feeder layer.
Modal chromosome number:	95 - 143, no mode.
Growth in nude mice:	Positive (see Chapter 6)
Plasminogen activator secretion:	50 u/10^6 cells/24 hr (Tissue-type PA) (see Chapter 3).

Cell Line: UCT-Mel 5

History

Tumour tissue was obtained from a 48 year old Caucasian female (SG), who noticed a mole on the left calf which rapidly increased in size over 6 months, but did not bleed. A wide excision in May 1976 revealed a malignant melanoma of Clark's Level IV. Over 2 years later, in October 1978, a left inguinal block dissection revealed melanoma in the lymph nodes. In October 1979 a well defined mass was removed from the right fronto-temporal region of the brain and proved to be metastatic melanoma. The line in tissue culture was established from this sample. No further

recurrence has occurred to date (August 1981).

The brain metastasis obtained for tissue culture contained no visible melanin. Histological examination confirmed the diagnosis of a malignant melanoma of mixed spindle and epithelioid type.

Initial culture

The biopsy specimen was minced finely and the tumour fragments were digested with trypsin. In this instance the most successful cultures obtained were those derived from spilled cells in the medium in which the biopsy specimen was transported to the laboratory. The trypsin derived cultures were contaminated with fibroblasts and the cell line originated from tumour cells released into the original biopsy bottle. The cells grew slowly at first. The initial culture dishes were passaged after 3 weeks and thereafter at bi-weekly intervals.

The cells had the following characteristics after approximately 10 serial passages in culture.

Morphology

The line grew as an adherent monolayer of triangular dendritic cells which generally had more dendritic projections than UCT-Mel 1 or 3 (Fig. 2.1g and h). At confluence the cells piled up, forming large multilayered clumps, and therefore failed to show contact inhibition.

Pigmentation

The cells were nonpigmented, even at high density.

Growth characteristics

Doubling time:	58 hr (DB-FC10)
	58 hr (RPMI-FC10)
Plating efficiency: Method (i):	2.0% when 5 x 10^3 cells/60 mm dish were seeded.
Method (ii):	85.2%
Saturation density:	1.1 x 10^5 (DB-FC10)
	3.1 x 10^5 (RPMI-FC10)
Cloning efficiency:	18.6% when 10^5 cells/60 mm dish were seeded.
Modal chromosome number:	72
Growth in nude mice:	Positive (see Chapter 6)
Plasminogen activator secretion:	7 u/10^6 cells/24 hr (Tissue-type PA) (see Chapter 3)

Cell Line: UCT-Mel 6

History

Tumour tissue was obtained from a 68 year old Caucasian female (GD).

In April 1977 a mole excised from the back of the left ankle of this patient was found to be a malignant melanoma, Clark's Level IV. In December 1979 some involved nodes were removed during a left inguinal lymph node dissection. In August 1980 a left inguinal region clearance was performed and the nodes again showed the presence of secondary melanoma. The sample for tissue culture was obtained at this juncture. Tumour nodules recurred in the femoral triangle and in the vicinity of the original graft, sometimes pigmented, sometimes containing no melanin. The patient

deteriorated and died in June 1981.

The sample obtained for tissue culture was nonpigmented. Histological examination confirmed deposits of secondary malignant melanoma of spindle morphology, with no melanin pigment being noted.

Initial culture

The biopsy had a firm consistency and was minced finely with scissors. Large numbers of viable cells were obtained both from the trypsin and the collagenase treated fragments. This cell line was derived from cells released by fragments which had been sequentially trypsinized on two occasions and then incubated in a collagenase solution at 4°C overnight. The primary culture was passaged after 7 days and the cells grew continuously and were passaged at weekly intervals. These cells had the following characteristics after approximately 20 serial passages in culture.

Morphology

The line grew as an adherent monolayer of predominantly epithelioid cells (Fig. 2.2e and f) which became crowded and appeared spindle-shaped on reaching confluence. The cells did not exhibit contact inhibition.

Pigmentation

The cells were nonpigmented even at high density.

Growth characteristics

Doubling time:	74 hr (DB-FC10)
	33 hr (RPMI-FC10)
Plating efficiency: Method (i):	0.8% when 5×10^3 cells/60 mm dish were seeded.

Method (ii):	96.0%
Saturation density:	1.4×10^5 cells/sq cm (DB-FC10)
	2.8×10^5 cells/sq cm (RPMI-FC10)
Cloning efficiency in soft agar:	0% when 3×10^5 cells/60 mm dish were seeded, with or without a fibroblast feeder layer.
Modal chromosome number:	81
Growth in nude mice:	Negative (see Chapter 6)
Plasminogen activator secretion:	68 u/10^6 cells/24 hr. (Urokinase-type PA) (see Chapter 3).

Cell Line: UCT-Mel 7

History

Tumour tissue was obtained from a 52 year old Negro female (NM).

In March 1980 a chronic ulcer on the left heel of this patient was removed and diagnosed as a malignant melanoma, Clark's Level V. Two months later, a left femoral gland dissection revealed nodes infiltrated by melanoma. This patient was subsequently lost to follow up.

The sample for tissue culture comprised a nonpigmented femoral lymph node which, on histological examination, showed the presence of secondary malignant melanoma with a spindle cell morphology and minimal melanin production.

Initial culture

The specimen had a hard rubbery consistency and was minced finely, after which the fragments were digested with trypsin and collagenase. Sparse cultures of slow growing cells were obtained from the enzymatically treated fragments. Fibroblast growth was vigorous and tumour cells released into the supernatant medium were collected and reseeded on a new culture dish. The cells grew slowly and were passaged for the first time after 8 weeks in culture. They continued growing slowly for 6 months during which time they were passaged four times at approximately 6 week intervals. The rate of growth steadily increased until the cells could be passaged at two weekly intervals. These cells had the following characteristics after approximately 20 serial passages in culture.

Morphology

The line grew as an adherent monolayer of cells with a distinctive spindle cell morphology (Fig. 2.2g and h), which frequently showed a "herringbone" type of pattern. At confluence the cells piled up, forming large multilayered clumps, and therefore failed to show contact inhibition.

Pigmentation

The cells were nonpigmented, even at high density.

Growth characteristics

Doubling time:		119 hr (DB-FC10)
		95 hr (RPMI-FC10)
Plating efficiency:	Method (i):	not determined as dividing cells detached from existing colonies and reattached, forming new colonies.
	Method (ii):	97.5%

Saturation density:	1.6×10^5/sq cm (DB-FC10)
	1.6×10^5/sq cm (RPMI-FC10)
Cloning efficiency in soft agar:	1.8% when 1.5×10^5 cells/60 mm dish were seeded, with a fibroblast feeder layer.
Modal chromosome number:	65
Growth in nude mice:	Positive (see Chapter 6)
Plasminogen activator secretion:	9 u/10^6 cells/24 hr (Tissue-type PA) (see Chapter 3)

Mycoplasma contamination

All cell lines were tested by the method described in Appendix A.7 and were found to be free of contamination by mycoplasmas.

Summary

For the sake of convenience and to emphasize similarities and differences between the various melanoma cell lines, I have collated the essential facts relating to their origins and their characteristics in Tables 2.1, 2.2 and 2.3 and Figs. 2.1, 2.2, 2.4 and 2.5.

Additional features

In addition to the studies that were completed on all the cell lines and that are summarized in the preceding tables and pages a number of investigations were performed on certain of the cell lines in order to characterize them in more detail. These included the study of growth on the chorioallantoic membrane (CAM) of the fertile hen's egg, serum

FIGURE 2.4

FIGURE 2.4

Growth curves of human melanoma cell lines *in vitro*.

Cells were seeded in RPMI-FC10 on 35 mm dishes as follows: A) UCT-Mel 1 at 1.2×10^5 cells/dish; B) UCT-Mel 2 at 1.4×10^5 cells/dish; C) UCT-Mel 3 at 1×10^5 cells/dish; D) UCT-Mel 5 at 2×10^5 cells/dish.

Twenty four hr after seeding, the medium was aspirated and fresh RPMI-FC10 (■---■) or DB-FC10 (▲——▲) was added to the cultures. Thereafter, the culture medium was changed every 48 hr. At the indicated times, replicate cultures were trypsinized and the cells were counted in a Coulter counter. In the case of UCT-Mel 3, nonadherent cells were pooled with adherent cells.

Note that in each case a period of exponential growth is followed by a diminished growth rate as higher saturation densities are reached. In each case, a faster growth rate and/or higher saturation density is shown by cells cultured in RPMI-FC10 as opposed to DB-FC10.

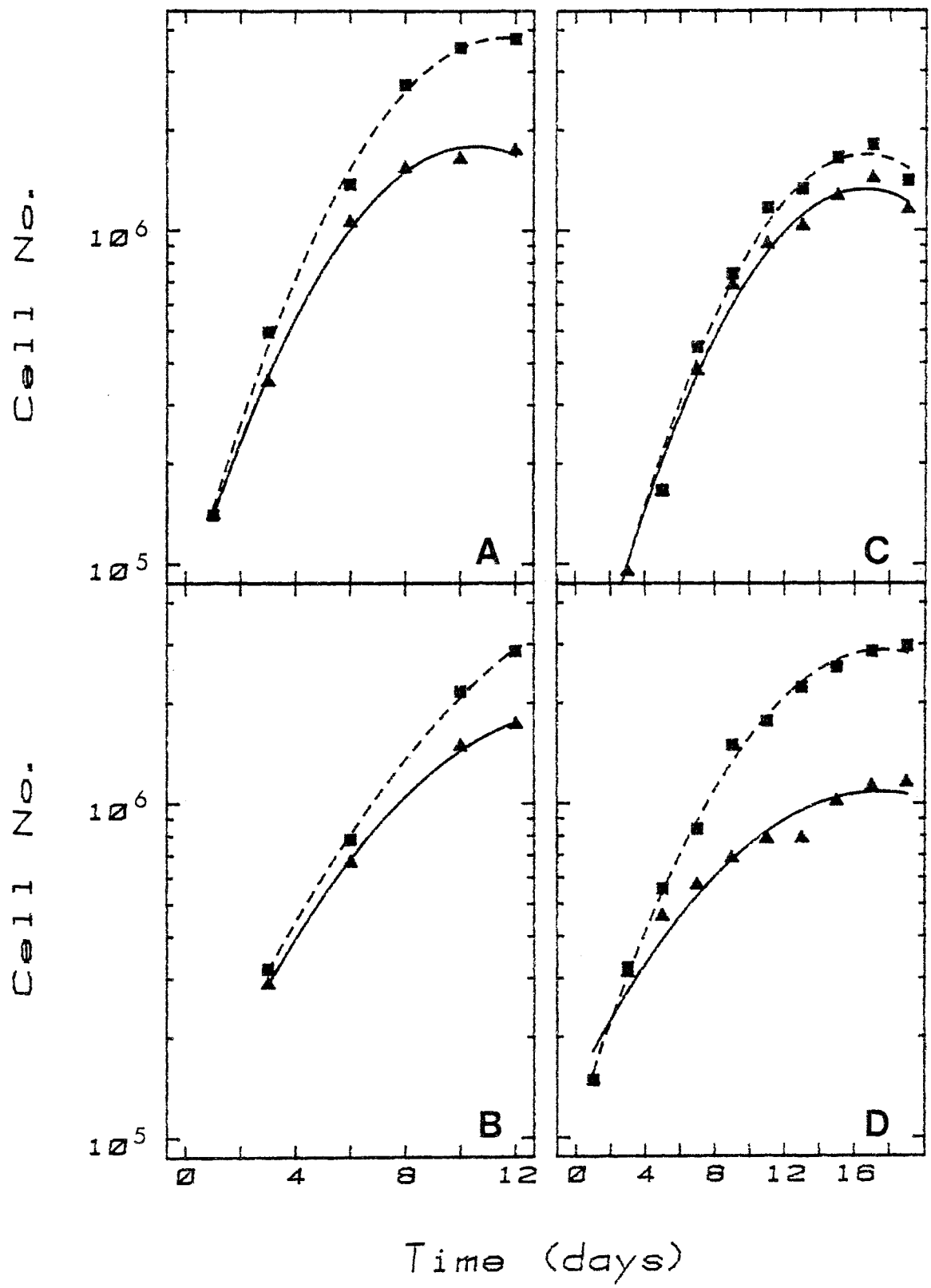


FIGURE 2.4

FIGURE 2.5

FIGURE 2.5

Growth curves of human melanoma cell lines *in vitro*.

Cells were seeded in RPMI-FC10 on 35 mm dishes as follows: A) UCT-Mel 4a at 1×10^5 cells/dish; B) UCT-Mel 4b at 1×10^5 cells/dish; C) UCT-Mel 6 at 5×10^4 cells/dish; D) UCT-Mel 7 at 2×10^5 cells/dish.

The medium was changed 24 hr later to either RPMI-FC10 (■---■) or DB-FC10 (▲——▲) and thereafter at 48 hr intervals. At the indicated times, replicate cultures were trypsinized and the cells counted in a Coulter counter.

Note the pronounced inhibitory effect of DB-FC10 on UCT-Mel 4a (A). In all other instances, exponential growth in both media was noted. Growth rates of cells tended to be faster in RPMI-10 and a higher saturation density was observed in this medium. The exception was UCT-Mel 7 (D) which grew equally well in both media.

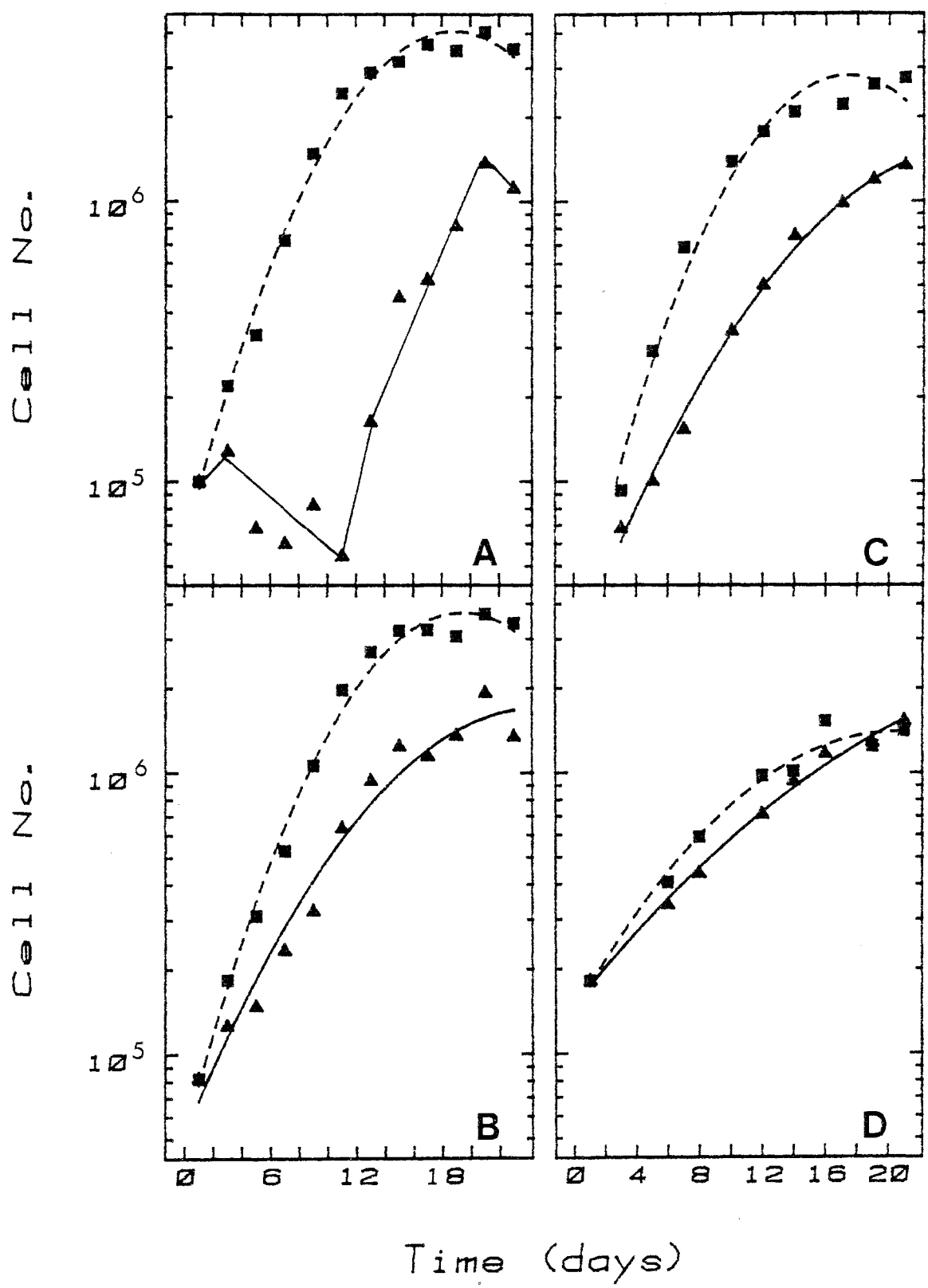


FIGURE 2.5

requirement for growth, tyrosinase synthesis, and modulation of cellular release of PA.

a) Growth on CAM of fertile hen's egg.

These studies were confined to UCT-Mel 1 cells. Early and late passage cultures were used in this experiment since it had been shown in the chick embryo system that cells which have been serially propagated in culture lose their ability to metastasize from the CAM to the lung (Ossowski and Reich, 1980b).

UCT-Mel 1 cells varying in number from 2×10^6 to 2×10^7 were placed on CAM's of 10-day old chick embryos as described in the Methods section and indicated in Table 2.4. The eggs were then returned to the incubator. After 7 days of incubation pigmented tumours were visible growing on all CAM's inoculated.

The tumours were divided in two. One half was examined histologically while the other was homogenized for assay of human PA content (using human plasminogen) and chicken PA content (using chicken plasminogen).

To examine the embryo lungs for evidence of metastases, these were removed and minced finely with scissors. One half of the resulting mince was assayed for human plasminogen. The other half was applied to a fresh CAM to examine for tumour growth after a further 7 day period of incubation.

In all cases, the initial tumour that grew on the CAM could be identified as a locally invasive human melanoma (Fig. 2.6).

In no case did secondary explants of lung tissue give rise to tumour growth, indicating that metastatic spread could not be detected by this method.

The results of the PA assays are summarized in Table 2.4, from which it can be seen that the primary tumours contained human PA, whereas

TABLE 2.4

GROWTH OF UCT-MEL 1 ON THE CHICK CHORIOALLANTOIC MEMBRANE

Passage No. of UCT-Mel 1	No. of cells inoculated	Tissue assayed	% Trypsin Released: Human PA	% Trypsin Released: Chick PA
6	$2x10^6$	Tumour on CAM	7	0
	$5x10^6$	"	11	0
	$5x10^6$	"	13	1
	$10x10^6$	"	15	1
81	$5x10^6$	"	67	5
	$20x10^6$	"	59	2
6	$2x10^6$	Chick lung	4	7
	$5x10^6$	"	3	5
	$5x10^6$	"	4	5
	$10x10^6$	"	5	7
81	$5x10^6$	"	4	8
	$20x10^6$	"	4	10

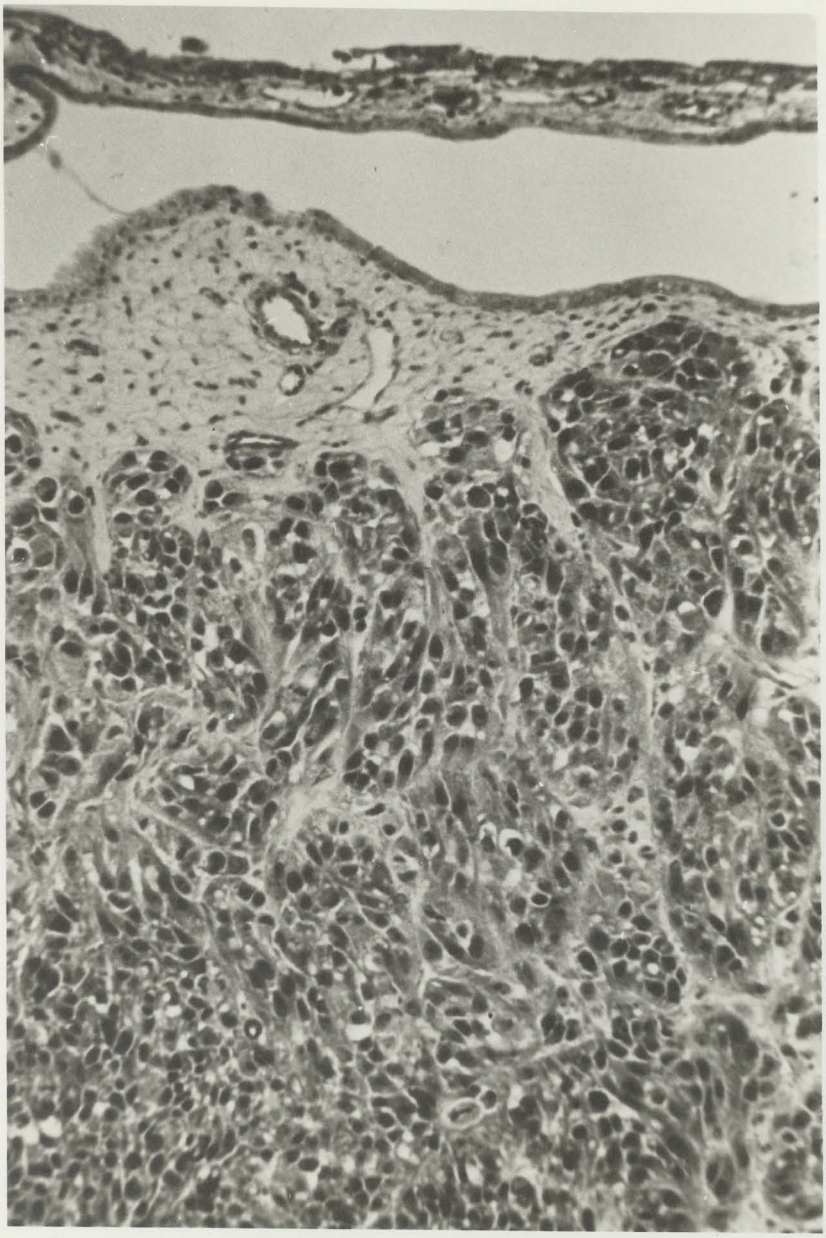


FIGURE 2.6

Growth of UCT-Mel 1 on the chick CAM.

The CAM of a 10 day old chick embryo was inoculated with 2×10^7 UCT-Mel 1 cells. The above microphotograph shows the histological appearance of a section through the invasive tumour growing on the CAM. x 200.

the embryo lungs contained small amounts of chicken PA. By these enzymatic criteria, therefore, metastases were similarly not apparent in the chick embryo lungs.

b) Cellular Tyrosinase content

Cellular extracts were assayed for tyrosinase activity using the fluorimetric procedure described in Appendix A.15. All of the cell lines were assayed in this way, and only two (UCT-Mel 1 and 2) contained significant amounts of the enzyme. These were the only two lines that pigmented spontaneously.

Both UCT-Mel 1 and 2 showed increased pigmentation and tyrosinase content as they became more confluent (Fig. 2.3). The magnitude of the density-dependent induction of tyrosinase activity was related to the medium in which the cells were cultured. UCT-Mel 1 and 2 contained 0.25 u/µg protein and 1.71 u/µg protein of tyrosinase respectively when grown in DB-FC10. Corresponding values for cells grown in RPMI-FC10 were not determined, but the cells were visually markedly less pigmented.

c) Serum requirement for growth

The extent to which growth of UCT-Mel 1 was dependent upon the presence of serum in the medium was examined in an experiment in which replicate cultures of these cells were established in medium containing 1% FCS. The following day, the medium was removed and replaced with fresh medium containing FCS in varied concentrations from 0% to 20%, as indicated in Fig. 2.7. The results of this experiment showed that UCT-Mel 1 cells were able to proliferate in the presence of concentrations of FCS as low as 1%, even though this growth was not as vigorous as that observed with higher concentrations of serum, and was more limited in duration.

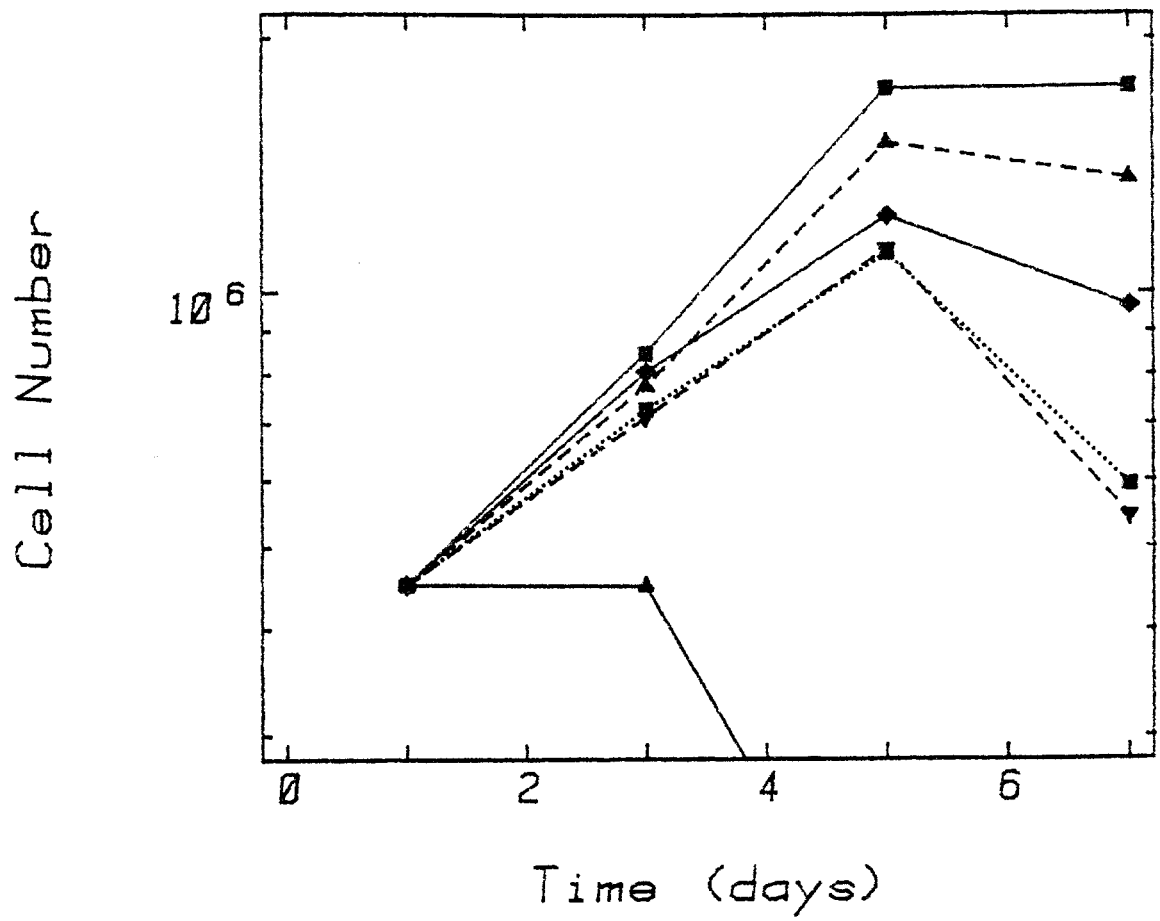


FIGURE 2.7

Serum requirement for growth of UCT-Mel 1.

UCT-Mel 1 cells were seeded at 4.5 x 10^5 cells/60 mm dish in DB-FC10. Twenty four hr later the cultures were rinsed with serum-free medium and DB containing FCS at the following concentrations was added: 0% (▲——▲); 1% (▼- - -▼); 2% (■·····■); 5% (◆——◆); 10% (▲- - -▲); 20% (■——■). No further medium changes were performed.

Note that the cells rapidly degenerated in the absence of serum, but that concentrations of FCS as low as 1% were able to support proliferation for up to 5 days.

d) Modulation of cellular PA release

Since hormones such as estrogens, progesterone, testosterone and dexamethasone are known or believed to be implicated in cellular growth and function, and since PA release is a modulatable function that is influenced by hormones and has circumstantial associations with transformation (for reviews see Roblin et al, 1975; Christman et al, 1977; Reich, 1978) it was of interest to examine the seven melanoma cell lines for their responsiveness to steroid hormones. For similar reasons, the effects of the tumour promoter TPA and the ubiquitous "second messenger", cAMP, were also studied.

Retinoic acid came into consideration in view of recent interest in this compound as an inducer of cellular differentiation (Strickland and Mahdavi, 1978; Strickland, 1979; Lotan and Lotan, 1980). Melanocyte stimulating hormone was chosen for study for obvious reasons.

The experiments that were performed to examine the effects of hormones or other compounds adhered fairly rigidly to the following protocol.

Cells were seeded at semi-confluence in full medium. The following day, the test compounds were added at the concentrations indicated in Table 2.5. The cultures were exposed to the compounds in the presence of serum for 48 hr, with one medium change after 24 hr. The cultures were then washed and 24 hr harvest fluids were prepared for PA assay in the presence of compounds. The results of the experiments are summarized in Table 2.5 where the value observed for PA release in each case is expressed as a percentage of the control value observed with cells that were not exposed to the compound.

Generally speaking, the melanoma cells proved resistant to the effects of estradiol, progesterone or testosterone. UCT-Mel 1 was stimulated to a modest extent by 10^{-6}M estradiol and by 10^{-6}M testosterone,

TABLE 2.5

MODULATION OF CELLULAR PA RELEASE[a]

LINE	Estrogen 10^{-6}M	Prog-esterone 10^{-6}M	Test-osterone 10^{-6}M	TPA 10ng/ml	RA 10^{-6}M	Dexa-methasone 10^{-6}M	dBcAMP 10^{-3}M	MSH $5x10^{-7}$M
UCT-Mel 1	172	100	168	27	164	143	135	62
UCT-Mel 2	100	100	100	100	29	143	174	100
UCT-Mel 3	100	100	100	100	100	100	183	140
UCT-Mel 4a	100	100	100	208	208	100	n.d.	n.d.
UCT-Mel 4b	100	100	100	157	264	100	n.d.	n.d.
UCT-Mel 5	100	100	100	0	290	100	21	68
UCT-Mel 6	100	100	100	100	208	34	n.d.	n.d.
UCT-Mel 7	100	100	100	100	143	78	n.d.	n.d.

(a) Results expressed as % Control.

(b) n.d. = not determined.

whereas none of the other lines were appreciably stimulated or suppressed by these two hormones. Neither UCT-Mel 1 nor any of the other lines were affected by 10^{-6}M progesterone.

Dexamethasone (10^{-6}M) stimulated PA release by 43% in the case of UCT-Mel 1 and 2. Enzyme release by UCT-Mel 6 and 7 was suppressed by this hormone, while the other lines were unaffected.

The effects of dBcAMP at 10^{-3}M on PA release by 4 lines were studied and in 3 cases (UCT-Mel 1, 2 and 3) protease release was stimulated by 35 - 83%. In one case (UCT-Mel 5) enzyme release was suppressed to 21% of the control value.

Melanocyte stimulating hormone at 5 x 10^{-7}M caused stimulation of PA release in the case of UCT-Mel 3; it was without effect in UCT-Mel 2, and suppressed enzyme release by approximately 35% in the case of UCT-Mel 1 and UCT-Mel 5.

The effects of TPA (10 ng/ml) and RA (10^{-6}M) proved, as was the case with the other compounds, to be variable. TPA suppressed enzyme release in two cases (UCT-Mel 1 and 5). It stimulated UCT-Mel 4 a and b and had no effect on the other lines.

Retinoic acid at 10^{-6}M was perhaps the most interesting compound in that only one line (UCT-Mel 3) proved refractory to stimulation or to suppression by this vitamin. Plasminogen activator release by UCT-Mel 2 was markedly suppressed by RA, whereas in all other cases the rate of enzyme release was increased by 43 - 200%.

During the course of these experiments I examined the cell cultures each day, and quite fortuitously, made the observation that certain of the compounds caused an apparent change in cellular morphology. These changes were most prominent when TPA was added to UCT-Mel 1 and 7 and when RA was added to UCT-Mel 2 (see Chapter 4).

Cell counts done at the end of the 72 hr experimental period

on control dishes and dishes to which compounds had been added revealed no significant or consistent effects that could be interpreted as indicating stimulation or inhibition of proliferation.

DISCUSSION

The culture of malignant melanomas was first described by Burrows (1914) in his report of a large series of tissue cultures which included two melanomas. This was followed by numerous reports of short-term studies of melanoma cells in tissue culture (Grand et al, 1935; Cobb and Walker, 1960; Wellings et al, 1960; Lane Brown et al, 1966; Rose 1963).

In this chapter I describe the establishment of eight human melanoma cell lines, two of which were sublines derived from the same metastatic deposit in one patient. These came from a total of 21 biopsies received representing a success rate in the establishment of cell lines of 33%. This success rate was 41% when only biopsies of secondary melanomas were considered. This compares well with the success rates of 11% (Gerner et al, 1975) 25% (Romsdahl and Hsu, 1972) and 39% (Giovanella et al, 1976) reported by other workers.

The major factor in failure to establish permanent cell lines was overgrowth by fibroblast cells. This occurred in all four of the samples of primary melanoma that I received. In the 7 biopsies from which neoplastic lines were established, tumour cell growth was sufficiently vigorous to enable me to select for these cells and for them to outgrow the fibroblasts which were present in the initial cultures.

In some cases (UCT-Mel 3 and 7) melanoma cell growth was so vigorous that many viable tumour cells could be collected from the supernatant fluid of primary cultures and used to create fibroblast-free

melanoma cell lines. Although derived from floating cells these cell lines, as found with the others, grew as monolayer cultures. This tendency for melanoma cells to adhere to the substratum was consistent with the experience of most others who have developed such cell lines, although there have been reports of melanoma cell lines which grew in suspension culture (Oettgen et al, 1968; Shimoyama et al, 1971).

With the exception of the UCT-Mel 5 cell line, all lines originated from enzyme-digested fragments of tumour tissue. Cobb and Walker (1960) also found a far more rapid growth rate of tumour cells after trypsinisation as opposed to the rate of outgrowth seen after implantation of a tumour fragment in a plasma clot. Trypsinisation may result in a cell population with a higher viability than that isolated by mechanical means, as found by Slocum et al (1981). Brief treatment with proteases may also stimulate cell proliferation (Quigley et al, 1979).

The greater ease with which cell lines could be established from metastatic deposits rather than from primary melanomas, probably relates to the selection at the time of metastasis *in vivo* of cells having a higher growth potential than most of the other cells in the primary tumour. It is of interest to note that all of the cell lines were derived from melanomas obtained from females, most of whom were Caucasian, and all of whom were post-menopausal. Unfortunately, I did not design this study in such a way that my results could be used to correlate the likelihood of success of *in vitro* establishment of a cell line with such clinical features as race, sex or age of the tumour donor, or with the aggressiveness with which the tumour grew in the patient. I am therefore unable to draw any valid conclusions from this observation.

Although the seven melanoma cell lines retained their individual characteristics faithfully through many *in vitro* passages, and despite the fact that the tumours from which they were derived were confidently diagnosed

as being melanomas, the cell lines differed considerably from one another in many respects. Thus two of them pigmented, while five failed to do so. Furthermore, they varied in morphology from epithelioid, through triangular, to dendritic and spindle-shaped. In agreement with Cobb and Walker (1960) and Romsdahl and Hsu (1967), who found that the dendritic form was seen in the most pigmented cultures, one of the pigmenting cell lines (UCT-Mel 2) was of dendritic morphology. The other pigmenting line,(UCT-Mel 1), had a triangular morphology, although large dendritic cells were occasionally seen, especially after treatment with tetradecanoylphorbol acetate (see Chapter 4).

The cell lines were all aneuploid, yet differed in their modal chromosome number, frequently showing a wide variation in the number of chromosomes per metaphase spread in any given cell line. I found modal chromosome numbers of between 61 and 81. These values are in fairly good agreement with the observations of other workers who have reported modal chromosome numbers in melanoma cells ranging from 43 to 78 (Romsdahl and Hsu, 1967, 1972), 58 (Tveit et al, 1980b) 45 (Chen and Shaw, 1973) 46-87 (Liao et al, 1975) 44-108 (Giovanella et al, 1976) and 68 (Nishihira et al, 1979). The near triploid number found in many melanomas is due mainly to hyperreplication of chiefly the C group chromosomes(Romsdahl and Hsu, 1972; Miles, 1967) and in some cases also of the A group chromosomes (Giovanella et al, 1976; Nishihira et al, 1979).

All of the melanoma cell lines released plasminogen activator, although at differing rates. Generally speaking, and within fairly wide limits, cell lines were consistent in the amount of PA they produced.

In view of the striking effects that low concentrations of hormones (Beers et al, 1975; Troll et al, 1975; Ossowski, 1979; Lacroix et al, 1979) anti-inflammatory steroids (Wigler et al, 1975; Rifkin and Pollack, 1976; Vassalli et al, 1976, 1977; Granelli-Piperno et al, 1977)

TPA (Wigler and Weinstein, 1976; Loskutoff and Edgington, 1977; Vassalli et al, 1977; Granelli-Piperno et al, 1977; Wilson and Reich, 1979) and RA (Wilson and Reich, 1978; Miskin et al, 1978) have upon release of PA by other cells cultured *in vitro*, it came as somewhat of a surprise to find that melanoma cells responded relatively modestly, if at all, to these compounds. If one considers the many clinical and epidemiological studies suggesting that the incidence or natural history of human melanomas is influenced by physiological situations such as puberty, pregnancy or sex (Pack and Scharnagel, 1951; Nathanson et al, 1967a; George et al, 1960; Byrd and McGanity, 1954; Allen, 1955; Sadoff et al, 1973; Lerner et al, 1979b; Shiu et al, 1976; Rampen and Mulder, 1980; Ellerbroek, 1968) it is perhaps disappointing that I was unable to find a more convincing effect of estradiol, progesterone or testosterone with the experimental protocol that I used.

The results obtained with dexamethasone were interesting in that at 10^{-6}M, this steroid had either no effect on PA synthesis by most of the melanoma cell lines or stimulated it to a slight degree. A notable exception was the case of UCT-Mel 6, where the anti-inflammatory steroid suppressed PA release by 66%. It was this cell line that released UK-type PA, whereas all of the other cell lines released the tissue-type enzyme. Roblin and Young (1980) have suggested that it is only the synthesis of PA's of the UK-type that is suppressible by dexamethasone, whereas other PA's are not. My results are consistent with this suggestion.

As far as the other compounds were concerned, their effects on PA synthesis were variable and, taken in isolation, rather uninteresting. UCT-Mel 1 and 2 showed features of differentiation (in that they pigmented and could be induced to undergo morphological changes) and tended to respond most convincingly to the addition of compounds by varying the

rate of PA release. I therefore felt it would be of value to abandon further study of *in vitro* cellular modulation of the other cell lines and to concentrate on these two. The experiments that I performed with this object in view are reported substantively in Chapter 4.

It is appropriate, at this point, to draw attention to the anomalous finding of urokinase-type PA release by the UCT-Mel 6 cells - anomalous since in all other melanoma cell lines that have been studied in this laboratory (Wilson et al, 1980) or by others, PA of the tissue-type has been released. UCT-Mel 6 was also unusual in that it failed to grow in soft agar or in nude mice. The clinical and histological diagnosis, however, appeared to be beyond doubt, and I have had to accept, on these grounds, that UCT-Mel 6 was a melanoma-derived cell line. Monoclonal antibodies such as those that have been reported as recognizing melanoma-specific determinants (Koprowski et al, 1978) may have been useful adjuncts to the definitive identification of these cells, had I had access to them.

The secretion of the urokinase-type PA by UCT-Mel 6 has added significance in view of the recent observations by Markus (personal communication) who found that melanomatous tissues taken directly from patients contained a mixture of urokinase and tissue-type enzymes with the former predominating. This may have been due to contamination of the biopsy of melanoma tissue with normal stromal or other cells that contributed the urokinase-type PA. On the other hand there may indeed be melanoma cells that do synthesize the urokinase-type enzyme but that proliferate at a disadvantage in tissue culture. My finding of a melanoma line that did secrete urokinase-type enzyme may be the exception that supports the more general rule suggested by the data of Markus.

The seven melanoma cell lines described also displayed a number of characteristics associated with malignancy or transformation. The saturation density of the lines was uniformly high (from three to eight

times higher in RPMI than the saturation density attained by normal adult skin fibroblasts). All grew to a higher saturation density than 1×10^5 cells per sq cm, which Dodson et al (1981) define as a low saturation density. High saturation density has a positive correlation with tumorigenicity (Stoker and Rubin, 1967; Holley and Kiernan, 1968; Aaronson and Todaro, 1968). The higher saturation density found in RPMI may have been due to the difference in pH in these cultures (Ceccarini and Eagle, 1971).

The plating efficiency measured by the ability of single cells to form colonies in liquid medium was relatively low, ranging from 0 to 12%. Other workers, too, have found a wide variability in plating efficiency, ranging from 0 to 86% (Liao et al, 1975; Giovanella et al, 1976). However, the ability of all cell lines to adhere to the surface of a tissue culture dish was high, with the lowest value found being 67% after 24 hr in the case of UCT-Mel 2.

Another characteristic property of transformed cells is the ability to grow in semi-solid media. Freedman and Shin (1974) showed that the ability to grow in methyl cellulose was the cellular characteristic which correlated best with *in vivo* tumorigenicity in nude mice. In addition, Carney et al (1980) found an excellent correlation between the ability of fresh tumour specimens to form colonies in agarose, and the presence in these specimens of histologically identifiable metastatic tumour. However, this correlation did not hold with my seven melanoma cell lines. Although the efficiency of colony formation in soft agar in these lines varied from 0 to 63%, there was no correlation between colony forming efficiency and rate of growth in nude mice (cf Chapter 6). The one cell line which did not form tumours in nude mice (UCT-Mel 6) also did not exhibit anchorage-independent growth, but the two sublines (UCT-Mel 4a

and 4b) which also did not form colonies in soft agar were nevertheless tumorigenic in nude mice.

A number of authors have found similar anomalies between growth in soft agar and growth in the nude mouse. Marshall et al (1977) reported one cell line which grew in nude mice but did not clone in soft agar, and Creasey et al (1979), in contrast, established three melanoma cell lines which grew in semisolid medium only one of which was tumorigenic in immunosuppressed mice. Hastings and Franks (1981) found that different chromosomal abnormalities were associated with *in vivo* tumorigenicity in nude mice and growth in soft agar. LT-2 cells, derived from an epithelial squamous cell carcinoma, readily form tumours in nude mice, although these cells are serum dependent, have a low saturation density, exhibit strict monolayer growth and cannot proliferate in soft agar, even after passage in the nude mouse (Dodson et al, 1981).

The phenotype of tumour formation *in vivo* and such correlates of tumorigenicity as anchorage-independent growth are therefore clearly separate. It is not clear what cell properties are actually being assayed by either the *in vivo* or the *in vitro* assay. Even though most tumours will clone in soft agar, the intertumour cloning efficiency may vary by a factor of 10 000 (Shin et al, 1975). It is therefore clear that, although determination of the properties of saturation density, plating efficiency and cloning in soft agar are important for the complete characterisation of a cell line in culture, these properties can rarely be used to predict the tumorigenic potential of the cells *in vivo*. The most reliable test of tumorigenicity therefore appears to be growth in the nude mouse.

The doubling times found for these 7 melanoma lines varied from 33 to 119 hr, which agrees well with the range of population doubling times found by workers who have tested a large number of human melanoma cell lines (Gerner et al, 1975; Liao et al, 1975; Giovanella et al, 1976).

In 1980, Ossowski and Reich reported a method for studying human carcinoma metastasis in the chick embryo. This model system closely parallels the events that must occur in *in vivo* metastasis i.e. it requires cells to detach from a growing tumour, be transported in the circulatory system and become established in a distant organ. Other advantages are rapidity, convenience, and the fact that the 10 day-chick embryo is unable to mount an immune response. I accordingly tested UCT-Mel 1 for growth and metastatic potential in the chick embryo. Tumour growth on the CAM was readily observed, but I was unable to detect metastases in the chick embryo lung, either by assaying for the presence of human PA or by re-inoculating fresh CAM with lung mince. Using this approach, Ossowski and Reich (1980a) found metastases with cell inoculi of as low as 1×10^4 cells, with a success rate of 25%; 5×10^4 or more cells always produced lung metastases and 5×10^3 cells and below were never observed to form metastases. Since up to 2×10^7 of UCT-Mel 1 cells were inoculated and failed to metastasize, the chick embryo system is clearly unsuitable for the study of metastasis of this melanoma line. It is not possible to state whether UCT-Mel 1 cells are of low metastatic potential or whether an unknown factor in the chick embryo system allows only certain tumours to metastasize.

In the two melanotic cell lines, pigmentation manifested itself as the cell cultures became confluent and proliferation slowed. Pigmentation was not seen in the other cell lines, even in extremely dense cultures.

The medium in which the pigmenting cells were grown proved to be an important factor since cells grown in DB showed a greater degree of melanization than did the same cells in RPMI. It therefore appears that melanization depends on the one hand, on the innate ability of the cells to synthesize the pigment. This is a genetic trait (Jimbow et al, 1976) and determined by the presence, within the cell, of functional tyrosinase and the wherewithal for synthesis of melanosomes. On the other hand,

in a cell with the correct genetic constitution, the cellular environment should be conducive to pigment synthesis. The "cellular environment" embraces a large number of factors, including the presence of excess substrate, absence of inhibitors of tyrosinase, pH, presence of sugars, cell contact, serum deprivation and rate of proliferation or degree of metabolic activity in the cells.

Although the exact mechanism of action of most of these factors is unknown, their influence on pigmentation in tissue culture has been well documented. The medium effect that I observed may well be attributable to the fact that DB contains far higher concentrations of tyrosine and phenylalanine than does RPMI. Calcium, which has been implicated in differentiation in many systems (Levenson and Housman, 1981) is also present at a higher concentration in DB than in RPMI.

In addition, RPMI contains glutathione, whereas DB contains none. This sulfhydryl reagent is known to be an inhibitor of tyrosinase (Lerner et al, 1950). Finally, glucose is thought to function as an inhibitor of tyrosinase (Nakayasu et al, 1977). This sugar is present in RPMI at concentrations twice those found in DB.

The inverse relationship between rate of cell proliferation and pigmentation has been reported in mouse melanoma systems (Silagi, 1969; Kitano and Hu, 1970; Hu, 1972) and in human melanoma cultures (Cobb and Walker, 1960; Romadahl and Hsu, 1972; Giovanella et al, 1976). Similarly, increased cell contact resulting from cell crowding (Kitano and Hu, 1970) or cell aggregation (Cobb and Walker, 1960) resulted in greater pigmentation. The relative importance of these two factors is difficult to ascertain, as the attainment of a high cell density is usually associated with a reduction in the rate of cell proliferation, even in cells which are not contact inhibited. Hu (1972) found that co-cultivation of mouse melanoma cells with heterogeneous cells such as monkey retinal cells or fibroblasts,

appeared to result in more rapid pigmentation in the mouse cells. The phenomenon of differentiation being associated with cell confluence or crowding has been noted in other systems as well, for example the differentiation of murine mammary cells to adipose cells (Hiragun et al, 1980) and the differentiation of rat transitional epithelial cells (Chlapowski and Haynes, 1979).

Since melanin is a distinctive product of the mature melanocyte, one may regard melanization as a manifestation of the "differentiated" phenotype. Furthermore, although seldom explicitly stated, it is generally accepted that, in order to differentiate, cells are required to arrest in G_1, to cease proliferating, and to commit themselves to the terminal function which they are genetically destined to complete.

The observations I have made showing increased pigmentation with confluence and a diminished rate of proliferation would be consistent with this concept, and one might be tempted to assume that pigmentation and proliferation were mutually exclusive cellular functions. The situation, however, is more complex than that. Exponentially growing cells are able to pigment, as shown by Giovanella et al (1976) and myself (Fig. 2.3) and pigmented cells are quite clearly capable of dividing or re-entering a phase of active division (Kitano and Hu, 1970).

The definition of differentiation is therefore not entirely straightforward. In the extreme case, as outlined above and as represented by the HO melanoma cell line (Huberman et al, 1979) and the C_3 clone of B-16 melanoma (Mufson et al, 1979) differentiation is a terminal cellular event that precludes further division. In the more usual case, however, it is probably best considered as an inducible state that is represented by a *relative* commitment to synthesis of unique cell products and a correspondingly *relative* decrease in the rate of cellular proliferation. I expand on these concepts in Chapter 4.

In the pigmenting melanoma cell lines that I have established, the transition from a deeply pigmented population at confluence to a rapidly dividing, depigmented population after trypsinization and reseeding was a striking one. The cells therefore offer a useful opportunity for documenting the relationships between melanization and proliferation, and studying the factors that control these two cellular functions. I hope to pursue these studies at some later stage.

CHAPTER 3

PLASMINOGEN ACTIVATORS SYNTHESIZED BY MELANOMA CELLS CULTURED *IN VITRO*

Largely due to the initiative and the observations of workers in Dr. Reich's laboratory in the Rockefeller University, New York (Vassalli et al, 1976; Granelli-Piperno et al, 1977; Unkeless et al, 1973, 1974; Ossowski et al, 1973a, b; Rifkin et al, 1974; Wilson and Reich, 1978) interest in serine proteases that function as plasminogen activators was stimulated by suggestions that this group of enzymes provided regulated local proteolysis by which various physiological and pathological processes were mediated. Thus ovulation (Beers et al, 1975), post-lactational mammary involution (Ossowski et al, 1979), trophoblast implantation (Strickland et al, 1976), pro-hormone to hormone conversion (Virji et al, 1980), and tumour spread by proteolytic digestion of barriers to local invasion (Ossowski et al, 1975; Ossowski and Reich, 1980b) are some of the many functions that have been ascribed to these enzymes. This matter has recently been the subject of a number of comprehensive and informative reviews (Reich, 1978; Christman et al, 1977).

My own interest in plasminogen activators came from the fact that their cellular synthesis and release are modulatable functions under the control of genetic events in the cell. The ease with which the enzymes can be measured; the exquisite assay sensitivity that is provided by the amplification of proteolytic activity by plasminogen; and the inducible nature of plasminogen activator release, have combined to provide an extremely useful experimental system for the study of biological compounds and their effects upon human cells. Without the need to ascribe any teleological function to the induction or suppression of proteolytic activity one is able to use this system to document and quantitate the

effects of such substances as hormones, vitamins, drugs, growth factors or other chemical compounds upon cultured cells. Furthermore, one has the certain knowledge that these effects are exerted by genetic mechanisms that require transcription and translation of mRNA.

In the second instance, my interest in these enzymes was sustained by a fairly early observation that human cells cultured *in vitro* release two immunochemically distinct forms of the enzyme, typified by urokinase- and tissue-type plasminogen activator. In an extensive survey of various cell types in which I participated (Wilson et al, 1980) we were able to show that melanoma cells, unlike cells derived from most other tissues, were consistent in their secretion of the tissue-type enzyme. This seemingly close association between melanoma cells and the type of enzyme they released suggested interesting biochemical correlations with cellular differentiation and function that warranted further study.

Fianlly, numerous reports have appeared in the literature to associate release of plasminogen activator with the expression of the transformed or neoplastic phenotype. These observations had obvious implications for the study of cells as highly malignant as metastatic melanoma cells.

For these three sets of reasons, I embarked on a fairly extensive series of experiments designed to characterize the molecular species of plasminogen activators released by melanoma cells cultured *in vitro* and the processes that regulated this release. In this chapter, I report the results of the first of these sets of experiments.

MATERIALS AND METHODS

For the sake of convenience and brevity, I have described most of the analytic and preparative methods in detail in the Appendix. It is, however, appropriate to give a brief account of these procedures at this point.

Preparation of Samples for Plasminogen Activator Assay.

Adherent cell monolayers were washed several times with pre-warmed, serum-free medium to remove serum and serum inhibitors of plasmin or plasminogen activator. The monolayers were then covered with a measured volume of serum-free medium and returned to the incubator for several hours. At the end of this time, the "conditioned" medium was collected as a "harvest fluid" and aliquots of this were assayed for PA content. The results obtained were used to calculate cellular secretion of PA. Before assay, the harvest fluids were clarified by centrifugation and adjusted to contain 0.4 mg/ml of PIF-BSA to stabilise the enzyme. After removal of the harvest fluids, the cells were trypsinized and counted to enable me to express the results as rate of enzyme secretion in units/ 10^6 cells/24 hr. Samples could be stored frozen at $-20^{\circ}C$ for several weeks without appreciable loss of activity.

Intracellular PA content was measured on cell lysates prepared as follows:

Adherent cells on 60 mm dishes were washed 3 times with 3 ml of ice-cold PBS. Two ml of PBS was added to the monolayer which was removed with a teflon policeman and transferred to a plastic centrifuge tube. The culture dishes were then rinsed with 2 ml of ice-cold PBS which was added to the original suspension. The cells were centrifuged at 350 g for 5 min at $4^{\circ}C$. The cell pellet was washed once with PBS, drained and lysed

by the addition of 100µl of 0.5% Triton X-100 in water per 10^6 cells. The lysate was incubated on ice for 1 hr, then stored at -20°C.

Plasminogen Activator Assay.

Plasminogen activator activity in harvest fluid or cell lysate samples was measured using a radioenzymatic assay described in detail in Appendix A.13. Briefly, this involved measuring, as a function of time, the plasminogen-dependent release of radioactive fibrin degradation peptides from solid phase ^{125}I-fibrin coated onto the surface of plastic tissue cultured wells. Standard urokinase samples assayed at the same time enabled me to express the results in terms of Ploug units (or simply "units") of enzyme activity.

Immunochemical Identification of PA type.

Plasminogen activators present in harvest fluids or cell lysates were identified as UK- or tissue-type enzyme by measuring residual enzyme activity after incubation with specific inhibiting antibody to each PA species.

Rabbit IgG antibody against human UK or tissue activator was prepared and purified as described in Appendix A.14.1 and A.14.2. Serial two-fold dilutions of these antibodies were prepared in PBS and 50 µl of each dilution was incubated with an equal volume of harvest fluid for 1 hr at 4°C. At the end of that time, PA activity of 20 µl of the mixture was measured in the ^{125}I-fibrin assay. Before addition of antibody, the harvest fluid was diluted so that in the final assay system, 10 µl would release approximately 50% of the insoluble radioactive fibrin in 2 hr.

Electrophoretic Identification of PA type.

Plasminogen activators present in harvest fluids and cell lysates were analysed by electrophoresis in 11% polyacrylamide gel slabs containing SDS. Detailed procedures are given in Appendix A.14.4. After electrophoresis, bands of enzyme activity were detected zymographically as plasminogen-dependent zones of fibrinolysis in fibrin-plasminogen-agar indicator underlays and their apparent molecular weights calculated by reference to co-electrophoresed molecular weight marker proteins.

Combined electrophoretic and immunochemical procedures were used to identify individual electrophoresed enzyme bands as follows. Mixtures to be analysed were electrophoresed in adjacent tracks on a slab gel. After electrophoresis the polyacrylamide slabs were washed in nonionic detergent (2.5% Triton X-100 in water) to remove the SDS and layered on fibrin-plasminogen-agar underlays in such a manner that one of the tracks lay parallel and in close proximity to a trough in the underlay to which specific antibody had been added several hours before the polyacrylamide gel was applied. In this way electrophoresed bands adjacent to the trough were exposed to a relatively high inhibitory concentration of diffused antibody. Enzyme bands one track distant from the trough diffused into antibody-free indicator gel.

RESULTS

Plasminogen-dependence of Cellular Fibrinolysin.

Whenever fibrinolytic activity was observed in harvest fluids or cell lysates, whether this was by the ^{125}I-fibrin assay or by the fibrin-plasminogen-agar underlay procedure, this was invariably plasminogen-dependent (Figs. 3.1 and 3.2). The enzymes that I consider in this chapter therefore, may legitimately be regarded as activators of plasminogen.

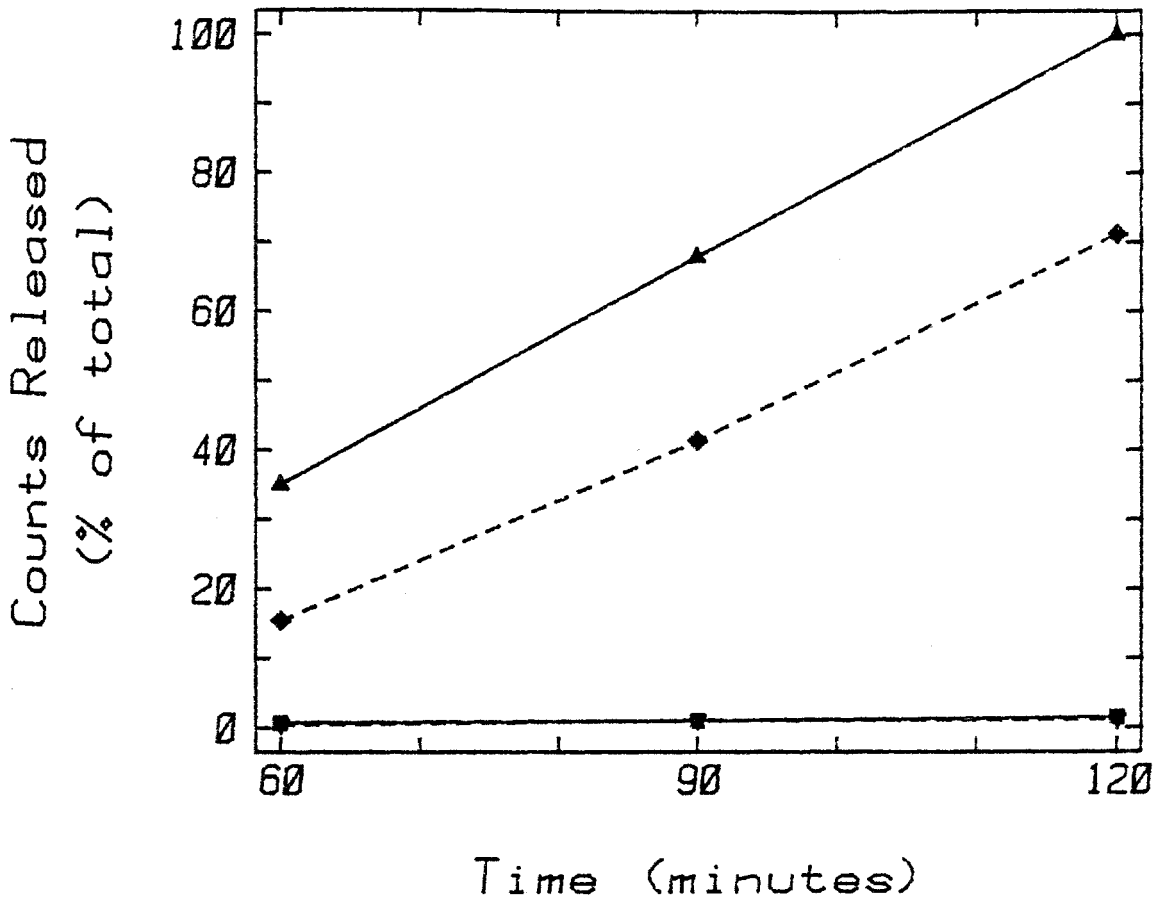


FIGURE 3.1

Plasminogen-dependence of ^{125}I-fibrin release.

UCT-Mel 1 harvest fluid and cell lysate were tested for PA activity in the ^{125}I-fibrin assay (Appendix A.13), in the presence or absence of added plasminogen. Note that in the presence of plasminogen both the melanoma harvest fluid (▲——▲) and cell lysate (◆--◆) released ^{125}I-fibrin. In the absence of plasminogen, neither the harvest fluid (■——■) nor the cell lysate (▼--▼) released more than 2% of the total radioactivity from the surface of the well. Similar results were obtained with the other melanoma cell lines.

FIGURE 3.2

FIGURE 3.2

Plasminogen-dependence of fibrin-plasminogen-agar lysis.

Harvest fluid of UCT-Mel 1, and cell lysates of UCT-Mel 1 and 7 were electrophoresed as described (Appendix A.14.4) and layered on fibrin-agar indicator gels containing plasminogen-free fibrinogen (Appendix A.20) together with added plasminogen (Tracks 1, 2 and 3) or with Tris-HCl buffer in place of plasminogen (Tracks 4, 5 and 6). As can be seen, UCT-Mel 1 cell lysate (Track 1) or harvest fluid (Track 2) and UCT-Mel 7 cell lysate (Track 3) produced lysis in the presence of plasminogen. No lysis was apparent in Tracks 4, 5 and 6. Fibrinolysis in all other melanoma harvest fluids and cell lysates was similarly plasminogen-dependent.

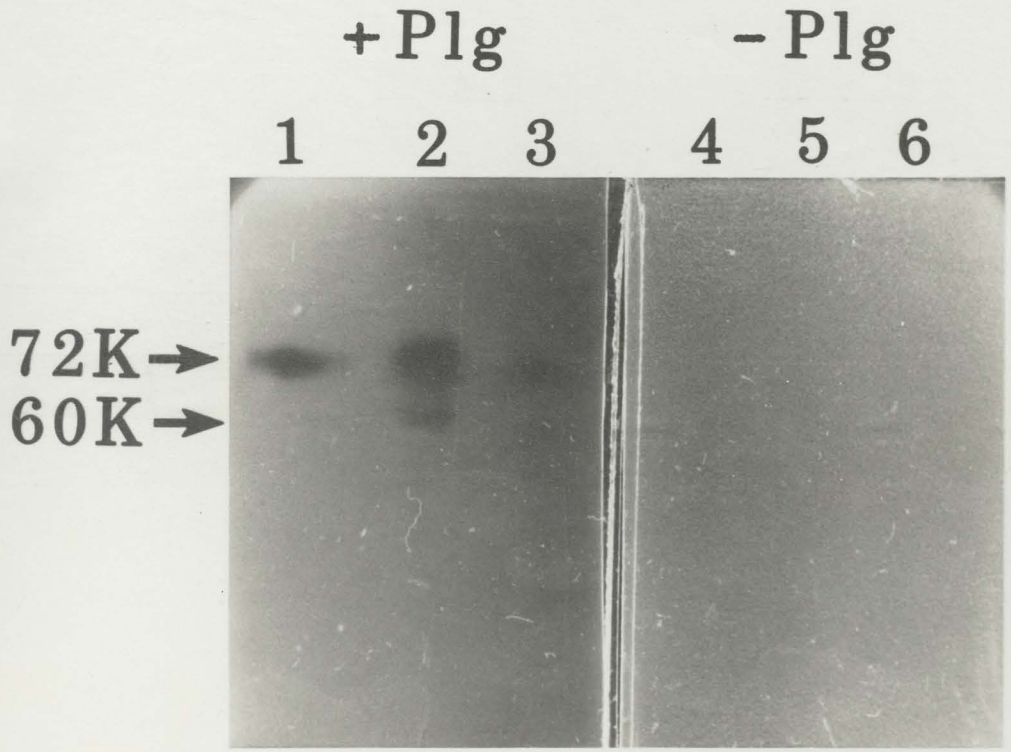


FIGURE 3.2

Kinetics of PA Release by Melanoma Cells.

All of the melanoma cells tested released PA's into the serum-free harvest fluid at rates that varied rather widely between melanoma cell lines. When rates of PA release were measured on different occasions with any given cell line some variation was also observed but this was less marked; each cell line tended to be relatively consistent in the amount of PA it produced. The results summarized in Table 3.1, illustrate this fact. UCT-Mel 2 secreted the least enzyme (4.3 u/10^6 cells/24 hr) whereas UCT-Mel 6 secreted enzyme at the greatest rate (67.4 u/10^6 cells/24 hr).

The rate of enzyme release was linear with time up to 24 hr (Fig. 3.3). In lines such as UCT-Mel 1, the amount of enzyme released over a 2 hr period could be quantitated with reasonable accuracy.

Having established that enzyme release was linear with time, it was necessary to examine for linearity with cell number, since cellular enzyme secretion was to be expressed in terms of units released per 24 hr per 10^6 cells. For comparative purposes, this form of expression implied linearity with time and cell number.

Harvest fluids were therefore collected from 60 mm dishes containing different numbers of UCT-Mel 1, 2 and 3 cells and PA release was measured. The graphs shown in Figs. 3.4 and 3.5 summarize the results obtained.

The amount of PA released by UCT-Mel 1 and 3 was not directly proportional to the number of cells on the dish and a plot of cellular enzyme release as a function of cell density showed that the secretion of PA by UCT-Mel 1 and 3 cells was inhibited by increased cell density.

A similar, but far less pronounced, density-dependent inhibition of PA release was observed in the case of UCT-Mel 2.

In the initial experiment in which I demonstrated the density-

TABLE 3.1

RELATIVE AMOUNTS OF PA SYNTHESIZED AND RELEASED BY MELANOMA CELL LINES

Cell Line	Plasminogen activator[a]	
	Intracellular (u/mg protein)	Extracellular (u/10^6 cells/24 hr)
UCT-Mel 1	13.1 ± 0.9 (98)	18.6 ± 1.8 (62)
UCT-Mel 2	3.5 ± 1.2 (4)	4.3 ± 0.4 (61)
UCT-Mel 3	2.5 ± 1.5 (4)	9.1 ± 1.1 (43)
UCT-Mel 4a	9.9 ± 4.9 (4)	30.7 ± 4.5 (16)
UCT-Mel 4b	8.7 ± 2.0 (4)	50.3 ± 11.4 (9)
UCT-Mel 5	8.4 ± 3.3 (4)	6.8 ± 2.2 (16)
UCT-Mel 6	122 ± 86 (4)	67.4 ± 9.2 (9)
UCT-Mel 7	96.8 ± 51.0 (4)	8.9 ± 2.4 (25)

(a) Results are presented as the mean ± standard error of the mean. Figures in parentheses represent the number of values from which the mean was obtained.

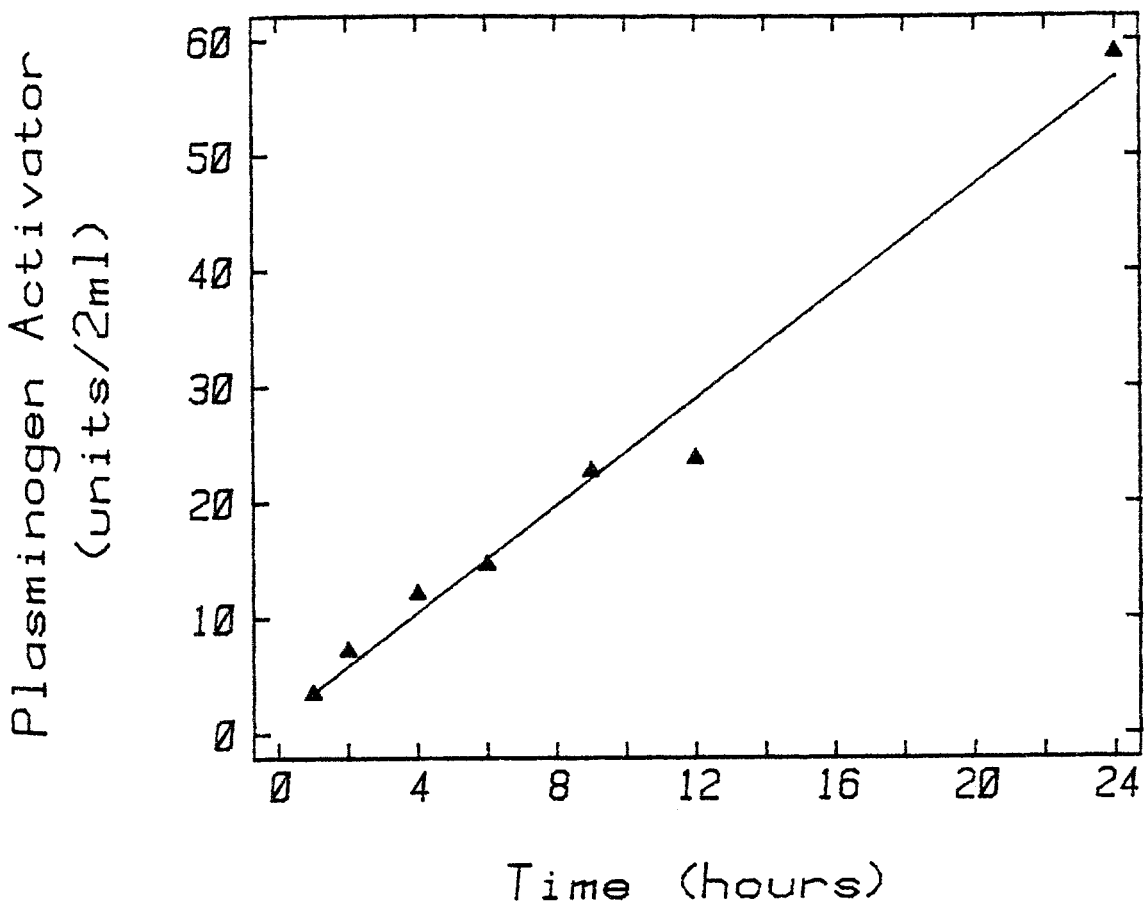


FIGURE 3.3

Release of PA was linear with time.

UCT-Mel 1 cells were seeded at 3 x 10^5/60 mm dish. Forty eight hr later, the cells were prepared for harvest fluids (see Methods) and the release of PA into the harvest fluid was monitored as a function of time. Results presented represent the mean of duplicate samples. Note that PA release was linear over 24 hr.

FIGURE 3.4

FIGURE 3.4

Density-dependence of PA release by UCT-Mel 1.

UCT-Mel 1 cells were seeded at 1.5 x 10^5/60 mm dish. Fresh medium (DB-FC10) was added daily for 10 days. At intervals between day 1 and day 10, 24 hr harvest fluids were collected from replicate cultures (a) and cell lysates were prepared from companion cultures (b), and assayed in the ^{125}I-fibrin assay. Plasminogen activator activity was calculated as total PA released by the given number of cells (u/dish/24 hr) or (u/dish) and as activity adjusted for the cell number (u/10^6 cells/24 hr) or protein concentration (u/mg protein). Note that in the case of released PA (a) the total PA activity (■—■) rose with increasing cell number, while the PA activity per 10^6 cells (▲—▲) dropped sharply over the same range of cell concentration. In the case of intracellular PA (b), a similar increase in total PA was observed with increasing protein concentration (■—■). The amount of PA per mg protein, however, remained constant (▲—▲). Each point represents the mean of duplication determinations.

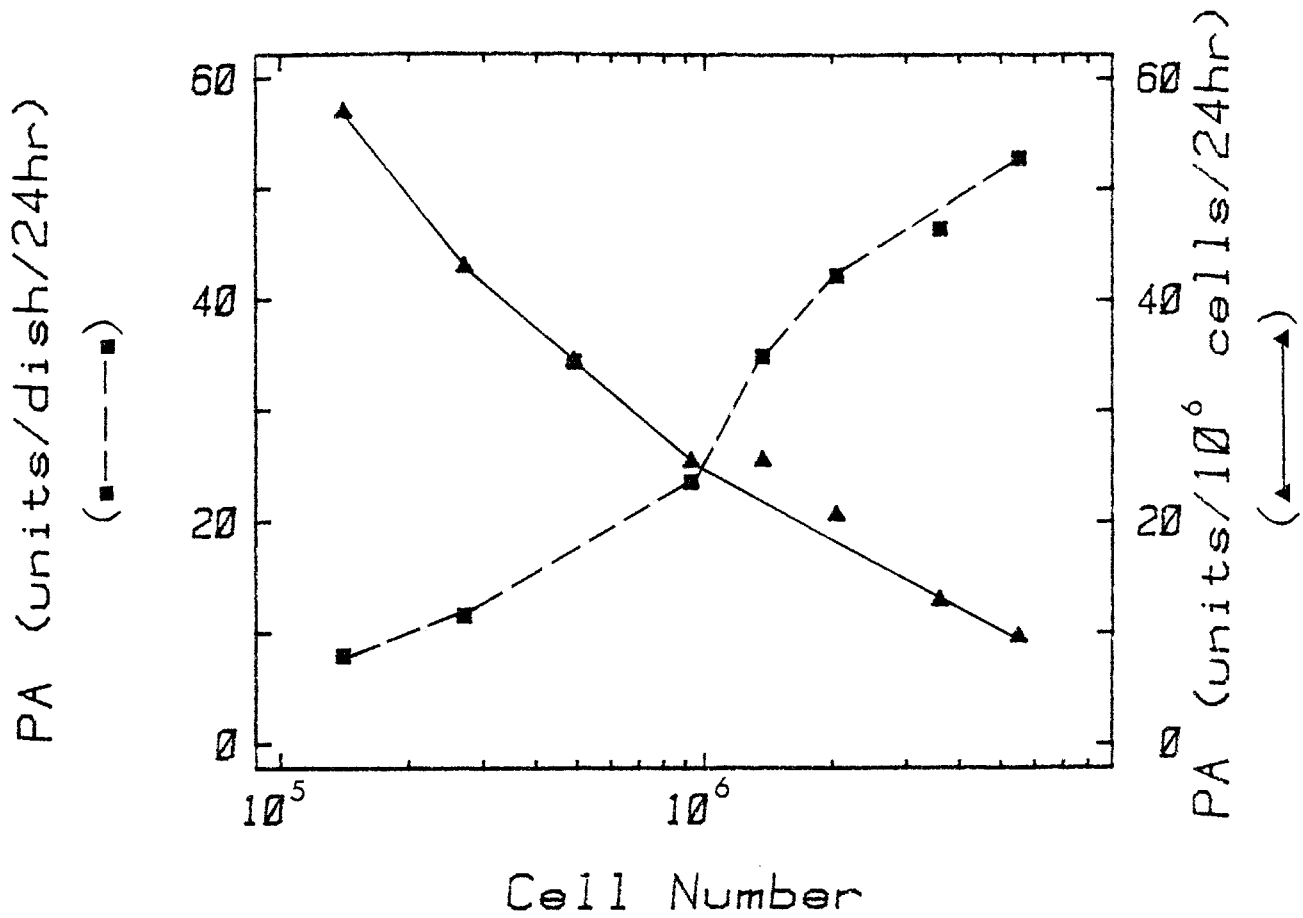


FIGURE 3.4a

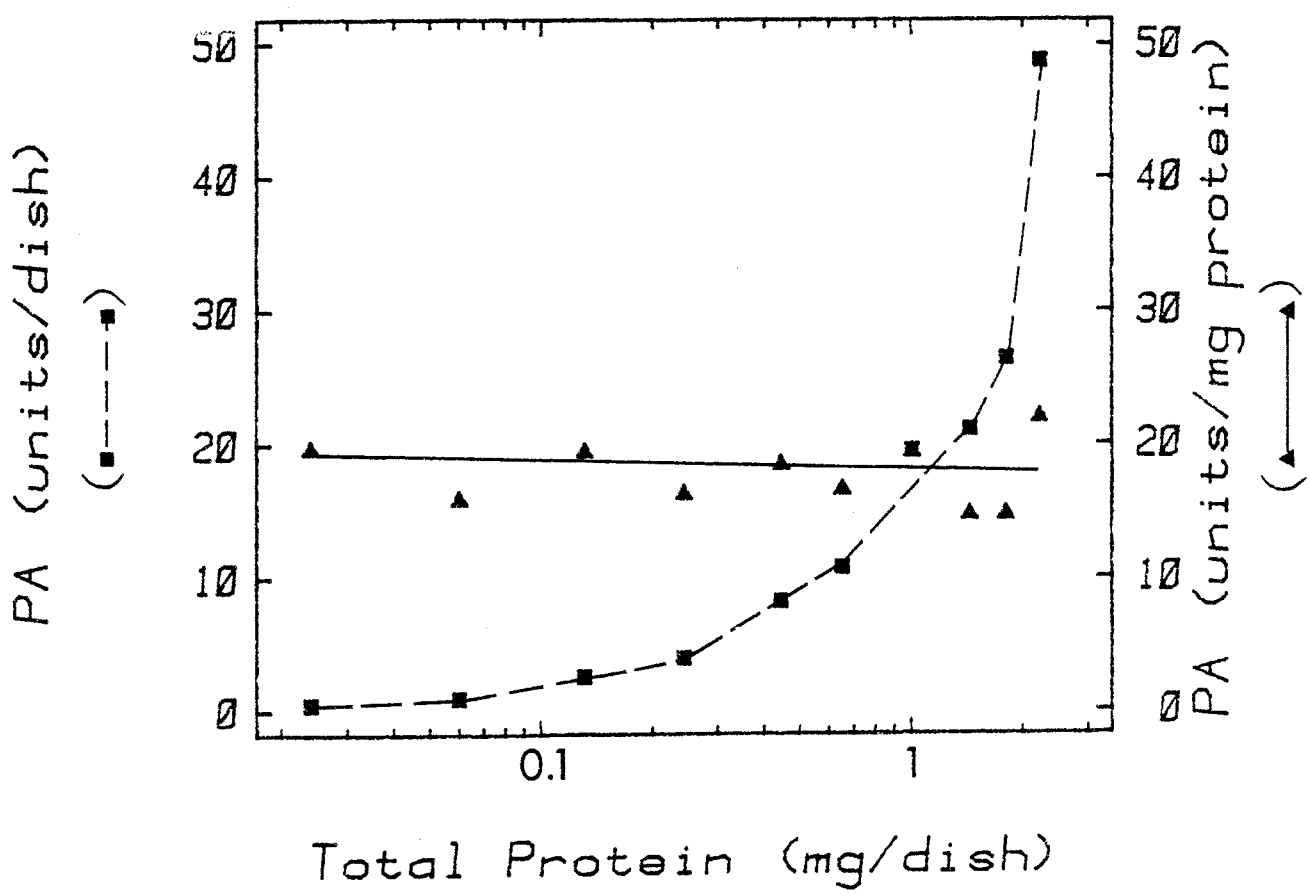


FIGURE 3.4b

FIGURE 3.5

FIGURE 3.5

Density-dependence of UCT-Mel 2 and 3.

The above graphs, (a) and (b) combine data from a large number of PA assays on UCT-Mel 2 and 3 cells at various cell densities. Note that in the case of UCT-Mel 2 (a) the total PA activity per dish (■—■) rose with increasing cell number, while the PA released per 10^6 cells (▲—▲) remained relatively constant, dropping slightly only at very high cell concentrations. In the case of UCT-Mel 3(b), the total PA released was similar at low and high cell densities (■—■) due presumably to the marked decrease in PA per 10^6 cells with increasing cell density (▲—▲).

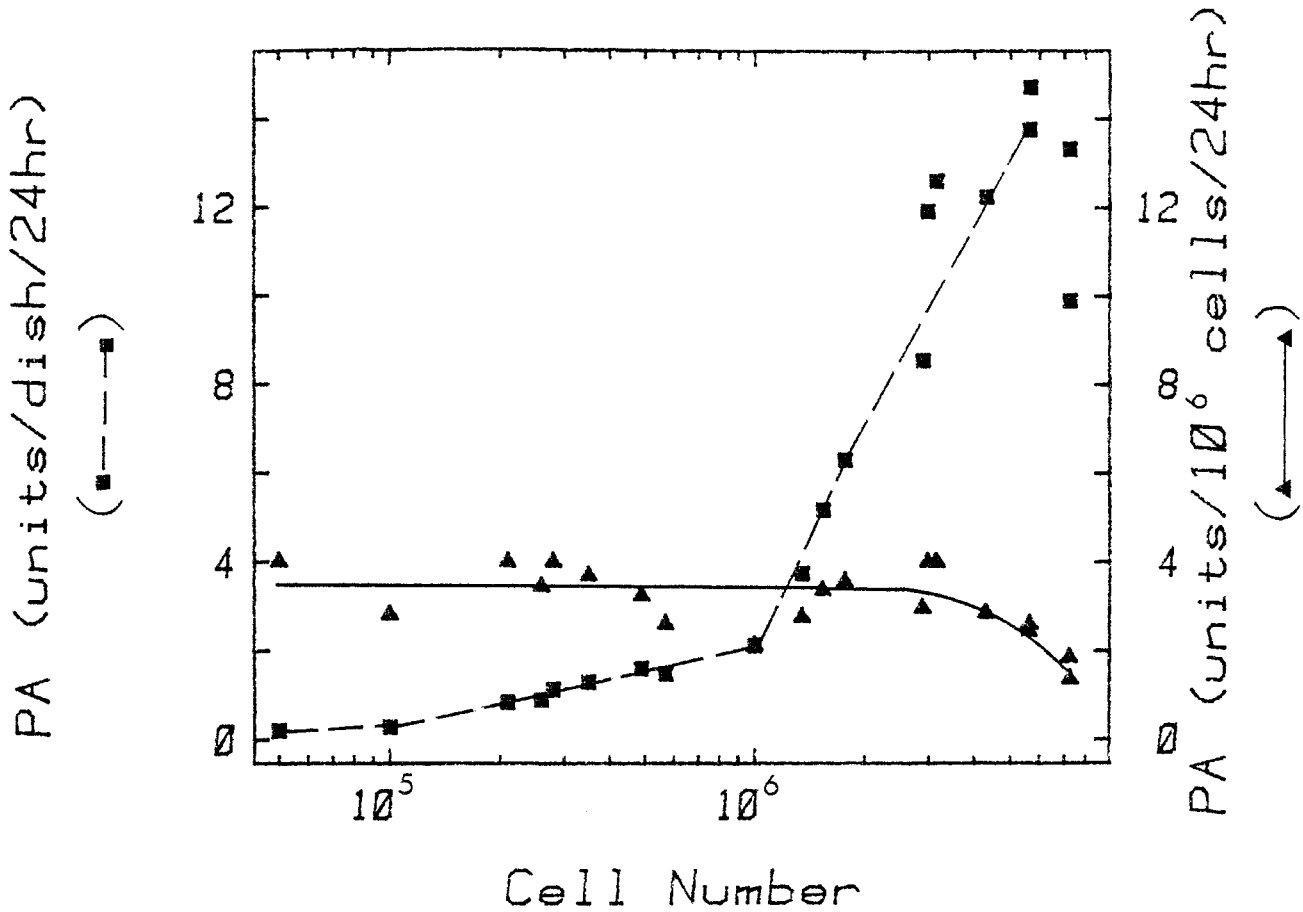


FIGURE 3.5a

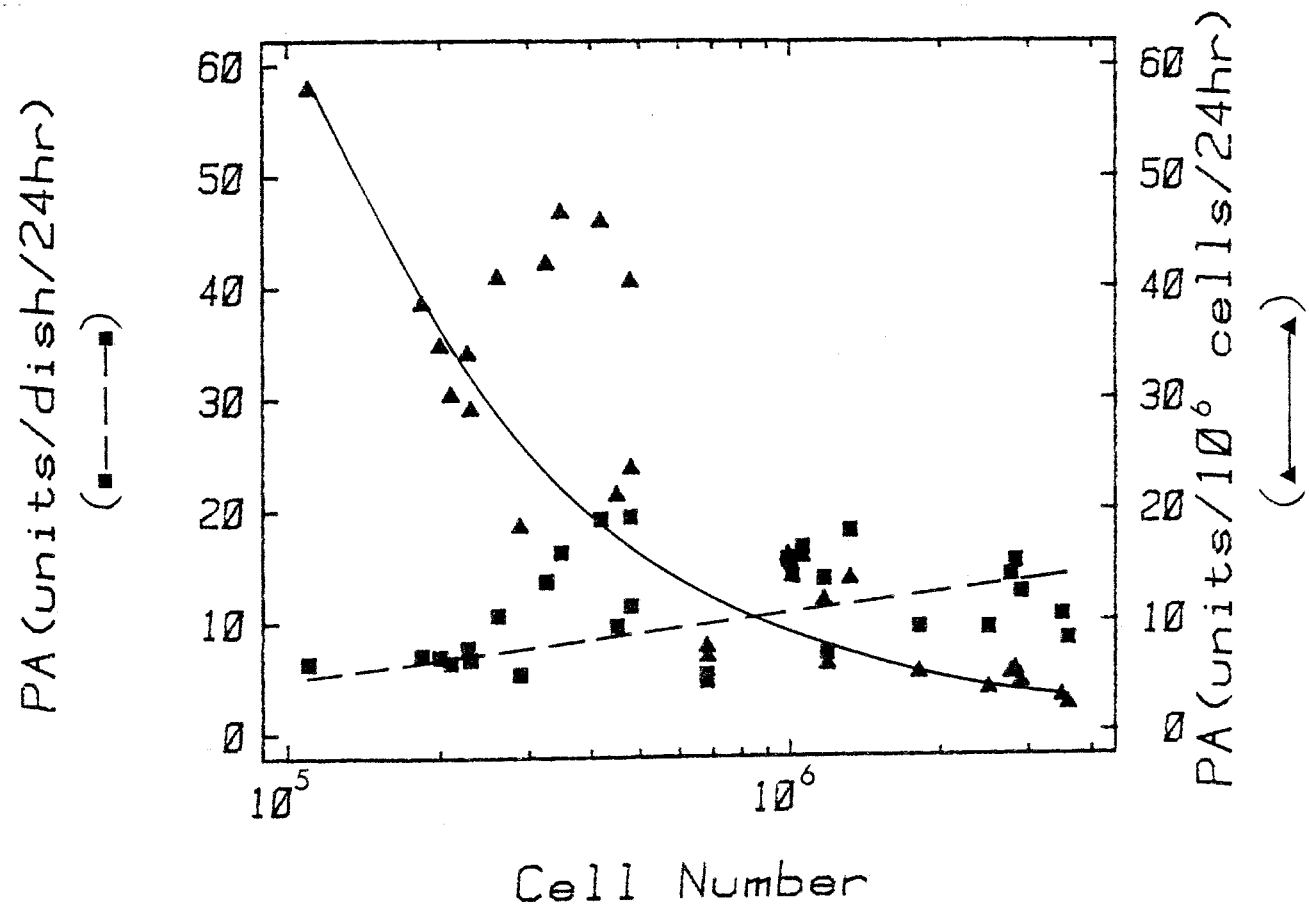


FIGURE 3.5b

dependent inhibition of PA release with UCT-Mel 1, adherent monolayers at varying densities were obtained by seeding cultures at the same density and taking harvest fluids after varying times in culture. It was conceivable, therefore, that the inhibition of PA release may have been due to aging in culture rather than cell density. This possibility was excluded by the experiment summarized in Fig. 3.6 in which UCT-Mel 1 cells were seeded simultaneously at densities varying from 4×10^5 to 32×10^5 and cellular PA release was then monitored in these cultures as a function of time. The results illustrate the fact that inhibition of enzyme release was related to cell density and not to time in culture.

Reversibility of density-dependent inhibition of PA release by UCT-Mel 1 cells could be shown by dispersing confluent, inhibited cultures and demonstrating a return to high rates of cellular enzyme release when the cells were reseeded at low density. This was noted whether adherent monolayers were dispersed with trypsin or EDTA.

Since it seemed possible that the fall in the cellular rate of PA release with increasing cell density might reflect a regulatory process in which the final proteolytic product (i.e. plasmin) exerted negative feedback repression on activator synthesis, an experiment was performed in which the effects of cell density were studied in the absence of plasminogen. Omission of plasminogen from the growth medium did not influence the density-dependent effect.

To examine for the possible presence of an inhibitor for plasmin or PA in harvest fluids collected from confluent cultures, mixing experiments were performed in which such harvest fluids were added to harvest fluids collected from sparse cultures. No such inhibitors could be found to explain the density-dependent effect.

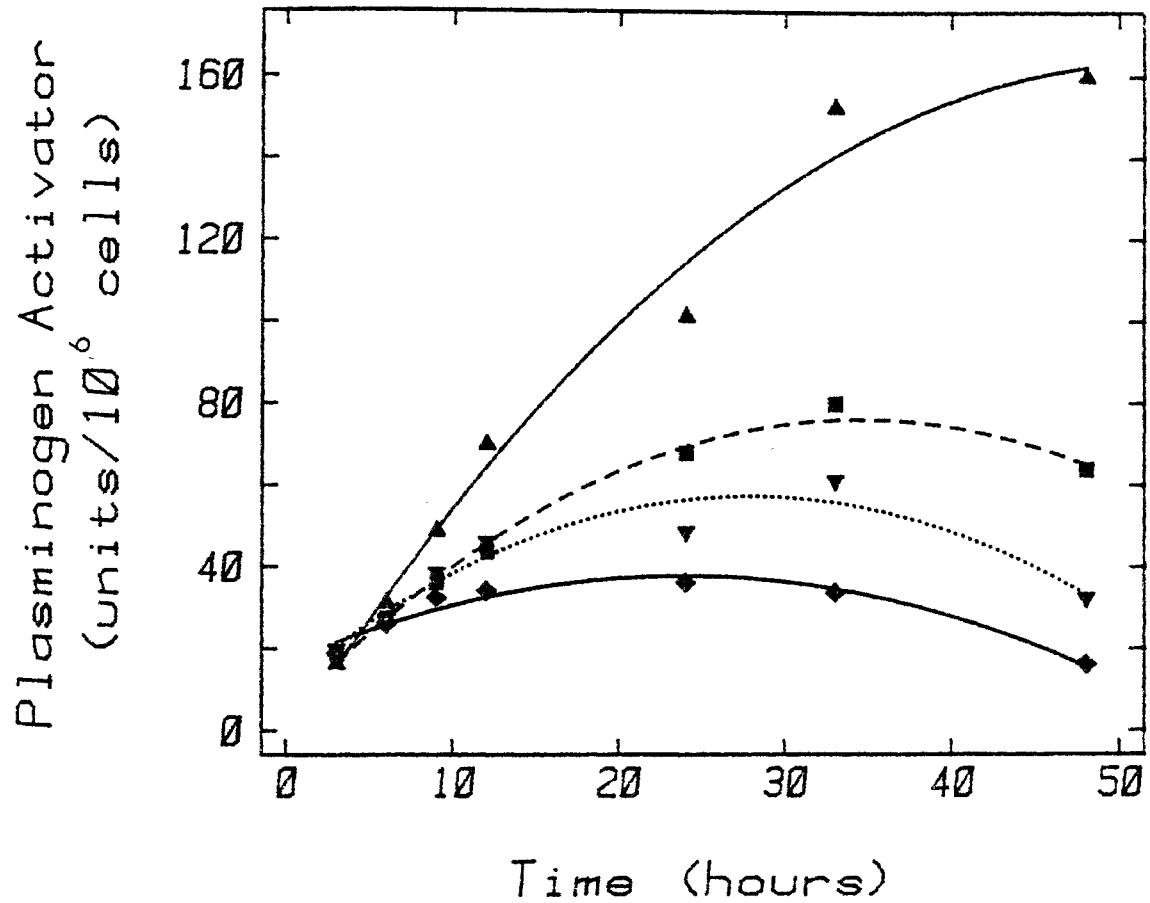


FIGURE 3.6

Decreased PA release as a function of cell density.

UCT-Mel 1 cells were seeded at 4 x 10^5, 13 x 10^5, 20 x 10^5 and 32 x 10^5/60 mm dish and prepared for harvest fluids 24 hr later. Plasminogen activator released into this harvest fluid was monitored by removing aliquots of medium at the indicated times for assay. Results of duplicate samples were averaged and corrected firstly for cell number and secondly to represent the total PA released into the harvest fluid at the indicated time. Note that 4 x 10^5 cells (▲——▲) released large amounts of PA when compared with 13 x 10^5 (■- - -■), 20 x 10^5 (▼·····▼) or 32 x 10^5 (◆——◆) which released progressively less PA. The downward slope seen with higher cell numbers after 24 or 33 hr is presumably due to the PA in the harvest fluid being degraded at a more rapid rate than it was released.

Intracellular PA Content.

PA could be detected in the cell lysates of all melanoma cell lines studied. Concentrations expressed in terms of units of PA per mg of cell protein, varied widely, from 2.5 u/mg in the case of UCT-Mel 3 to 122 u/mg in the case of UCT-Mel 6 (Table 3.1).

Although in the case of UCT-Mel 2 and 6, there appeared to be a correlation between the rate of PA release and intracellular PA content, this correlation did not hold good for the other cell lines.

Increasing cell density had a marked inhibitory effect on the PA release by UCT-Mel 1 but no such effect on intracellular PA content (Fig. 3.4).

Molecular Species of PA's Synthesized by Melanoma Cells in Tissue Culture.

The molecular forms of PA's found in the harvest fluids and cell lysates obtained from melanoma cells were identified by antibody titration, electrophoresis and zymography and a combination of electrophoresis and antibody inhibition. Representative results of these procedures are presented in Fig. 3.7 to 3.10.

The results obtained when these procedures were applied to all of the melanoma lines are summarized in Table 3.2.

The data in Table 3.2 include results obtained from 3 other melanoma cell lines that were imported into the laboratory.

The results are noteworthy in the following respects:

(i) Immunochemical analyses, whether applied as titrations or to electrophoretically separated enzymes, invariably showed that cells released or contained one type of enzyme only (UK- or tissue-type) and never a mixture of the two.

(ii) For any given cell type the enzyme that was released was of the same type as the enzyme that was found in the cell lysate.

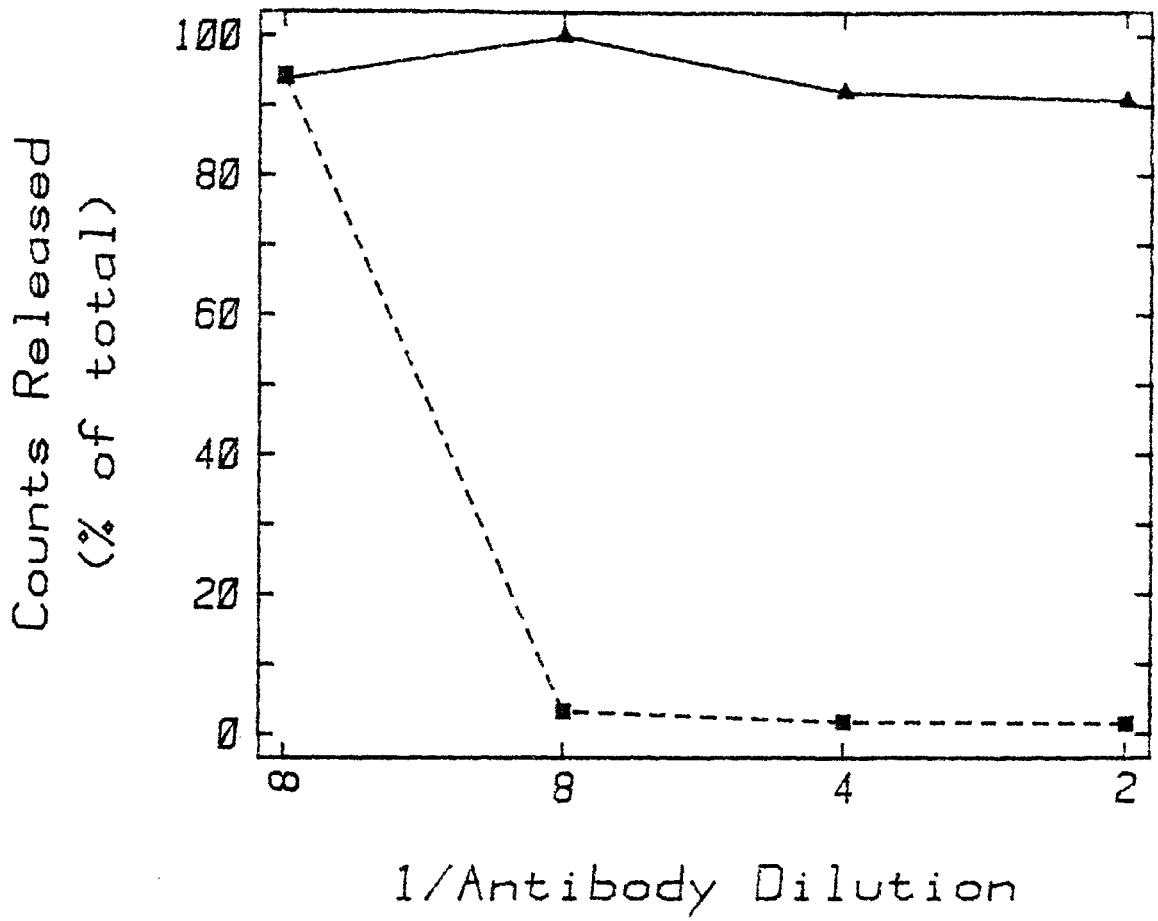


FIGURE 3.7

Inhibition of melanoma PA by specific antibody in the ^{125}I-fibrin assay.

UCT-Mel 5 harvest fluid was incubated with serial 2-fold dilutions of anti-UK IgG (▲—▲) and anti-melanoma PA IgG (■--■) as described in Appendix A.14.3 and assayed for residual fibrinolytic activity. Results are expressed as percentage of total counts released. Note that anti-UK IgG has no inhibitory effect on melanoma PA, while anti-melanoma PA IgG inhibits activity by over 95% at an antibody dilution of 1/8.

FIGURE 3.8

FIGURE 3.8

Fibrin-plasminogen-agar zymograms of melanoma plasminogen activators.

This figure depicts zymograms of secreted and intracellular PA in the 7 melanoma lines reported in this thesis. In each instance, (a) and (b) represent PA found in cellular harvest fluids, and (c) and (d) represent PA found in cell lysates. Early stages in the development of lysis bands are illustrated in (a) and (c), and later stages in (b) and (d). The zymograms do not allow for quantitative comparison of the amounts of PA synthesized by the different melanoma cell lines as photographs were taken at times designed to illustrate the molecular species of the lysis bands. This figure demonstrates the relative quantities of the PA species synthesized by each melanoma line, and should be considered in conjunction with Table 3.2, in which the totality of my results with the melanoma PA's is considered.

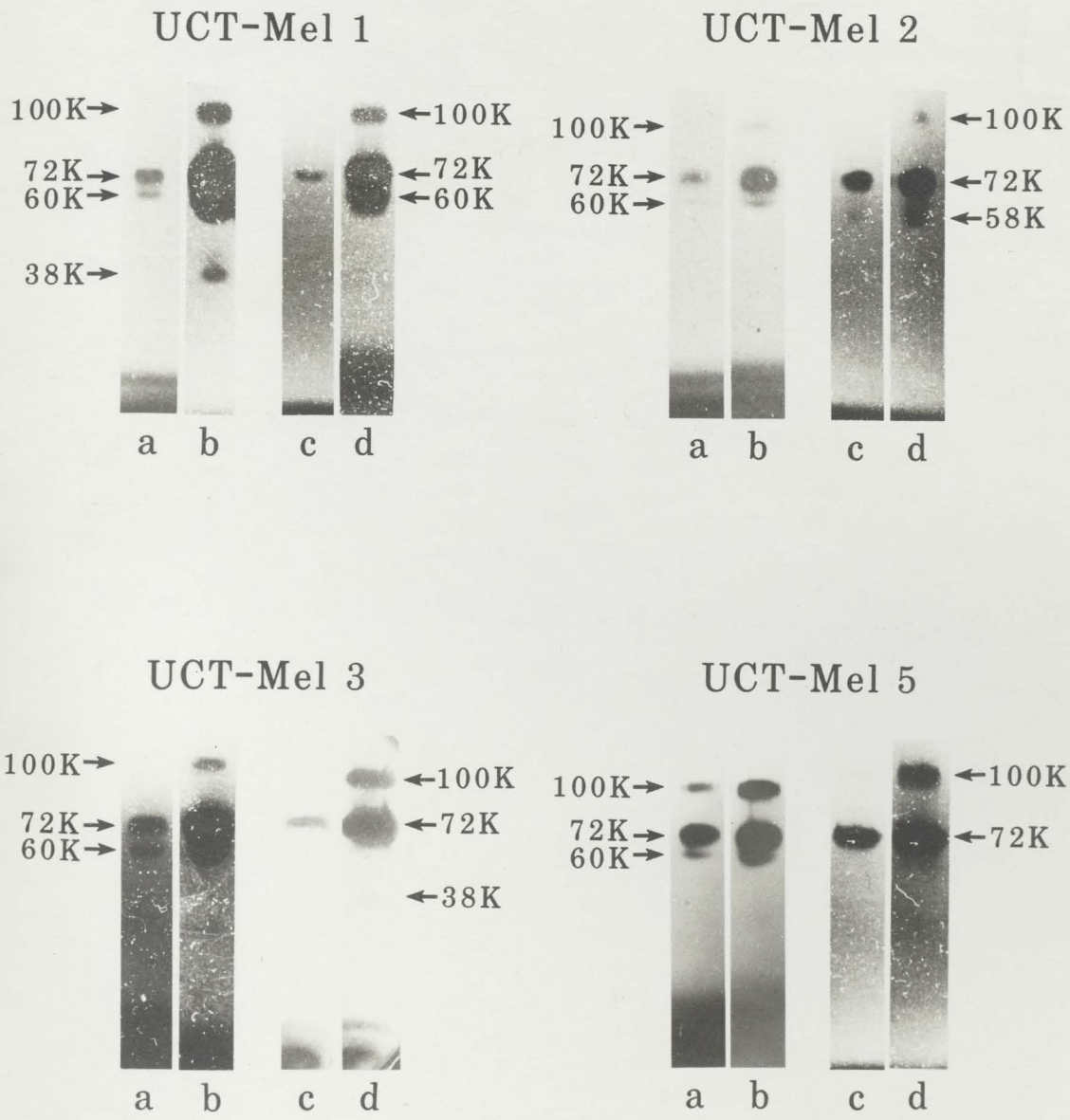

UCT-Mel 1
100K→
72K→
60K→
38K→
←100K
←72K
←60K
a b c d
UCT-Mel 2
100K→
72K→
60K→
←100K
←72K
←58K
a b c d
UCT-Mel 3
100K→
72K→
60K→
←100K
←72K
←38K
a b c d
UCT-Mel 5
100K→
72K→
60K→
←100K
←72K
a b c d

FIGURE 3.8

FIGURE 3.9

FIGURE 3.9

Fibrin-plasminogen-agar zymograms of melanoma plasminogen activators.

This figure depicts zymograms of secreted and intracellular PA in the 7 melanoma lines reported in this thesis. In each instance, (a) and (b) represent PA found in cellular harvest fluids, and (c) and (d) represent PA found in cell lysates. Early stages in the development of lysis bands are illustrated in (a) and (c), and later stages in (b) and (d). The zymograms do not allow for quantitative comparison of the amounts of PA synthesized by the different melanoma cell lines as photographs were taken at times designed to illustrate the molecular species of the lysis bands. This figure demonstrates the relative quantities of the PA species synthesized by each melanoma line, and should be considered in conjunction with Table 3.2, in which the totality of my results with the melanoma PA's is considered.

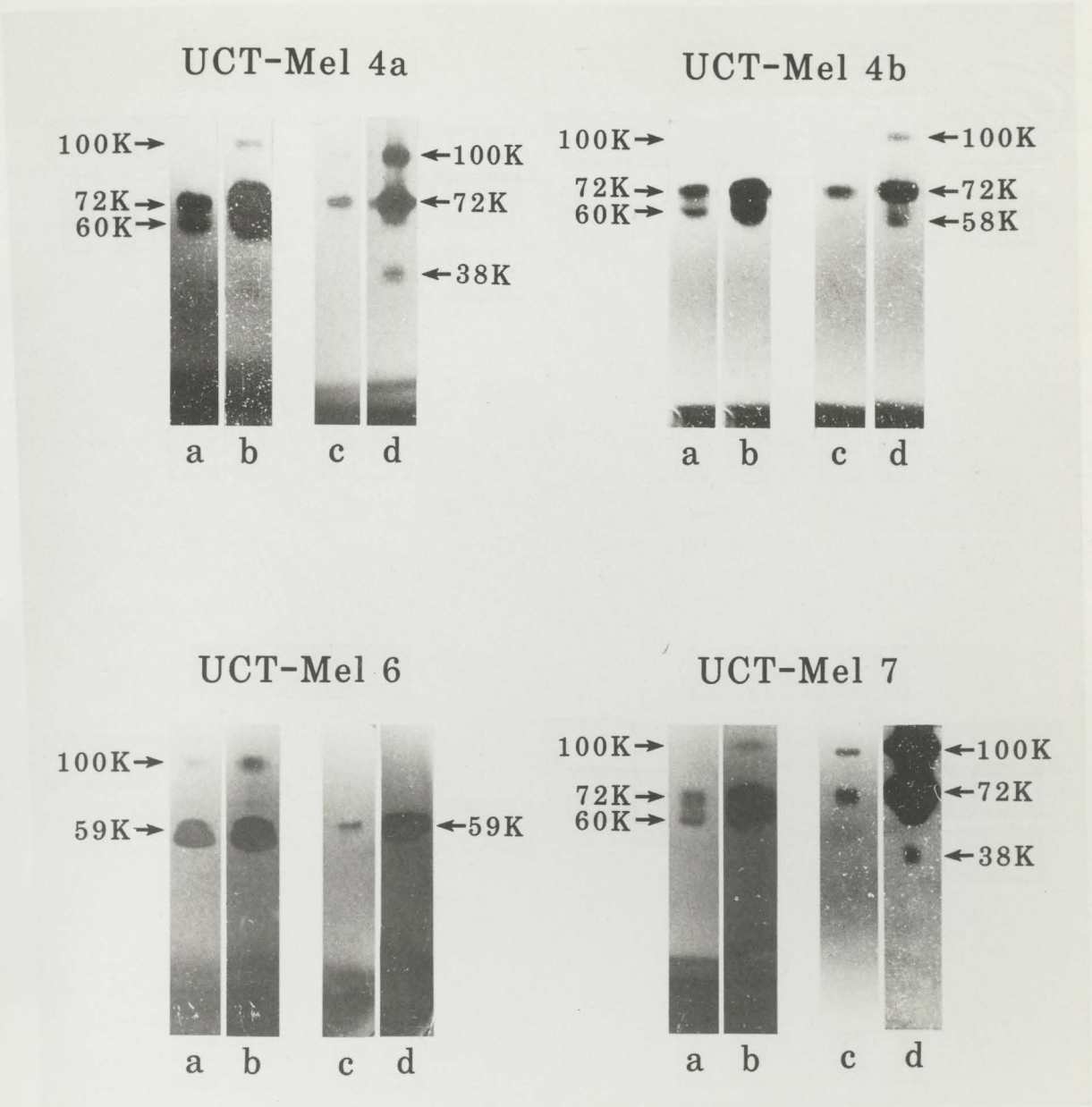

UCT-Mel 4a
100K→
72K→
60K→
←100K
←72K
←38K
a b c d
UCT-Mel 4b
100K→
72K→
60K→
←100K
←72K
←58K
a b c d
UCT-Mel 6
100K→
59K→
←59K
a b c d
UCT-Mel 7
100K→
72K→
60K→
←100K
←72K
←38K
a b c d

FIGURE 3.9

FIGURE 3.10

FIGURE 3.10

Inhibition of melanoma PA by specific antibody in fibrin-plasminogen-agar indicator gels.

The figure depicts 2 separate experiments. In the gel on the left, anti-UK antibody was added to the trough in the fibrin agar indicator gel. In the gel on the right, anti-melanoma PA antibody was added to the trough (see Methods). Samples of PA which were electrophoresed and layered onto the underlay were as follows: Tracks 1 and 2, human urokinase; Tracks 3 and 4, UCT-Mel 1 harvest fluid; Tracks 5 and 6, UCT-Mel 5 harvest fluid; Tracks 7 and 8, UCT-Mel 1 harvest fluid. Note that anti-UK antibody inhibits UK (Track 2) but has no effect on melanoma PA (Track 3). All molecular weight components of melanoma PA are inhibited completely by anti-melanoma PA antibody (Tracks 6 and 7).

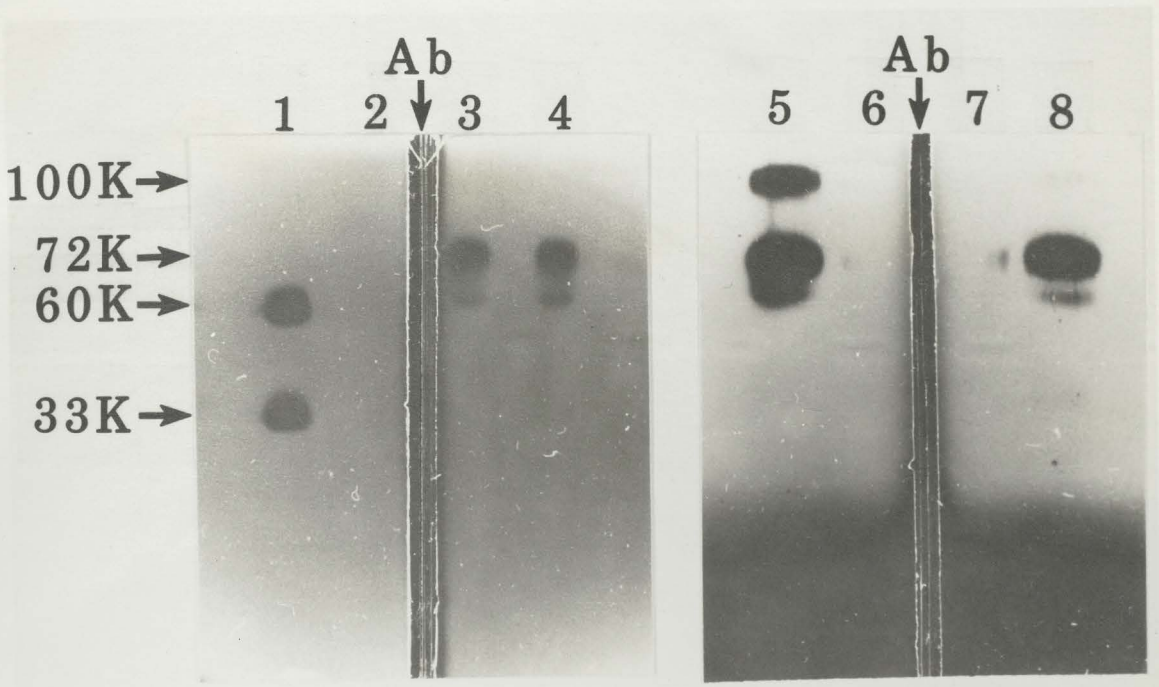


FIGURE 3.10

TABLE 3.2

MOLECULAR SPECIES OF PA SYNTHESIZED BY MELANOMA CELLS

	Type[b]	Molecular weight[a] 100K	72K	60K	58K	38K	33K
Extracellular PA							
UCT-Mel 1	Tiss	+[c]	+++	++		±	
UCT-Mel 2	Tiss	+	+++	+			
UCT-Mel 3	Tiss	+	+++	++			
UCT-Mel 4a	Tiss	+	+++	++		±	
UCT-Mel 4b	Tiss	+	+++	++		±	
UCT-Mel 5	Tiss	++	+++	++		±	
UCT-Mel 6	UK	+		+++			±
UCT-Mel 7	Tiss	+	+++	++		±	
RPMI-7272	Tiss	+	+++	+			
M-127	Tiss	+++	++				
M-170	Tiss	+	+++				
Intracellular PA							
UCT-Mel 1	Tiss	++	+++	+			
UCT-Mel 2	Tiss	+	+++		+		
UCT-Mel 3	Tiss	+	+++			±	
UCT-Mel 4a	Tiss	++	+++			±	
UCT-Mel 4b	Tiss	++	+++		+	±	
UCT-Mel 5	Tiss	++	+++				
UCT-Mel 6	UK			+++			
UCT-Mel 7	Tiss	+	+++			±	

(a) Molecular weights given are approximate

(b) The immunochemical type of PA is expressed as tissue-type (Tiss) or UK-type (UK)

(c) Entries are intended to convey the relative abundance, on the scale ± to +++, of the various molecular species present in each sample based upon the area of the fibrinolytic zone and the time taken for an area of lysis to appear in the indicator gel.

(iii) All of the melanoma cell lines examined, with the exception of UCT-Mel 6, released the tissue-type enzyme.

(iv) Melanoma cells that synthesized the tissue-type PA, released and contained one or more of four molecular weight species. These could be identified as follows:

(a) A 72K dalton band was the most prominent species, both intracellularly and extracellularly, in all cases but one (M-127). The relative abundance of this enzyme could readily be judged by the area of lysis in the fibrin underlay and the rapidity with which fibrinolysis became apparent. In some cases the 72K dalton band appeared as a very closely-spaced doublet. Usually the resolving power of the indicator gel was such that this doublet could not be discerned.

(b) A band with an approximate molecular weight of 60K daltons. This was observed in harvest fluids collected from 7 of the 9 melanomas, where it usually appeared as the second most abundant species. It was found in only 1 of the cell lysates, and then in trace amounts.

The 60K dalton band of the tissue-type PA corresponded, in electrophoretic mobility, to the upper component of the prominent 60K dalton doublet that characterizes PA's of the urokinase type. In the case of UCT-Mel 2 and 4b, a 58K dalton band of the tissue-type PA was observed with an electrophoretic mobility corresponding to the lower member of the urokinase doublet. Although this was found on many occasions it was seen only with these two melanoma cell lines and only in the cell lysate.

(c) A band, with an approximate molecular weight of 100K daltons, that was present in all of the harvest fluids and cell lysates as

the second or third most abundant component.

(d) A faint and inconsistent band corresponding to an enzyme of approximately 38K daltons was observed in most of the harvest fluids and a number of the cell lysates. It usually required prolonged incubation of the gel to demonstrate this band and cultures of any given cell line appeared to release it erratically.

(v) UCT-Mel 6 - the exceptional line that released urokinase-type enzyme showed a prominent "60K dalton" doublet with trace amounts of 100K dalton and 33K dalton enzyme. The cell lysate contained the 60K doublet and a trace of 33K enzyme, with no sign of the 100K component. Despite prolonged incubation of the gel on the fibrin-plasminogen-agar underlay, no sign of PA with a molecular weight of 72K daltons could be found.

DISCUSSION

Human plasminogen activators can be broadly classified into two distinct groups on the basis of their immunochemical similarity to urokinase or tissue-type PA. Each group encompasses multiple molecular weight forms of PA, but the two groups, generally speaking, consist of (a) urokinase-like enzymes which have the 60K doublet as their most prominent component and (b) enzymes which have the 72K doublet as the predominant species. These two groups are inhibited by their homologous antibodies and do not cross-react (Kucinski et al, 1968; Bernik and Kwaan, 1969; Kok, 1979; Rijken et al, 1979a; Vetterlein et al, 1979; Wilson et al, 1980; Markus et al, 1980; Corasanti et al, 1980; Camiolo et al, 1981).

The enzymes in the first group include urokinase itself, and the PA's secreted by the majority of normal and malignant cells in culture (Astedt and Holmberg, 1976; Granelli-Piperno and Reich, 1978; Vetterlein

et al, 1979; Wilson et al, 1980). The second group includes the vascular activator (Aoki and Von Kaulla, 1971; Binder et al, 1979), tissue activator (Kok and Astrup, 1969; Rijken et al, 1979b) and the PA's released by melanoma cell cultures (Vetterlein et al, 1979, 1980; Roblin and Young, 1980; Wilson et al, 1980; Rijken and Collen, 1981). In certain tissues e.g. lung (Markus et al, 1980) and colon(Corasanti et al, 1980) synthesis of the urokinase-like PA appeared to be associated with the malignant state, and PA of a type not inhibited by anti-UK antibody was found only in adjacent normal tissue.

Distinct antigenic forms of PA have also been described in hamster cells (Christman et al, 1975c). In this chapter and in previously reported results (Wilson et al, 1980) I found that all but one of the 11 melanoma cell cultures studied released the 72K doublet as the most abundant species. Relatively less of the 100K, 60K and even less of the 38K enzyme was produced, and none of these molecular weight species cross-reacted with urokinase. The activity of these enzymes was totally dependent upon plasminogen. Melanoma-derived PA's with similar molecular weights have been reported (Danø and Reich, 1978; Granelli-Piperno and Reich, 1978) and Tucker et al (1978) noted that the PA secreted by cerebral melanoma cells in culture was not inhibitable by anti-UK antibody. In addition, Vetterlein et al (1979, 1980) reported a PA of 73K that was non-inhibitable by anti-UK IgG and which was secreted by three human melanoma-derived cultures. Roblin and Young (1980) tested 6 melanoma cell cultures and found the 73K molecular weight form in all 6. In only 2 cases (both of which had some 60K activity) was some inhibition by anti-UK IgG observed, with a maximum inhibition of 19% in one culture. The purified PA from human melanoma cells in culture, reported by Rijkin and Collen (1981) has a molecular weight of 72K and is unrelated to urokinase.

The findings with melanoma cells, therefore, are sufficiently consistent to permit the generalization that secretion of an enzyme immunochemically distinct from UK is a characteristic feature of this neoplasm.

It is not, however, an exclusive attribute of these tumours since an enzyme with similar physical and immunochemical properties has been identified in a number of malignant cell types, including carcinomas and sarcomas (Wilson et al, 1980; Roblin and Young, 1980), and in harvest fluid collected from a culture of early embryonic fibroblasts (Wilson et al, 1980). Furthermore, 72K anti-UK-resistant enzyme is not necessarily a distinctive product of the malignant or early embryonic cell as most normal tissues, normal adult oesophageal fibroblasts and at least one primary culture of normal adult bladder urothelium (Wilson et al, 1980) have been shown to contain this enzyme. Conversely, not all melanomas, when analysed as freshly removed tumours, contain exclusively the tissue-type enzyme (Markus, personal communication) and I appear to have identified a melanoma-derived cell line that is an exception to the general rule in that it produces the UK-type enzyme. I have discussed this matter at some length in Chapter 2.

Much speculation has surrounded the relationship that exists between the multiple forms of plasminogen activator that are observed. Lesuk et al (1967) found that urinary PA's with molecular weights significantly below 54K arise from the proteolytic degradation of native UK indicating that molecular heterogeneity may be explained, in part, by partial proteolysis. It is unlikely that the 72K anti-UK IgG-resistant PA-type is a precursor of the smaller antibody-sensitive, urokinase-related forms, as suggested by Vetterlein et al (1979). In those cases where the melanoma cells secreted the 60K and 38K species, I found that enzyme activity in these lower molecular weight species was unaffected by anti-UK IgG, and was completely inhibited by antibody to the tissue-type enzyme. These findings show clearly that any molecular weight forms that may have arisen by proteolytic cleavage of a larger precursor molecule maintained their antigenic identity.

The 60K and 38K molecular weight species of the tissue activator that I observed, were barely detectable or absent in the cellular lysate of the melanoma cell lines. This observation is consistent with the explanation that less proteolysis or spontaneous breakdown to lower molecular weight forms would occur in a cell lysate prepared from fresh cells and frozen within approximately 20 min, than might be expected in an extracellular harvest fluid collected over a period of 24 hr at 37°C.

Within either the tissue-type or UK group the relationships that exist between the 100K dalton molecular weight species and their corresponding lower molecular weight enzymes are uncertain. Assuming that these molecular weight species represent post-translational modifications of one gene product a number of possibilities clearly exist.

The first and most obvious of these would be to suggest that the enzyme is synthesized as a 100K molecular weight protein and is converted, by a series of limited proteolytic steps, to the lower molecular weight forms without damage to the active site.

Although there is good evidence for believing that the 60K urokinase doublet is the parent, by proteolysis, of the 33K species (Lesuk et al, 1967) and that the UK-like enzyme in plasma is present in a "latent form" that implies the existence of the pro-enzyme (Wun, Schleuning and Reich, in press 1981) definitive studies to show conversion of the 100K dalton species to lower molecular weight species have not, to my knowledge, been completed.

An alternative proposal would be to regard the 72K enzyme (or, in the case of the UK-type, the 60K enzyme) as the product that is translated in the first place. The 100K molecular weight enzyme would then be derived by complex formation with an inhibitor or other binding protein by way of an SDS-resistant bond. A complex such as this, that retained

enzymatic activity in the fibrin-plasminogen-agar underlay system, was shown by Granelli-Piperno and Reich (1978) to be formed between plasmin and α_2-macroglobulin. Furthermore, as I shall demonstrate in Chapter 5, the melanoma activator complexes with a component on fibroblasts. This results in an apparent change in molecular weight of the enzyme from 72K to over 100K. On balance therefore my preference is for this latter explanation, although further work is clearly required to resolve the issue.

As the different immunochemical PA types do not appear to be derived from each other and since each cell line is restricted to expression of only one type, it appears likely that two different genes code for the two series of plasminogen activators. The structural gene for the urokinase-type of PA appears to reside on chromosome 6 (Kucherlapati et al, 1978). Similar studies to determine the chromosomal locus for the tissue-type form will have to precede any definitive pronouncement on the molecular genetics of plasminogen activators.

When, for each cell line, intracellular PA content was considered in relationship to the release of enzyme into the medium no clearly defined correlation was observed (Table 3.1). Unfortunately, uncertainties regarding the presence of intracellular inhibitors - e.g. such as those described by Loskutoff and Edgington (1977) in endothelial cells - made it difficult for me to draw any interesting conclusions from this cell-to-cell variation. This difficulty was compounded by my lack of knowledge concerning the relative amounts of the various molecular species of the PA's, inside and outside the cells, and of the kinetic parameters of these various species. It may well be that cells with a high intracellular content and a relatively low rate of secretion may prove to have characteristics that are not possessed by cells which maintain the opposite relationship between intracellular accumulation and release. As yet the system is insufficiently characterised for me to define these differences.

Of more interest, perhaps, was the striking change that I observed in the apparent rate of release of PA by UCT-Mel 1 and 3 cells as they approached confluence. In UCT-Mel 1 this occurred without a corresponding depletion of intracellular enzyme to suggest inhibition of synthesis. Nor could I detect a concomitant accumulation of intracellular PA to indicate that transport of the enzyme out of the cell had been blocked. The only other observation that I was able to make with confidence regarding this density-dependent effect relates to its reversibility. Dispersal of confluent cultures with EDTA or trypsin and reseeding at a lower density rapidly restored PA release.

It was at this point in the work that I made the observation with fibroblasts that indicated that they were much more effective than melanoma cells in preventing the accumulation of tissue activator in the medium. I then diverted into the study of melanoma cell-fibroblast co-cultivation that is presented in Chapter 5 and abandoned further work on the effect of confluence *per se*.

Although one may suppose that the fibroblast effect and the density-dependent effect shown by UCT-Mel 1 and 3 were qualitatively similar but quantitatively different, with fibroblasts being more effective, the case for believing this is an uncertain one. Further study may show that confluence, as a biochemical expression of "contact inhibition", does indeed influence the economy of PA synthesis and release in certain melanoma cells.

Chou et al (1977) found a similar decrease in the secretion of PA as their mouse 3T3 cultures became confluent and density-dependent. The intracellular levels of PA in the 3T3 cells remained uniformly high. In contrast, dense cultures of SV40-transformed 3T3 cells did not show this density-dependent effect but maintained their customary secretion of relatively large amounts of plasminogen activator. The authors therefore

suggested that the ability to decrease secretion of PA as cells become dense may be characteristic of cells which demonstrate density-dependent inhibition of cell growth.

A similar density-dependent decrease in PA release per mg cell protein was found with transformed adult pig kidney (APK) cells by Mochan et al (1975).

It is of interest that untransformed mouse cells (3T3), transformed pig cells (APK) and malignant human cells (UCT-Mel 1 and 3) display very similar patterns of PA secretion, indicating that all possess a common regulatory mechanism which is activated by, or associated with, increasing cell density. As I have suggested, such a mechanism might involve (a) a decrease in PA synthesis, resulting in less PA being exported into the medium; (b) an alteration in the process by which PA is secreted into the extracellular medium, resulting in feedback inhibition on the synthesis of PA and a constant intracellular concentration of PA being maintained; or (c) synthesis of a protease inhibitor or increased synthesis of inhibitors normally produced by the cells, so that the resulting imbalance between PA and inhibitor would manifest itself in a decreased overt secretion of PA.

The third possibility (c) is supported by evidence that PA activity in the medium represents a balance between PA and PA inhibitor secretion (Roblin et al, 1978). A cell-derived inhibitor was found to be responsible for the decrease in detectable PA in APK cells (Mochan et al, 1975). When I examined sparse and dense cultures of UCT-Mel 1 I was unable to find any evidence to support this explanation. Mixing experiments did not show the secretion of an inhibitor by dense cultures.

The second possibility (b) applies to the decreased secretion seen in 3T3 cells. Jaken and Black (1979) found no evidence for a PA inhibitor in 3T3 cells. In view of the fact that the PA from growing cells was found mostly in the light, plasma membrane-enriched fraction, while

the PA from confluent cells was found mostly in the heavy membrane fractions, they concluded that the decrease in PA release by confluent cultures was due to the inability of the cells to release the PA extracellularly. In growing cultures an increased rate of insertion into the plasma membrane may be related to an increased rate of loss from the plasma membrane (Jaken and Black, 1979).

If a similar situation existed *in vivo* with respect to the pattern of PA secretion by tumour cells, it may be postulated that a single cell breaking free from the primary mass (and therefore representing a "sparse" culture) would secrete high levels of PA which could provide either a selective advantage for the passage of the tumour cell to vascular or lymphatic channels, or the proteolytic apparatus for conditioning the environment and generating growth factors. A malignant cell in the "dense" environment of an established tumour deposit would not benefit from the high secretion of PA and release of this enzyme may thus be inhibited.

Such induction and suppression of proteolytic potential would have considerable relevance to the survival and proliferation *in vivo* of tumour cells. Further study of these processes is clearly warranted.

CHAPTER 4

THE EFFECTS OF TETRADECANOYL PHORBOL ACETATE AND RETINOIC ACID ON UCT-MEL 1 AND UCT-MEL 2

The preliminary experiments reported in Chapter 2 demonstrated quite clearly that two of the melanoma cell lines (UCT-Mel 1 and 2) showed certain features of the differentiated melanocyte phenotype and responded to biologically active compounds that have relevance to cellular proliferation or differentiation.

At the time at which I started this work, the tumour promoter TPA and the vitamin-related retinoic acid appeared to be the most interesting compounds for further study in this regard. Tetradecanoyl phorbol acetate, it had been reported, would induce cells in culture to display a number of the features of the transformed phenotype (Driedger and Blumberg, 1977; Rifkin et al, 1979; Wilson and Reich, 1979; Quigley, 1979; review by Diamond, 1980) and, in the case of other cells, would inhibit differentiation (Rovera et al, 1977; Yamasaki et al, 1977; Fibach et al, 1978; Diamond et al, 1977; Ishii et al, 1978; Sisskin and Barrett, 1981; Cohen et al, 1977; Lowe et al, 1978). Melanization of mouse melanoma cells could be delayed (Mufson et al, 1979) and melanogenesis of chick embryo melanoblasts inhibited (Payette et al, 1980) by TPA.

Conversely, retinoids had been found to reverse squamous metaplasia (Clamon et al, 1974; Sporn et al, 1976) in epithelial tissue and the role of this class of compounds in inducing normal epithelial differentiation *in vivo* was well known. In addition, retinoids had been reported to inhibit chemical carcinogenesis in many systems (Bollag, 1974; Verma and Boutwell, 1977; Verma et al, 1979; Moon et al, 1977; Port et al, 1975;

Sporn et al, 1977) and had been used to treat human carcinomas (Bollag and Ott, 1971; Micksche et al, 1977). Sporn (1977) suggested that retinoid therapy be considered for the prevention of epithelial cancers.

It could be predicted therefore, that my pigmenting melanoma lines would cease to pigment on exposure to TPA and would differentiate with increased pigmentation, diminished proliferation and the acquisition of the dendritic morphology of the mature melanocyte when exposed to RA. The experiments that I report in this chapter were designed to examine this prediction. The experiments were given added cogency by subsequent observations indicating that, under certain circumstances, retinoids could promote tumour growth (Polliack and Levij, 1969; Polliack and Sasson, 1972) and could act synergistically with TPA and the Rous sarcoma gene to induce cellular transformation (Wilson and Reich, 1978). The usefulness of retinoids as agents for the therapy of malignant disease therefore came into question (Schroder and Black, 1980). I hoped that systematic studies with differentiating human cell lines would contribute to the resolution of this uncertainty.

MATERIALS AND METHODS

The experiments described in this section were designed to examine the effects of TPA and RA on UCT-Mel 1 and 2 cells cultured *in vitro*. Details of the techniques that were used are described in the Appendix and need not be repeated here.

For the sake of clarity, however, it is appropriate that I should give a broad outline of the protocols that I used.

All experiments followed the same basic plan in which compounds were added at different concentrations to adherent monolayer cultures of UCT-Mel 1 and 2. Both TPA and RA were dissolved in absolute ethanol to give stock solutions such that the highest final concentration required a dilution of at least 1/10 000. The concentration of solvent in the final

medium was therefore negligible and could be shown in control experiments to have no effect.

Unless specifically called for by the experiment (in which case this is stated in the results section) all experiments were started by seeding replicate cultures in full serum containing medium without compounds. Twenty-four hours later, when the cells had adhered, the medium over the monolayers was replaced with serum-containing medium to which compounds at the desired concentration had been added.

Thereafter, the medium was replaced every 48 hr with fresh medium containing test compounds at the desired concentration.

Replicate cultures were used at appropriate time points to construct growth curves; to assay for intracellular PA, LDH or tyrosinase; or to measure cellular release of PA.

The rate of activator release was assayed by measuring the amount of enzyme released over a timed period into serum-free harvest fluids containing compounds and prepared, collected and stored as described in Appendix A.8.

The results of measurements of cellular functions were calculated and expressed on the basis of viable cell number or, in the case of intracellular variables, on the basis of mass of cell protein. In the experiments in which modulation of tyrosinase activity was studied, changes in intracellular content of this enzyme were related to cellular LDH activity - this being a constitutive enzyme in most human cells.

RESULTS

Effects of TPA

Growth in vitro.

Tetradecanoyl phorbol acetate inhibited the growth of UCT-Mel 1 in a dose-dependent manner when added at concentrations between 0.1 and 10

ng/ml. When added at 0.01 ng/ml, it had no effect (Fig. 4.1). In contrast the growth of UCT-Mel 2 was stimulated by TPA. This effect was observed over the range of concentrations from 0.1 to 30 ng/ml and was maximal between 0.1 and 1 ng/ml. At higher concentrations, the stimulatory effect diminished (Fig. 4.2).

Tyrosinase synthesis

The effect of TPA (10 ng/ml) on intracellular tyrosinase content was measured in a number of separate experiments on UCT-Mel 1 and 2, and in neither case could a significant or consistent effect of the drug be demonstrated (Fig. 4.3). Care was taken in all cases to ensure that experimental and control cultures were at the same cell density when intracellular tyrosinase concentrations were compared. This was important since, as described in Chapter 2, intracellular tyrosinase content was critically affected by cell density in both cell lines and any effects of TPA might have been explained simply on the basis of its effects on cell number had this difficulty not been borne in mind.

Plasiminogen activator synthesis and release

In the case of UCT-Mel 2 cells, TPA (10 ng/ml) had no significant effects on the rate of PA release. This line was therefore not studied further in this regard.

When added to UCT-Mel 1 cells, TPA consistently and reproducibly diminished intracellular content of PA (Fig. 4.4) and inhibited the release of this enzyme into the medium (Fig. 4.5). The inhibitory effects of TPA (10 ng/ml) became apparent approximately 8 hr after addition to the cells and reduced both intracellular content and rate of PA release by approximately 60% after 24 hr.

Inhibition of PA synthesis was dose-dependent over the concentration range 0 to 3 ng/ml TPA where maximum inhibition was reached and no further fall in rate of enzyme synthesis occurred with higher concentrations, up to 30 ng/ml (Fig. 4.6).

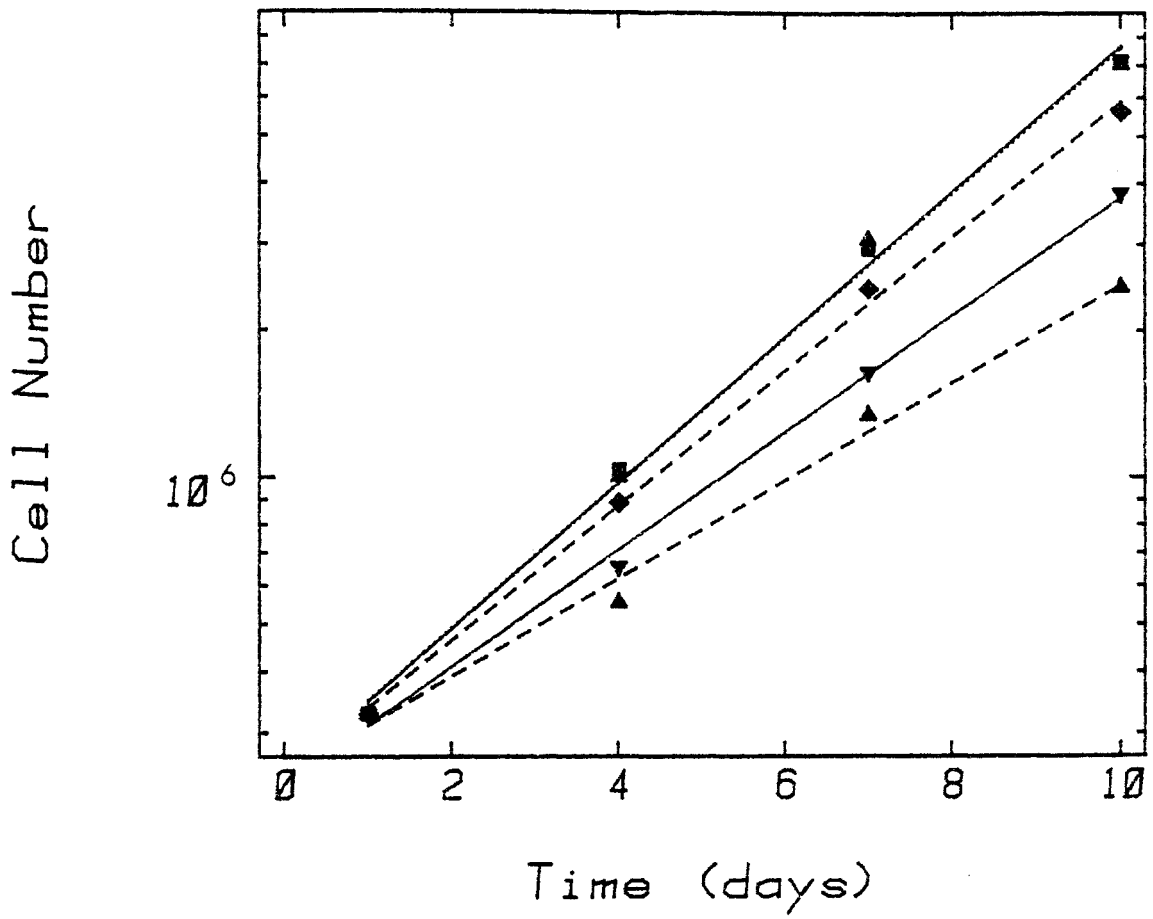


FIGURE 4.1

Growth inhibition of UCT-Mel 1 by TPA.

UCT-Mel 1 cells were seeded at 3 x 10^5/60 mm dish. Twenty four hr later, TPA was added at 0.1 (■·····■), 1 (◆- -◆), 3 (▼——▼) and 10 ng/ml (▲- -▲). Control dishes received no TPA (▲——▲). Fresh medium containing the above concentrations of TPA was added daily. At the times indicated, duplicate dishes were trypsinized and the cells counted in a Coulter counter. The means of these results are presented. Note that while 0.1 ng/ml TPA had no inhibitory effect on the growth of these cells, higher concentrations inhibited proliferation in a dose-dependent manner.

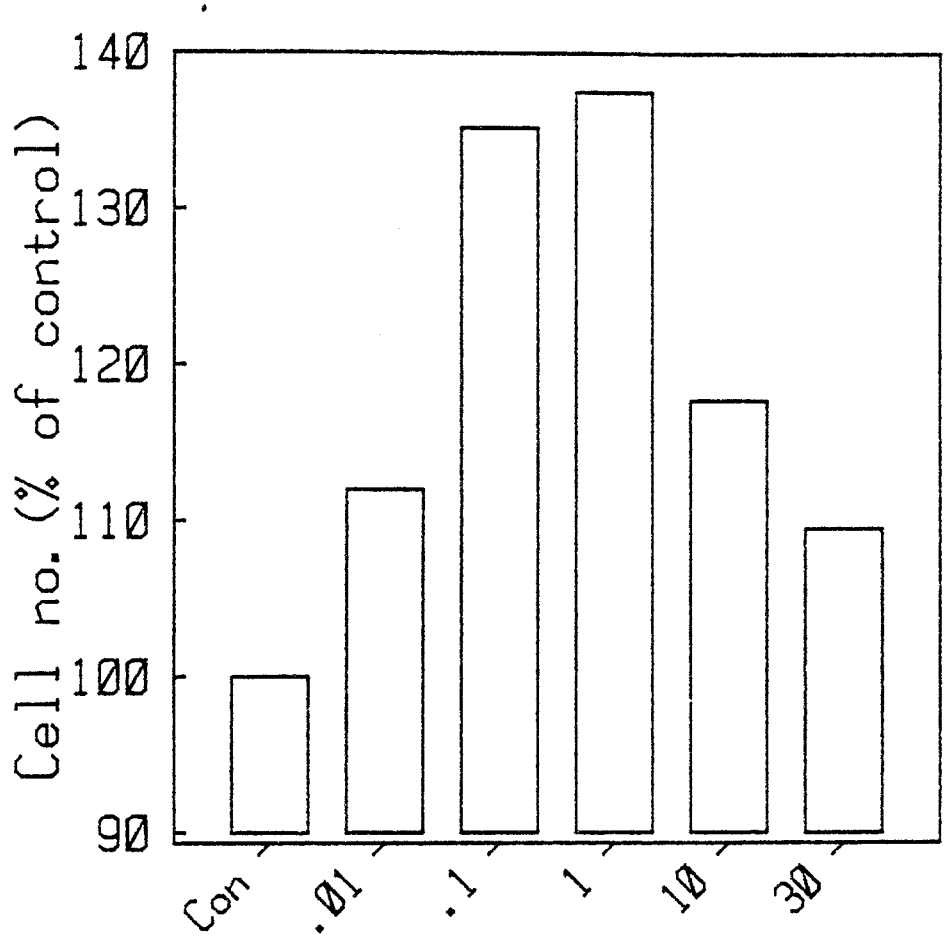


FIGURE 4.2

Growth stimulation of UCT-Mel 2 by TPA.

UCT-Mel 2 cells were seeded at 2 x 10^5/60mm dish. Twenty four hr later, TPA was added to replicate cultures at concentrations ranging from 0.01 ng/ml to 30 ng/ml, as indicated. Fresh medium containing the appropriate concentration of TPA was added at 48 hr intervals. Control cultures received fresh medium without TPA. On day 9, replicate cultures were trypsinized and counted in a Coulter counter. The mean results of two similar experiments are presented as a percentage of the cell number in control dishes. Note that all the concentrations of TPA tested stimulated proliferation of UCT-Mel 2, and that this stimulation was maximal between 0.1 and 1 ng/ml TPA.

FIGURE 4.3

FIGURE 4.3

Effect of TPA on tyrosinase levels in UCT-Mel 1 and 2.

Results presented were obtained from dishes with approximately the same total protein concentration, for reasons detailed in the text.

(a) UCT-Mel 1: tyrosinase activity in extracts of cells grown in the absence of TPA (Con) or in the presence of 10 ng/ml TPA (TPA) for 9-13 days. (Total protein per 60 mm dish was 1.5 mg $\pm$ 10%).

(b) UCT-Mel 2: as for (a), except that TPA was present for 11-13 days. (Total protein per 60 mm dish was 1.2 mg $\pm$ 10%).

In both (a) and (b), each line and set of points represents the mean of duplicate results obtained in a separate experiment. Note the slight but inconsistent tyrosinase stimulation by TPA seen in UCT-Mel 1, and the absence of effect in UCT-Mel 2. The scale chosen for the figures was the same as that seen in Figure 4.14.

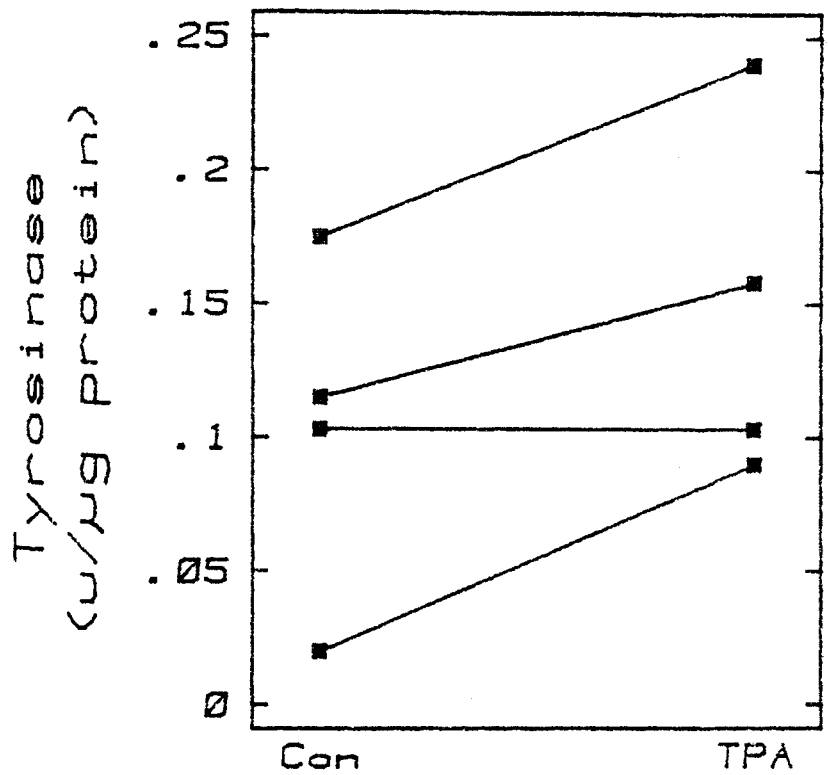


FIGURE 4.3a

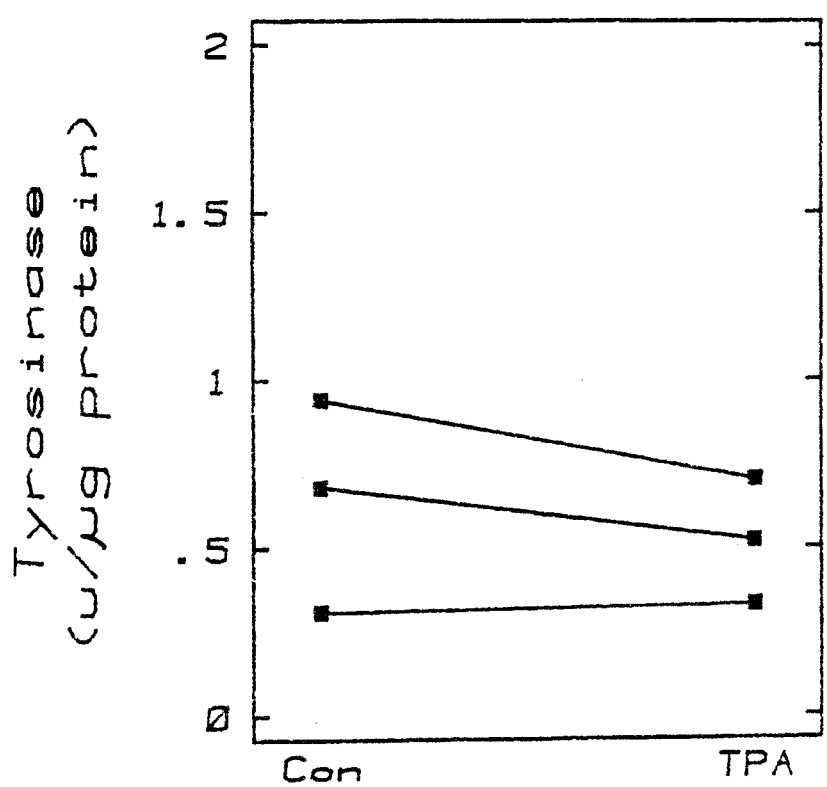


FIGURE 4.3b

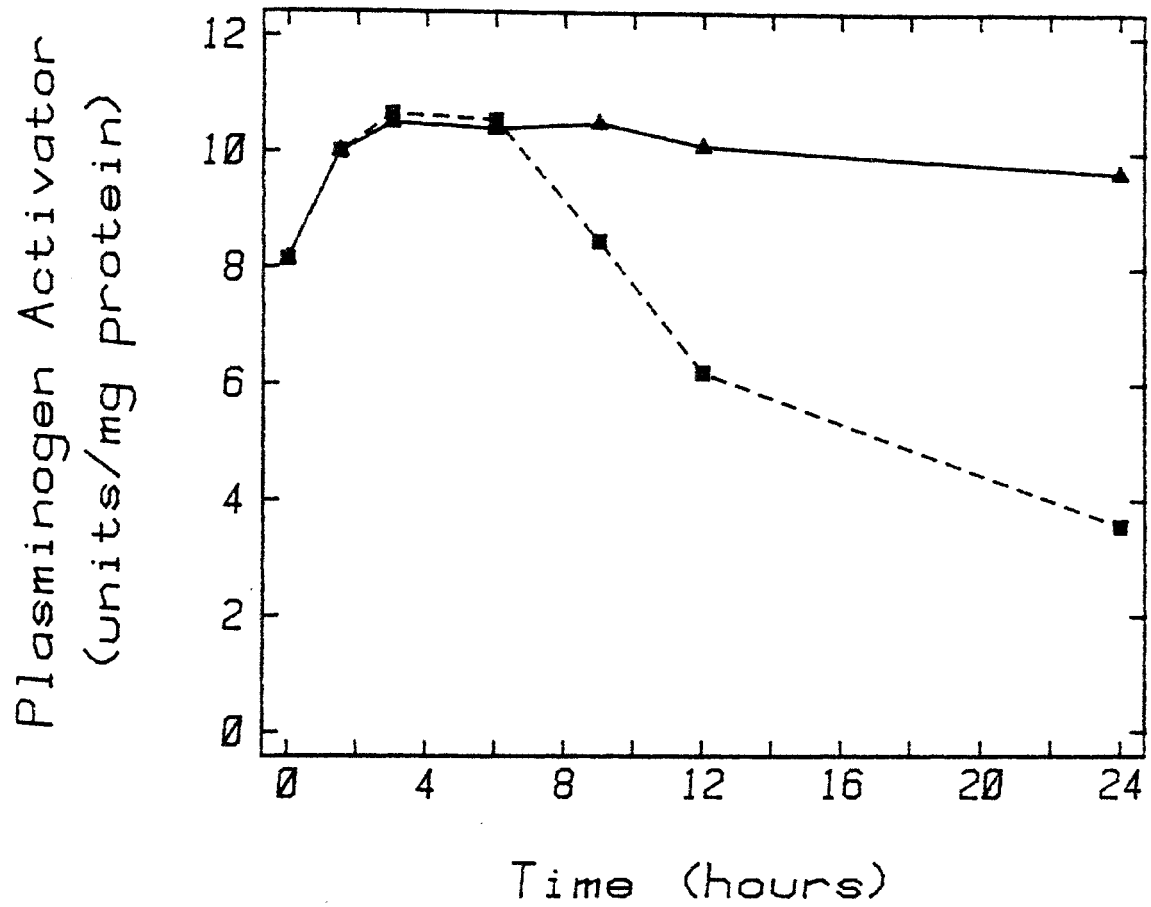


FIGURE 4.4

Inhibition of intracellular PA in UCT-Mel 1 by TPA.

UCT-Mel 1 cells were seeded at 8 x 10^5/60 mm dish. After 48 hr, at time 0, fresh medium without TPA (▲——▲) or containing 10 ng/ml TPA (■---■) was added. Cell lysates (Appendix A.9) were prepared from replicate cultures at the indicated times and assayed for PA content. Note that PA activity began to fall after approximately 6 hr in the presence of TPA, and PA content was inhibited by 63% after 24 hr.

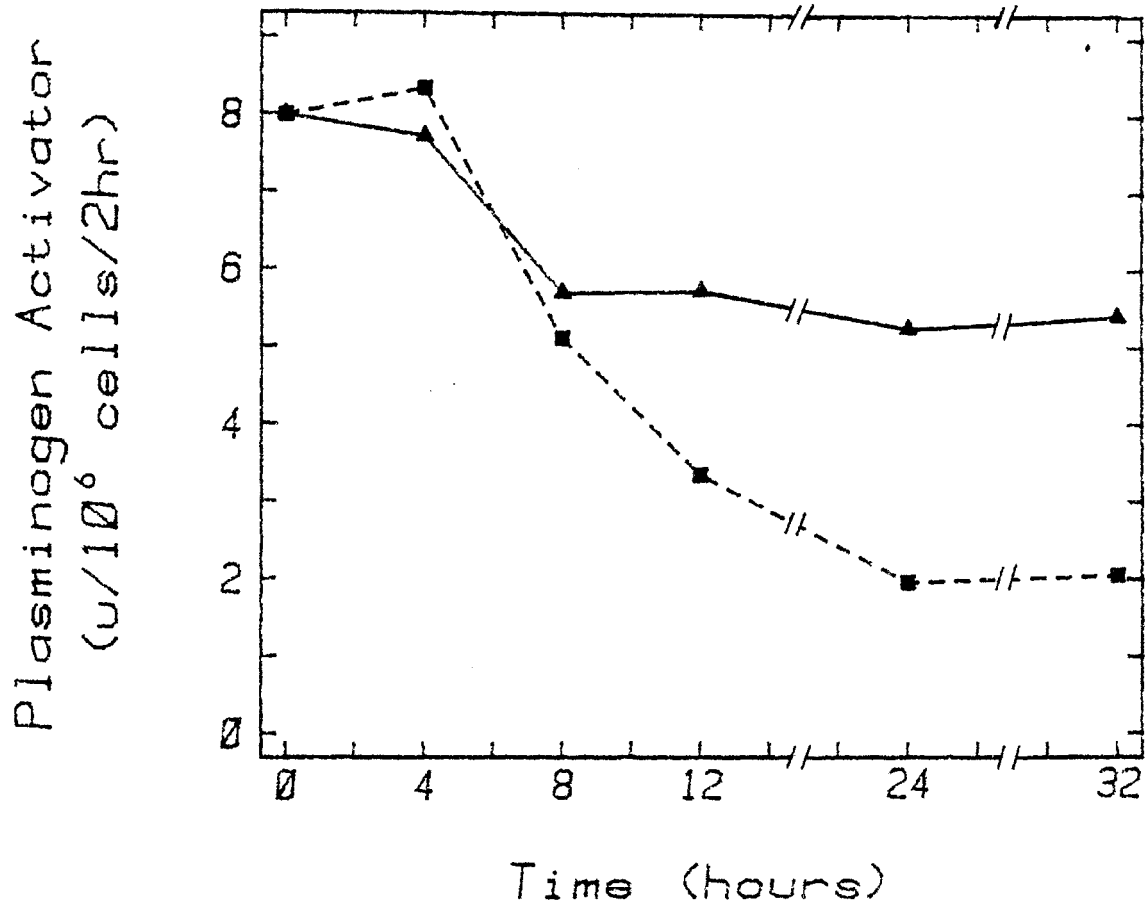


FIGURE 4.5

Inhibition of released PA in UCT-Mel 1 by TPA.

UCT-Mel 1 cells were seeded at 2 x 10^5/35 mm dish. After 48 hr, at time 0, 10 ng/ml TPA was added to cultures and the release of PA by control (▲——▲) and TPA-treated cultures (■- -■) was monitored by collecting 2 hr harvest fluids. The times indicated represent the midpoint of this 2 hr collection period. Note that PA inhibition was evident after 8 hr in the presence of TPA, and PA release was inhibited by 63% after 24 hr, closely following the kinetics of intracellular PA inhibition (see Fig. 4.4).

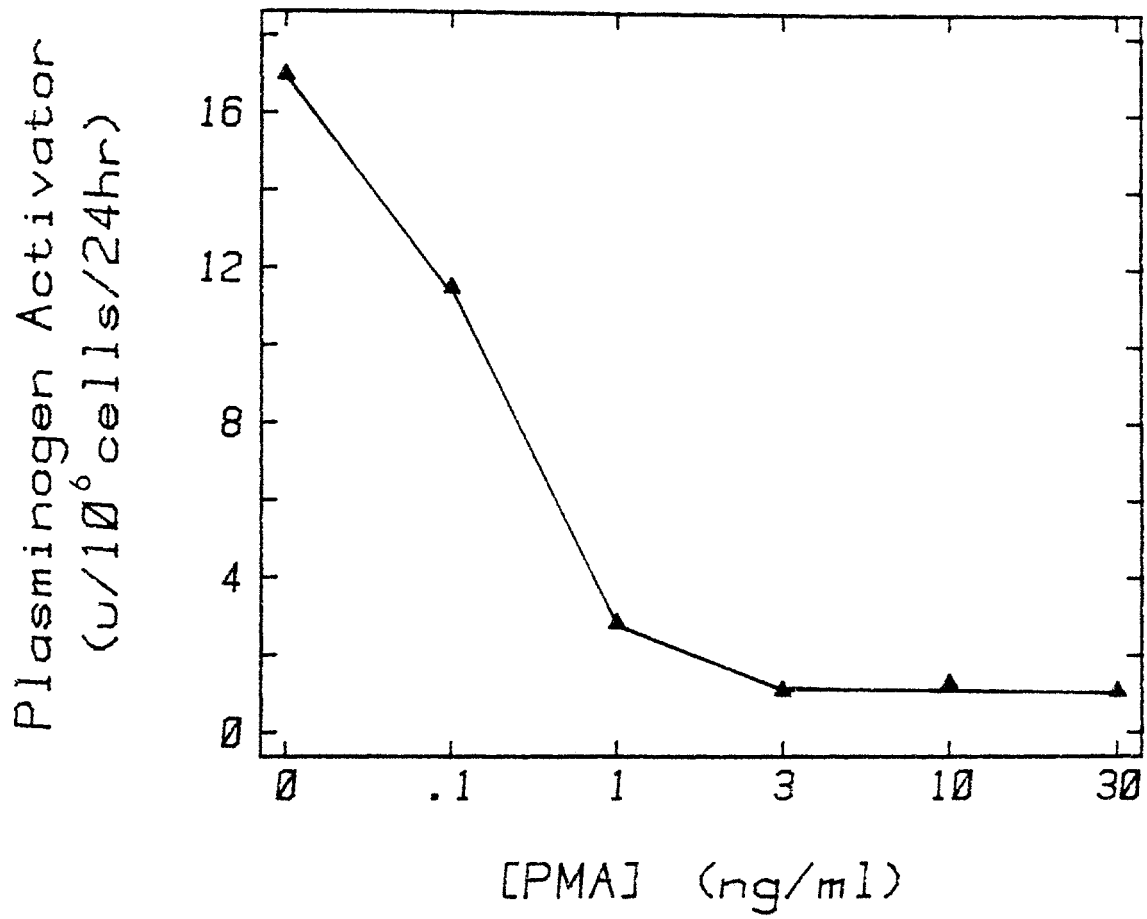


FIGURE 4.6

Dose-dependence of TPA inhibition of PA in UCT-Mel 1.

UCT-Mel 1 cells were seeded at 8 x 10^4/35 mm dish. Twenty four hr later, TPA was added at concentrations ranging from 0 to 30 ng/ml, as indicated. Fresh medium with or without TPA was added at 24 hr intervals and harvest fluids were prepared after the cells had been exposed to TPA for 72 hr. Note that inhibition of PA release was dose-dependent over the concentration range 0-3 ng/ml TPA. At higher concentrations of TPA, no further inhibition was observed.

As can be seen from the data illustrated graphically in Fig. 4.7, inhibition of PA synthesis by TPA at 10 ng/ml was reversible. Cells in which release of the enzyme had been inhibited by exposure to 10 ng/ml of TPA for 24 hr recovered some measure of enzyme synthesis 36 hr after removal of the co-carcinogen and returned to approximately normal levels of enzyme release after 72 hr.

In order to determine whether or not prolonged *in vitro* exposure to TPA would select for the emergence of a less responsive cellular subpopulation or would lead to the slow induction of a TPA-refractory state, an experiment was performed in which cells were maintained in culture in the continuous presence of 10 ng/ml TPA for 6 passages. Cells from the same initial culture were maintained in parallel without TPA to serve as controls.

As is evident from the data summarized diagrammatically in Fig. 4.8, control cells released approximately 20 units of PA/10^6 cells/24 hr. Control cells to which TPA at 10 ng/ml was added for 72 hr released 2 units of PA/10^6 cells/24 hr, indicating that these cells were susceptible to inhibition by the co-carcinogen. After 6 passages in the presence of TPA, cellular release of PA had recovered spontaneously to control levels despite the presence of 10 ng/ml of the inhibitor. When TPA was removed from chronically exposed cells and enzyme synthesis was measured 48 hr later, it was observed that these cells released PA at a rate 3 times that of control cells. Similar results were observed with cells maintained in 10 ng/ml TPA for 10 and 17 passages (data not shown). Despite the fact that cells recovered their ability to synthesize PA after 6 passages in the presence of TPA, the growth of these cells was still inhibited by approximately 30% relative to control cells after 8 days (Fig. 4.9). This inhibition was completely reversed when TPA was removed from the cells, but no "overshoot" in the growth rate was observed as in the case of PA.

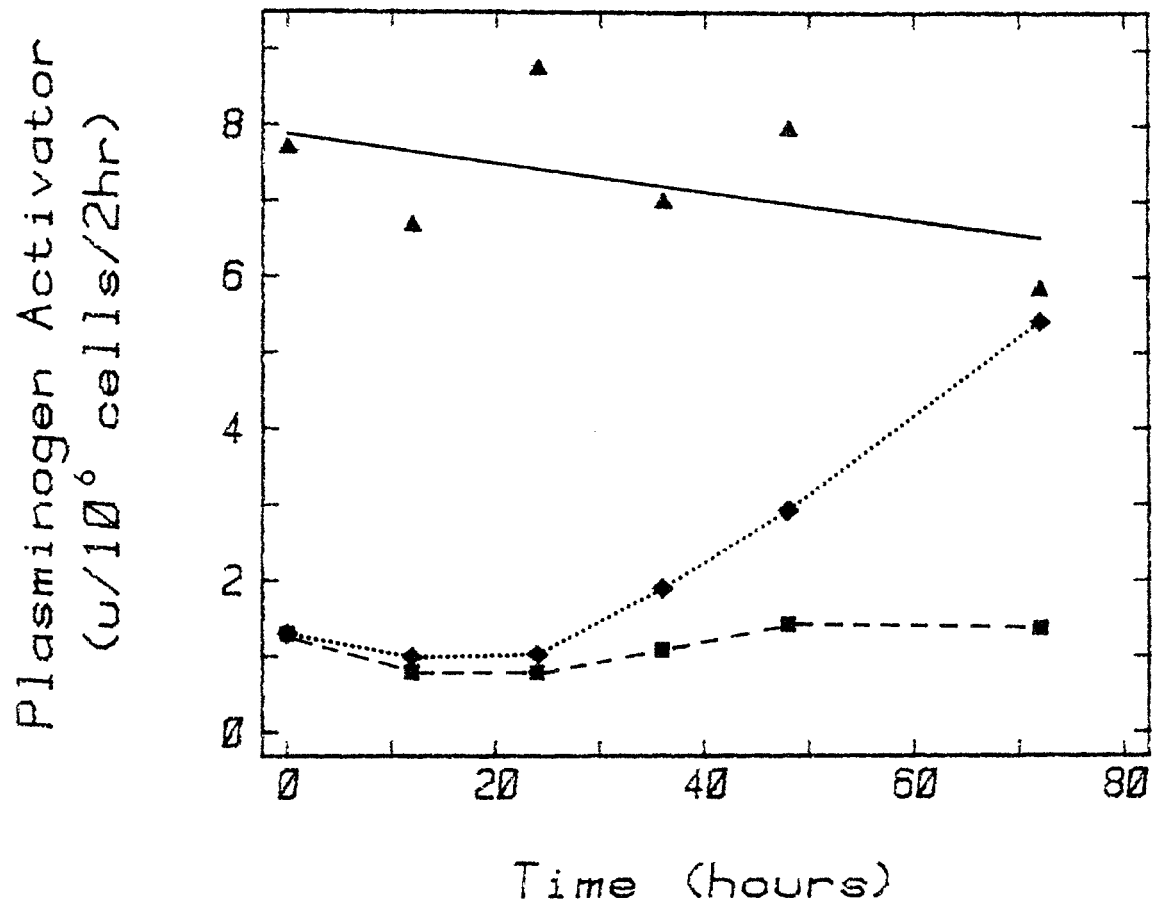


FIGURE 4.7

Inhibition of PA by TPA in UCT-Mel 1 was reversible.

UCT-Mel 1 cells were seeded at 6 x 10^4/35 mm dish. Forty eight hr later, fresh medium was added to the cells, either alone (▲——▲) or containing 10 ng/ml TPA (■- -■). Twenty four hr later (time 0) fresh medium was added to the cells, and TPA was removed from some of the cells (◆·····◆) that had been cultured for 24 hr in the presence of 10 ng/ml TPA. The release of PA from duplicate cultures was monitored by collecting 2 hr harvest fluids. The results are presented as the midpoint of this 2 hr collection period. Note that inhibition of PA persisted for 24 hr in the absence of TPA, after which enzyme synthesis recovered and approached normal levels after 72 hr.

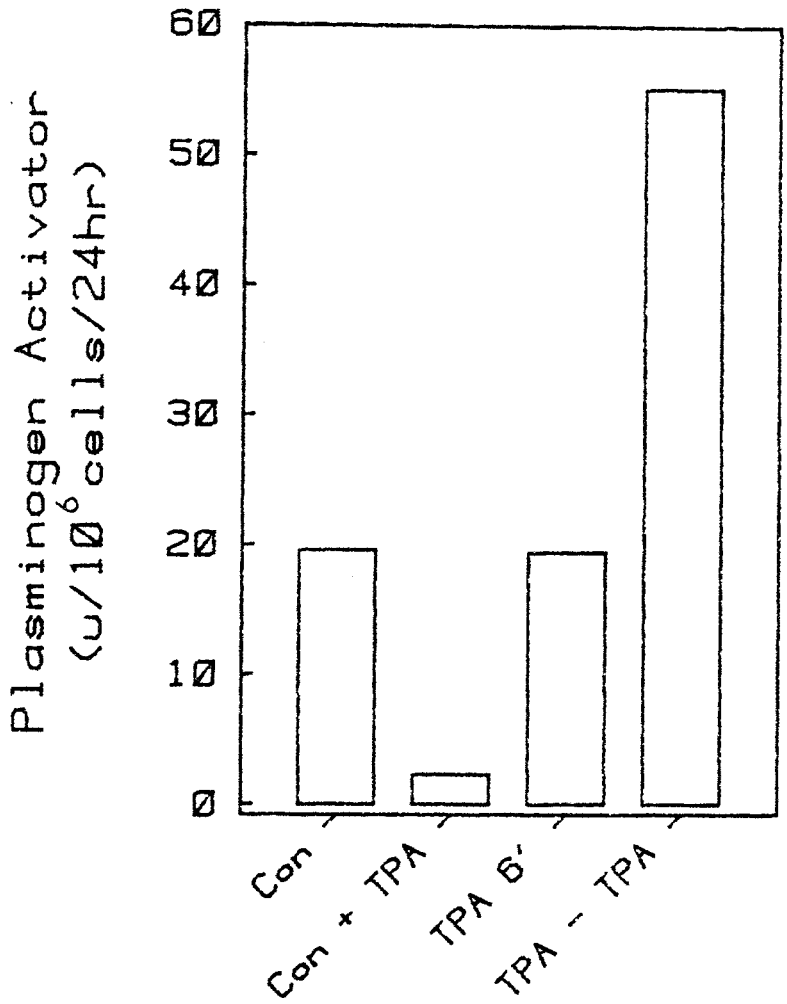


FIGURE 4.8

Release of PA by TPA-accustomed melanoma cells.

UCT-Mel 1 cells were seeded at 3 x 10^5/60 mm dish. Cells used had either never been exposed to TPA (Con) or had been cultured in the presence of 10 ng/ml TPA for 6 passages (TPA 6'). These TPA-accustomed cells were seeded in medium containing 10 ng/ml TPA. Twenty four hr after seeding, fresh medium containing TPA at 10 ng/ml was added to some of the control cultures (Con + TPA) and medium lacking TPA was added to some of the TPA-accustomed cells (TPA-TPA). Forty eight hr later, harvest fluids were prepared and assayed for PA activity.

Note that, while the addition of 10 ng/ml TPA to control cells resulted in inhibition of PA, the PA secretion of TPA-accustomed cells had become refractory to inhibition. When TPA was removed from these TPA-accustomed cells, the cellular release of PA was found to be increased by approximately 3-fold.

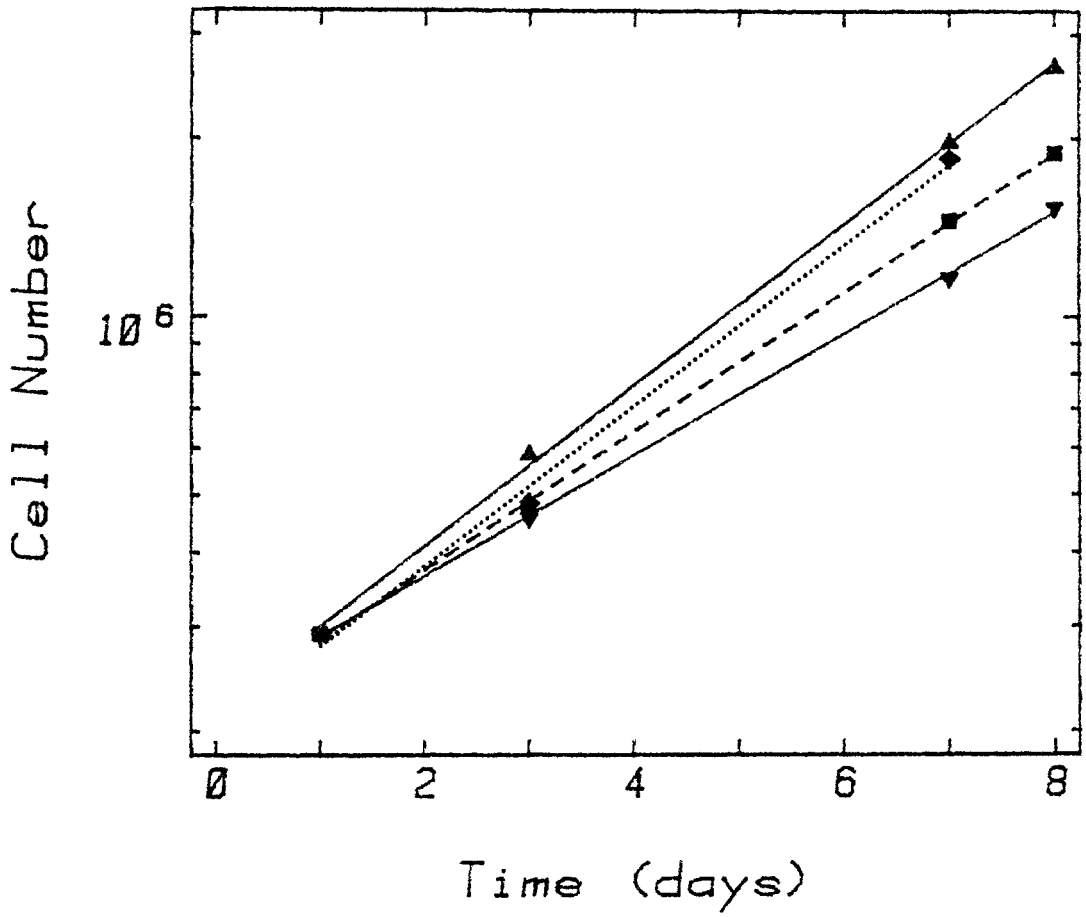


FIGURE 4.9

Growth rate of TPA-accustomed melanoma cells.

Cells used were as described in the legend to Fig. 4.8. At the indicated times, duplicate cultures were trypsinized and the cells counted in a Coulter counter. Note that the growth rate of TPA-accustomed UCT-Mel 1 cells (■- -■) was inhibited by approximately 30% relative to control cells (▲——▲), but was less inhibited than the growth of control cells to which TPA was added on day 1 (▼——▼). When TPA was removed from TPA-accustomed cells (◆·····◆) the growth rate recovered to that of control cells.

Morphology

The addition of TPA to UCT-Mel 1 consistently induced a morphological change which became apparent about 8 hr after the addition of 10 ng/ml TPA and progressed over the ensuing 24 hr to involve approximately 80% of the cells on the dish. These changes were marked in the initial stages by loss of the usual triangular or polygonal morphology as the cells became elongated and spindle-like (Fig. 4.10d). As time passed, a small percentage of these cells spread themselves on the surface of the dish and developed dendritic processes very similar to the processes that characterize mature cutaneous melanocytes *in vivo* (Fig. 4.10e). Similar changes were not observed in UCT-Mel 2, nor, incidently, were they observed in the other melanoma lines save in the case of UCT-Mel 7 - a non-pigmenting melanoma that showed no other noteworthy responses to added compounds to justify its inclusion in this chapter.

The morphological changes in UCT-Mel 7 that were brought about by TPA, although different from those observed with UCT-Mel 1, were nonetheless striking. When these cells were exposed to TPA at 10 ng/ml, they retained the spindle-like, elongated morphology that they manifest under control conditions but ceased to be distributed randomly over the surface of the dish. Within a matter of hours after addition of the compound, they could be seen to be associated into distinct clumps of cells from which fascicles of other cells protruded (Fig. 4.10b).

With UCT-Mel 1, the morphological changes induced by TPA were entirely reversible even when cells had been exposed to 10 ng/ml TPA for a number of passages. Within hours of removing the compound, the appearance of the cells was noticeably closer to that of the control cultures and by the end of 52 hr, all traces of the TPA effect had disappeared (Fig. 4.11).

The requirement for mRNA synthesis in the TPA-induced morphological change in UCT-Mel 1 was shown by adding actinomycin D (1 µg/ml

FIGURE 4.10

FIGURE 4.10

Effect of TPA on the morphology of UCT-Mel 1 and 7.

UCT-Mel 1 and 7 cells cultured in the present of TPA showed altered morphological appearances. Microphotographs (a) and (b) show UCT-Mel 7 cells: a) Cells cultured in medium without added compound; b) Cells cultured in the presence of 10 ng/ml TPA for 24 hr. Microphotograph (c), (d) and (e) show UCT-Mel 1 cells:
c) Cells cultured in medium without added compound;
d) Cells cultured in the presence of 10 ng/ml TPA for 24 hr;
e) Cells cultured in the presence of 10 ng/ml TPA for 5 passages, where a small percentage of cells exhibit a stellate morphology. (a)(b)(c) and (d) are at the same magnification. The scale markers in (d) and (e) represent 50 μm. Note that incubation with TPA resulted in cell clumping in UCT-Mel 7 (b), while UCT-Mel 1 cells became elongated with dendritic processes (d and e).

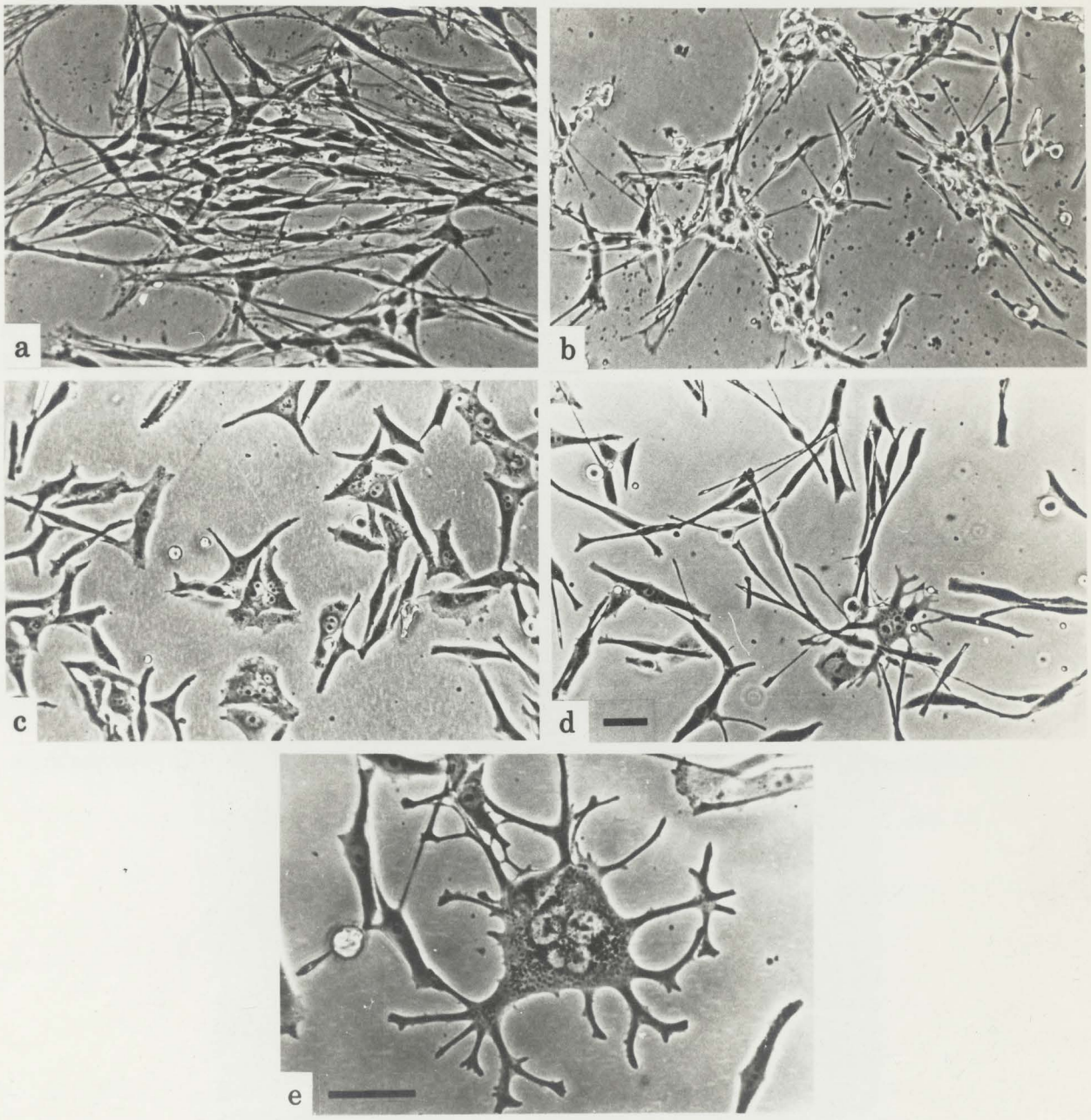


FIGURE 4.10

FIGURE 4.11

FIGURE 4.11

Reversal of TPA-induced morphological change in UCT-Mel 1.

The microphotographs illustrate the morphological appearance of UCT-Mel 1 cells (a) cultured in the absence of TPA; (b) cultured for 48 hr in the presence of 10 ng/ml TPA; and (c) cultured in the absence of TPA for 24 hr following exposure to TPA for 5 passages over 5 weeks. Note the normal triangular morphology of UCT-Mel 1 cells in (a), the dendritic TPA-induced morphology in (b), and the intermediate morphology of the cells in (c). The scale marker in (c) represents 50 μm. All photographs are at the same magnification.

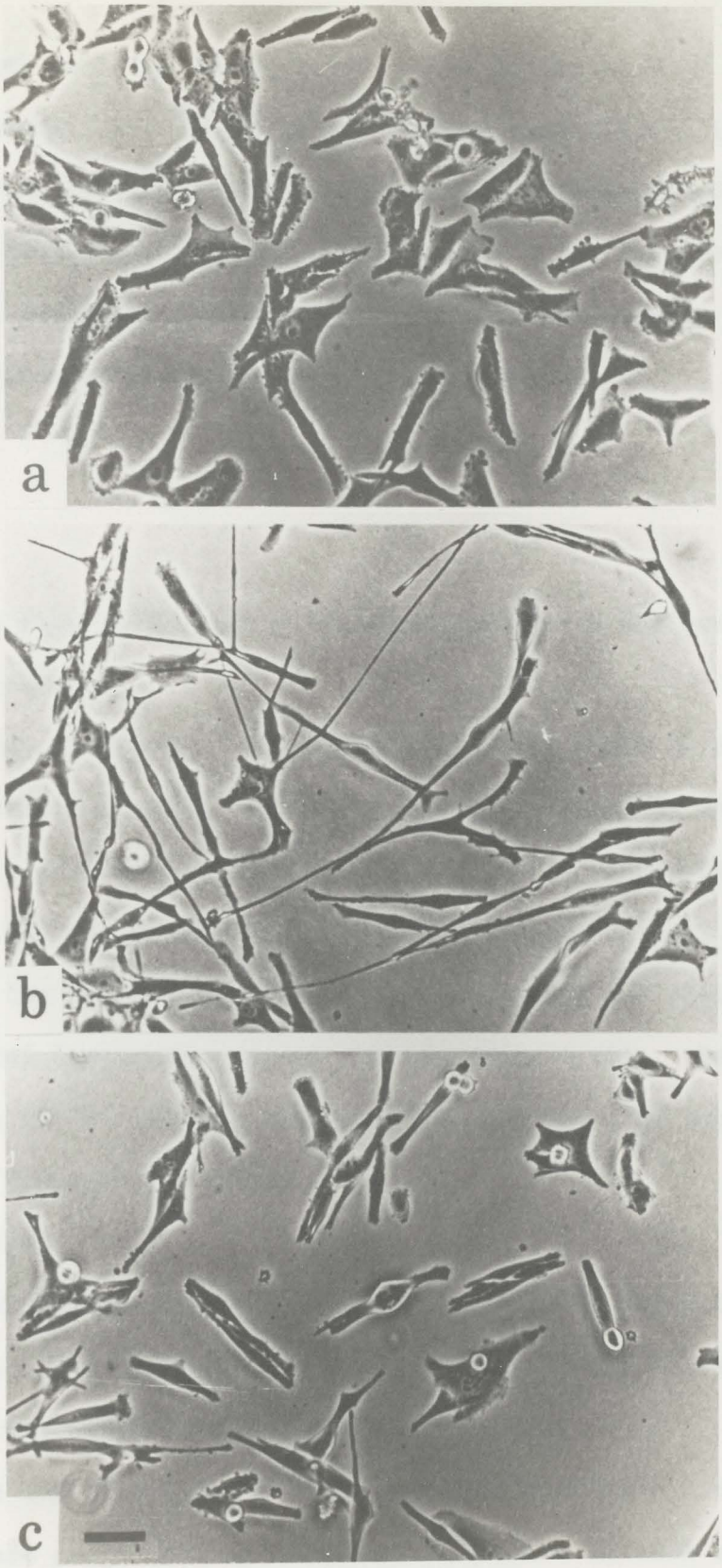


FIGURE 4.11

final concentration) to cells 30 min prior to the addition of 10 ng/ml TPA. After 17 hours, when the dendritic changes were evident in cultures treated with TPA alone, those treated with actinomycin D and TPA exhibited the normal non-dendritic morphology. The results of a similar experiment designed to examine the effect of actinomycin D on TPA-induced inhibition of PA synthesis were inconclusive.

Effects of RA

Growth in vitro

Retinoic acid at 10^{-6} or 10^{-7}M had no effect on the growth rate of UCT-Mel 1 (Fig. 4.12a).

The growth of UCT-Mel 2 was inhibited by 48% after 15 days culture in the presence of 10^{-6}M retinoic acid; no inhibition was observed with 10^{-7}M retinoic acid (Fig. 4.12b). The inhibition of growth seen with 10^{-6}M RA was not due to cytotoxicity as no decrease in cell viability was observed over the two week period of the experiments and cell numbers recovered to control levels when RA was removed (Fig. 4.13). Retinol and retinol acetate at 10^{-6}M inhibited the growth of UCT-Mel 2 by approximately 30% after 11 days (results not shown).

Tyrosinase synthesis

Retinoids had no effect on tyrosinase levels in UCT-Mel 1 (Fig. 4.14a). They had, however, a profound effect on the intracellular concentration of this enzyme in UCT-Mel 2 (Fig. 4.14b).

Retinoic acid added at 10^{-6}M to cultures of UCT-Mel 2 cells inhibited tyrosinase production by 70% after 13 days. Retinol acetate and retinol at 10^{-6}M were also potent inhibitors of tyrosinase (Fig. 4.15). Retinoic acid at 10^{-7} or 10^{-8}M had no inhibitory effects on this enzyme.

It will be recalled from Chapter 2 (Fig. 2.3) that UCT-Mel 2 (as with UCT-Mel 1) pigmented in a density-dependent manner, and that this

FIGURE 4.12

FIGURE 4.12

Effect of RA on growth rates of UCT-Mel 1 and 2.

a) UCT-Mel 1 cells were seeded at 1 x 10^5/35 mm dish. Twenty four hr later, 10^{-6}M RA was added to half the cultures (■- -■) while control cultures received no RA (▲——▲). Fresh medium was added at 48 hr intervals. Duplicate cultures were trypsinized and counted at the times indicated. No inhibition of growth was detected by 10^{-6}M RA or by 10^{-7}M RA (results not shown). Results presented are the means of duplicate determinations in two separate experiments.

b) UCT-Mel 2 cells were seeded at 1.2 x 10^5/35 mm dish. Twenty four hr later, RA at 10^{-6}M (■- -■) or 10^{-7}M (◆·····◆) was added to cultures. Control cultures received no RA (▲——▲). Determination of growth rate was carried out as in (a). Note that 10^{-7}M RA produced no inhibition of growth, while 10^{-6}M RA inhibited growth by 48% at day 15. Results presented are the means of duplicate determinations in six separate experiments.

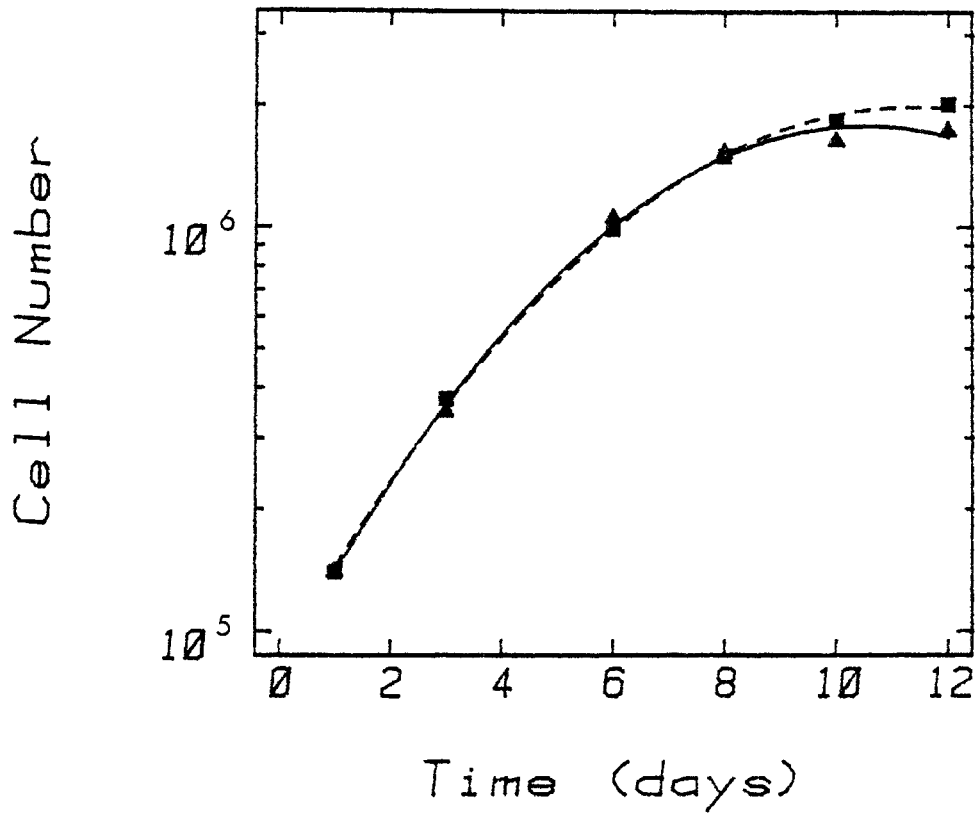


FIGURE 4.12a

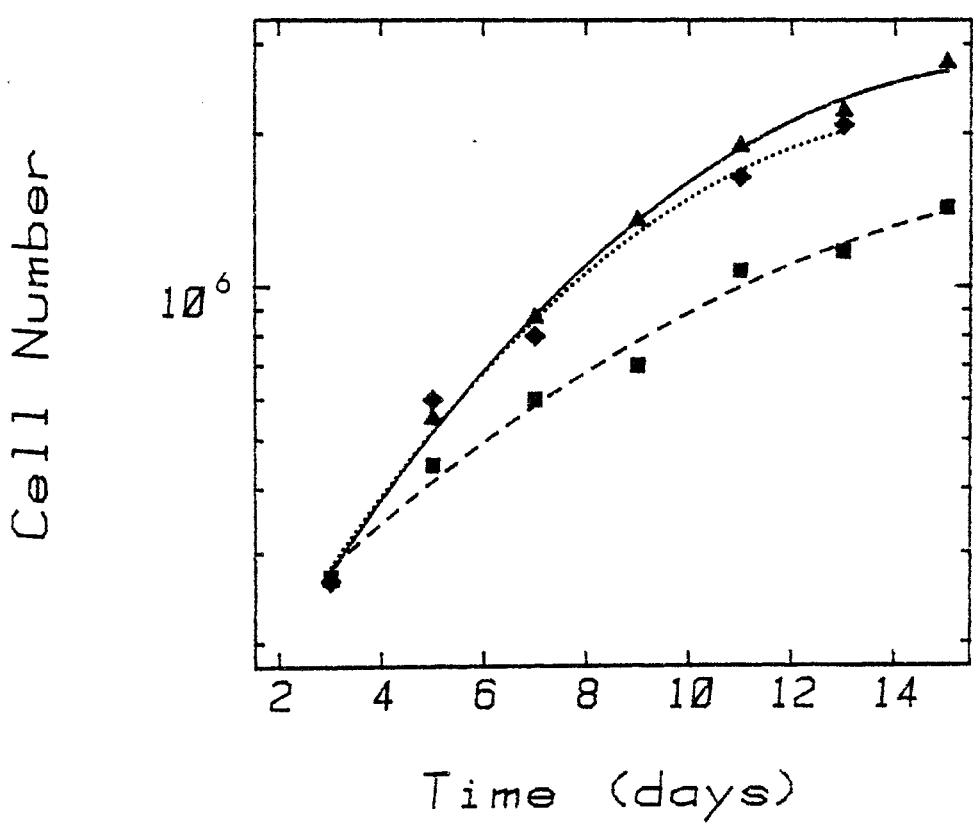


FIGURE 4.12b

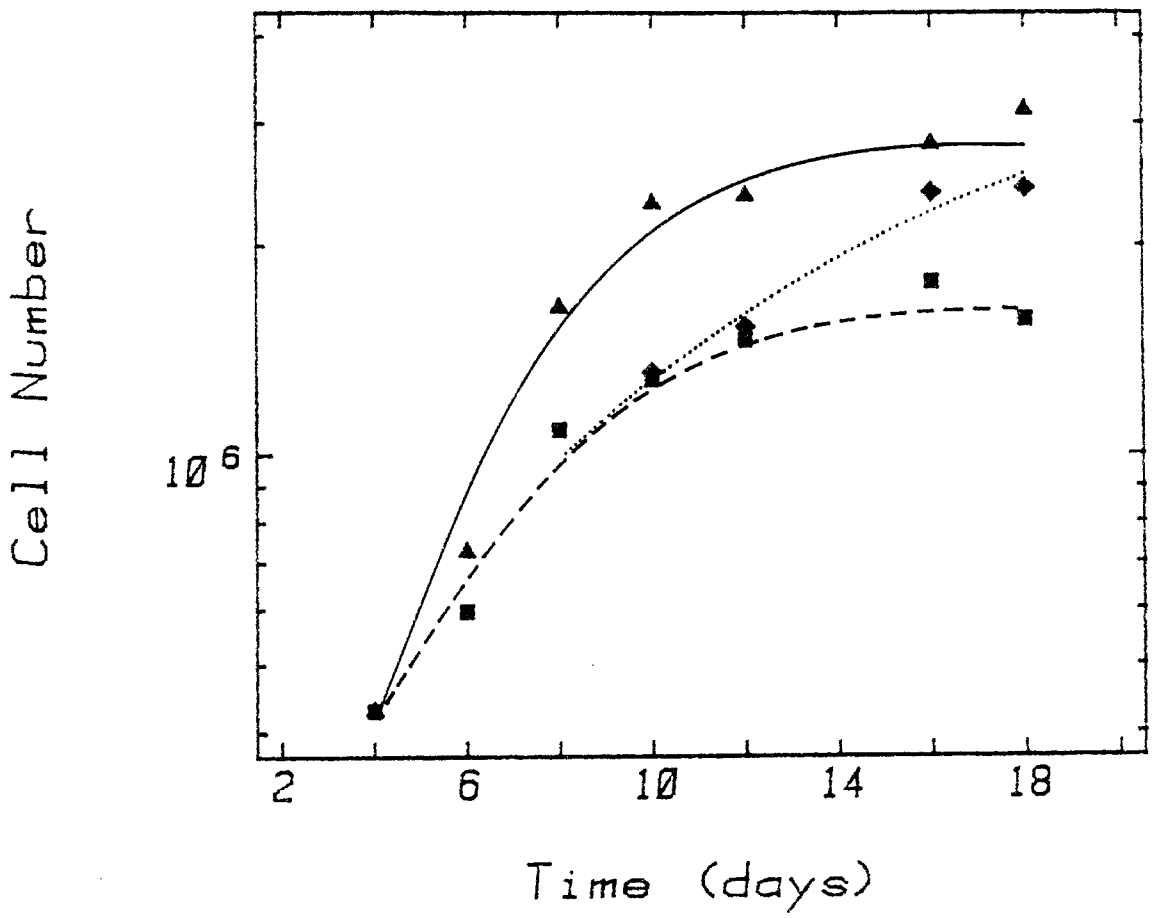


FIGURE 4.13

Reversal of RA-induced growth inhibition of UCT-Mel 2.

UCT-Mel 2 cells were seeded at 1 x 10^5/35 mm dish and 10^{-6}M RA was added to a number of dishes (■--■) 24 hr later (day 1). Fresh medium was added at 48 hr intervals. Control cells received no RA (▲—▲). On day 8, RA was removed from a number of cultures (◆·····◆) which had been exposed to RA from day 1. Duplicate cultures were trypsinized and counted at the times indicated. Not that the cell numbers in the cultures from which the RA had been removed, had recovered to approximately control levels after a 10 day period in the absence of RA.

FIGURE 4.14

FIGURE 4.14

Effects of RA on tyrosinase levels in UCT-Mel 1 and 2.

Results presented were all obtained from dishes with approximately the same total protein concentration, for reasons detailed in the text.

a) UCT-Mel 1: tyrosinase activity in extracts of cells cultured in the absence of RA (Con) or in the presence of 10^{-6}M RA (RA) for 8-11 days. (Total protein per 60 mm dish was 1.2 mg $\pm$ 10%).

b) UCT-Mel 2: as in (a), except that RA was present for 10-15 days. (Total protein per 60 mm dish was 2.2 mg $\pm$ 10%). In both (a) and (b), each line and set of points represents the mean of duplicate results obtained in a separate experiment. Note the marked inhibition of tyrosinase levels induced by RA in UCT-Mel 2, while UCT-Mel 1 cells were unaffected. The scale chosen for the figures was the same as that in Fig. 4.3.

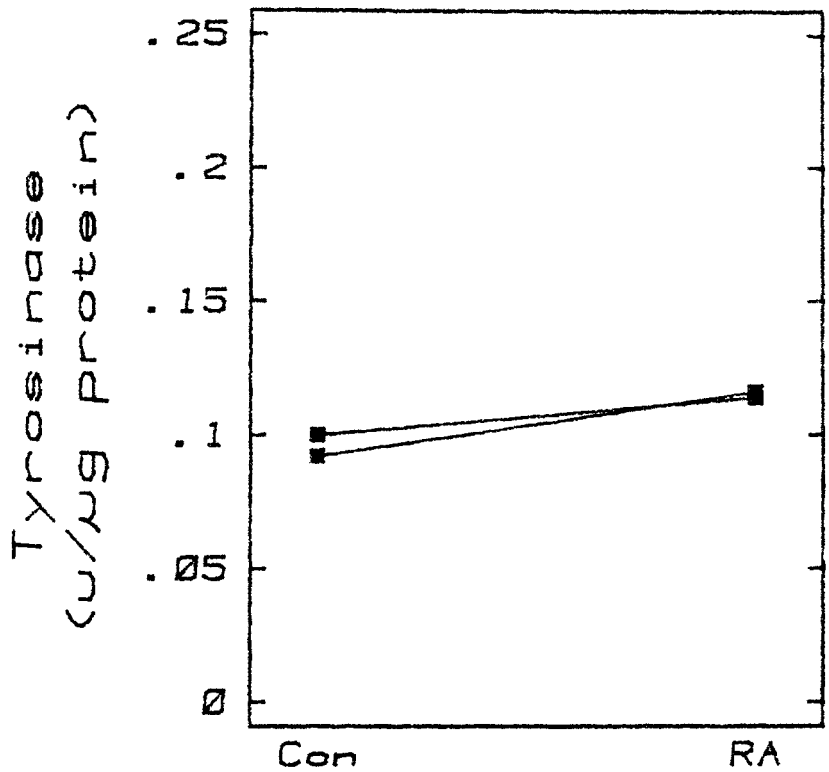


FIGURE 4.14a

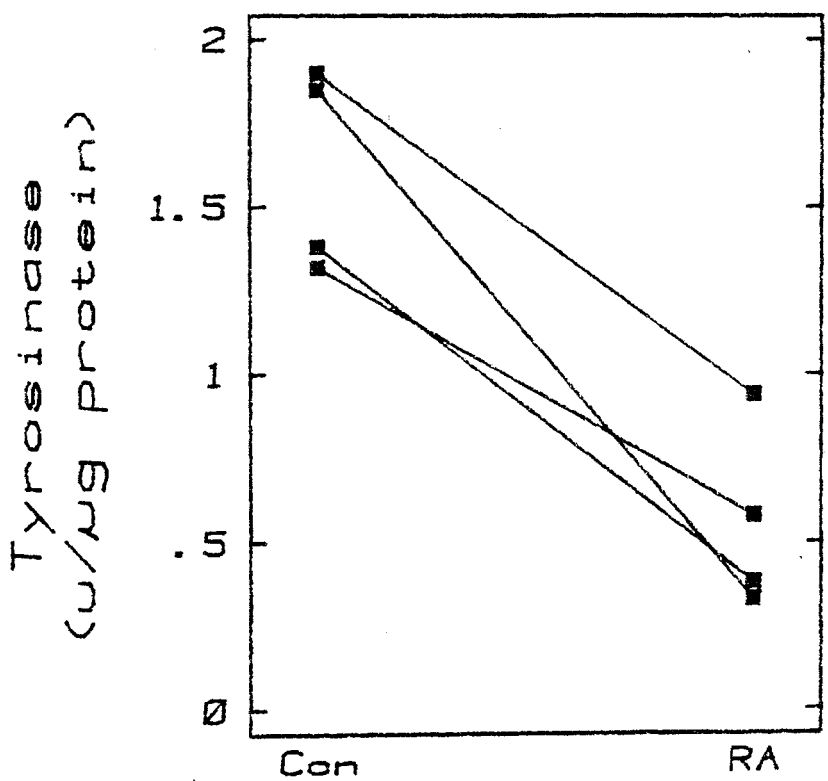


FIGURE 4.14b

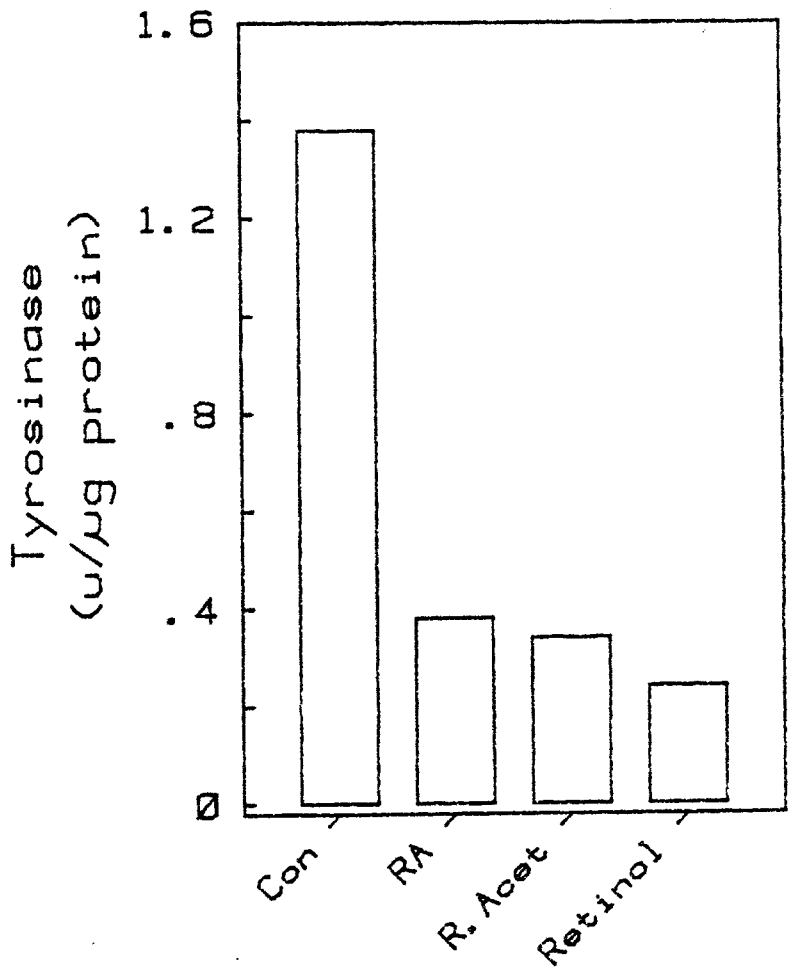


FIGURE 4.15

Inhibition of tyrosinase in UCT-Mel 2 by retinoids.

UCT-Mel 2 cells were seeded at 3.5 x 10^5/60 mm dish (control dishes) or at 5 x 10^5/60 mm dish (test cultures). Twenty four hr later, retinoic acid (RA), retinol acetate (R.Acet) or retinol, all at 10^{-6}M, were added to duplicate test cultures. These were seeded at a higher density in order to compensate for the inhibition of growth caused by retinoids (cf. Fig. 4.12b). Fresh medium was added at 48 hr intervals, and cell lysates were prepared after 14 days. The total protein concentration per dish was approximately 2 mg in all cultures. Note that all three retinoids tested produced marked inhibition of tyrosinase levels in UCT-Mel 2 cells.

pigmentation was preceded by a spontaneous rise in tyrosinase levels as cultures became confluent. The effect of RA on tyrosinase levels in UCT-Mel 2, therefore, was best observed when the compound was added to sparse cultures so that its action could be measured as an inhibition of the increase in tyrosinase levels that occurred spontaneously with increased cell density. The density dependence of the tyrosinase levels also meant that valid comparisons between control and experimental cultures could be made only when they contained equivalent numbers of cells per dish.

Mufson et al (1979) found that TPA delayed the onset of melanogenesis in the C_3 clone of B-16 melanoma by about 2 days. This inhibitory effect was diminished if TPA was added 24 hr or later after plating. In the case of UCT-Mel 2, however, no difference in the inhibition of tyrosinase by RA was found between cultures to which RA had been added either 30 min or 30 hr after seeding.

The inhibition of intracellular tyrosinase accumulation that resulted from exposure to retinoic acid was a reversible phenomenon. Twelve days after removal of the compound tyrosinase levels were completely restored to those observed in control cells and frequently to levels that were slightly higher (Fig. 4.16). Several nonpigmenting melanomas, (UCT-Mel 3, 4, 6 and 7) failed to show any increase in tyrosinase content when exposed to 10^{-6}M retinoic acid for up to 11 days.

Plasminogen activator synthesis and release

Addition of 10^{-6}M retinoic acid to UCT-Mel 1 cells increased the rate of PA secretion from a mean of 36 u/10^6 cells/24 hr to 59u/10^6 cells/24 hr. Although relatively modest, this increase was consistent. It was readily apparent after 72 hr and could still be detected after 12 days. Intracellular concentrations of PA were increased to approximately the same extent by RA when measured 72 hr after addition of the compound (from 28 u/mg protein to 52 u/mg protein).

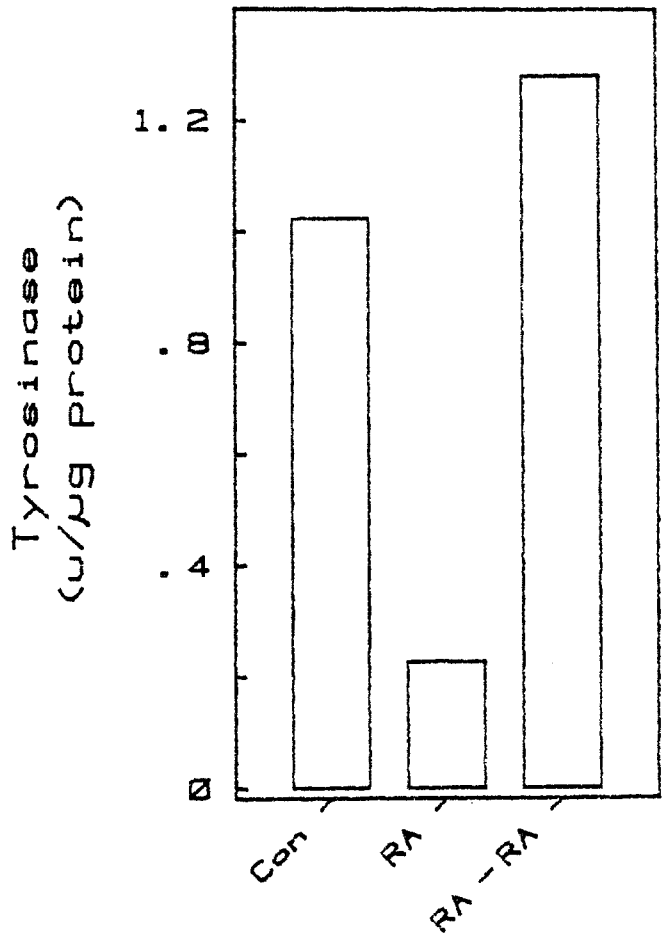


FIGURE 4.16

Reversal of RA-induced tyrosinase inhibition in UCT-Mel 2.

UCT-Mel 2 cells were seeded at 1 x 10^5/35 mm dish. Twenty four hr later, 10^{-6}M RA was added to a number of cultures (RA) and control cells were cultured free of RA (Con). Fresh medium was added at 48 hr intervals. On day 5, RA was removed from some of the cultures which had been exposed to RA for 96 hr (RA-RA). Ten days later cell lysates were prepared from the Con and RA-RA dishes, and 12 days later, from the RA dishes. This protocol yielded total protein concentrations in all dishes in the region of 1.0 mg. Note that, while RA inhibited tyrosinase levels in UCT-Mel 2 cells, the activity in cells from which RA had been removed was restored to levels slightly higher than those observed in control cells. Results presented are the means of duplicate determinations. Similar results were obtained in two experiments.

When cultures of UCT-Mel 2 cells were exposed to 10^{-6}M retinoic acid, the effect on PA release depended upon the duration of the exposure. When enzyme secretion was measured 72 hr after addition of RA, inhibition to 29% of control levels was observed. Some inhibition was still apparent by the sixth day after which, despite the continued presence of RA, enzyme release recovered so that its rate slightly exceeded control levels by day 9, and exceeded control levels by a factor of approximately 8-fold by day 18. This biphasic response with initial inhibition followed by marked stimulation of enzyme release was consistently observed in 3 experiments and is shown in Fig. 4.17.

Retinoic acid at 10^{-7}M, retinol and retinol acetate had an inhibitory effect similar to that observed with 10^{-6}M retinoic acid when studied at day 5, although this was not as marked (Fig. 4.18). The stimulatory effects of retinoic acid were reversible in that, when the compound was removed from the cultures on day 5 or day 8, a fall in the rate of PA release to approach control values was observed after a further 10 days (Fig. 4.19).

Morphology

Retinoic acid at 10^{-6}M and 10^{-7}M had a pronounced effect on the morphology of UCT-Mel 2. The usual dendritic appearance of the cells became accentuated so that after 3 days in cultures they appeared frankly neuritic in character (Fig. 4.20). Retinol and retinol acetate at 10^{-6}M had a similar, but less marked effect.

Despite prolonged exposure to 10^{-6}M retinoic acid, UCT-Mel 1 cells showed no obvious morphological change. The morphology of the non-pigmenting melanoma lines (UCT-Mel 3, 4, 5, 6 and 7) were similarly unaffected by exposure to the compound.

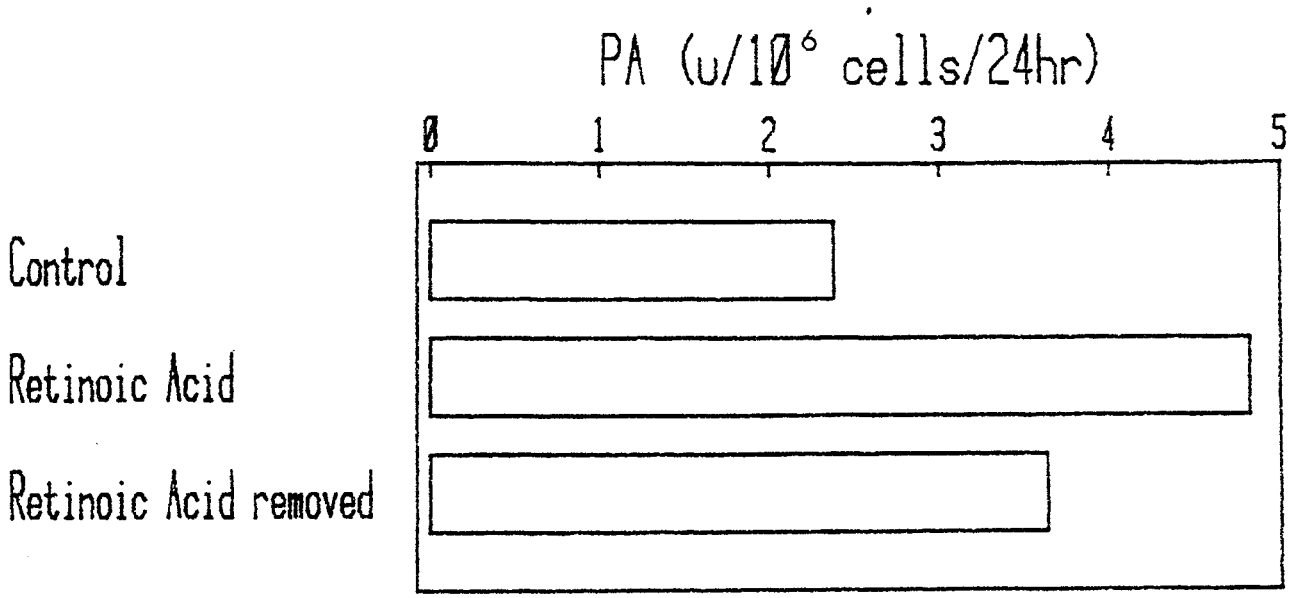


FIGURE 4.19

Reversal of PA stimulation in UCT-Mel 2 by RA.

UCT-Mel 2 cells were seeded at 1 x 10^5/35 mm dish. Retinoic acid at 10^{-6}M was added 24 hr after seeding. Control dishes were not exposed to RA. Fresh medium was added at 48 hr intervals. On day 5, RA was removed from duplicate cultures that had been exposed to RA from day 1. Ten days later, harvest fluids were prepared from these three sets of cells. Note that the RA-induced stimulation of PA levels was still evident in cells from which the RA had been removed for 10 days, but that the release of PA by these cells approached control levels.

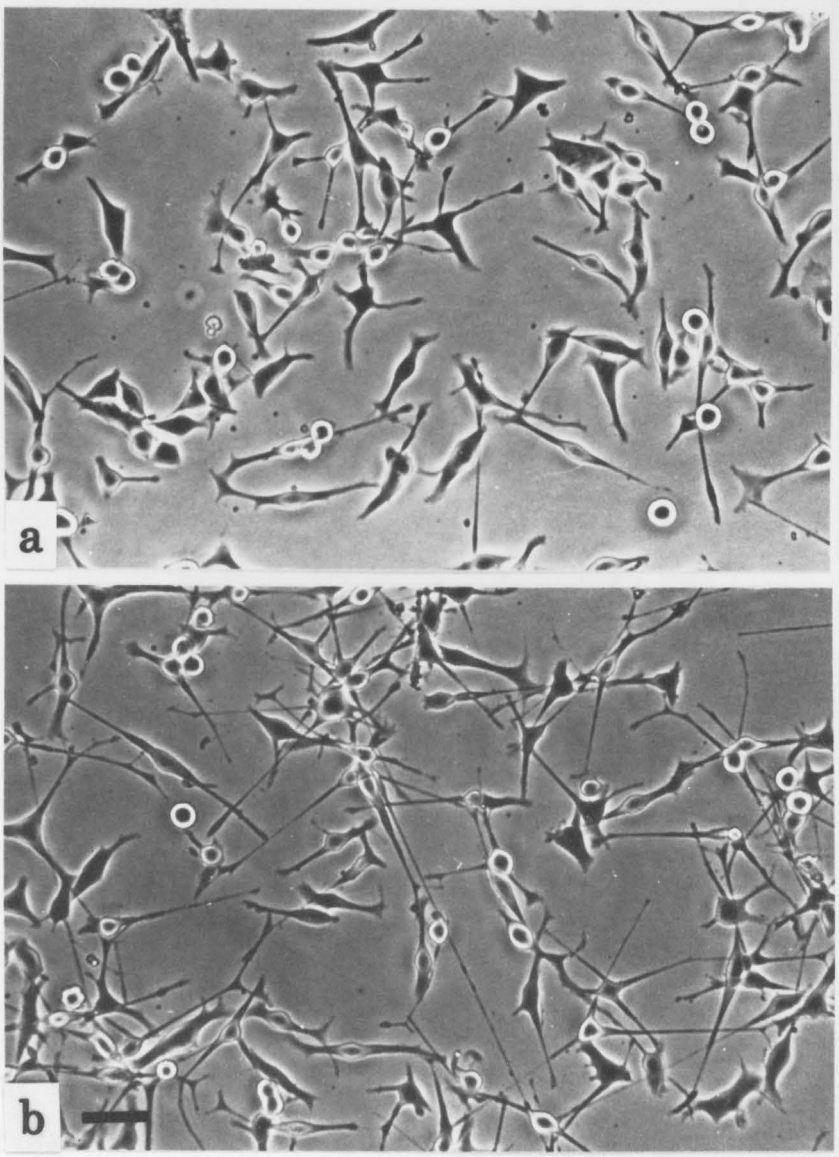


FIGURE 4.20

Effect of retinoic acid on the morphology of UCT-Mel 2

The above microphotograph shows the morphological appearance of UCT-Mel 2 cells cultured in (a) DB-FC 10 medium; or (b) in DB-FC 10 medium containing 10^{-6}M RA for 48 hr. The scale marker in (b) represents 50 μm. Both photographs are at the same magnification. Note the induction of a dendritic morphology by RA.

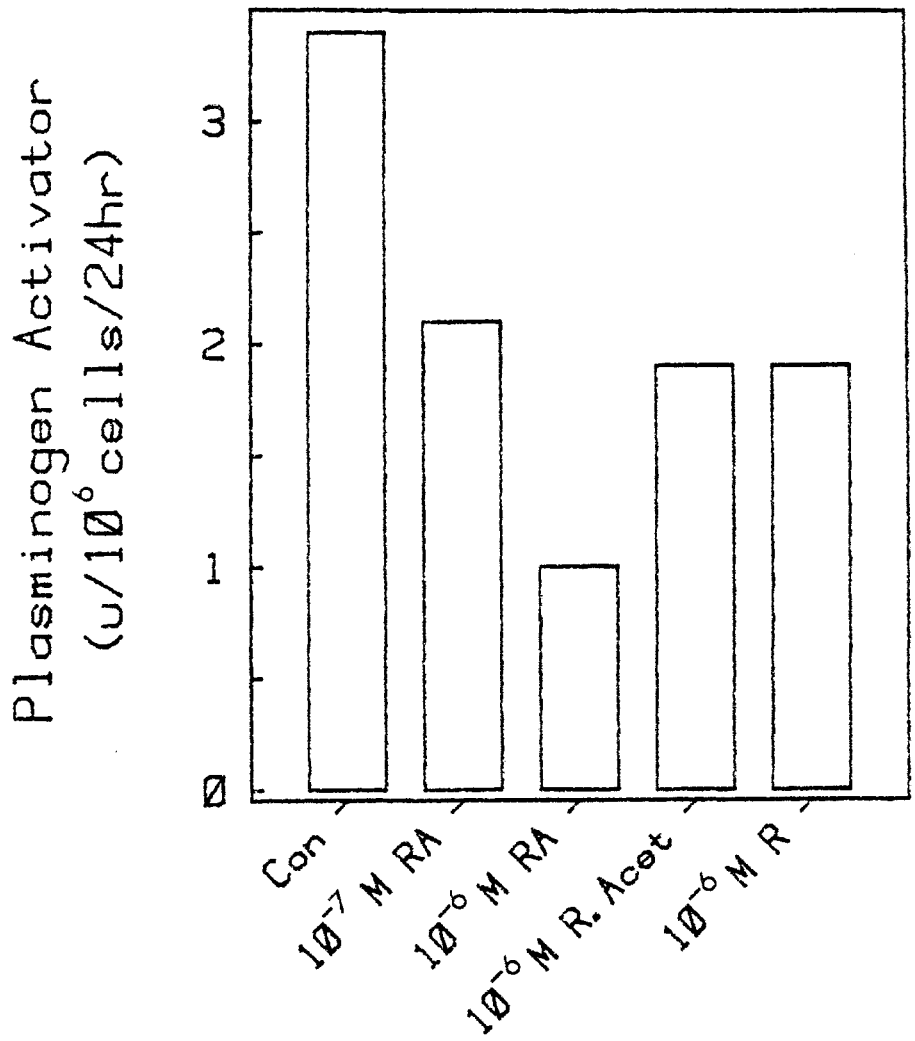


FIGURE 4.18

Effect of retinoids on PA in UCT-Mel 2.

UCT-Mel 2 cells were seeded at 1.2 x 10^5/35 mm dish. Twenty four hr later, retinoic acid (RA), retinol acetate (R.Acet) or retinol (R) was added at the concentrations indicated. Control cultures (Con) were not exposed to retinoids. Fresh medium was added at 48 hr intervals. Harvest fluids were prepared after a further 72 hr. Note that the inhibition of PA by retinoic acid was dose-dependent, and that retinol and retinol acetate were less effective inhibitors of enzyme secretion.

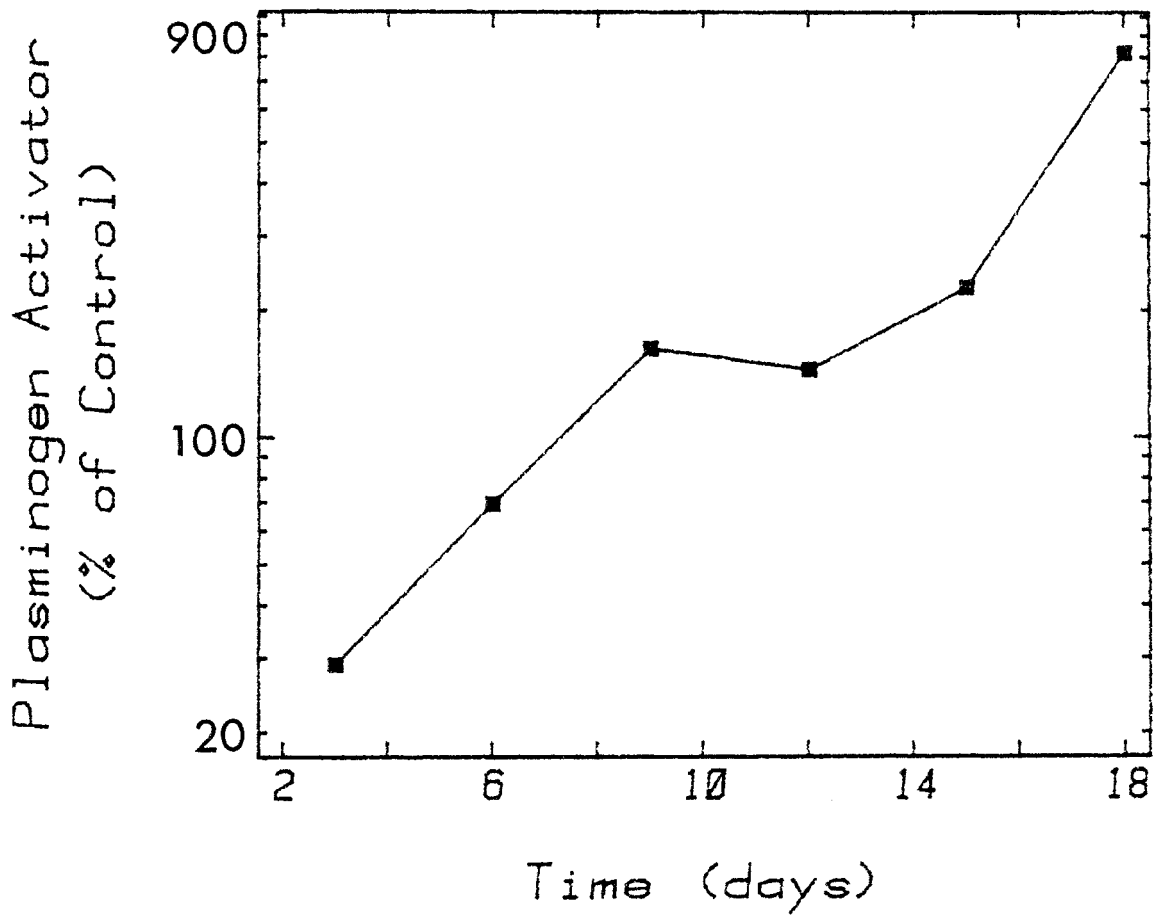


FIGURE 4.17

Effect of RA on PA levels in UCT-Mel 2 cells.

Retinoic acid was added to UCT-Mel 2 cells 24 hr after seeding, and at the indicated times, 24 hr harvest fluids were collected from control cultures and cultures exposed to RA. Fresh medium was added at 48 hr intervals. Results obtained for RA-treated cultures are expressed as the percentage of the control PA levels determined over the same 24 hr period. The mean results of duplicate determinations from 3 separate experiments are presented. Note the biphasic response of PA levels to RA characterised by a marked inhibition relative to control levels in the early stages, and progressing to marked stimulation in the later stages.

Lactate dehydrogenase

To evaluate the significance of the effects of RA on cellular enzyme economy in terms of the specificity of the response observed, effects upon the constitutive cellular enzyme LDH, were measured at the same time. In six experiments in which 10^{-6}M RA was present for 10-17 days, no significant effect on intracellular LDH levels could be measured (Fig. 4.21).

DISCUSSION

The experimental results I present in this chapter were intended to examine the feasibility of using melanoma cells as models for *in vitro* differentiation. This proved to be difficult, and the successes that I achieved were preliminary and limited - largely because melanoma cells themselves are so variable, and differentiation, as a biological phenomenon is difficult to define and to understand. It frequently happens that in developing an experimental approach to a complex subject, one is compelled to reduce its complexities to proportions that are manageable, not only observationally and technically, but also in the conceptual sense.

Nowhere is this need for practical and conceptual reductionism more prominent than in the formulation of constructive approaches to the question of how it is that higher organisms generate diverse and individually specialized sets of cellular progeny from a single stem cell.

In focusing on the mechanisms involved in the acquisition of mature and distinctive characteristics by melanoma cells in culture, I have chosen to base my approach on the assumption that "differentiation" involves the following:

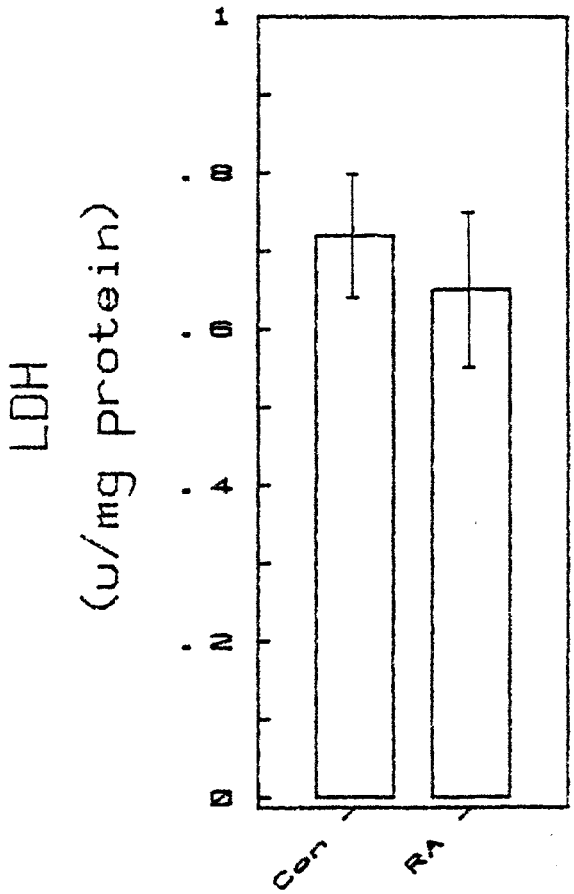


FIGURE 4.21

Effect of RA on LDH levels in UCT-Mel 2.

Lactate dehydrogenase values were determined in the same cell lysates used to assay tyrosinase activity. The histogram represents the means of 24 determinations each on control and RA-treated cell lysates. The vertical bars represent 2 x standard error of the mean. Note that there is no significant difference in LDH levels between control and RA-treated cells.

- *the induction of the biochemical and organellar apparatus required for melanin synthesis.*

This is a unique and distinctive feature of the melanocyte that involves the synthesis of tyrosinase and melanosome assembly. This subject has been extensively reviewed (Breathnach, 1969; Pawelek, 1976; Jimbow et al, 1976; Hearing et al, 1980) and there would seem to be little doubt that tyrosinase is the rate-limiting enzyme in cellular melanin synthesis. Measurement of this enzyme may therefore be justifiably regarded as a reliable means of measuring the extent to which commitment to differentiation has taken place.

- *the acquisition of a dendritic morphology that is characteristic of the mature melanocyte.*

Epidermal pigment cells are known to arise from the primitive neural crest from which they migrate in early embryonic life to establish themselves permanently in junctional regions. Their neural provenance is evident in mature cells in their content of S-100 protein (Gaynor et al, 1980), a gene product that is believed to be exclusively expressed by cells of neuro-ectodermal origin; and by their characteristic shape with ramifying processes extending from the cell body. Since I was unable to measure S-100 protein content, which may correlate with differentiation in melanoma cells as it does in the nervous system (Herschman et al, 1971) I relied upon the less definitive, but nevertheless readily discernible, differentiation marker of morphological change.

- *a change in the kinetics of cellular proliferation.*

Although less well defined and seldom explicitly regarded as a necessary concomitant of cellular differentiation, it is apparent from the writings of many who have studied this problem, that differentiation and proliferation are regarded to a greater or lesser extent, as mutually exclusive phenomena (Silagi, 1969; Hu, 1972; Schachtschabel et al, 1970).

Examples of the extreme situation are provided by the mature granulocyte in the peripheral blood or the fully differentiated neuronal cell, both of which have specific, well-defined functions to perform, but neither of which is able to divide under any circumstances.

An intermediate position is exemplified by the epidermal basal stem cell or the normal erythroid precursor in bone marrow that is differentiated to the extent that its function is to divide *asymmetrically*. By this I mean a pattern of cellular division in which one of the progeny is committed to terminal differentiation while the other daughter cell retains the proliferative potential of the parent.

One might consider, at the other extreme, a normal cell, such as a fully differentiated renal or hepatic cell, that is triggered to divide "symmetrically" and therefore exponentially, by injury and the need for repair. During the period of regeneration, both daughter cells derived from division would retain the ability to multiply and yet would continue to express features of the differentiated progenitor cell. These features may be obvious and readily detectable (e.g. the expression of a specific protein) or they may be subtle and evident only as the ability of the exponentially dividing cells to respond to appropriate regulatory stimuli that would induce a return to asymmetrical division or to G_1 arrest.

Accepting these three patterns, therefore, one may have as a *concomitant* of differentiation rather than a manifestation of the process, growth that is exponential, asymmetrical or arrested. Generally speaking, however, a move towards further differentiation would be likely to be associated with a move towards a diminished growth rate, and I have hence used this as a criterion for induction of differentiation.

Some of the above patterns are considered by Levenson and Housman (1981) in their discussion of commitment.

- *changes in the rate of PA synthesis.*

The grounds for regarding PA synthesis as a cellular function that is in any way related to differentiation are slender. As I have indicated earlier in this thesis the rate of synthesis of this enzyme is an inducible cellular function that has been circumstantially associated with tumorigenesis and hence, by inference, "dedifferentiation" (Rifkin et al, 1974; Pollack et al, 1974; Christman et al, 1975a,b; Jones et al, 1975; Mak et al, 1976; Silagi, 1976; Nagy et al, 1977). Of possibly greater value is the fact that, in general, compounds that affect the rate of enzyme synthesis do so by acting at a genetic level, and hence one can ascribe effects of drugs that affect PA release to processes that occur at the significant level of transcription and translation of new mRNA.

If one accepts these simplified concepts of differentiation as valid, the results of experiments that I report are interesting and, in some ways, conflicting.

In the first instance, they emphasize yet again the variability between different melanoma cell lines that was apparent not only in the extent to which they displayed differentiated characteristics, but also in the extent to which they responded to TPA and RA. Thus, UCT-Mel 1 was much more susceptible to the actions of TPA than was UCT-Mel 2 whereas the reverse was true in the case of RA. The other melanoma cell lines that were studied in less detail, showed similar responses that varied from one line to the next.

Secondly, when it has been applied to both human and animal cells in culture, TPA has usually induced an increased synthesis of PA (Wigler and Weinstein, 1976; Loskutoff and Edgington, 1977; Vassalli et al, 1977; Granelli-Piperno et al, 1977; Weinstein et al, 1977). In the experiments I report TPA inhibited PA release by UCT-Mel 1 cells in a manner that was

dose-dependent and reversible. This somewhat paradoxical effect of TPA and melanoma cells may be related to the molecular species of PA that they synthesize. Christman et al (1978) found that TPA enhanced the production of one antigenic type of PA in hamster cells, and had no effect on PA of a different antigenic form in these cells, and Jaken and Black (1981a, b) showed a preferential enhancement of 49K as opposed to 75K PA activity in TPA-treated 3T3 cells. Furthermore, it has been suggested by Roblin and Young (1980) that the tissue-type activator is less modulatable by dexamethasone than is the UK-type enzyme. Thus the two immunochemical types of human PA may respond differently to TPA. The urokinase type enzyme appears to be stimulated by TPA and in some instances, such as UCT-Mel 1, the melanoma enzyme is inhibited by this agent.

The reversibility of TPA effects has also been observed by other workers (Wigler and Weinstein, 1976; Yamasaki et al, 1977).

Being a cocarcinogen, one might have expected TPA to cause dedifferentiation. Conversely, one might have expected RA, as an agent that causes ectodermal differentiation, to have induced changes in melanoma cells that were characteristic of maturation.

The results that I obtained neither confirmed, nor entirely refuted, these predictions. Tetradecanoyl phorbol acetate caused UCT-Mel 1 to *differentiate* as measured by decreased proliferation, diminished PA release, and the acquisition of a dendritic morphology. On the other hand, it had only a slight stimulatory effect on tyrosinase synthesis. The compound caused a modest increase in the growth rate of UCT-Mel 2 but had no effect upon any of the other parameters of differentiation. Recently, a number of reports have indicated that TPA may stimulate differentiation in some malignant or transformed cells (Miao et al, 1978; Nagasawa and Mak, 1980; Huberman and Callaham, 1979; Rovera et al, 1979a, b; Newburger et al, 1981)

including two human melanoma cell lines (Huberman et al, 1979; Tveit et al, 1980b). The differentiation induced by TPA in the HO line (Huberman et al, 1979) included morphological changes, cessation of cell growth, and stimulation of melanin synthesis.

The morphological changes in UCT-Mel 1 may have been due to an unrelated effect. Payette et al (1980) found that the TPA-induced processes elaborated by chick embryo neural crest cells differed in their cyto-skeletal structure from the dendritic processes of normal melanocytes.

The effects of RA were, in many ways, similarly conflicting. While it had very little effect on UCT-Mel 1, the compound induced apparent differentiation in UCT-Mel 2 with retardation of *in vitro* proliferation and the assumption of a dendritic morphology. On the other hand, RA caused a profound inhibition of intracellular tyrosinase and led to a greater than 8-fold increase in the rate of PA release. As far as effects of RA on PA are concerned, reports in the literature have shown both decreases (Vassalli et al, 1976) and increases (Wilson and Reich, 1978; Miskin et al, 1978; Strickland, 1979; Wilson and Dowdle, 1980) in the rate of release of this enzyme after exposure to retinoids, depending upon the cell type studied. With regard to the "biphasic" response seen with PA secretion in RA-treated UCT-Mel 2 cells, a similar variation of fibrinolytic activity during the cell growth curve was noted in 3T3 and SV3T3 cell cultures (Chou et al, 1974). They found that, depending on whether the cells were growing or quiescent, the fibrinolytic activity of the SV40-transformed 3T3 cells was less than, equal to, or greater than that of untransformed 3T3 cells.

The growth rates of melanoma cells *in vitro* may be either inhibited (Lotan et al, 1978; Patt et al, 1978; Meyskens and Fuller, 1980) or stimulated (Lotan, 1979) by culture with retinoids. My findings are therefore not entirely unusual in these respects.

On the other hand, other authors who have studied the effects of RA on human melanoma cells, have reported an increase in melanogenesis

that was consistent with the canonical view of retinoids as agents of differentiation. The results that I obtained were quite clearly contrary to those of others (Lotan, 1979; Lotan and Lotan, 1980; Tveit et al, 1980b; Meyskens and Fuller, 1980) in this respect.

Although one cannot justifiably extrapolate from the results of experiments of this sort under artificial *in vitro* conditions to the clinical situation that obtains *in vivo*, my findings do indicate that melanoma cells differ, and this may have important applications for the use of retinoids in the management of this disease.

CHAPTER 5

THE INTERACTION BETWEEN MELANOMA PLASMINOGEN ACTIVATOR AND SKIN FIBROBLASTS

At a fairly early stage in this work I made the observation that, as UCT-Mel 1 cells approached confluence *in vitro*, their cellular release of PA diminished, tyrosinase increased and pigmentation ensued (Chapters 2 and 3). I had no difficulty in showing that the apparent decrease in PA release with confluence was a function of cell density and not time in culture. I therefore assumed that physical cell-cell interactions were required for this phenomenon which I saw as a biochemical counterpart to "contact-inhibition".

Kyner et al (1975, 1978) reported a similar interaction between cells derived from a PA-producer clone of the B16 murine melanoma and a clone that did not produce PA. When co-cultivated, the PA-nonproducer cells inhibited release of PA by the PA-producer cells by a mechanism that required intimate cellular contact. These observations, taken together with the knowledge that direct intercellular communication involving the transfer of small molecules between cells through gap junctions was a well-described phenomenon (Gilula et al, 1972; Fentiman and Taylor-Papadimitriou, 1977), led me to propose that the fall in PA release I had observed involved the intercellular passage of "regulatory" molecules that suppressed enzyme synthesis and release.

Although, as I show later in this section, this idea proved to be erroneous, it was nevertheless useful in that it led me to perform the experiments to determine whether or not other cell types would substitute for melanoma cells as inhibitory partners in co-cultivation. These experiments showed that fibroblasts were in fact far more effective in

this respect and I accordingly embarked upon a programme of research that was designed to study this phenomenon.

In this chapter I record the results of these experiments more or less in the chronological order of the way in which they were conceived and performed. My earlier experimental approach was dictated by the misguided notion that fibroblasts elaborated a labile "hormone-like" substance that acted upon melanoma cells at a genetic level to suppress PA synthesis by mechanisms involving transcription and translation of mRNA.

The later phase of the work comprises experiments that were performed after the true nature of the phenomenon had become apparent, and I realised that the fall in PA content of medium covering melanoma cells as a result of fibroblast co-cultivation was due to the fact that fibroblasts had a particular tendency to bind PA present in the medium.

RESULTS

Co-cultivation of normal adult skin fibroblasts with melanoma cells led to an apparent diminution in the rate of melanoma PA release.

The initial experiments that established the existence of the "fibroblast inhibitory phenomenon" gave results which are summarized in Fig. 5.1. In these experiments, melanoma cells from three different lines (UCT-Mel 1, 2 and 3) were seeded in duplicate and at 3 different densities ranging from 5×10^4 to 7×10^5 cells per 35 mm culture dish. An additional set of duplicate dishes received melanoma cells at the lowest density together with 7×10^5 normal adult skin fibroblasts. The cells were covered with complete medium and cultured for 48 hr, after which serum-free harvest fluids were prepared for PA assay.

As can be seen from the results, UCT-Mel 1 and 3 showed a

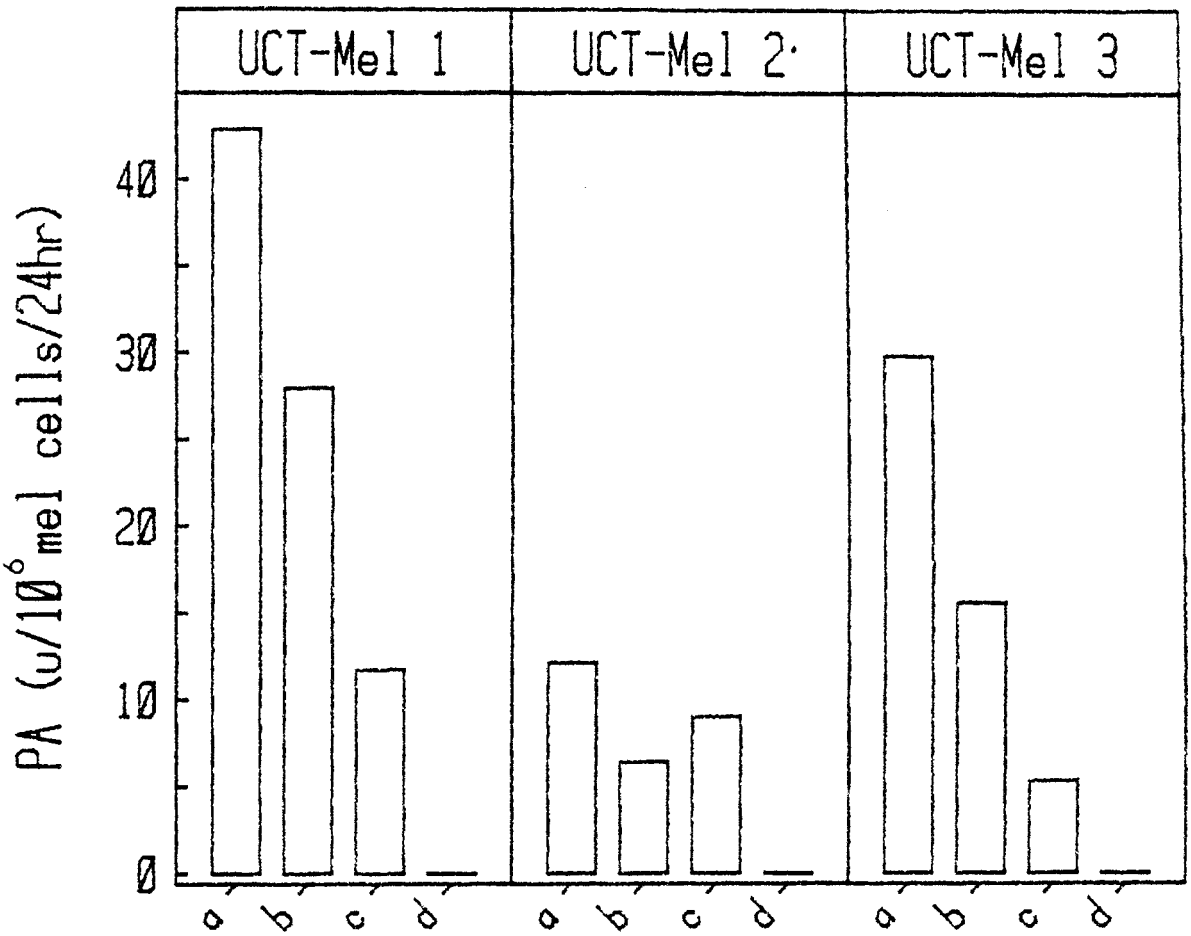


FIGURE 5.1

Cocultivation of fibroblasts with melanoma cells.

UCT-Mel 1, 2 and 3 cells were seeded in duplicate at 7 x 10^5 (a), 2 x 10^5(b) and 5 x 10^4(c) cells per 35 mm dish. On additional duplicate dishes 5 x 10^4 melanoma cells were seeded together with 7 x 10^5 skin fibroblasts (d). The above figure depicts the complete inhibition of the melanoma PA in all three cell lines at this cell ratio. Note that UCT-Mel 1 and 3 exhibit a decrease in PA secretion with increasing cell number, while UCT-Mel 2 does not show this effect.

characteristic decrease in cellular activator release with cell density. This effect was not observed with UCT-Mel 2. In all three cases, however, the presence of fibroblasts in the adherent cellular monolayer rendered PA activity in the serum-free harvest fluid undetectable.

This effect could not be ascribed to inhibition of melanoma cell growth by fibroblasts since viable melanoma cells and fibroblasts were clearly distinguishable under phase contrast microscopy in the monolayers. Furthermore, the total number of cells trypsinized from the co-cultivation dishes was equal to the sum of the cells released when melanoma cells and fibroblasts were cultivated independently.

The magnitude of the fibroblast effect depended on the fibroblast to melanoma cell ratio.

In order to define the fibroblast-melanoma cell interaction in quantitative terms, experiments were performed in which UCT-Mel 1 or 2 cells were seeded at the same density into 35 mm tissue culture dishes together with varying numbers of fibroblasts to give melanoma to fibroblast cell ratios (M:F) ranging from 1:1 to 1024:1.

The cells were incubated in complete medium for 24 hours after which serum-free harvest fluids were prepared for PA assay. As can be seen from the results summarized in Figs. 5.2 and 5.3 fibroblasts appeared to inhibit PA release in a concentration-dependent manner. In the case of UCT-Mel 1, the effect was detectable at an M:F of 16:1 and complete at an M:F of 1:1. Similar results were observed with UCT-Mel 2. These cells, however, were approximately four-fold more sensitive than were UCT-Mel 1 cells in that the inhibition was detectable at an M:F of 64:1 and was complete at an M:F of 4:1.

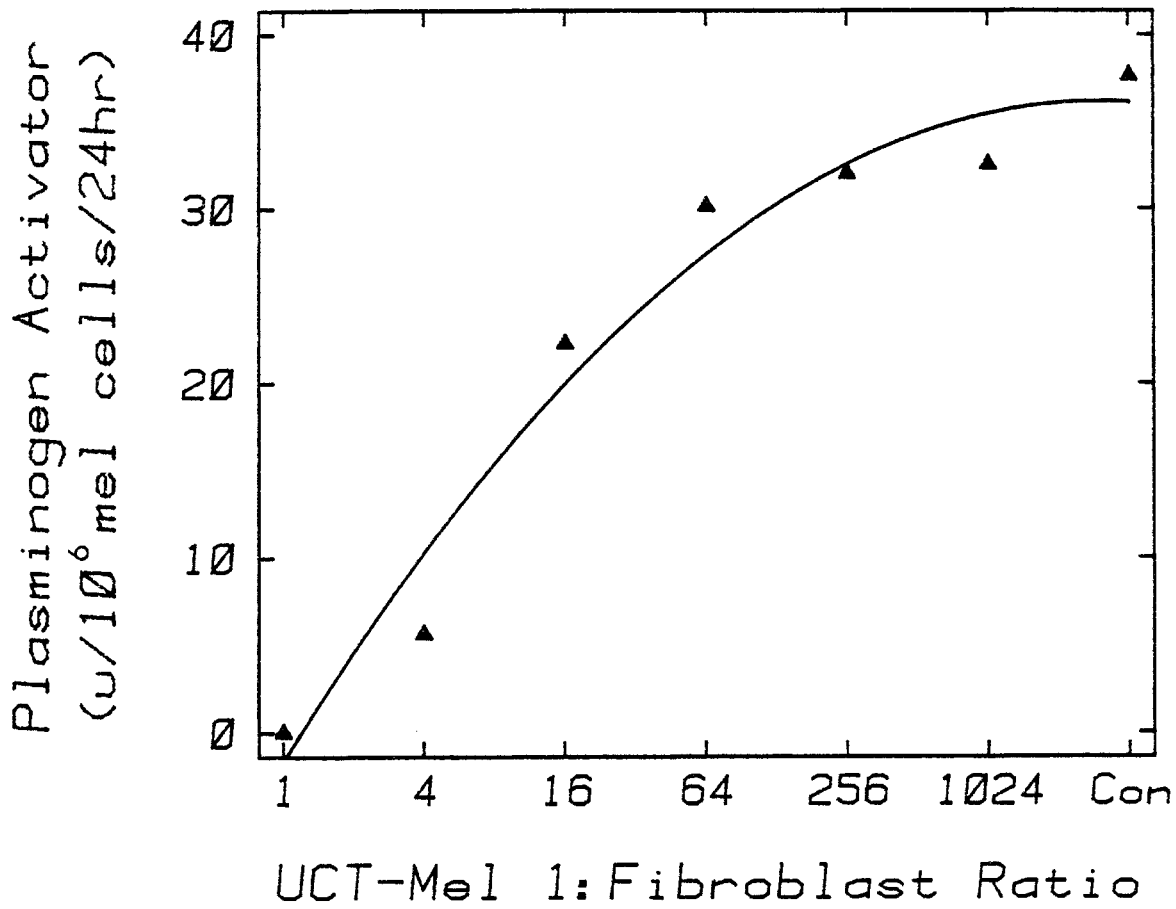


FIGURE 5.2

The magnitude of the fibroblast effect depended on the ratio of UCT-Mel 1 cells to skin fibroblasts.

UCT-Mel 1 cells were seeded at 5 x 10^4 cells/35 mm dish. Skin fibroblasts were cocultivated with the melanoma cells, at 5 x 10^4 cells/35 mm dish, and four-fold dilutions thereof, resulting in melanoma to fibroblast ratios ranging from 1:1 to 1024:1, as indicated. Control cultures consisted of UCT-Mel 1 cells seeded alone. Note that the degree of inhibition depended on the M:F ratio. An M:F ratio of 1:1 resulted in complete inhibition of melanoma PA, and inhibition was discernible at an M:F ratio of 16:1.

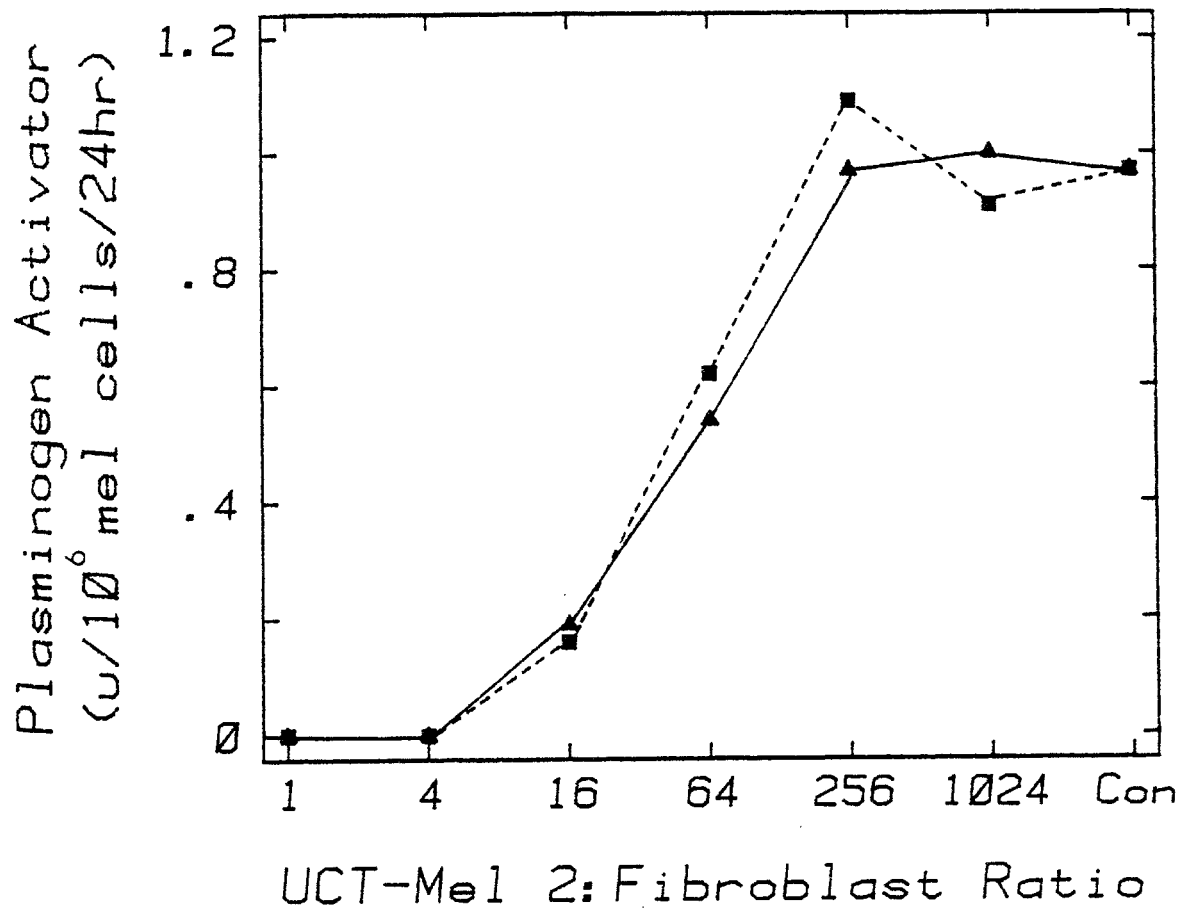


FIGURE 5.3

The magnitude of the fibroblast effect depended on the ratio of UCT-Mel 2 cells to adult skin fibroblasts or foreskin fibroblasts.

UCT-Mel 2 cells were seeded at 5 x 10^6 cells/35 mm dish. Adult skin fibroblasts (▲—▲) or foreskin fibroblasts (■--■) were cocultivated with the melanoma cells, at 5 x 10^4 cells/35 mm dish, and four-fold dilutions thereof, resulting in melanoma to fibroblast ratios ranging from 1:1 to 1024:1 as indicated. Control cultures consisted of UCT-Mel 2 cells seeded alone. Note that, as in Fig. 5.2, the degree of inhibition depended on the M:F ratio. UCT-Mel 2 cells, however, were 4-fold more sensitive to inhibition than were UCT-Mel 1 cells (see Fig. 5.2). An M:F ratio, in this case, of 4:1 resulted in complete inhibition of melanoma PA, and inhibition was detectable at an M:F ratio of 64:1.

The inhibitory effect was not confined to skin fibroblasts but was demonstrable with fibroblasts derived from other tissues.

Having observed the effects with normal adult skin fibroblasts, it was of interest to determine whether or not fibroblasts from other tissues would be equally effective in their ability to inhibit PA release.

Two sets of experiments were performed to examine this question. In the first, UCT-Mel 1 cells at a constant density per dish were seeded with serial four-fold dilutions of neonatal foreskin fibroblasts, human embryo skin fibroblasts and skin fibroblasts obtained from an adult patient at the time of removal of a primary melanoma. UCT-Mel 1 cells were also co-cultivated with human embryo lung and human embryo heart fibroblasts at M:F's of 1:1 and 7:1 respectively.

In the second set of experiments, UCT-Mel 2 cells were co-cultivated with serial dilutions of foreskin fibroblasts.

Of all the fibroblasts used in these two sets of experiments, only human embryo lung fibroblasts released detectable PA. Since this was of the urokinase-type and did not cross-react immunochemically with melanoma-type PA, an antiserum to urokinase could be used in the assay for melanoma-type PA in the serum-free harvest fluid.

The results of these experiments are summarized in Figs. 5.3 and 5.4 from which it can be seen that all of the fibroblasts tested were approximately equally effective in their ability to produce apparent inhibition of the melanoma cell PA release.

Cells other than fibroblasts were less effective in inhibiting PA.

To determine whether or not the ability to inhibit melanoma cell activator was an exclusive property of fibroblasts, three other cell types were tested. Two of these, UCT-Ca 2 and UCT-Gli 1 were cell lines derived

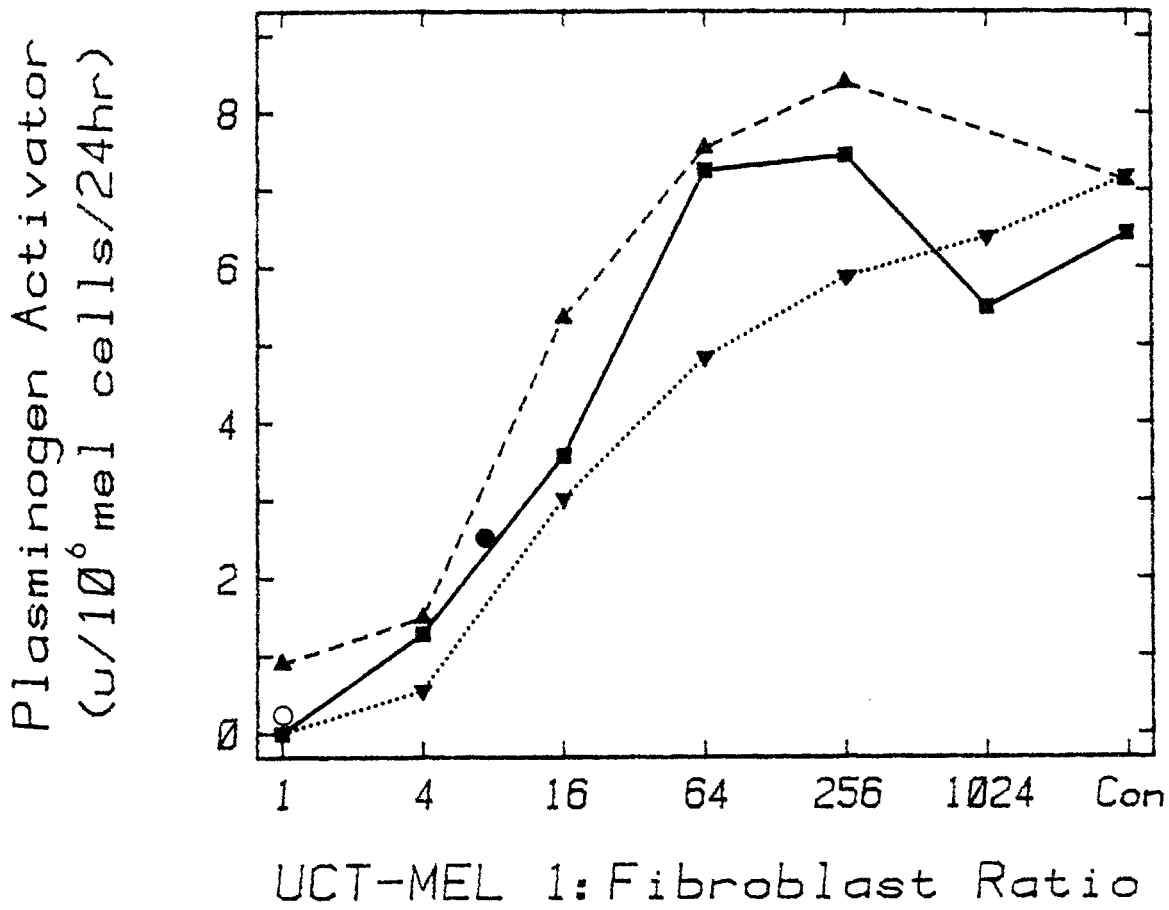


FIGURE 5.4

The inhibitory effect was not confined to skin fibroblasts.

UCT-Mel 1 cells were seeded at 5 x 10^4 cells/35 mm dish. Foreskin fibroblasts (▲--▲), human embryo skin fibroblasts (■—■) or adult skin fibroblasts derived from a patient with primary melanoma (▼·····▼) were cocultivated with the melanoma cells as described in the legend to Fig. 5.2 and 5.3. Control cultures, consisting of UCT-Mel 1 cells seeded alone, released less PA in this experiment than in the experiment seen in Fig. 5.2. Direct comparison with the inhibitory effect of adult skin fibroblasts was therefore not possible. Note that the degree of inhibition seen, however, and the M:F cell ratios at which inhibition was evident were similar to those seen in Fig. 5.2. Inhibition obtained with human embryo lung (O) and human embryo heart (●) fibroblasts, is indicated at the M:F ratios of 1:1 and 7:1 respectively. There was no consistent difference in the inhibitory capacity of the different fibroblast types.

respectively from a secondary carcinoma in bone where the primary site was unknown, and a malignant glioblastoma. The third cell type (HEK) was epithelial in nature and derived from a culture of human embryo kidney cells. When any of these cell types released PA, this was of the urokinase type which could be blocked by specific antibody in the assay.

The results obtained may be summarized as follows:

Cells added	Melanoma: added cell ratio	% Inhibition of UCT-Mel 1 PA
UCT-Gli 1	1:1	39
UCT-Ca 2	1:7	40
HEK	2:1	49

From these results, it is evident that these cells were inhibitory, but to a far lesser extent than were fibroblasts. At M:F ratios equal to or higher than the ratios shown in the table, fibroblast inhibition of PA was total.

The fibroblast effect was not exerted only on melanoma cells.

Since it is well-known that there are two distinct species of PA (Wilson et al, 1980; Vetterlein et al, 1979; Markus et al, 1980; Rijken et al, 1979a) and that it is not only melanoma cells that release the tissue-type activator, it was of interest to examine fibroblasts for their ability to inhibit PA release by cells other than melanoma cells.

An experiment was accordingly performed in which fibroblasts were examined for their ability to inhibit PA release by human embryo kidney cells (HEK) and cell lines derived from a human hypernephroma (UCT-Hyp 1), a human glioblastoma (UCT-Gli 1), and a human breast carcinoma (UCT-Br 1).

The results obtained are summarized in the following table.

Cells tested	PA-secreting cell:fibroblast ratio	Type of PA released	% Inhibition of PA
HEK	1:3	Urokinase	0
UCT-Hyp 1	1:1	Urokinase	30
UCT-Gli 1	1:1	Urokinase	42
UCT-Br 1	1:1	Tissue	100

As is evident from these results PA released by most of the cells types studied was inhibited by fibroblasts. The enzyme released by the breast cell line (i.e. tissue activator) was inhibited to a much greater extent than was the urokinase-type enzyme produced by the other cell types.

The kinetics of PA inhibition by skin fibroblasts.

UCT-Mel 1 cells were seeded alone or together with fibroblasts at a ratio of 1:1 and the inhibition of PA was measured as a function of time. It can be seen from the results summarized in Fig. 5.5 that the inhibition of melanoma PA was detectable within 2 hr of adding freshly trypsinized fibroblasts and was complete after 24 hr.

Direct contact between fibroblasts and melanoma cells was not necessary for inhibition.

The initial experiments with UCT-Mel 1 cells alone and those in which M:F ratios were varied suggested that inhibition of PA release required intimate cell contact between PA-releasing and inhibitory cells.

To examine the need for such contact, a series of experiments was performed in which monolayers of fibroblasts and melanoma cells were cultivated in close proximity in common medium by plating them respectively

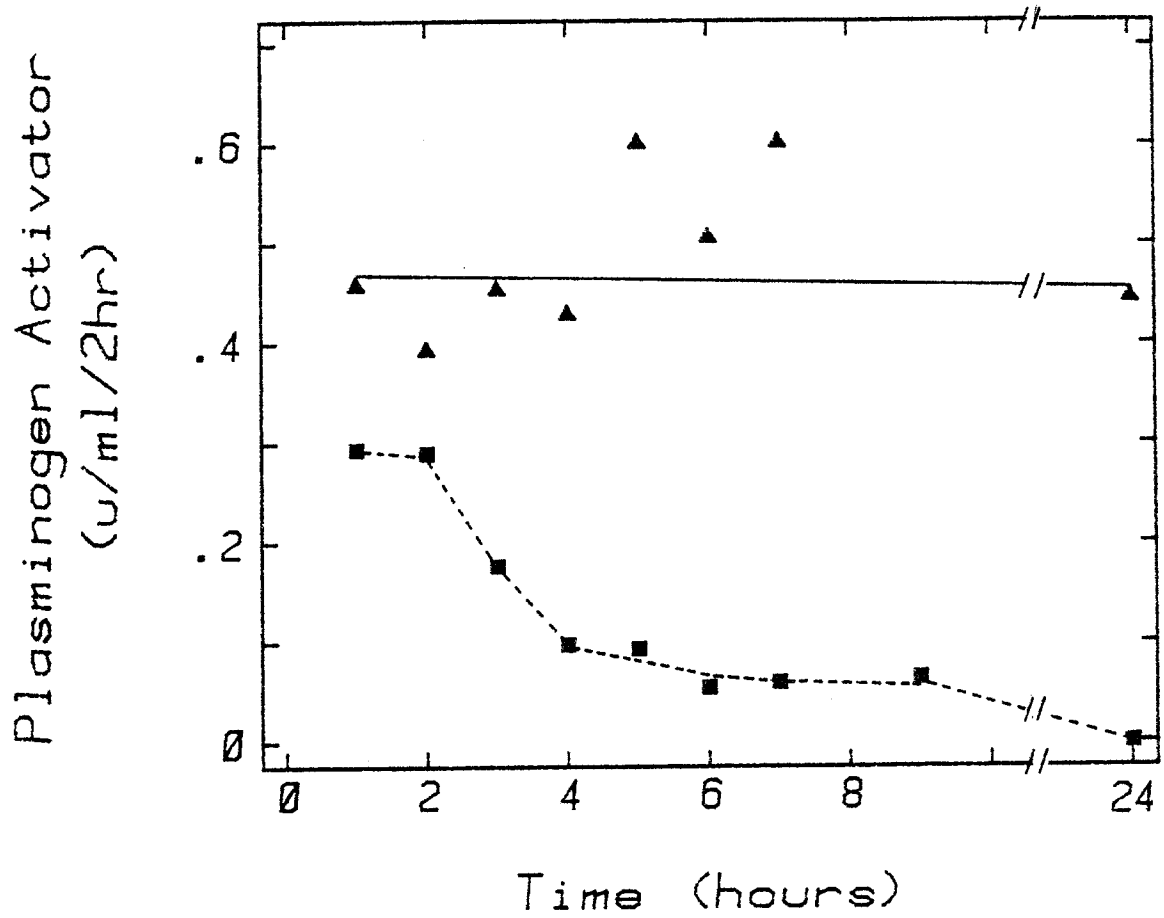
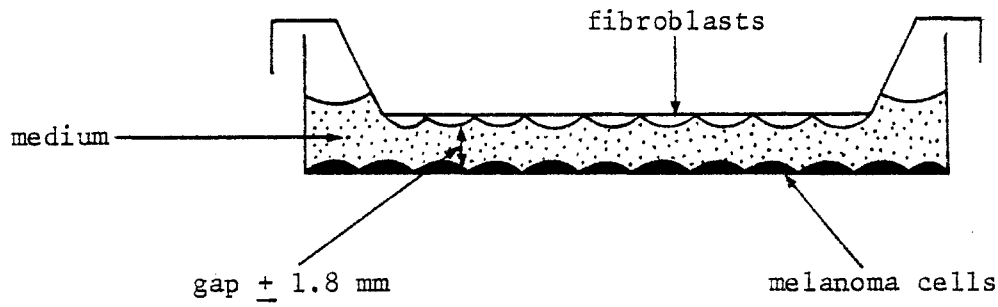


FIGURE 5.5

Timecourse of PA inhibition by skin fibroblasts.

UCT-Mel 1 cells were seeded at 5 x 10^4 cells per 35 mm dish. Twenty four hr later, these cells were washed three times with 1 ml of serum-free DB. At time 0, 1 ml of DB was added to control cultures (▲—▲) and 1 ml of DB containing 5 x 10^4 skin fibroblasts was added to test dishes (■--■). Duplicate cultures treated in this way were incubated for harvest fluids, while FCS to a final concentration of 10% was added to the remaining culture dishes. At 2 hr intervals cultures were prepared for harvest fluids which were collected after 2 hr and the time indicated in the figure is the midpoint of each 2 hr harvest fluid collection period. Note that inhibition is apparent within 2 hr and is complete by 24 hr.

on the undersurface of the lids and the bases of Cooper dishes (Falcon No. 3009). The experimental system in which Cooper dishes were used is depicted in the following diagram:



This experimental configuration was achieved as follows.

UCT-Mel 1 cells were seeded at 1.5 x 10^5 cells per 60 mm Cooper dish base in DB with 10% FCS and covered with conventional lids. At the same time, 1 x 10^5 skin fibroblasts were seeded on inverted Cooper dish lids in serum-free DB to expedite adherence to the tissue culture surface. Once the fibroblasts had attached, the lids were used to cover 60 mm dishes containing sufficient (5 ml) DB with 10% FCS to ensure that the suspended fibroblast monolayer was submerged in medium. After 24 hr, the lids on which skin fibroblasts were plated were combined with bases containing adherent UCT-Mel 1 cells and 5 ml of complete medium. After co-cultivation, for 48 hr, the cell layers were rinsed three times with serum-free medium. The lids were then restored to their respective bases with 4 ml of serum-free medium (the minimum volume that would allow contact between the 2 layers of cells). Control UCT-Mel 1 dishes were covered with Cooper dish lids on which no cells had been plated.

Despite the fact that fibroblasts and melanoma cells were separated by a gap of approximately 1.8 mm, the inhibition of PA release was still observed. This is evident from the following results.

Cooper dish base (no. of cells)	Cooper dish lid (No. of cells)	PA ($u/10^6$ UCT-Mel 1 cells)
UCT-Mel 1 ($8x10^5$)	-	38
UCT-Mel 1 ($8x10^5$)	Skin fibroblasts ($4x10^5$)	0

On examination of the Cooper dish cultures under phase-contrast, the cultures appeared healthy and floating cells were very rarely seen. In the extremely unlikely event that fibroblasts detached from the lids and settled to contaminate the melanoma cells below, the extent of this contamination would certainly have been well below that required to have produced the degree of inhibition seen.

Fibroblast inhibition was reversible.

The use of Cooper dishes presented two major advantages. Firstly, at the conclusion of an experiment, the cell numbers on the base and lid of the dish could be determined separately and therefore accurately. Secondly, the two cell types could be separated or reunited at will, at any stage of co-cultivation. This second advantage was used to study the kinetics of reversal of PA inhibition by skin fibroblasts.

A Cooper dish lid on which skin fibroblasts were plated was removed from co-cultivation with melanoma cells which were seeded on a Cooper dish base, and the time course of the reversal of inhibition was monitored (Fig. 5.6).

This experiment showed that the inhibitory effect exerted by fibroblasts was reversible, with small quantities of PA appearing in the harvest fluid after 8 hr. The amount of PA gradually increased until, at 36 hr, the enzyme activity in the medium covering fibroblast-treated cells had recovered to reach the same level as that observed with UCT-Mel 1 cells cultured alone.

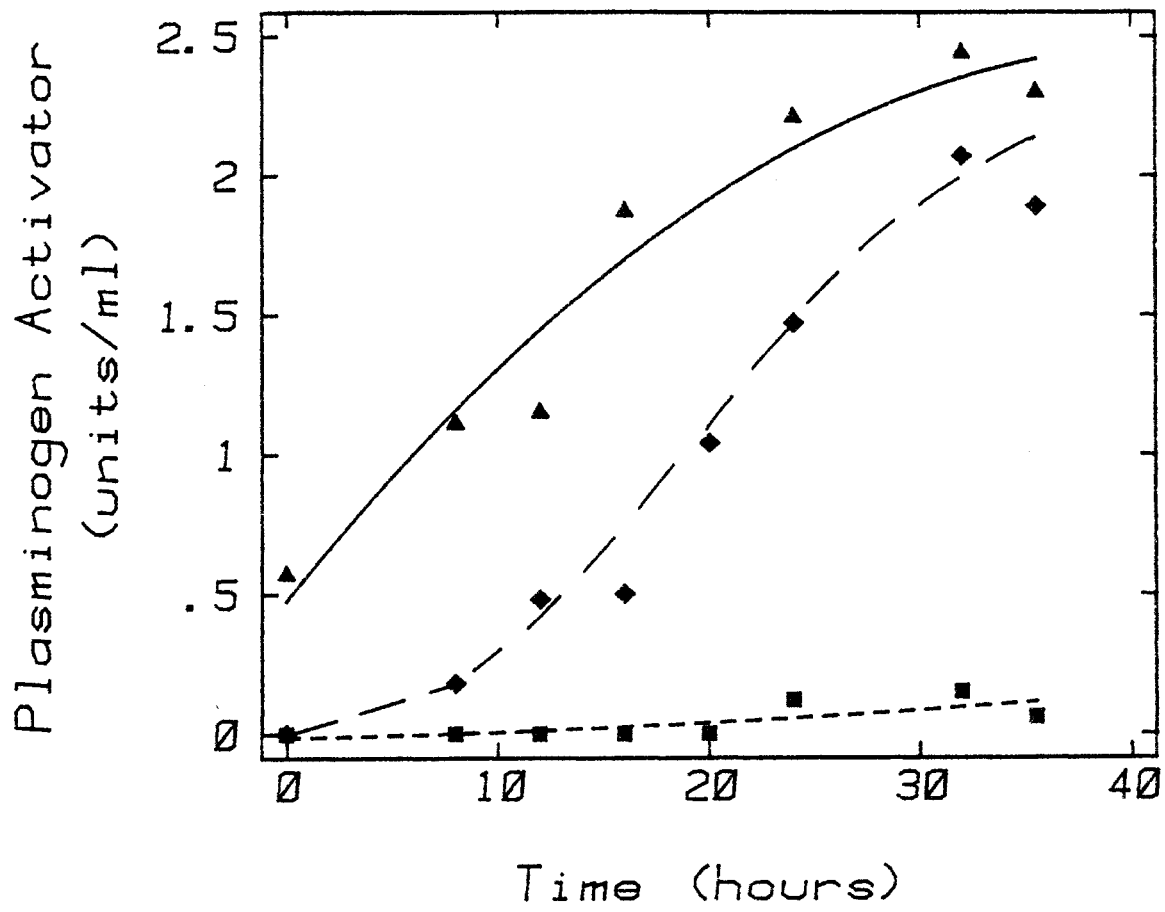


FIGURE 5.6

Reversibility of fibroblast inhibition.

UCT-Mel 1 cells were seeded at 1.5 x 10^5 cells per Cooper dish base and skin fibroblasts were seeded at 1 x 10^5 cells per Cooper dish lid. Twenty four hr later the medium was changed to 5 ml of DB containing 3% FCS, and the fibroblasts and melanoma cells were placed together. After 12 hr cocultivation i.e. at time 0, lids with skin fibroblasts were removed from duplicate UCT-Mel 1 cultures, and replaced with lids on which no cells were plated (◆—◆). Control UCT-Mel 1 cells were incubated throughout with lids on which no cells were plated (▲—▲) and other UCT-Mel 1 cells were incubated throughout with lids on which skin fibroblasts were plated (■---■). At the indicated times 100 µl aliquots were removed and stored at -20°C prior to assaying for PA. Note that the inhibitory effect of fibroblasts was reversible, with some recovery evident at 8 hr after removal of the fibroblasts, and complete reversal evident at 36 hr.

Inhibition could not be attributed to intracellular accumulation of PA or inhibition of PA synthesis in melanoma cells.

The preceding experiments might have been interpreted as indicating that the fibroblasts suppressed synthesis and/or release of PA.

To examine this possibility the following experiment was performed.

UCT-Mel 1 cells were seeded at 3×10^5 cells per Cooper dish base, and skin fibroblasts at 2×10^5 cells per Cooper dish lid. After 24 hr, the cells were co-incubated and after a further 24 hr, cell lysates of the melanoma cells were prepared (Appendix A.9). As a control, UCT-Mel 1 cells alone were incubated with a lid on which no skin fibroblasts were plated. Measurement of intracellular PA in the UCT-Mel 1 cell lysates showed no difference between the melanoma cells that had been co-incubated with skin fibroblasts or melanoma cells incubated alone (10.0 u/mg protein and 10.1 u/mg protein respectively).

Fibroblasts did not secrete an inhibitor of PA, or PA synthesis and release.

Since intimate fibroblast-melanoma cell contact was not required for the inhibitory effect it was obviously possible that the fibroblasts released an inhibitor of melanoma PA or that they released a soluble factor that acted directly upon melanoma cells to affect enzyme synthesis and release. To investigate the first of the possibilities, mixing experiments were performed in which serum-free harvest fluids from confluent fibroblast cultures were mixed with melanoma cell harvest fluids and the effect on enzyme activity was noted. Control melanoma harvest fluid samples were similarly treated with DB which had not been exposed to fibroblasts.

The results obtained are summarized in Table 5.1 and indicated that fibroblasts did not release a soluble inhibitor of melanoma PA or

TABLE 5.1

FIBROBLASTS DO NOT SECRETE AN INHIBITOR OF PA

Plasminogen Activator		PA activity (u/ml)[a]	
Source	Type	After incubation with DB	After incubation with fibroblast harvest fluids
UCT-Mel 1	Tissue	33.1	28.0
UCT-Mel 2	Tissue	2.7	2.8
UCT-Mel 3	Tissue	36.7	26.9
UCT-Mel 6	Urokinase	709.9	1044.2
UCT-Ca 2	Urokinase	7.6	7.0

(a) Plasminogen activator-containing harvest fluids were mixed with equal volumes of DB or skin fibroblast harvest fluid in Falcon tubes (Cat. No. 2054) and incubated for 30 min in a 37°C waterbath. After incubation the samples were assayed for residual PA activity.

urokinase-type PA. Similar experiments were carried out in which UCT-Mel 1 harvest fluid was mixed with (a) conditioned serum-free medium from skin fibroblasts collected over 3 days; (b) a 3-day serum-free harvest fluid collected from skin fibroblasts which had previously been incubated for 3 days in serum-free medium to remove all traces of residual serum inhibitors, and (c) a 3-day serum-free harvest fluid prepared from foreskin fibroblasts.

In no case was I able to demonstrate inhibition of PA by released fibroblast products.

In order to test for the presence of a cellular inhibitor in fibroblast-conditioned medium, I added serum-free harvest fluid from fibroblasts directly to melanoma and other cells and monitored the release of PA.

Serum-free conditioned medium was prepared by rinsing fibroblasts three times with serum-free medium, followed by incubation with the medium for 24 hr. This conditioned medium was added to the test cells listed below for a 24 hr period, after which it was aspirated and a second, freshly-prepared batch of fibroblast-conditioned medium was added to the test cells for a further 24 hr period. The amount of PA secreted into this second addition of conditioned medium was measured and compared with the PA secreted into fresh serum-free medium by untreated melanoma cells. A similar experiment was performed using fibroblast-conditioned medium which had been diluted 1:1 with fresh medium in order to compensate for any effects due to medium depletion. The effect of fibroblast-conditioned medium was tested on UCT-Mel 1, 2, 3,6 and UCT-Ca 2. All cells were seeded at 5×10^4 per 35 mm dish. The results obtained are presented in Table 5.2.

Although in the case of UCT-Mel 2 and UCT-Ca 2, serum-free fibroblast-conditioned medium produced a significant decrease in the

TABLE 5.2

FIBROBLASTS DO NOT SECRETE AN INHIBITOR OF PA SYNTHESIS AND RELEASE

Cell line	PA(u/10^6 cells/24 hr)[a]		
	Control	Fibroblast CM (diluted)	Fibroblast CM
UCT-Mel 1	39.8	35.3	33.0
UCT-Mel 2	4.6	2.8	2.3
UCT-Mel 3	29.5	26.5	21.8
UCT-Mel 6	239.5	266.0	185.0
UCT-Ca 2	44.0	24.3	20.8

(a) Cultures of the cells listed were covered with serum-free medium (control) or medium that had been conditioned by fibroblast monolayers, and that was used either undiluted or diluted with an equal volume of fresh medium.

Release of PA into harvest fluids prepared in this way was measured over a 24 hr period as described in the text.

release of cellular PA, this was not observed with other cell lines. Furthermore, the decrease in PA release observed with UCT-Mel 2 was far less than would have been expected had UCT-Mel 2 cells been cultivated with fibroblasts at a corresponding cell ratio.

Fibroblast monolayers inactivated PA present in melanoma cell harvest fluids.

In view of the fact that the inhibition phenomenon did not require fibroblast-melanoma cell contact, nor was it mediated by a soluble factor released by fibroblasts into the tissue culture medium, I examined fibroblast monolayers for their ability to inactivate or remove PA from melanoma cell harvest fluids.

The experiment was performed as follows: Serum-free melanoma harvest fluids were pooled to give a solution containing approximately 4 units of PA/ml. Adult skin fibroblasts were seeded to give sparse cultures in replicate 35 mm dishes in complete medium. Control dishes, prepared at the same time, received 1 ml of foetal calf serum without cells. After 24 hr, the adherent fibroblasts and the control dishes were washed free of serum and to each was added a 1 ml aliquot of the melanoma harvest fluid. Plasminogen activator content in fibroblast and control dishes was monitored as a function of time.

As can be seen in Fig. 5.7, PA disappeared progressively from the medium covering the fibroblasts, whereas it remained present at a constant level in the control dishes. By 4 hr, the fibroblasts had removed 84% of the melanoma activator from the harvest fluid.

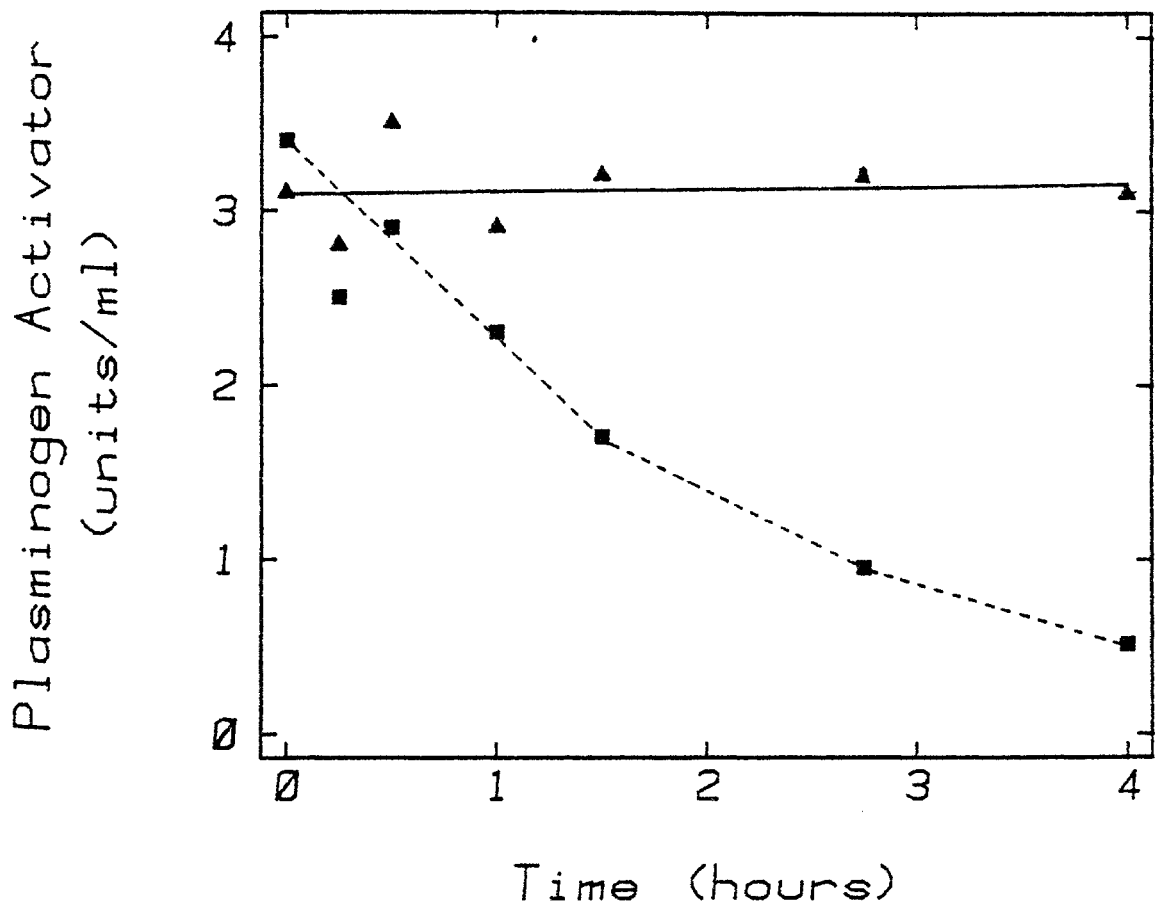


FIGURE 5.7

Released melanoma PA is inactivated by skin fibroblasts.

UCT-Mel 1 cells were seeded at 5 x 10^4 cells per 35 mm dish and harvest fluids were prepared 24 hr later. Skin fibroblasts were seeded at 5 x 10^4 cells per 35 mm dish. Control dishes were incubated with 1 ml of FCS overnight. After 24 hr, dishes seeded with skin fibroblasts or incubated with FCS were rinsed three times with 1 ml DB. The harvest fluids were removed from the dishes of UCT-Mel 1 and incubated with either the dish pre-treated with FCS (▲——▲) or the dish seeded with skin fibroblasts (■----■). Aliquots of 100 µl were removed at the indicated times and assayed for PA activity. Note the steady fall in PA activity following addition of the harvest fluid to the fibroblasts. Fibroblasts removed 84% of the melanoma PA from the medium during a 4 hr incubation period.

Inactivation of melanoma PA was directly related to fibroblast number.

As shown in co-cultivation experiments (Fig. 5.2) the magnitude of the fibroblast inhibitory effect depended on the number of fibroblasts present. In order to determine whether a similar relationship held when melanoma harvest fluid was incubated with a fibroblast monolayer, the following experiment was performed in which a constant volume of UCT-Mel 1 harvest fluid was incubated with different numbers of skin fibroblasts. After incubation residual PA activity in the medium was measured. The results presented in Fig. 5.8, indicated that removal of PA from the medium was directly related to the number of fibroblasts to which the medium was exposed.

Using the values obtained when zero, 2×10^3, or 7×10^3 fibroblasts were present in each dish, it could be calculated that these cells inactivated PA at a rate of between 80 and 120 units per 10^6 fibroblasts per 4 hr.

The ability of skin fibroblasts to inactivate PA was limited.

To determine whether or not the fibroblast-enzyme interactions that led to PA inactivation were saturable, I performed an experiment in which samples of melanoma harvest fluid were added repeatedly to the same fibroblast monolayer, until the inhibitory capacity of the fibroblasts was exhausted.

The detailed protocol for this experiment is given in the legend to Table 5.3, in which the results are summarized. Repeated addition of enzyme-containing harvest fluids for the times indicated, up to 25 hr, resulted in fibroblast inactivation of the enzyme. At some stage, however, between 12 and 25 hr, the fibroblast activity became saturated and no further inactivation was observed.

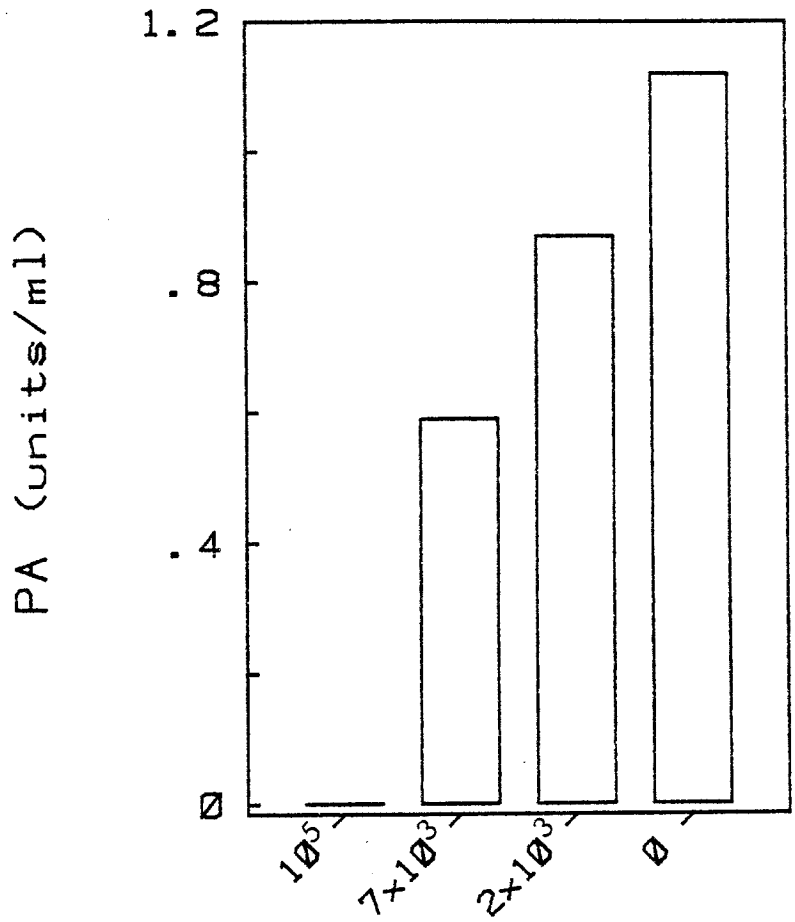


FIGURE 5.8

The fibroblast inhibition of melanoma PA in harvest fluids is dose dependent.

An arbitrary dilution of UCT-Mel 1 harvest fluid was incubated with skin fibroblasts at 2 x 10^3, 7 x 10^3 and 10^5 cells per 35 mm dish. The harvest fluid was removed after 4 hr incubation and assayed for residual PA activity. Note that the degree of PA inhibition was directly related to the number of fibroblasts with which the melanoma harvest fluid was incubated.

TABLE 5.3

THE ABILITY OF SKIN FIBROBLASTS TO INACTIVATE PA WAS LIMITED[a]

Time (hrs)	PA present in harvest fluid		
	Before incubation	After incubation	PA adsorbed
	(u/ml)	(u/ml)	(u/ml)
0-4	4.6	1.8	2.8
4-8	5.1	3.6	1.5
8-12	6.4	5.1	1.3
12-25	7.1	4.0	3.1
25-29	5.8	5.9	0
			8.7

(a) Harvest fluids were prepared from melanoma cultures and added to fibroblast monolayers. After incubation at 37°C for the periods indicated, residual PA present in the harvest fluid was measured and compared with the amount present before incubation. Fresh harvest fluid was then added. This procedure was repeated until the ability of the fibroblasts to adsorb PA was saturated.

The results of this experiment allowed the conclusion that, using this procedure, approximately 170 units of PA could be removed from the medium per 10^6 fibroblasts before saturation was observed.

Fibroblast inactivation of melanoma PA appeared to be irreversible.

If, as seemed entirely possible, fibroblasts were inactivating melanoma PA by selective adsorption of the enzyme from the medium, it was appropriate to ask if the fibroblast-PA complex was dissociable.

To answer this question, an experiment was performed in which skin fibroblasts that had been subject to prolonged exposure to melanoma PA by co-cultivation with melanoma cells in Cooper dishes were rinsed thoroughly with DB and covered with serum-free medium. The cells were returned to the incubator for 24 hr after which the medium was assayed for PA activity that may have been released by the fibroblasts. None was found in the ^{125}I-fibrin assay.

The fibroblast effect was unaffected by inhibitors of RNA and DNA synthesis and only partially affected by inhibition of protein synthesis.

Inhibitors of macromolecular synthesis were used to define the extent to which fibroblast inactivation of melanoma PA required the synthesis of DNA, RNA or protein.

Preliminary experiments established that incubation of fibroblasts with mitomycin C at 2 µg/ml for 18 hr inhibited ^{3}H-thymidine incorporation by more than 90%. Actinomycin D at 1 µg/ml for 1 hr inhibited incorporation of ^{3}H-uridine by more than 90%, and cycloheximide at 5 µg/ml inhibited protein synthesis as measured by ^{3}H-leucine incorporation by 88%. Since the effect of cycloheximide was readily reversible, its presence was maintained throughout the experiment.

The experiment was performed by exposing adult skin fibroblasts to inhibitors as determined by the preliminary experiments summarized in the previous paragraph. The cells were then washed and covered with melanoma cell harvest fluid containing a measured amount of melanoma PA. After 4 hr, the harvest fluid was assayed for residual PA activity. Similar 4 hr exposures of inhibitor-treated cells to melanoma harvest fluid were used at 24 and 46 hr after initial exposure to the inhibitors to assay the fibroblasts for their ability to inactivate PA.

The results are depicted graphically in Fig. 5.9 where each point represents residual PA activity as a function of the midpoint of the 4 hr incubation period. As can be seen from the results neither actinomycin D nor mitomycin C had an appreciable effect upon the ability of fibroblasts to inactivate PA in the medium, even when present for as long as 48 hr.

Cycloheximide appeared to have a slight effect on fibroblast ability to remove PA activity from the medium. The effect of cycloheximide increased slightly with time so that by 48 hr, cycloheximide-treated fibroblasts had removed only 66% of the melanoma PA activity as compared to untreated fibroblasts which removed 95%.

Fibroblasts that had been fixed with ethanol or frozen and thawed retained their ability to inactivate PA.

Since macromolecular synthesis was not required for the fibroblast effect, it was appropriate to ask whether cell viability was necessary. This question was addressed in the following experiment.

Pooled melanoma cell harvest fluid was diluted with an equal volume of fresh DB and the PA content of this solution was measured. One millilitre samples were then added to duplicate 35 mm plastic tissue culture dishes containing semiconfluent monolayers of fibroblasts that had been treated in one of the following ways:

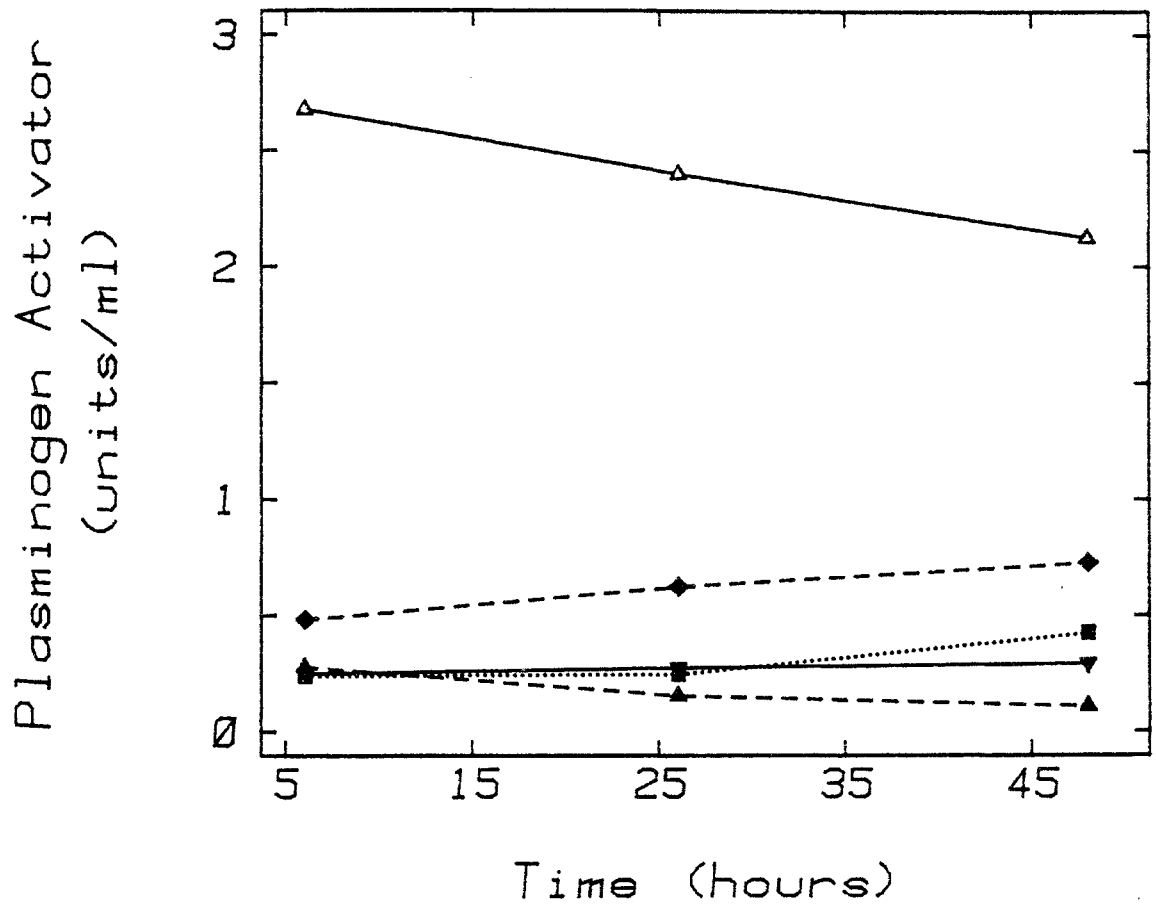


FIGURE 5.9

Partial inhibition of fibroblast inhibitory effect by cycloheximide.

Skin fibroblasts were seeded at 5 x 10^4 cells per 35 mm dish. The inhibitors of macromolecular synthesis were added at time 0, at the following concentrations: 2 µg/ml mitomycin C; 1 µg/ml actinomycin D; 5 µg/ml cycloheximide. At the times indicated, harvest fluids from UCT-Mel 1 cells were added to untreated fibroblasts (▲--▲) or fibroblasts treated with mitomycin C (▼——▼), actinomycin D (■·····■) or cycloheximide (◆--◆) for a period of 4 hr, after which the harvest fluid was removed and stored at -20°C until assay. Harvest fluid added to empty dishes served as a control (△——△). Cycloheximide was present during the 4 hr incubation period with melanoma harvest fluids as its effect on protein synthesis is reversible. Mitomycin C and actinomycin D were not present during this period. Note that the inhibitory capacity of fibroblasts pre-treated with cycloheximide was diminished by approximately 30%.

(i) the dishes were washed three times with 1 ml of DB.

(ii) the dishes were washed as above, after which the cells were fixed with two changes of 70% ethanol in water and allowed to air dry. The dishes were then washed twice more with DB.

(iii) the dishes were washed as above. The cells were then lysed by freezing at -80°C for 2 hr and thawing slowly at room temperature.

Control dishes contained no cells. These were incubated at 37°C with 1 ml of FCS for 2 hr, after which they were washed three times with 1 ml of DB.

The melanoma harvest fluids were removed after 6 hr of incubation for measurement of residual PA activity. The results are presented in Table 5.4. Cells killed by freezing and thawing were as effective as live fibroblasts. Ethanol fixed and air-dried fibroblasts removed 86% of the PA activity.

Fibroblasts inactivated different molecular types of PA to different extents.

Since, as described in Chapter 3, PA's released by cells in culture may be classified into two broad groups according to their immunochemical relationship to urokinase or tissue activator, it was of interest to see whether the urokinase- and tissue-type proteases were inactivated by fibroblasts to the same extent.

An experiment was accordingly performed in which fibroblast-monolayers in 35 mm dishes were covered with 1 ml samples of serum-free conditioned medium, harvested from cultures of five cell lines known to release urokinase-type enzyme (UCT-Ca 2, UCT-Hyp 2, UCT-Sq 1, UCT-Sarc 1 and UCT-Mel 6).

For comparison, identical cultures were covered with 1 ml samples

TABLE 5.4

EFFECT OF ETHANOL-FIXATION AND FREEZE-THAW LYSIS OF FIBROBLASTS

Contents of dish[a]	Residual PA Activity (mu/ml)[b]	PA taken up by fibroblasts (% inactivation)
Untreated fibroblasts	0.3	100.0
Ethanol-fixed fibroblasts	114.3	85.9
Freeze-thawed fibroblasts	0.6	99.9
Nil (control)	808.5	-

(a) Fibroblast containing dishes were seeded with 5×10^4 fibroblasts per 35 mm dish in complete medium and were used 24 hr later. All monolayers were washed free of serum and used without further treatment, after ethanol-fixation, or after freezing and thawing as described in the text. Control dishes were incubated with 1 ml of FCS for 2 hr at 37°C, after which they were washed three times with DB.

(b) One millilitre of melanoma harvest fluid diluted with an equal volume of DB and containing approximately 1000 milliunits (mu) of PA was added to each dish. Residual activity was measured after 6 hr. Each value represents the mean of duplicate dishes.

harvested from cultures of five melanoma cell lines (UCT-Mel 1, 2, 3, 5 and 7).

In both sets, the harvest fluids were adjusted by dilution to contain approximately the same concentration of PA activity.

Residual PA activity in the medium was measured after 2 and 4 hr of incubation and compared with that in cell-free control dishes, treated with FCS alone.

The results are presented in Fig. 5.10 where the percentage of the original PA activity remaining in each dish is plotted as a function of time. A remarkably consistent pattern was observed in which tissue-type PA was removed from the medium by the fibroblasts more rapidly and to a significantly greater extent than the urokinase-type PA.

Thrombin did not significantly influence the ability of fibroblasts to inactivate PA.

While this work was in progress, two papers from the same laboratory appeared in the literature (Baker et al, 1980; Low et al, 1981) in which it was reported that cultured fibroblasts released a "component" which linked thrombin and plasminogen activator and mediated their binding to cells. This component, referred to as "protease-nexin" (PN), they claimed also regulated the internalization and degradation of serine proteases. The authors referred briefly in their second paper to observations indicating that PA lost its fibrinolytic activity when linked to protease nexin.

When these authors incubated ^{125}I-UK with human foreskin fibroblast-conditioned medium they found that the radioactive enzyme formed a complex with PN present in the medium so that it subsequently electrophoresed in SDS-polyacrylamide gel with an apparent molecular weight of 72K as opposed to free enzyme which had an apparent molecular weight of

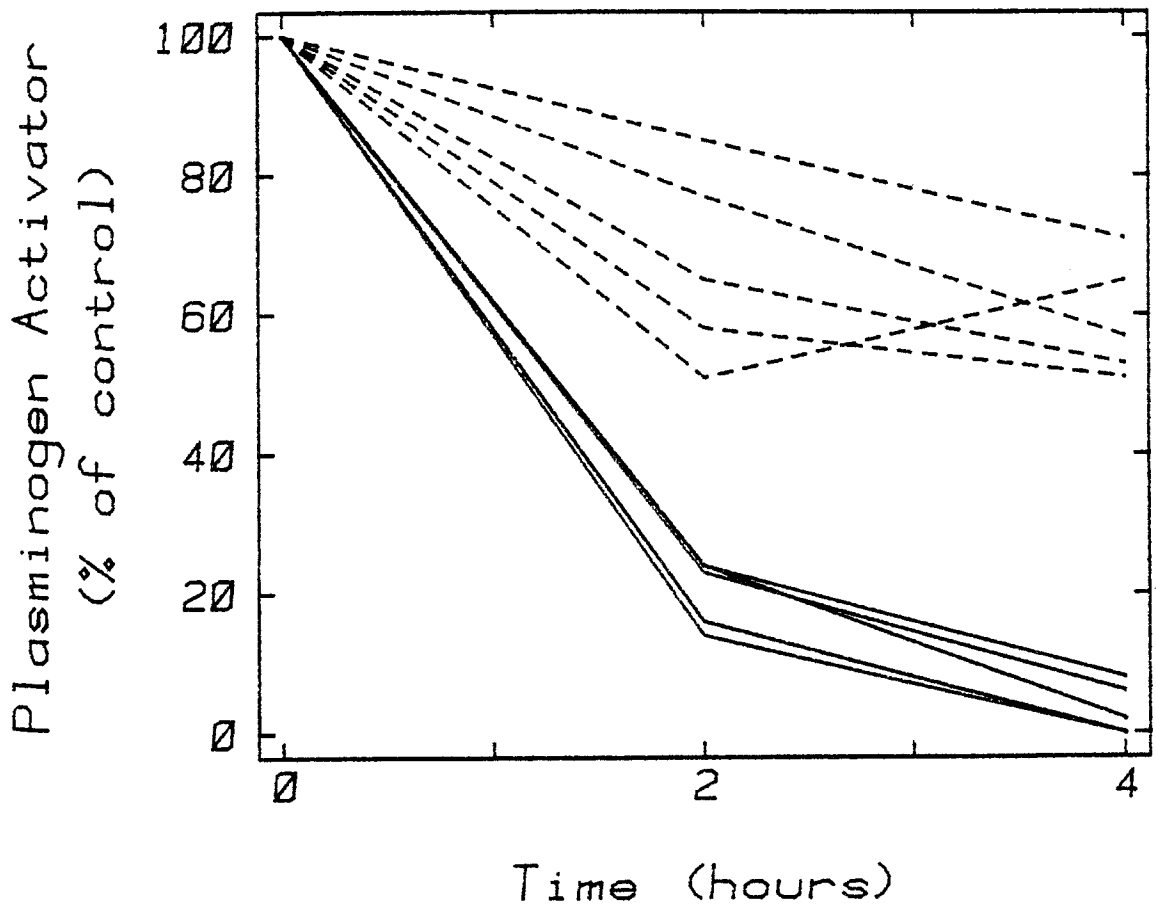


FIGURE 5.10

Fibroblasts inactivated different molecular types of PA to different extents.

Harvest fluids from UCT-Mel 1, 2, 3, 5 and 7 which secrete the tissue-type PA (solid lines) and from UCT-Ca 2, UCT-Hyp 2, UCT-Sq 1, UCT-Sarc 1 and UCT-Mel 6, which secrete urokinase-type PA (broken lines) were diluted so that all contained approximately the same specific activity as measured in a ^{125}I-fibrin assay (Appendix A.13). The above figure depicts the inhibition produced when these harvest fluids were added to cultures of skin fibroblasts seeded at 5 x 10^4 cells per 35 mm dish. Aliquots of the harvest fluids were removed at the indicated times and assayed for PA activity. The residual activity detected in harvest fluids incubated with fibroblasts was expressed as a percentage of the activity found in culture dishes containing no cells.

35K. They proposed that the protease-PN complex then bound to the cell membrane as part of a specific mechanism for internalization and degradation of proteases. The binding of the protease to PN was inhibitable by thrombin. The binding of the protease-PN complex to the cell was inhibitable by heparin.

It was clearly conceivable that protease-nexin was responsible for the phenomenon that I had observed, in which case fibroblast inactivation of PA should have been inhibitable by thrombin. To examine this prediction, fibroblasts were tested for their ability to inactivate melanoma PA either after pre-incubation of the cells with thrombin or when an estimated excess of thrombin over PA was present in the medium.

The results, presented in Table 5.5, showed that thrombin did not inhibit the fibroblast inactivation of PA with either of these two protocols.

Melanoma PA bound to fibroblasts by forming a complex with a fibroblast-associated molecule.

The results of all the experiments performed thus far were consistent with the possibility that melanoma PA was removed from the medium by binding to a fibroblast component.

An experiment was accordingly performed in which melanoma harvest fluid was added to ethanol-fixed fibroblast monolayers. After incubation for 3 hr, the fibroblasts were washed extensively with PBS to remove unbound melanoma PA and scraped from the plastic surface with a teflon policeman. The cells were then washed once more with PBS by centrifugation and lysed by resuspension in approximately 100 µl of 2% SDS in PBS. After incubation for 10 min at 37°C to complete disaggregation, the lysate was electrophoresed in an 11% polyacrylamide gel slab containing 1% SDS. After electrophoresis the slab was prepared for zymography on a plasminogen-

TABLE 5.5

THROMBIN DID NOT SIGNIFICANTLY INFLUENCE THE ABILITY OF FIBROBLASTS TO ADSORB PA[a]

Experiment	PA (u/ml) (I)[b]	PA (u/ml) (II)[c]
UCT-Mel 1 HF	1.04	3.20
UCT-Mel 1 + fibroblasts	0.51	0.89
UCT-Mel 1 + fibroblasts + thrombin	0.51	0.75
UCT-Mel 1 HF + thrombin	n.d[d]	2.34

(a) Vials of freeze-dried thrombin (Sigma) containing 10 NIH units per vial were reconstituted with 1 ml of distilled water to yield a solution containing 10 mg/ml BSA and 0.15M NaCl. Further dilution was carried out by adding the required amount of phosphate buffered saline (PBS). Control solution consisted of an equal concentration of BSA in PBS.

(b) Fibroblasts were pre-treated with a solution containing 7.5 units/ml of thrombin and 7.5 mg/ml of BSA. After one hour at 37°C, this solution was aspirated and a harvest fluid of UCT-Mel 1 in DB was added to the fibroblasts. An aliquot of harvest fluid was added to a dish which had been pre-incubated with 1 ml of FCS, then rinsed three times with DB (UCT-Mel 1 HF). After 4 hr incubation at 37°C, the harvest fluids were removed and stored until assay.

(c) Harvest fluid from UCT-Mel 1 was diluted 1:1 with either PBS containing 10 mg/ml BSA, or 0.15M NaCl containing 10 mg/ml BSA and 10 units/ml thrombin. These solutions were incubated with dishes pre-treated with FCS as described in (b), or with dishes containing fibroblasts. After 4 hr incubation at 37°C, the diluted harvest fluid solutions were removed and stored until assay.

(d) n.d. = not determined.

fibrin-agar underlay as described in Appendix A.14.4. To identify the plasminogen activator immunochemically, the lysate was electrophoresed in adjacent lanes, one of which was set next to a trough in the fibrin agar underlay to which antibody to melanoma PA had been added.

A photograph of the gel is shown in Fig. 5.11, from which it can be seen that untreated fibroblasts contained no PA, whereas lysates prepared from fibroblasts that had been exposed to melanoma PA contained plasminogen activators with apparent molecular weights of 72K and 115K, both of which were inhibited by antibody to melanoma PA.

No evidence of the 115K band was found in the harvest fluid to which the fibroblasts were exposed.

These results allow the conclusion, therefore, that the melanoma PA bound to a fibroblast component to give an SDS-stable complex which retained enzymatic activity in this particular system.

An additional feature of interest but uncertain significance is evident in Fig. 5.11. It can be seen that the melanoma PA initially added to the fibroblasts contained two electrophoretic species that originated as a closely-spaced doublet in the 72K region and a band of approximately 60K whereas enzyme detected in the same area in the lane in which the melanoma-treated fibroblast lysate was run, consisted chiefly of the 72K doublet with relatively little of the 60K component.

When the same experiment was performed with viable, unfixed fibroblasts, similar results were obtained but the results were inconsistent and far less PA could be detected in the gels, presumably owing to degradation of the enzyme by the viable cells.

FIGURE 5.11

FIGURE 5.11

Melanoma PA formed a complex with a fibroblast-associated molecule.

Ethanol fixed skin fibroblasts were incubated with UCT-Mel 1 derived harvest fluid as described in the text. Fibroblast lysates were electrophoresed in an 11% SDS-polyacrylamide gel which was then layered onto a fibrin-plasminogen-agar indicator gel containing a central trough into which anti-melanoma IgG had been allowed to diffuse (Appendix 14.4 and Chapter 3 - Materials and Methods).

The lysis bands indicate the position in the gel of active PA enzyme. The electrophoresed samples were as follows: Track 1: UCT-Mel 1 harvest fluid; Tracks 2 and 3: lysate of skin fibroblasts after incubation with melanoma harvest fluids; Tracks 4 and 5: lysate of untreated skin fibroblasts. Note that (a) ethanol-treated skin fibroblasts have no endogenous PA activity (Tracks 4 and 5); (b) incubation of melanoma harvest fluid with fibroblasts leads to the formation of a melanoma PA-fibroblast component complex with an apparent molecular weight of approximately 115K daltons (Track 2). A band of activity at 72K was also evident, which coincided with the molecular weight of the upper band of the untreated UCT-Mel 1 harvest fluid; (c) the bands of PA activity seen in the fibroblast lysate were of the melanoma type, as shown by their inhibition by anti-melanoma PA IgG (Track 3).

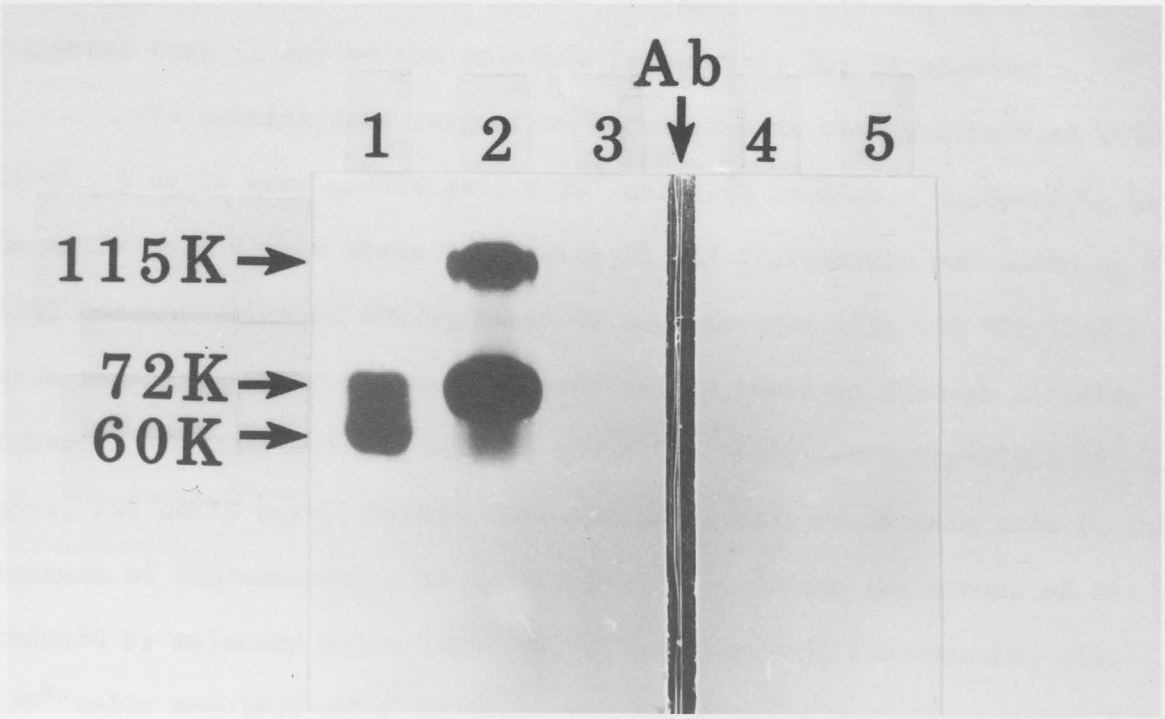


FIGURE 5.11

Plasminogen activator binding to fibroblasts did not appear to be mediated by fibronectin.

Fibronectin is a major pericellular matrix protein that has been identified on fibroblast surfaces. The well-documented existence of this protein and its circumstantial association with cellular transformation and PA release (Chen et al, 1976; Vaheri et al, 1978) suggested that it may be the molecule responsible for PA binding.

To examine this suggestion an experiment was performed in which UCT-Mel 1 cells were seeded at 3 x 10^5 cells/60 mm dish. Forty-eight hr later the cells were rinsed three times with DB and fibronectin was added at a final concentration of 100 µg/ml in DB supplemented with 10% FCS from which the plasminogen had been removed by two passages through a lysine-sepharose affinity column (Deutsch and Mertz, 1970). Forty-eight hr later, the cells were prepared for harvest fluids, which were made in the presence of fibronectin. No difference was found in the amount of PA produced by melanoma cells incubated with or without fibronectin, (14.7 u/10^6 cells and 15.2 u/10^6 cells respectively). A radioimmunoassay for fibronectin showed no difference in the amount of fibronectin in this solution before and after incubation with UCT-Mel 1 cells[(a)].

(a) I am indebted to Dr. L. Purves of the Department of Chemical Pathology, U.C.T. Medical School, Cape Town, for the generous gift of purified fibronectin and for performing the radioimmunoassay.

The fibroblast-associated inactivator of PA was not derived from serum.

In view of the presence of inhibitors of plasminogen activator in serum (Heimburger, 1975), it was conceivable that one or more of these inhibitors bound to the fibroblast cell surface and were responsible for the PA inactivation I observed.

In order to test this possibility, the following experiment was performed. Semi-confluent cultures of skin fibroblasts were cultured for 7 days in RPMI medium containing either no serum, 1%, 3% or 10% FCS, or 10% FCS that had been acid-treated in order to destroy protease inhibitors (Loskutoff and Edgington, 1977).

Serum-free melanoma harvest fluid was incubated with these fibroblasts for 6 hr and assayed for PA activity at the end of this period.

The results of two similar experiments are summarized in Table 5.6. As can be seen, the fibroblasts that were cultured in medium with no serum or with acid-treated FCS produced the greatest degree of melanoma PA inhibition. Although the inhibition produced by these cells was not significantly different from that produced by cells incubated with 10% of untreated FCS, the results illustrate clearly that acid-treatment, or the complete absence of FCS, did not adversely affect their ability to inactivate PA. In fact, the reverse was indicated.

DISCUSSION

The studies I have reported in this chapter have demonstrated the existence of a phenomenon whereby cultured human fibroblasts remove tissue-type PA from overlying serum-free medium. This phenomenon is mediated by binding of the PA to the cells followed, in all probability, by degradation of the enzyme.

TABLE 5.6

THE FIBROBLAST-ASSOCIATED INACTIVATOR OF PA IS NOT DERIVED FROM SERUM

Fibroblast medium[a]	PA inactivation (% of Control)[b]
RPMI - no serum	59
RPMI + 1% FCS	39
RPMI + 3% FCS	56
RPMI + 10% FCS	43
RPMI + 10% Acid-treated FCS	69

(a) Skin fibroblasts were cultured in the media indicated for a period of 7 days before being tested for ability to inactivate melanoma PA.

(b) Fibroblasts were rinsed three times with serum-free medium, and were then incubated with melanoma-derived serum-free harvest fluids for 6 hr. The results are expressed as a percentage of the control value, which was obtained by incubating melanoma harvest fluid for the same period of time in a dish which had previously been coated with FCS, then rinsed with serum-free medium.

Experiments performed to characterize this phenomenon have enabled me to conclude that fibroblasts obtained from diverse tissues or sources are equally effective in their ability to combine with PA. The phenomenon is not an exclusive attribute of fibroblasts since it could be demonstrated, albeit to a far lesser extent, with other cell types including human glioblastoma, carcinoma, and embryo kidney cells.

Fibroblast binding and inactivation of tissue PA appeared under physiological conditions of pH and ionic strength to be an irreversible phenomenon that was saturable.

It transpired that over the timecourse of the *in vitro* study (approximately 48 hr) the fibroblast phenomenon did not require DNA synthesis nor did it require transcription or translation of new mRNA, although inhibition of protein synthesis did result in a modest decrease in the fibroblast effect. If, as I discuss below, the binding of the enzyme to the cells involved a specific protein ligand on the cell surface, these inhibitor experiments would suggest that this protein has a relatively slow rate of turnover, with a half-time in excess of 48 hr indicating that metabolic activity or cell viability was not necessary for the initial stages of complex formation to take place. The ethanol-fixed fibroblasts provided a useful system for studying the binding reaction and could be used in association with SDS-polyacrylamide gel electrophoresis, plasminogen-fibrin-agar zymography and inhibition with specific antibody to show that tissue PA associated with the cells was present in two distinct forms - one with a molecular weight of approximately 115K daltons and the other with a molecular weight of 72K daltons. The enzyme present in the melanoma harvest fluid added to the fibroblasts migrated electrophoretically as two bands with molecular weights of approximately 72K daltons and 60K daltons.

The fact that fibroblast binding appeared to convert at least portion of the PA to a higher molecular weight species strongly suggested

the presence of a cell-associated binding molecule with a molecular weight of approximately 40-50K daltons. Furthermore, as found previously to be the case with α_2-macroglobulin (Granelli-Piperno and Reich, 1978) the fibroblast binding protein formed an SDS-stable complex with the PA that did not involve irreversible inhibition of the active site of the enzyme. One can state this with confidence since enzyme activity in the 115K dalton complex was readily demonstrable in the fibrin-agar underlays.

I am unable as yet to offer a definitive explanation for the fact that cell-associated PA included a 72K molecular weight component. This may reflect the fact that the 72K enzyme was able to bind directly, whereas the 60K enzyme associated with the specific binding molecule to give the 115K band. Alternatively, the binding of the enzyme might have proceeded in two stages with an intermediate low-affinity complex that was readily dissociable by SDS. This would then have produced a 115K "covalent" complex and a 72K band of dissociated enzyme. Finally, it is possible that the activator was degraded to a lower molecular weight species of approximately 35K and that this combined with the fibroblast component to produce the 72K complex.

In view of these observations, the question naturally arose as to whether or not cell-bound PA was in an enzymatically active form or not. Although activity was demonstrable in the underlays after electrophoresis this may have been induced by the mild denaturing effect of the SDS which, as has been demonstrated by others (Granelli-Piperno and Reich, 1978) may expose the active site of the enzyme in a protease-inhibitor complex. To have answered the question of whether or not the fibroblast-associated PA-binding molecule was an inhibitor or whether it served simply to concentrate proteolytic activity on the surface of the fibroblast would have required an assay for PA in fibroblast lysates that was linear

over a wide range of protein concentrations, that was uninfluenced by other possible intracellular inhibitors, and that allowed for confident quantitative interpretations of values for recovery of added enzyme. Unfortunately I have been unable to develop such an assay. I have, however, been able to demonstrate on two occasions, the presence of melanoma PA in lysates of fibroblasts after exposure to melanoma harvest fluid. In these experiments (results not presented) the enzyme was assayed in the standard ^{125}I-fibrin assay without exposure of the cells to detergents. These results would indicate that at least part of the cell-associated enzyme was still active. As indicated above, however, I am uncertain of the recoveries obtained and I am hence unable to give a quantitative estimate of the extent to which cell-associated enzyme was inhibited.

The nature of the fibroblast-associated PA binding molecule has not been completely defined except in the negative sense that it did not appear to be fibronectin, nor was it derived from serum since fibroblasts cultured for up to 7 days without serum or in acid-treated serum were as effective in the inhibition of melanoma-PA as fibroblasts cultured in the presence of 10% foetal calf serum.

The superficial similarities between the fibroblast effect that I observed and the phenomenon described by Baker et al (1980) and Low et al (1981) and ascribed to "protease-nexin" were striking. In both cases, a fibroblast product with an assumed molecular weight of approximately 40-50K daltons, complexed with PA and retarded its electrophoretic mobility in SDS gels. On reflection, however, it seems unlikely that the fibroblast-associated PA-binding molecule that I describe and protease nexin, were the same molecule.

Low et al (1981) reported that urokinase lost its fibrinolytic activity when linked to PN. As seen in the fibrin-agar zymograms, this was not the case with fibroblast-bound PA. Furthermore, thrombin could

competitively inhibit the binding of PN to urokinase, whereas thrombin did not appear to compete in any way with the binding of PA to fibroblasts. Finally, in the experiments of Low et al (1981) a major fraction of the fibroblast PN was released into the culture medium whereas I was unable to detect any difference in the activity of PA incubated with conditioned medium obtained from fibroblasts. Baker et al (1980) and Low et al (1981) did not examine PN for its ability to complex with tissue-type PA. I am hence unable to compare my results with theirs in this respect.

Despite the fact that the PN-mediated phenomenon and the fibroblast-tissue PA interaction that I describe are probably different, they may well both represent cell-protease interactions similar to those described between trypsin and murine cells (Kirschner et al, 1980); between fibrinolysin and rabbit vascular endothelial cells (Loskutoff and Edgington, 1977); and between thrombin and a number of cell types, such as mouse embryo cells or Chinese hamster lung cells (Simmer et al, 1979), chick embryo fibroblasts (Quigley et al, 1979), and bovine vascular endothelial cells (Isaacs et al, 1981).

It would be premature to do anything but speculate on the physiological role that such protease-cell interactions may have *in vivo*. It is, however, reasonable to suspect they may serve a regulatory purpose - with either the cell modulating proteolytic activity or conversely, proteolysis influencing cellular function and behaviour.

There is, for example, good reason to believe that malignant cells may influence surrounding cells by the secretion of proteases.

Jaken and Black (1979) expressed this concept well when they wrote: "Because PA is expressed extracellularly, and because outside the cell the amount of proteolytic activity can be amplified many-fold by the catalytic conversion of plasminogen to plasmin, it is reasonable to suggest that elaboration of PA by cells provides them with a potentially

powerful mechanism for interacting with and changing their environment". Proteases such as trypsin, pronase, or thrombin have indeed been found to have mitogenic activity in 3T3 cells (Burger et al, 1972) and chick embryo fibroblasts (Quigley et al, 1979; Zetter et al, 1976; Cunningham and Ho, 1975). Other changes have also been induced as, for example, increased agglutinability in human and chick embryo fibroblasts (Cunningham and Ho, 1975) after treatment with pronase or trypsin, or morphological changes with a reduction in cell-cell interactions when chick embryo fibroblasts were exposed to several proteolytic enzymes, including thrombin and trypsin (Zetter et al, 1976).

It is also possible that fibroblast receptors for serine proteases may mediate the stimulatory effects of such enzymes on cell division or may be involved in the transmission of signals from one cell to another. They might also serve to regulate the amount of extracellular PA in the immediate vicinity in which case these molecules could be considered as agents for the concentration of PA on the cell-surface prior to its internalization and degradation.

Finally, the intriguing possibility remains that the reaction of serine proteases with fibroblasts and other cells may foster growth or promote the expression of other phenotypic characteristics. Sirbasku and Benson (1979) for example, have proposed that estrogens produce promotion of tumour growth by inducing the production and secretion of growth factors from tissue distant to the tumour mass which then act on the tumour cells resulting in proliferation. In a similar fashion it is conceivable that the stimulus provided by PA binding to a fibroblast receptor molecule might trigger the production of growth factors which would stimulate adjacent tumour cells.

Any speculation on the role that fibroblast-associated PA-binding molecules might have should take into account the fact that they appear

to complex very much more efficiently and effectively with tissue-PA than they do with urokinase. It may well be that if distinct physiological roles for these two plasminogen activators can be defined a clearer understanding of the function of the fibroblast phenomenon will emerge.

Tissue PA and urokinase differ not only in their molecular weights and in their immunochemical characteristics, but also in their physiological distribution, the kinetics of their ability to hydrolyse synthetic substrates and the extent to which they bind to and are stimulated by fibrin (Thorsen, 1977; Wallen et al, 1977; Heussen and Dowdle, personal communication; Camiolo et al, 1981). The phenomenon that I have described documents one further respect in which these two serine proteases differ.

CHAPTER 6

THE GROWTH OF HUMAN MELANOMA CELLS IN THE NUDE MOUSE

Congenitally immunodeficient mice of use for the study of human neoplasms *in vivo* have been available since the late 1960's when Flanagan (1966) and Pantelouris (1968) described and characterised the "nude" athymic mutant that would accept xenografts. Animals homozygous for the recessive *nu* allele have now been extensively used for supporting growth of human neoplasms - so much so, in fact, that "tumorigenicity in the nude mouse" has become an accepted criterion of malignancy for cultured cell lines (Stiles et al, 1976b, c). There is certainly no better model for the laboratory study of metastatic potential of human tumours than the immunodeficient animal. Although, as I discuss later, the nude mouse is limited in its ability to serve as a host for the investigation of human tumour metastasis, it serves as a convenient and perhaps even a more valid laboratory animal for this purpose than the chick embryo or the immunosuppressed rodent.

The *nu* gene has been transferred to several other inbred strain backgrounds. Recently, Hansen (1978) developed a strain N:NIH(S)II - *nu/nu* that was doubly congenic with the athymic *nu/nu*-bearing strains and the CBA/N strains carrying an X-linked immunodeficiency. This provided a model with combined immunodeficiency (Azar et al, 1980) that has proved extremely susceptible to the grafts of human neoplasms.

A colony of these animals has been established in our animal house and I have used them to determine the tumorigenic nature of my melanoma cell lines, to characterize their growth as xenografts and to study the effects of co-injected human skin fibroblasts on their *in vivo* proliferation.

In this chapter I report the results of these experiments.

MATERIALS AND METHODS

N:NIH(S)II-*nu/nu* (NIH-2) mice

The mice used were of the recently developed strain (Azar et al, 1980), N:NIH(S)II-*nu/nu*. These mice have combined immunodeficiency, i.e. in addition to the thymic hypoplasia found in the original strain of *nu/nu* mice, both T-cell and B-cell zones of lymph nodes and the spleen are depleted of lymphocytes.

The NIH-2 mouse colony in this laboratory was established from two breeding pairs obtained from Dr. B.C. Giovanella at the Cancer Research Laboratory, St. Joseph Hospital, Houston, Texas. The colony was maintained by breeding pairs of the heterozygous females and homozygous *nu/nu* males. The homozygous (*nu/nu*) female offspring were used for experiments.

The mice were housed on open shelves in a specific pathogen-free environment, in isolation from other animals. The plastic cages were covered with polyester-fibre bonnets (Lab. Products Inc., Mainwood, N.J.) and mice were provided with autoclaved food, water and bedding. The drinking water was supplemented with vitamins (0.5 ml per litre of Pancebrin, Lilly Pharmaceuticals) and antibiotics (27 mg per litre of cefamandole nafate (Mandokef, Lilly Pharmaceuticals). To avoid any possible effects of antibiotics on tumour growth, cefamandole was discontinued 7 days before inoculation of tumour cells and was omitted from the drinking water for the remainder of the duration of the experiment.

The mice were handled under sterile conditions at all times and tumour cell inoculations, surgery and examinations were done in a laminar flow hood by personnel clothed in sterile gowns and wearing sterile rubber gloves. Mice kept under these conditions thrived. The growth rate of homozygotes was comparable to that of heterozygous littermates and their

lifespan approached the 2-year life span of normal mice.

Inoculation of Tumour Cells

Cells to be injected were removed from the tissue culture dish by treatment with 0.06% trypsin containing 0.02% EDTA in a buffered salt solution. Foetal calf serum was then added to a final concentration of 5% to neutralize the trypsin, and the cells were pelleted and washed once with serum-free medium.

The cells were resuspended in serum-free RPMI medium and adjusted to provide the requisite number for injection in a volume of 0.1 ml.

Cells were injected subcutaneously into the scapular region of 2 month-old female mice. All animals were observed for at least 3 months for the appearance of tumour growth.

The mice were weighed and their tumours were measured at weekly intervals. The product of the three major diameters was recorded as the tumour volume. All tumours were examined histologically.

In certain cases, to prevent death from the tumour that developed from the primary inoculum, tumours were removed under ether anaesthesia. The mice were then killed a minimum of 10 weeks later and examined macroscopically by dissection for evidence of metastases. Lymph nodes adjacent to the primary tumour, and the lungs were examined histologically for evidence of metastases.

RESULTS

Growth of Human Melanomas in Nude Mice

When melanoma cells were inoculated, in numbers ranging from 10^3 to 5×10^6, into nude mice, tumours developed at the site of inoculation and grew progressively. This general statement requires qualification in two respects. Firstly, smaller inocula (10^3 - 10^5 cells) did not always produce tumours. Secondly, one of the melanoma cell lines tested (UCT-Mel 6) consistently failed to produce tumours even when injected in large numbers into nude mice that were observed for 92-126 days.

When tumours did grow, a fairly consistent pattern of growth was observed. Following a latent period, during which time the tumour was growing but imperceptible, there was a period of overt growth which could be followed fairly accurately by estimating tumour volume as the product of 3 axial measurements and plotting this as a function of time. Although in many cases somewhat erratic, the growth rates observed could all be seen to have an exponential phase from which a "doubling time" could be derived (Table 6.1). The doubling time *in vivo* could be determined more easily and more reliably than could the latency period for the simple reasons (a) that the dividing line between perceptibility and imperceptibility is an arbitrary one and (b) that mice were examined only weekly, so that the time of appearance of a tumour could only be assigned with this degree of accuracy. When parallel growth curves were observed that extrapolated to different intercepts, I have concluded that the tumour whose growth curve was to the left had a shorter latency period.

With these qualifications, the latency period and *in vivo* growth rates of the 7 melanoma cell lines are presented in Tables 6.2 and 6.3 and Figs. 6.1a and b.

TABLE 6.1.

GROWTH OF HUMAN MELANOMAS IN NUDE MICE[a]

Cell Line	Key (Fig. 6.1)	Exponential Growth[b] Period (Days)		Doubling Time (Days)	
		Inoculum$_6$ = 1 x 10^6	Inoculum$_6$ = 5 x 10^6	Inoculum$_6$ = 1 x 10^6	Inoculum$_6$ = 5 x 10^6
UCT-Mel 1	△——△	12-41	6-42	3.2	3.2
UCT-Mel 2	▲- - -▲	30-55	28-50	4.1	3.3
UCT-Mel 3	▼·····▼	19-28	14-28	4.0	3.2
UCT-Mel 4a	▽——▽	42-98	27-71	15.6	8.0
UCT-Mel 4b	■·····■	57-118	36-71	9.0	5.5
UCT-Mel 5	□·—·-□	12-153	7-63	17.6	8.4
UCT-Mel 7	◆——◆	20-61	20-48	6.2	5.2

(a) Nude mice were injected with 10^6 or 5 x 10^6 melanoma cells and were subsequently observed as described in the legend to Fig. 6.1a and Fig. 6.1b respectively.

(b) Column entries indicate the first and last days of the exponential growth period that were used to calculate the doubling times given in the last column.

TABLE 6.2

TABLE 6.2

GROWTH OF HUMAN MELANOMAS IN NUDE MICE.

Cell Line	No. of Cells inoculated	Passage No.	(No. tumours/ No. mice)	Minimum Latency Period[a] (weeks)	Doubling Times *In vivo*[b] (days)	Doubling Times *In vitro* (hrs)
UCT-Mel 1	10^6	105;118	2/2	1	3.2	41
	5×10^6	105;118	2/2	1	3.2	
UCT-Mel 2	10^3	107	0/2	-	-	50
	10^4	107	1/2	9	4.7	
	10^5	88;96;107	2/6	6	4.2;9.6	
	10^6	88;96	4/4	2	4.1	
	5×10^6	83	2/2	4	3.3	
UCT-Mel 3	10^6	43;60	2/2	3	4.0;10.7	50
	5×10^6	43;60	3/3	1	3.2;7.0;8.3	
UCT-Mel 4a	10^6	23	1/1	6	15.6;10.0[c]	52
	5×10^6	23	1/1	1	8.0	
UCT-Mel 4b	10^6	21	1/1	1	9.0	54
	5×10^6	21	1/1	1	5.5	
UCT-Mel 5	10^6	8;16	3/3	1	17.6	58
	5×10^6	8;16	3/3	1	8.4	
UCT-Mel 6	10^6	20	0/1	-	-	33
	5×10^6	20;25	0/2	-	-	
	10^7	25;40	0/5	-	-	
UCT-Mel 7	10^6	10	1/1	3	6.2	95
	5×10^6	10	1/1	3	5.2	

TABLE 6.2

GROWTH OF HUMAN MELANOMAS IN NUDE MICE

(a) The minimum latency period or time taken for tumours to become apparent was recorded on the basis of weekly observations where a search was made by inspection and palpation for a detectable subcutaneous mass. Tumours were clearly evident when they had reached a volume of approximately 8 cu. mm.

(b) The *in vivo* doubling time was calculated from the period of exponential tumour growth as indicated in the legend to Fig. 6.1 and the footnote to Table 6.1. Where the tumour doubling times in individual mice differed by less than one day, the mean doubling time is given. In other cases, the individual doubling times are presented.

(c) In this instance, two distinct phases of exponential growth were evident. The two values presented represent the doubling times found in the first and second growth phases respectively.

TABLE 6.3

GROWTH OF HUMAN MELANOMAS IN NUDE MICE

Cell Line	No. of Cells inoculated	No.of tumours studied	Day at which tumour volume reached 500 cu.mm	Day tumour removed	Tumour Mass (g)
UCT-Mel 1	10^6	2	28 ; 45	42 ; 68	1.6 ; 1.9
	5×10^6	2	21 ; 21	34 ; 42	1.7 ; 4.1
UCT-Mel 2	10^4	1	76	98	3.7
	10^5	2	68 ; 118	77 ; 134	1.3 ; 1.4
	10^6	4	45;48;52;55(a)	55;55;62;70(a)	1.9;1.8;2.1;2.3(a)
	5×10^6	2	51	64	2.2 ; 2.6
UCT-Mel 3	10^6	2	24 ; 65	47 ; 107	2.4 ; 1.2
	5×10^6	3	23;50;50(a)	34;107;107(a)	1.5;2.1;1.7(a)
UCT-Mel 4a	10^6	1	159	185	1.4
	5×10^6	1	67	71	0.7
UCT-Mel 4b	10^6	1	99	133	2.7
	5×10^6	1	69	77	1.5
UCT-Mel 5	10^6	1	94	153	2.0
	5×10^6	2	49 ; 56	63	1.2
UCT-Mel 7	10^6	1	53	70	1.3
	5×10^6	1	46	56	1.2

(a) The sequence of results is similar in all three columns.

FIGURE 6.1

FIGURE 6.1

Growth of human melanomas in nude mice.

Mice were injected with either 10^6 (Fig. 6.1a) or 5×10^6 (Fig. 6.1b) melanoma cells on Day 0 and the tumour volume was estimated as the product of 3 axial measurements at the indicated times thereafter. Curves in the figure were constructed by plotting estimated tumour volume as a function of time for a single mouse in the case of UCT-Mel 3, 4a, 4b and 7, or by plotting the average tumour volume of two mice in the case of UCT-Mel 1, 2 and 5. It will be noted:

(a) in all cases a period of approximate exponential growth could be discerned from which a doubling time *in vivo* could be calculated. This was done from the data presented in the figure as summarized in Table 6.1.

(b) that the growth patterns varied, as represented by the two extremes, illustrated by UCT-Mel 1 and UCT-Mel 4a in Fig. 6.1a. The former grew exponentially from the time of its first appearance. The latter grew very slowly for approximately 50 days, after which it entered exponential growth. After a further 50 days, a lag period lasting approximately 30 days was observed, after which exponential growth resumed once more.

The tumours illustrated are as follows:

UCT-Mel 1 (△——△); UCT-Mel 2 (▲- - -▲); UCT-Mel 3 (▼·····▼); UCT-Mel 4a (▽——▽); UCT-Mel 4b (■·····■); UCT-Mel 5 (□—·—□); UCT-Mel 7 (◆——◆).

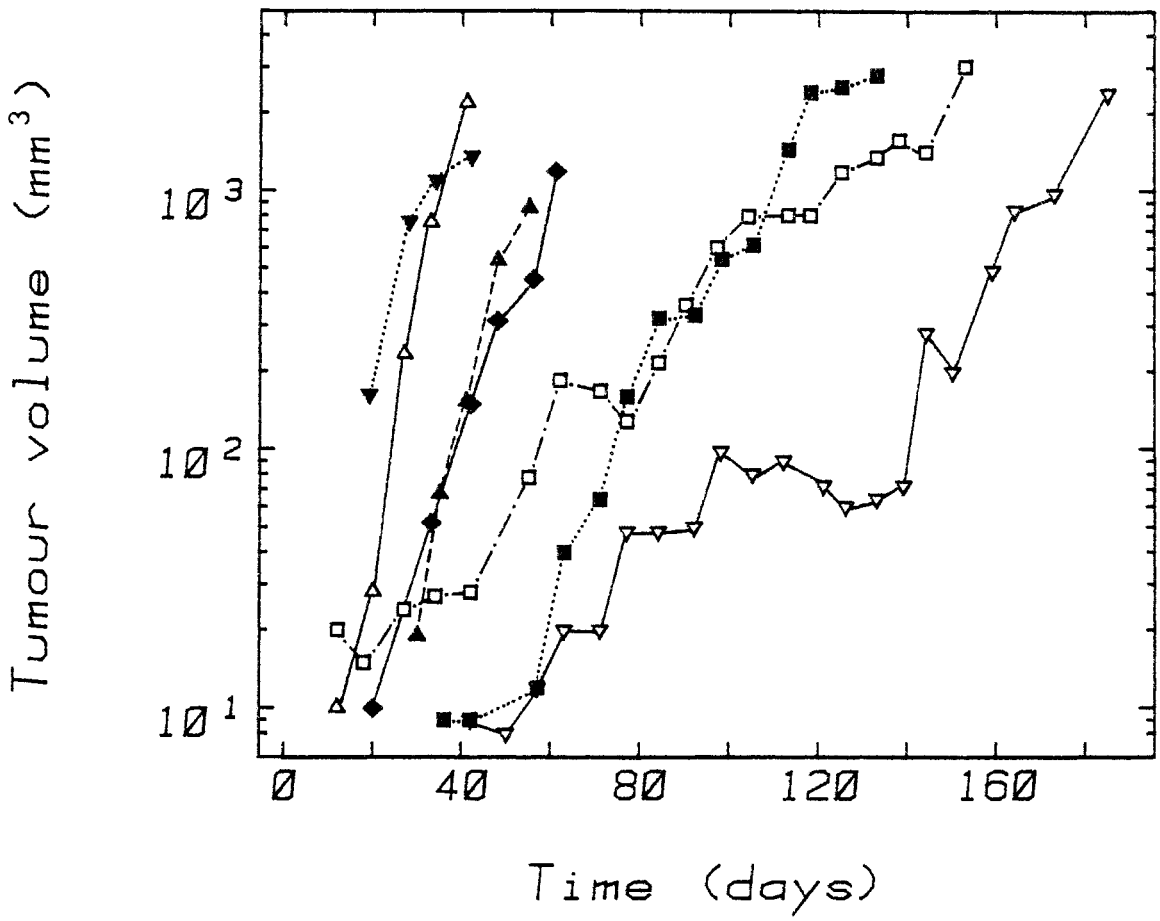


FIGURE 6.1a

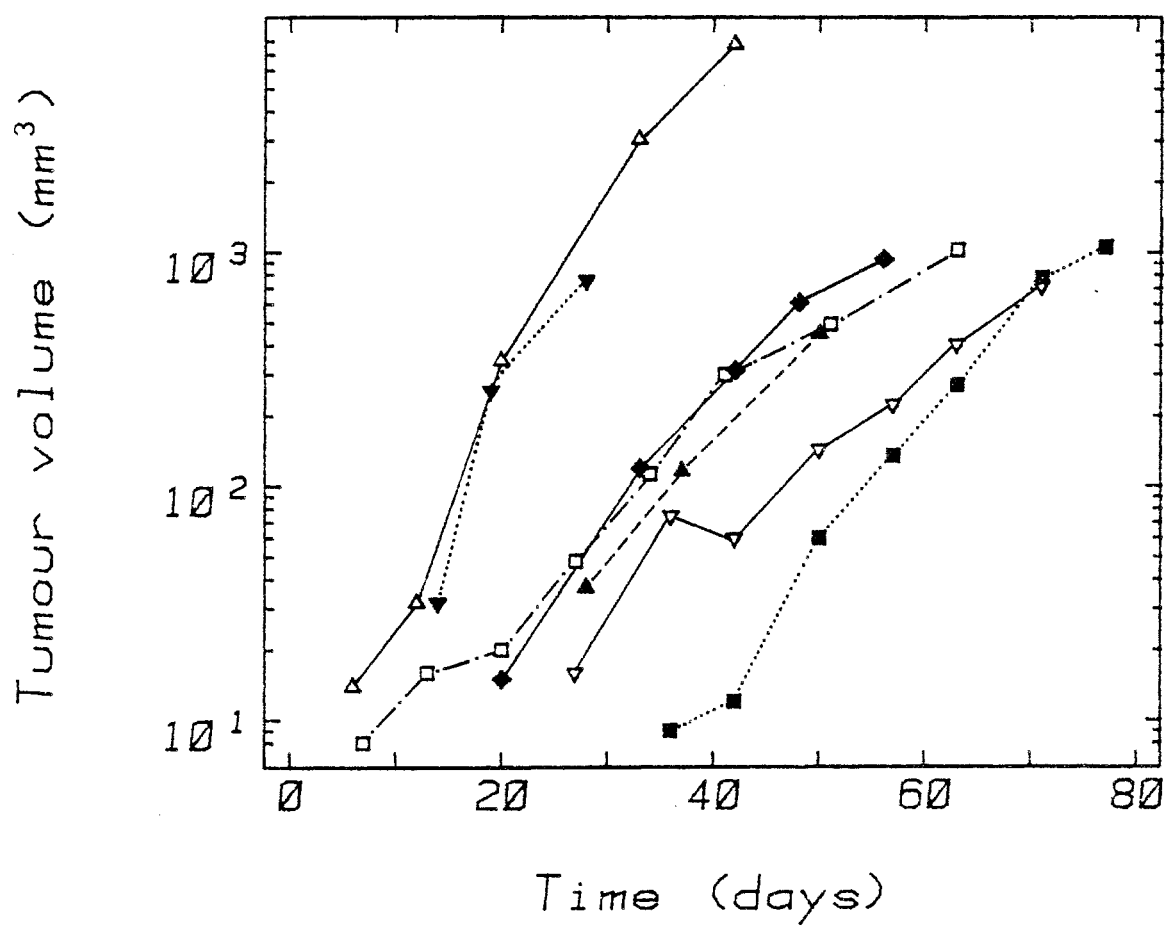


FIGURE 6.1b

The kinetics of the melanoma cell growth as I observed them were characterized most strikingly by their considerable variability with no obvious correlations between doubling time in the mouse, generation time *in vitro*, plating efficiency *in vitro* or ability to form colonies in soft agar. The data that support these observations are presented in Fig. 6.2 and Table 6.7. Doubling time *in vivo* varied from 3.2 days to 17.6 days and was generally unaffected by the size of the tumour inoculum (Fig. 6.3 and 6.4).

One consistent exception to this observation was provided by UCT-Mel 5, where the doubling time *in vivo* was inversely related to the inoculum size. In an experiment in which 3 mice were given 5×10^6 cells and 3 were inoculated with 1×10^6 cells, the tumours that arose from the larger inocula had *in vivo* doubling times of 8.1, 5.8 and 8.8 days, whereas doubling times of tumours from smaller inocula were 18.6 and 11.9 days.

Latency periods varied from one week to 12 weeks and in most instances the duration of the latency period was inversely related to inoculum size.

UCT-Mel 7 had a doubling time of 95 hours *in vitro* and a half time of approximately 6 days *in vivo*. By contrast, UCT-Mel 4a showed a half time of 52 hr *in vitro* with a doubling time *in vivo* of more than 8 days. Examples intermediate between these two extremes were also observed (Table 6.2).

The growth patterns of UCT-Mel 4a and 4b, although derived at the time of establishment from cells showing different morphological characteristics (see Chapter 2), were interestingly similar in that both showed a period of dormancy that interrupted periods of growth. In the case of UCT-Mel 4a, the tumour grew progressively for 48 days, remained dormant for approximately 40 days, then resumed growth at an increased rate. In the case of UCT-Mel 4b, periods of growth arrest of 35-50 days were observed,

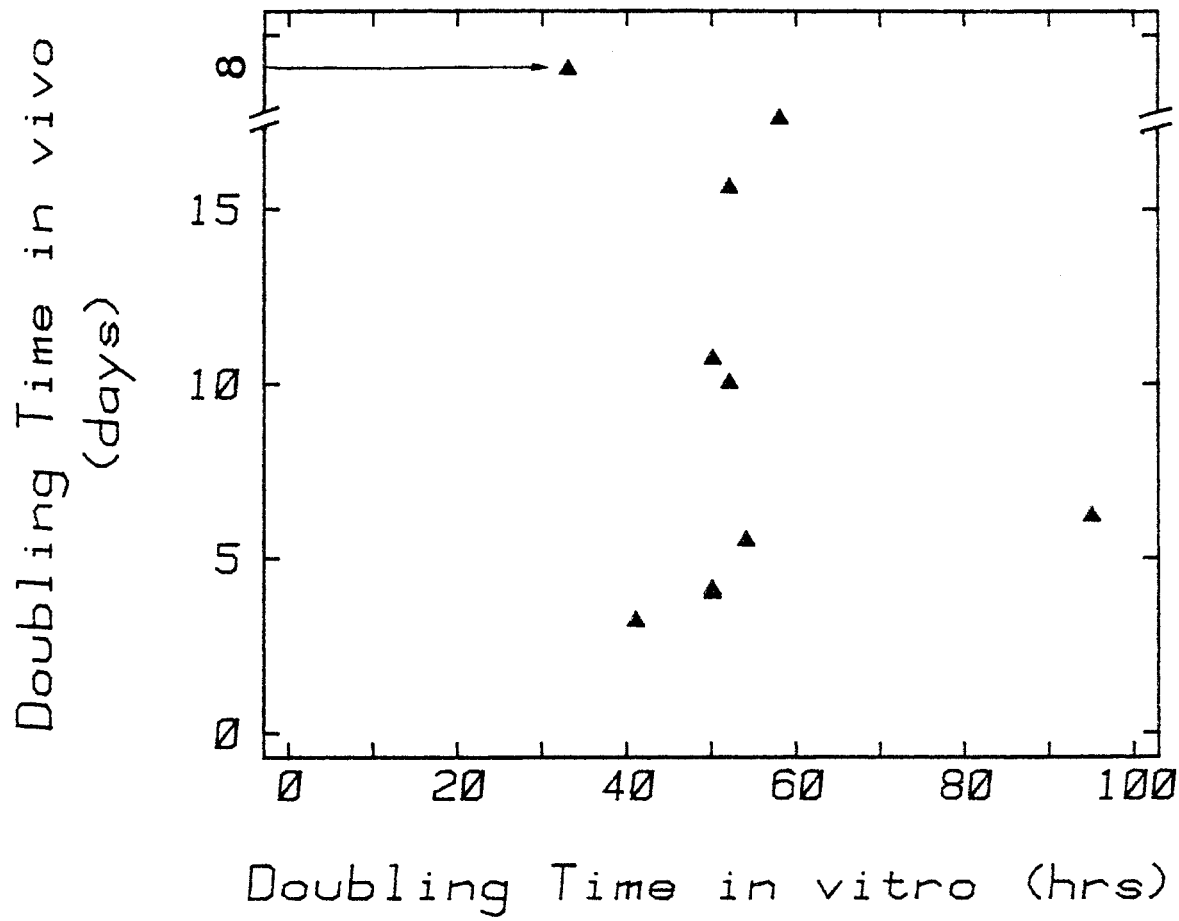


FIGURE 6.2

Relationship between proliferation *in vitro* and *in vivo*.

Nude mice were inoculated with 10^6 melanoma cells and *in vivo* growth patterns and doubling times were studied as shown in Fig. 6.1a. *In vitro* doubling times were obtained from growth curves for each of the melanoma cell lines as measured at the approximate passage number of the cells used for inoculation. Each point represents results obtained from a single mouse for a different melanoma line. In the case of UCT-Mel 3 and 4a, where widely differing *in vivo* doubling times were obtained (see Table 6.2) both *in vivo* doubling times were plotted.

Note the evident lack of correlation between *in vitro* and *in vivo* growth rates.

The arrow indicates the *in vitro* doubline time of UCT-Mel 6, the cell line which did not grow in nude mice.

FIGURE 6.3

FIGURE 6.3

Growth of human melanomas in nude mice: variability of growth patterns and effects of inoculum size.

Each line and set of points represents data accumulated from a single mouse. Cells were injected subcutaneously at either 1×10^6 (▲——▲) or 5×10^6 (■--■) cells per mouse, as follows: A) UCT-Mel 1; B) UCT-Mel 3; C) UCT-Mel 4a; D) UCT-Mel 4b. Tumour volume was estimated at the times indicated as the product of 3 axial measurements. Note that the inoculum size was inversely related to the latency period (except in (B)), but was unrelated to the doubling time.

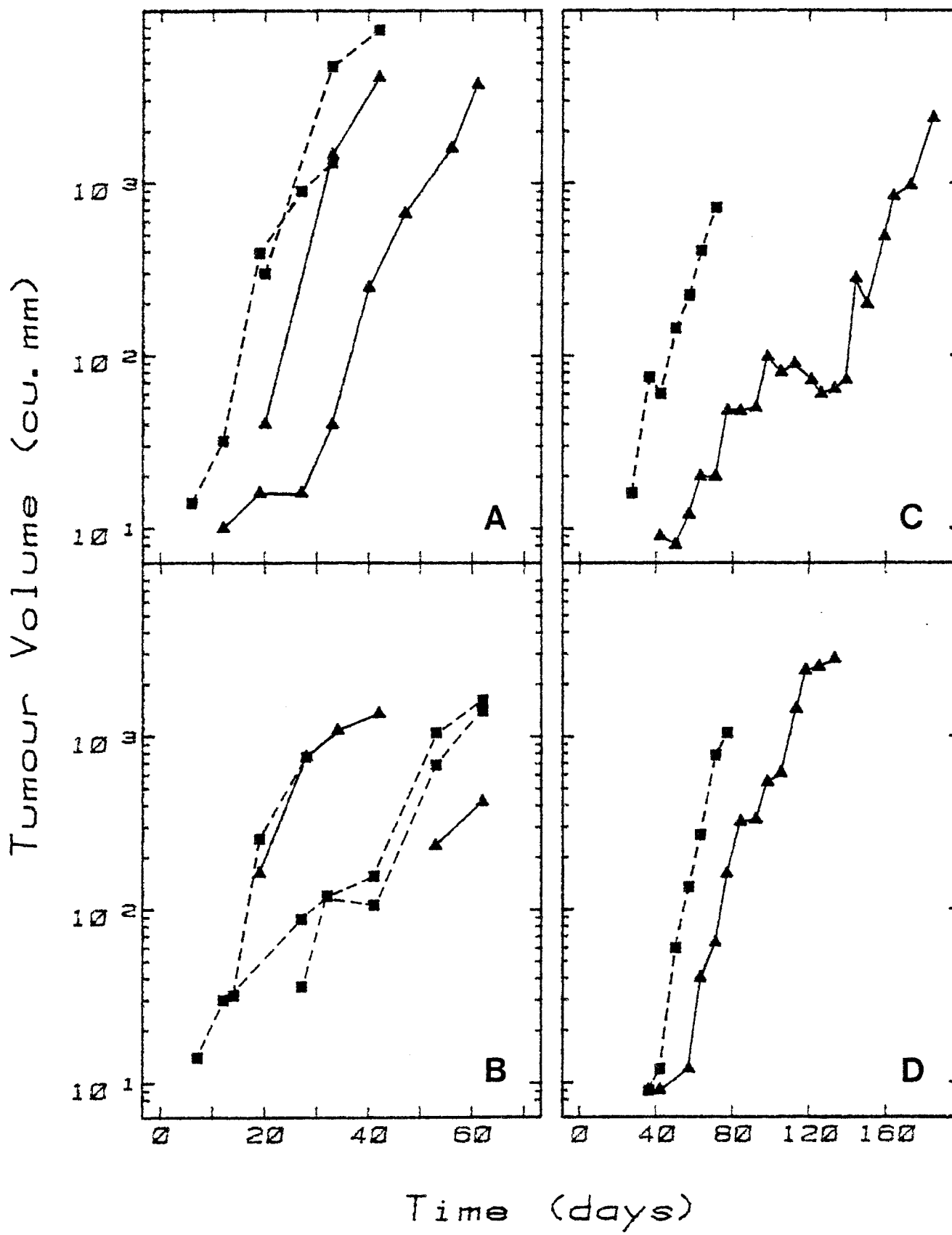

Tumour Volume (cu. mm)
10 3
10 2
10 1
A
C
B
D
0
20
40
60
0
40
80
120
160
Time (days)

FIGURE 6.3

FIGURE 6.4

FIGURE 6.4

Growth of human melanomas in nude mice: variability of growth patterns and effects of inoculum size.

A) UCT-Mel 2: mice were injected with UCT-Mel 2 cells as summarized in the following table:

No. of mice	No. of cells inoculated	Tumours	Symbol.
2	10^3	0	-
2	10^4	1	▼- - ▼
6	10^5	2	◆·····◆
4	10^6	4	▲——▲
2	5×10^6	2	■- - -■

B) UCT-Mel 5 and C) UCT-Mel 7: mice were injected with 10^6 (▲——▲) or 5×10^6 (■- - -■) cells. *In vivo* growth rates were recorded as described in the legend to Fig. 6.1. Note that in (A), tumour growth was uncertain when low inocula were used, and inoculum size was usually inversely related to the latency period but was unrelated to the doubling time. In (B), the tumour growth rate appeared to be directly related to the cell inoculum, and the latency times were not affected. In (C), little difference was noted between tumour latencies and growth rates with the different inocula.

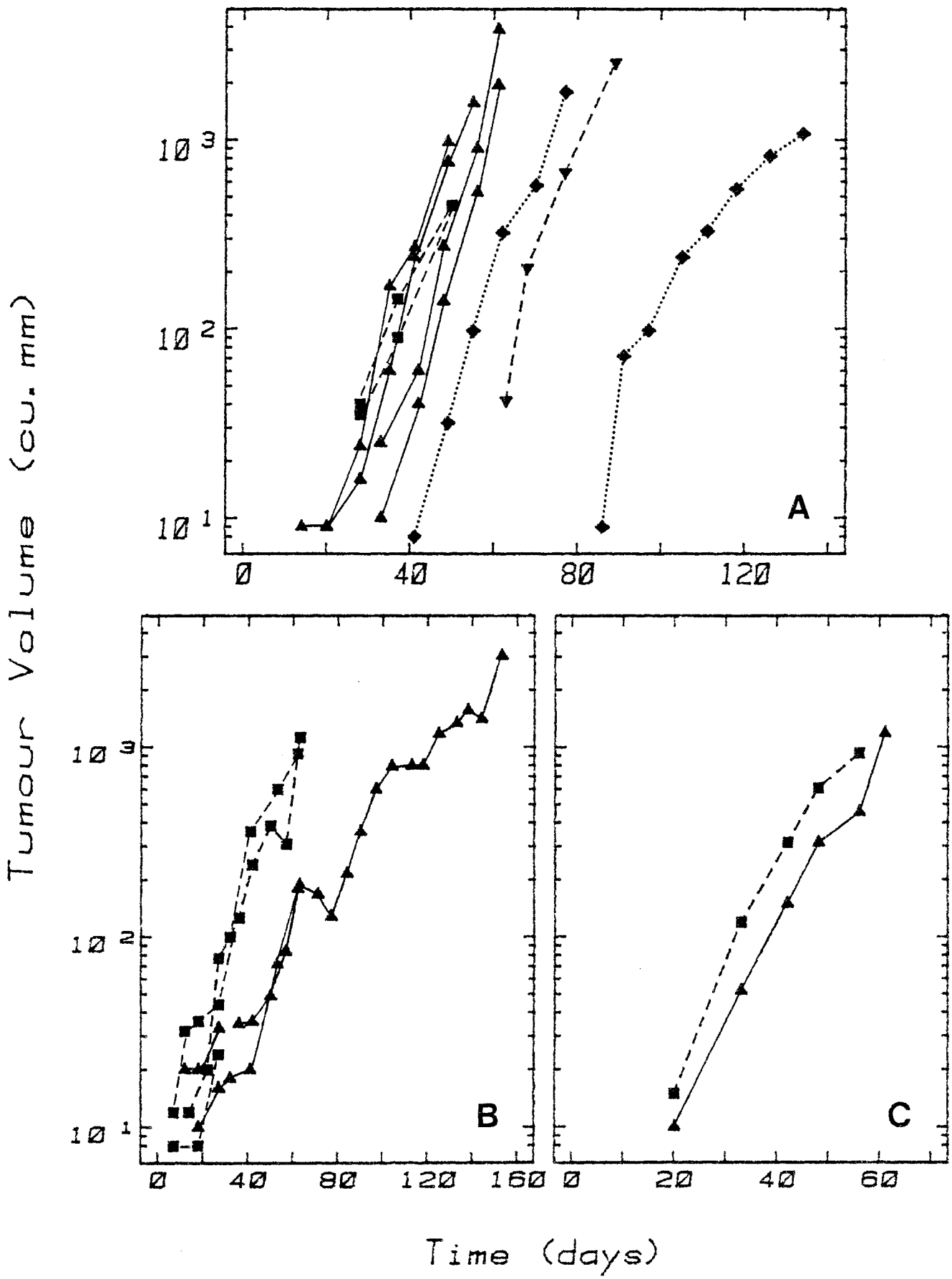

Tumour Volume (cu. mm)
10 3
10 2
10 1
0
40
80
120
A
B
C
160
20
60
Time (days)

FIGURE 6.4

that started only 8 days after inoculation when the tumours were barely visible. Following these dormant periods, rapid growth resumed with doubling times of 5-9 days.

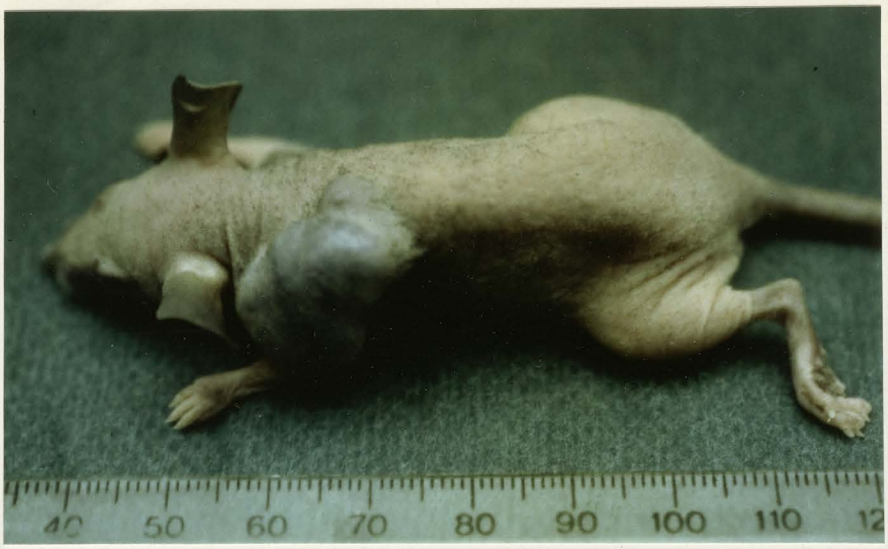
Pigmentation of Human Melanomas in Nude Mice

It is well known that human melanomas growing as primary or metastatic tumours show varying degrees of pigmentation (Clark et al, 1979) This variation is frequently observed in primary tumours and has, indeed, been described as a diagnostic feature of malignant melanomas that distinguishes them from benign pigmented lesions.

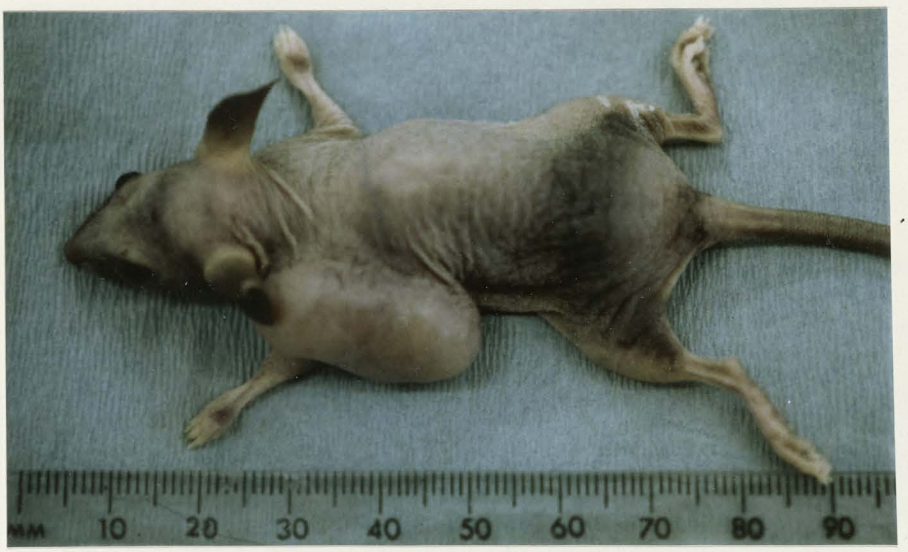
The variation between patients is even more pronounced with certain individuals having tumours that are entirely amelanotic and others having deeply pigmented lesions. The series of melanomas that I established were no exception to the rule in terms of the varying pigment that they displayed. Two of these (UCT-Mel 1 and 4) were reported on as pigmented, while two of them (UCT-Mel 5 and 6) were described as nonpigmented and three (UCT-Mel 2, 3 and 7) were described as slightly pigmented.

Since the cell lines that emerged showed a consistent tendency to pigment or not to do so, and since this did not necessarily correlate with the appearance of the tumour in the patient, it was of interest to observe macroscopically the tumours that grew in nude mice for the degree of pigmentation that they manifested.

For the most part, the results were unremarkable. UCT-Mel 1, which consistently pigmented *in vitro*, invariably grew as a deeply pigmented tumour in the nude mice (Fig. 6.5, 6.6 and 6.7). UCT-Mel 3, 4a, 4b, 5 and 7 did not pigment *in vitro*, neither did they show evidence of melanin pigmentation in the nude mouse (Fig. 6.5 and 6.6). On occasions, these tumours were highly vascular and contained blood, which gave the appearance of melanin pigment but proved not to be so (Fig. 6.6).



A



B

FIGURE 6.5

Representative examples of nude mice bearing human melanoma tumours.

Female nude mice at age 2 months were injected with 10^6 UCT-Mel 1 (A) or 10^6 UCT-Mel 2 (B) cells subcutaneously on Day 0. The above photographs show the appearance of the xenografts on Day 35 and 55 respectively. Note that pigmentation (A) or the lack of it (B) was clearly visible in the subcutaneous tumour. The scale markers indicate mm.

FIGURE 6.6

FIGURE 6.6

The appearance of tumours on removal from nude mice.

The gross appearance of human melanoma tumours removed from nude mice varied from nonpigmented to deeply pigmented.

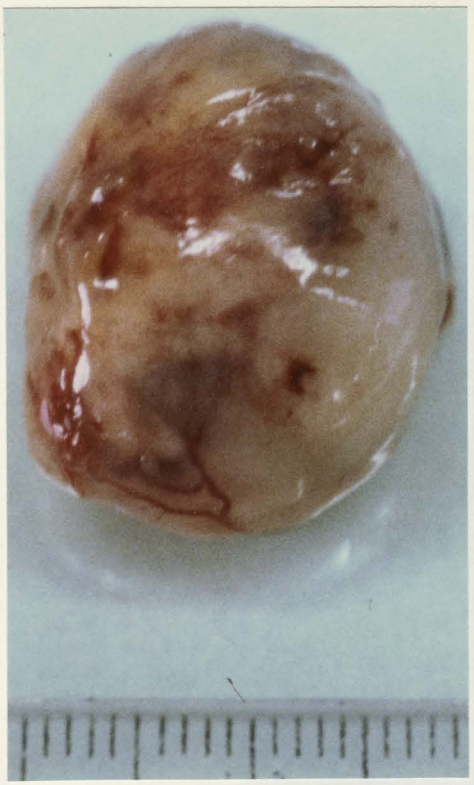
(A) - a nonpigmented tumour removed from a mouse injected with 1×10^6 UCT-Mel 2 cells

(B) - a partially pigmented recurrent tumour which followed resection of a pigmented tumour induced by the inoculation of 5×10^6 UCT-Mel 2 cells

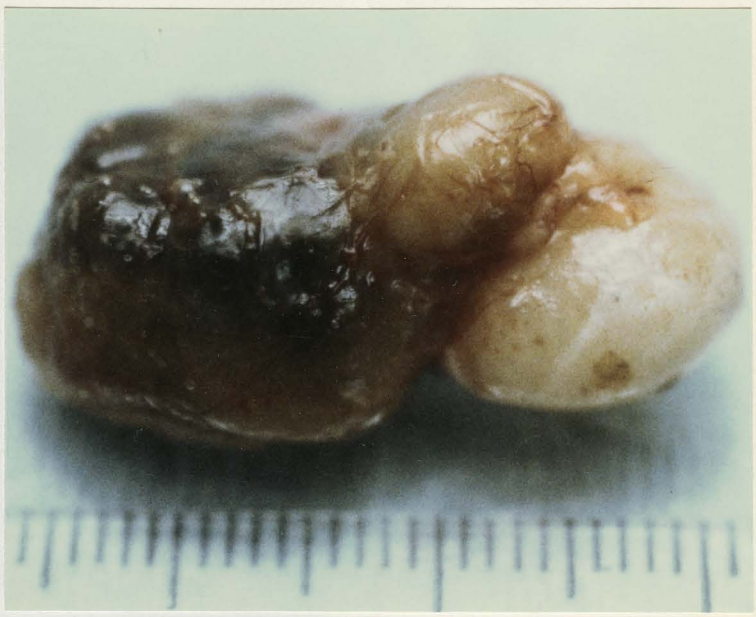
(C) - a deeply pigmented tumour removed from a mouse injected with 1×10^6 UCT-Mel 1 cells

(D) - an apparently pigmented tumour removed from a mouse injected with 5×10^6 UCT-Mel 3 cells. On dissection and histological examination, the dark colour was found to be due to blood.

Scale Marker: 1 small division represents 1 mm.



A



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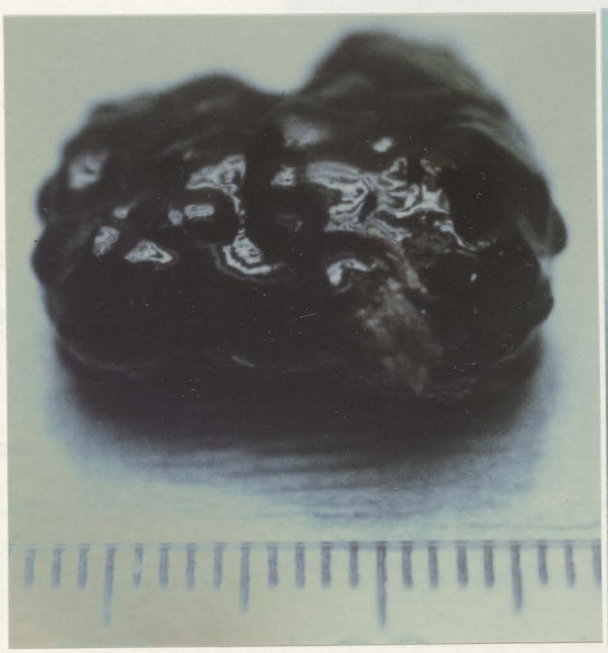
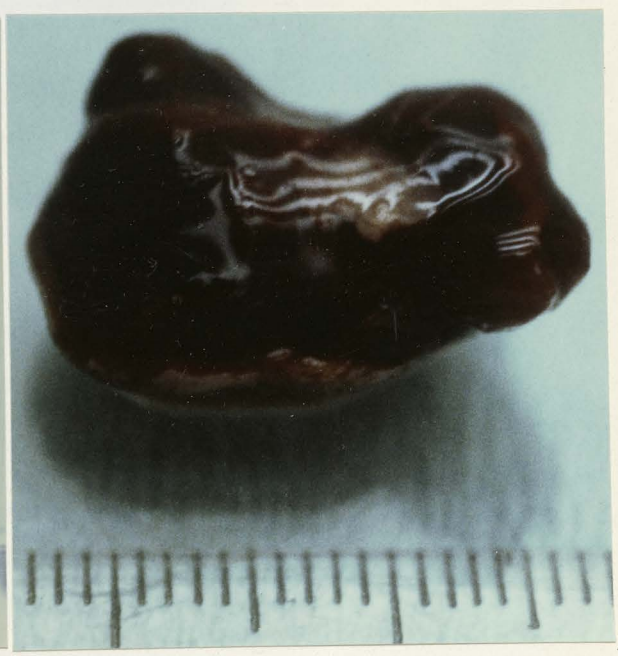


C



D

FIGURE 6.6

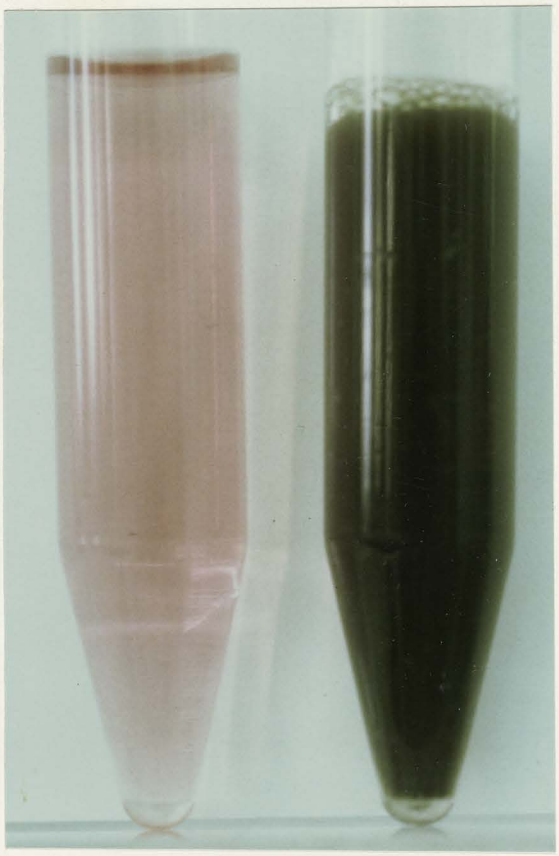


FIGURE 6.7

Pigmentation of UCT-Mel 1 in the nude mouse.

The tubes shown contain RPMI medium alone (left-hand side) or medium in which a tumour removed from a mouse injected with 1×10^6 UCT-Mel 1 cells had been minced (right-hand side).

The results with UCT-Mel 2, were of some interest. It may be recalled that this tumour was established from a biopsy specimen which was reported as being partially pigmented. When cultured *in vitro*, however, UCT-Mel 2 cells became heavily pigmented as they reached confluence. Tyrosinase activity in these cells was also modulatable inasmuch as it could be affected by retinoids (Chapter 4). In the nude mouse UCT-Mel 2 gave rise to tumours that were on occasions heavily pigmented; on other occasions the tumours, although of equal size, showed faint "speckling" or no pigmentation and on other occasions, gave rise to tumours with clearly visible confluent pigmented and nonpigmented areas (Fig. 6.6).

Examination of the assembled data from experiments with UCT-Mel 2 gave the distinct impression that the degree of pigmentation was directly related to the size of the inoculum and not to the mass of the tumour or its growth rate. These data are recorded in Table 6.4. Although I have not studied this phenomenon in greater detail, it clearly warrants further investigation at a later stage since it implies that cell density or cell-cell contacts at the inoculation site at the time of xenograft establishment are important determinants of differentiation. In some respects, these results are reminiscent of *in vitro* findings of increased pigmentation with increased cell density (Chapter 2).

Further experiments would require quantitative estimates of melanin content based upon measurements of tyrosinase and melanin, and experiments designed to establish whether or not the inoculum size selected in some way for proliferation of clones with different potential for pigmentation.

TABLE 6.4

TABLE 6.4

EFFECT OF INOCULUM SIZE AND CO-INOCULATION OF SKIN FIBROBLASTS ON PIGMENTATION OF UCT-MEL 2 IN THE NUDE MOUSE

(a) Pigment assessed visually on an arbitrary scale from 0 to +++ according to the following criteria:-

0	no pigmentation
+	fine uniformly distributed speckled pigmentation
++	confluent areas of distinct pigmentation
+++	heavily pigmented

(b) This was a recurrent tumour which appeared in a mouse from which a tumour had previously been removed. The first removal of the tumour is shown as Experiment 4a i.e the tumour removed 57 days post inoculation. Fifty-eight days later i.e. 115 days post inoculation, the recurrent tumour was removed (shown as Experiment 4b).

TABLE 6.4

EFFECT OF INOCULUM SIZE AND CO-INOCULATION OF SKIN FIBROBLASTS ON PIGMENTATION OF UCT-MEL 2 IN THE NUDE MOUSE

Experiment No.	Inoculum		Removed (Days post inoculation)	Mass (g)	Pigmentation[a]
	UCT-Mel 2	Skin fibroblasts			
1	5×10^6	-	57	2.6	+++
2	5×10^6	-	57	2.2	+++
3	5×10^6	5×10^6	48	2.5	+++
4a	5×10^6	5×10^6	57	5.2	+++
4b	5×10^6	5×10^6	115[b]	2.6	++
5	10^6	-	62	2.1	0
6	10^6	-	70	2.3	+
7	10^6	-	56	1.8	0
8	10^6	2.5×10^5	49	1.6	0
9	10^6	2.5×10^5	56	2.6	+
10	10^6	2.5×10^5	134	2.2	0
11	10^6	10^6	43	1.4	0
12	10^6	10^6	49	2.0	0
13	10^5	-	77	1.4	0
14	10^5	-	134	1.3	0
15	10^5	10^6	98	1.4	0
16	10^5	10^6	98	2.0	+
17	10^5	10^6	56	2.5	+
18	10^5	10^6	64	3.0	0
19	10^5	10^6	55	1.8	+
20	10^4	-	98	3.7	0
21	10^4	10^6	79	6.1	+
22	10^3	10^6	98	2.5	0
23	10^3	10^6	98	5.6	+

Histological Appearances of Melanomas in Nude Mice and Comparison with the Original Tumour.

The histological appearances of all tumours removed from the nude mice and the original tumours as they were seen after removal from the patients were evaluated by a senior and experienced histopathologist, Dr. J.A.H. Campbell, to whom I am grateful for the reports that I have included as Appendix A.21. In this section I felt it would be of interest to attempt to relate the histological appearances of the original tumour to those of the tumour that developed in nude mice after incoulation of cells that had been cultured for many passages *in vitro*. The data that draw these comparisons are summarized on the microphotographs presented in Fig. 6.8 and in Table 6.5, where the essential features have been tabulated.

Perhaps the feature of greatest interest to emerge from these comparative studies is the fact that in as many as 5 cases, there was a striking resemblance of the tumour xenografts to the original human tumours. This was evident not only in terms of cellular morphology, nuclear to cytoplasmic ratio, presence or absence of multinucleate cells, and the presence or absence of pigment but also, more significantly, in terms of the overall manner in which cells were organised and grew in relation to one another. This was particularly evident in the case of UCT-Mel 1, 3 and 7 (Fig. 6.8) where, in both the primary tumour and the tumour xenograft, the cells showed a similar appearance.

This similarity was evident despite the fact that the xenograft was derived from cells that had been maintained in culture for 10 - 118 passages over a period of 1-5 years.

FIGURE 6.8

FIGURE 6.8

Histological comparison of original tumours with tumours in nude mice.

The composite figure portrays microphotographs of the histological appearance of the tumours as removed from the nude mice (left half of each photograph) and the corresponding original tumours as removed from the patient (right half of each photograph). The tumours are as follows:-

(a) UCT-Mel 1; (b) UCT-Mel 2; (c) UCT-Mel 3; (d) UCT-Mel 4a; (e) UCT-Mel 4b; (f) UCT-Mel 5; (g) UCT-Mel 7.

The scale marker in (g) represents 60 μm. All photographs are at the same magnification.

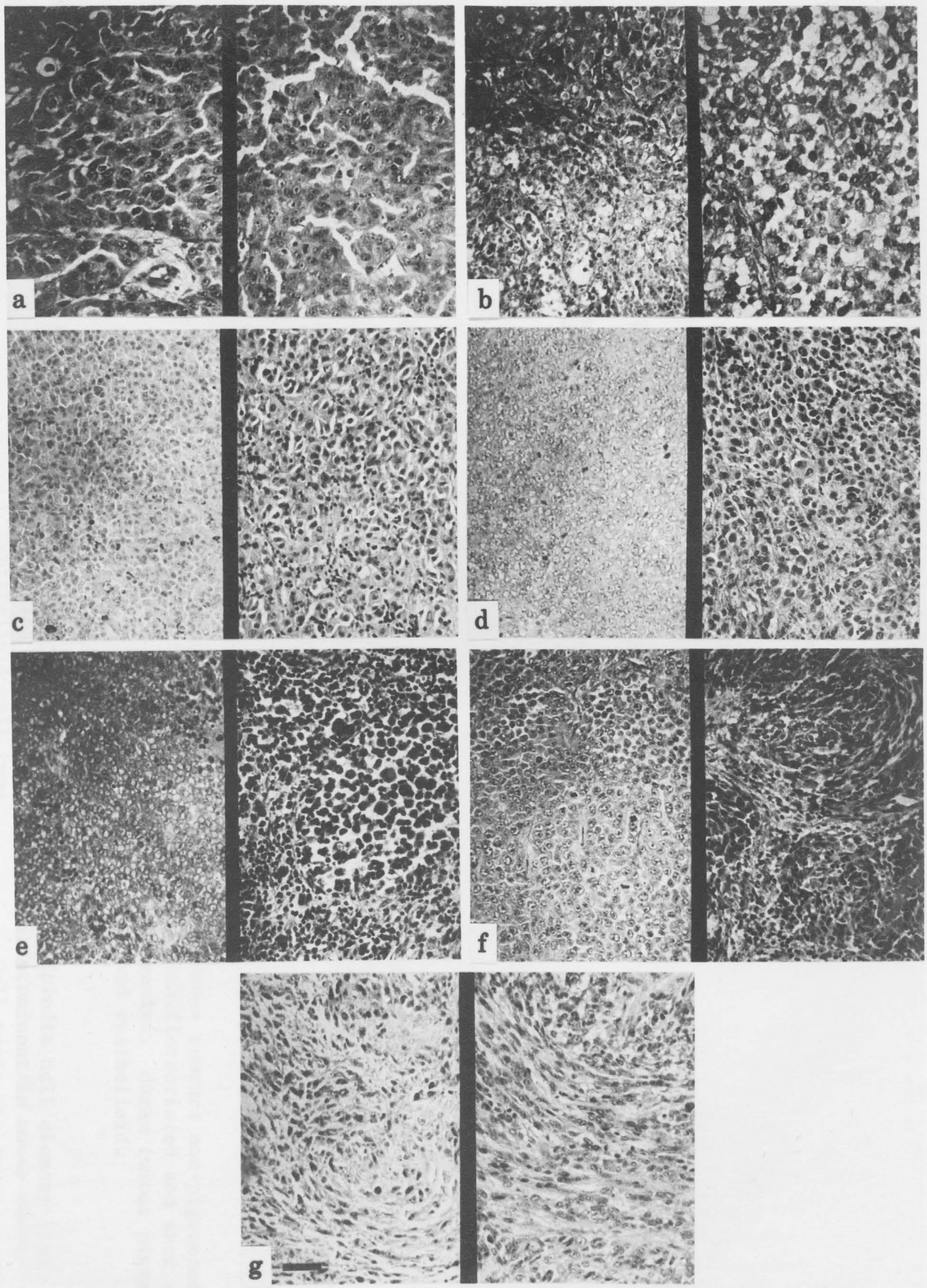


FIGURE 6.8

TABLE 6.5

HISTOLOGICAL APPEARANCES OF TUMOURS IN NUDE MICE

Cell Line	*In Vitro* Passage No. of inoculated cells	Comments: Similarities to original tumour	Comments: Differences from original tumour
UCT-Mel 1	105;118	Both poorly differentiated. Cells epithelioid and pigmented.	
UCT-Mel 2	88;96	Both mixed epithelioid with giant cells. Some pigmentation.	Mouse tumours had fewer giant cells.
UCT-Mel 3	43	Both epithelioid and non-pigmented.	
UCT-Mel 4a	23	Similarity only to undifferentiated parts of original tumour.	Mouse tumours non-pigmented and undifferentiated and show swollen nuclei. Human tumour pigmented and epithelioid.
UCT-Mel 4b	21		
UCT-Mel 5	8;16	Both non-pigmented and of mixed spindle and epithelioid cell type.	Spindle cell element less prominent in mouse tumour. Cell types not found in discreet areas.
UCT-Mel 7	10	Both non-pigmented and of spindle cell type.	

Invasion

Histological examination revealed invasive tumours in four instances.

In one tumour produced by the injection of 5×10^6 UCT-Mel 1 cells, the tumour was seen to be invading into adjacent voluntary muscle and subcutaneous adipose tissue. In this case, the primary tumour of 4.1 g was removed 42 days after inoculation. The tumour recurred and 20 days later a second mass of 2.3 g was removed. This second tumour proved to be invasive.

Another invasive tumour derived from the inoculation of 5×10^6 UCT-Mel 2 cells had a similar history. The primary tumour was excised 57 days after injection and weighed 5.2 g. The tumour recurred locally and a tumour weighing 2.6 g was removed after another 57 days. This tumour showed one pigmented and one nonpigmented lobe. Histological examination of this macroscopically nonpigmented lobe showed tumour cells invading voluntary muscle.

In the other two cases of invasion, the tumours involved were primary tumours with no history of surgical manipulation. A tumour induced by 10^6 UCT-Mel 2 cells was removed after 70 days and found to be invading voluntary muscle. This tumour weighed 2.3 g. Similarly 10^6 UCT-Mel 7 cells resulted in a tumour of 1.3 g which, when removed 70 days after inoculation, showed voluntary muscle being invaded in one area.

In all cases, the tumours could be dissected easily as well-defined masses separate from the muscles of the back or trunk. The voluntary muscle observed therefore was in all probability that of the panniculus carnosus - the thin sheet of striated muscle found in the head, neck and trunk regions that inserts on the fibres of the dermis at its boundary with the subcutis and allows movement or twitching of the skin under voluntary control.

Metastatic Spread of Human Melanomas in the Nude Mouse

A total of 55 mice were inoculated with inocula ranging from 10^3 to 5 x 10^6 cells from the various melanoma lines with or without a simultaneous inoculum of fibroblasts as indicated in Tables 6.2 and 6.9.

When the resulting tumours had grown to a size where they were arbitrarily considered to be life-threatening for the mouse (approximately 2000 - 6000 cu. mm.) or when the overlying skin developed small areas of necrosis, the mice were examined for evidence of metastatic spread by one of the following two protocols. Firstly, the mice were killed and the contents of the abdominal, thoracic and cranial cavities were examined macroscopically for metastases. In addition, the regional lymph nodes the lungs and the spleen were placed into 10% neutral buffered formalin for histological examination of representative paraffin sections, stained with haematoxylin and eosin. Where pigmented foci were observed, these were stained with a Masson-Fontana stain for melanin.

Secondly, the primary tumour was removed under light ether anaesthesia and the skin wound was closed with interrupted silk sutures. Care was taken at the time of excision to remove as much of the tumour as friability, anatomical location and welfare of the mouse would allow. The mouse was then allowed to recover and observed until local recurrence necessitated termination of the experiment by the criteria given above or until at least 70 days had elapsed. The mice were then killed and examined for metastases as indicated in the first protocol.

The second protocol was followed to allow time for metastases to develop before death of the mouse due to the local tumour burden or to complications such as haemorrhage in the primary tumour. This procedure was recommended by Tseng et al (1980).

True metastatic deposits were observed on only three occasions.

1) This mouse was inoculated with 5 x 10^6 UCT-Mel 1 cells. A tumour weighing 4.1 g was removed 42 days post-inoculation. The tumour recurred locally to give rise to a mass of 2.3 g which was removed after a further period of 20 days. The tumour removed on this occasion was re-examined histologically and found to be invading the panniculus carnosus. Following the second surgical procedure, the mouse remained apparently well and free of recurrence for 40 days after which small tumour nodules re-appeared in the region of the original tumour. After a further period of 35 days (i.e. 137 days after the original inoculation) the mouse seemed to be losing weight and was killed. At autopsy, the lungs were heavily infiltrated with pigmented tumour and small deposits of tumour were observed growing in the mesentery of the small bowel. Histological examination confirmed the diagnosis of metastatic malignant melanoma (Fig. 6.9).

2) An inoculum of 5 x 10^6 UCT-Mel 5 cells gave rise to a tumour which was removed after 63 days and, following local recurrence, after a further 102 days. The tumour recurred a second time and what appeared to be two blood-filled cysts appeared in the subcutaneous tissue on the back of the mouse's neck. The mouse was killed after a further 34 days (199 days after the original inoculation). Histological examination of the "cysts" demonstrated small groups of cells consistent with melanoma.

3) This mouse was injected with 1 x 10^6 UCT-Mel 3 cells. The primary tumour was removed after 47 days and no further local recurrence occurred. After a further 155 days (202 days after the primary inoculation) dissection of the mouse revealed "plaques" of tumour on the lungs and enlarged axillary lymph nodes. Histological examination confirmed the presence of metastatic melanoma in the lungs and lymph nodes.

In 5 of 5 tumours induced by the inoculation of UCT-Mel 1 cells and in 10 of 11 tumours induced by the inoculation of UCT-Mel 2 cells the

FIGURE 6.9

Metastasis of human melanoma cells in the nude mouse.

The photographs show the histological appearance of UCT-Mel 1 cells (a) in the original tumour removed from the patient; (b) in the recurrent subcutaneous tumour in the nude mouse at the time the mouse was killed and (c) in the nude mouse lung.

The tumour and lungs were removed at an autopsy done on the mouse 6½ months after the initial injection of UCT-Mel 1 cells. Tumours growing at the site of primary inoculation had been removed by surgical excision on 2 previous occasions, as described in the text. In both (b) and (c) large bizarre nuclei can be seen. The scale markers in (a), (b) and (c) represent 60 μm.

FIGURE 6.9

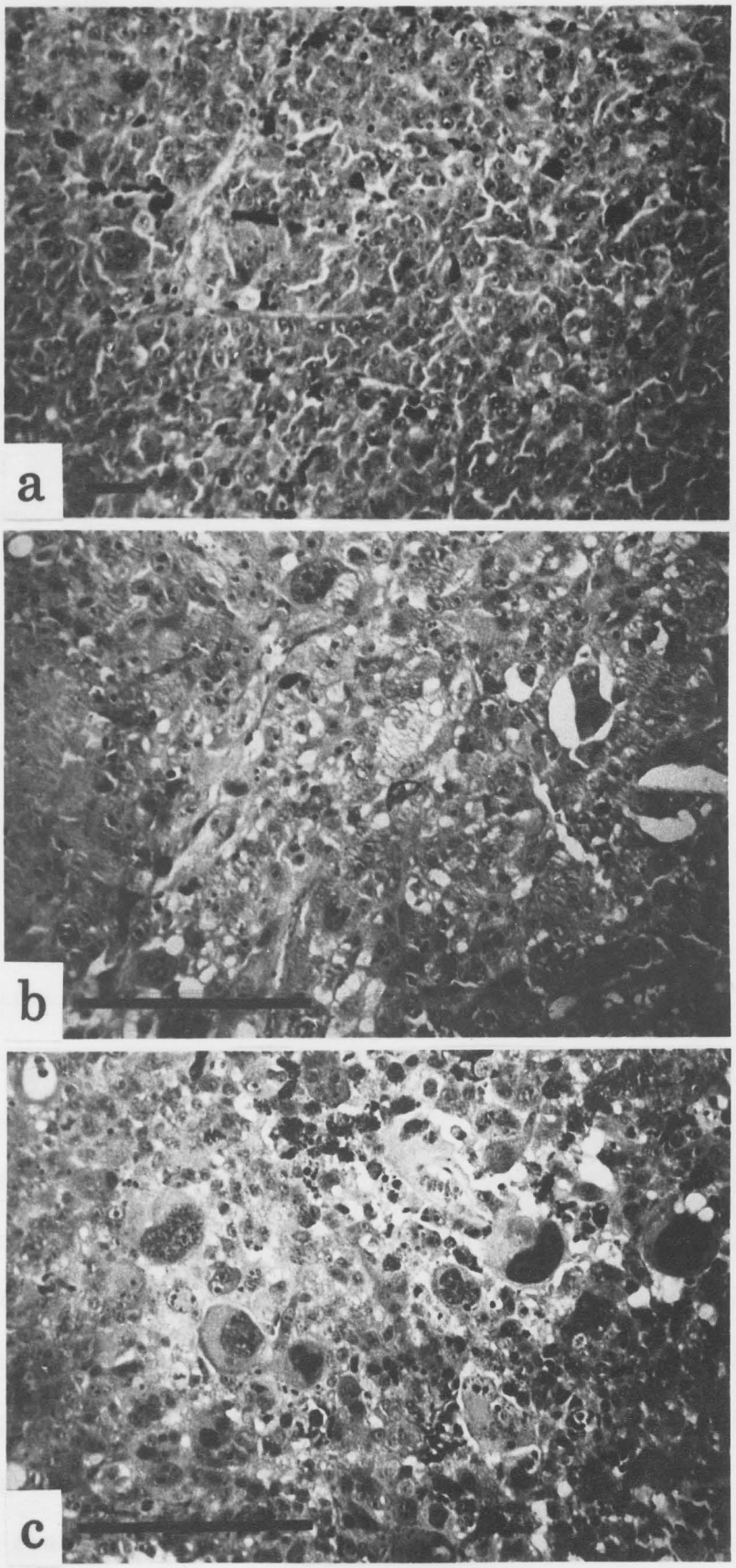


FIGURE 6.9

regional lymph nodes were found to contain melanin-laden histiocytes that frequently gave the macroscopic appearance of lymphoid metastases but proved histologically not to be so.

On occasions, the regional lymph nodes and the spleen showed "reactive" features.

In Vitro Properties of Cells Derived from Tumours in Nude Mice.

With the possibility in mind that the xenogeneic environment of the nude mouse may have selected for the growth of a particular clone or subpopulation of the inoculated human melanoma cells, I re-established *in vitro* cultures from the nude mouse tumours and compared these with the original cultures which were used to prepare the nude mouse inocula and which were maintained *in vitro* in the interim.

Thus cultures which had been passaged through nude mice and parallel cultures which had been maintained *in vitro* were examined for their viability, morphological appearance, plating efficiency, ability to grow in soft agar, and PA secretion and content.

In all cases, the viability of cells obtained from the tumours was assessed by trypan blue exclusion. As can be seen from Table 6.6 this varied over a wide range from 7% to 100%. The cells released mechanically by mincing and pipetting tended to contain a higher proportion of dead cells than did those released enzymatically by trypsinization. The viability of the cells released from the tumours did not correlate with the size of the tumour or the length of time it had been in the mouse.

The morphological comparisons are presented in Figs. 6.10 and 6.11. Generally speaking there was no obvious difference. In a number of cases moderate to heavy contamination of the melanoma cultures with mouse fibroblasts was observed (Figs. 6.11d and h). In other cases, contamination

TABLE 6.6

VIABILITY OF CELLS RELEASED FROM NUDE MOUSE-DERIVED TUMOURS[a]

Mouse	Cells injected (dose)	Tumour (Day removed)	Tumour Mass(g)	% Cells viable	
				Mechanically released	Trypsinized
	UCT-Mel 1				
1	1×10^6	42	1.9	78	n.d
	1×10^6	68	3.7	81	100
2	5×10^6	34	1.7	23	n.d
3	5×10^6	42	4.1	73	n.d
	5×10^6	62	2.3	9	80
	5×10^6 (lung metastasis)	137	n.d	91	n.d
	UCT-Mel 2				
4	1×10^5	77	1.4	100	98
5	1×10^6	62	2.1	7	78
6	1×10^6	70	2.3	94	88
7	5×10^6 (pigmented lobe)	115	2.6	24	n.d
	(non-pigmented lobe)	115	2.6	15	n.d
	UCT-Mel 3				
8	5×10^6	34	1.5	18	n.d

(a) n.d = not determined.

FIGURE 6.10

Morphology of melanoma cell lines before and after passage through nude mice.

The microphotographs display the phase contrast microscopic appearances of cells growing as adherent monolayers, before and after passage through nude mice. The protocol used for establishing the tumours and subsequent monolayers was as described in Chapter 2. The tumours are as follows: a & b) UCT-Mel 1; c & d) UCT-Mel 2; e & f) UCT-Mel 3.

In all cases, the photographs on the left (a, c and e) represent the morphological appearance of cells maintained *in vitro*, while the photographs on the right (b, d and f) represent the appearance after passage through nude mice. All photographs are at the same magnification. The scale marker in (f) equals 50 μm.

FIGURE 6.10

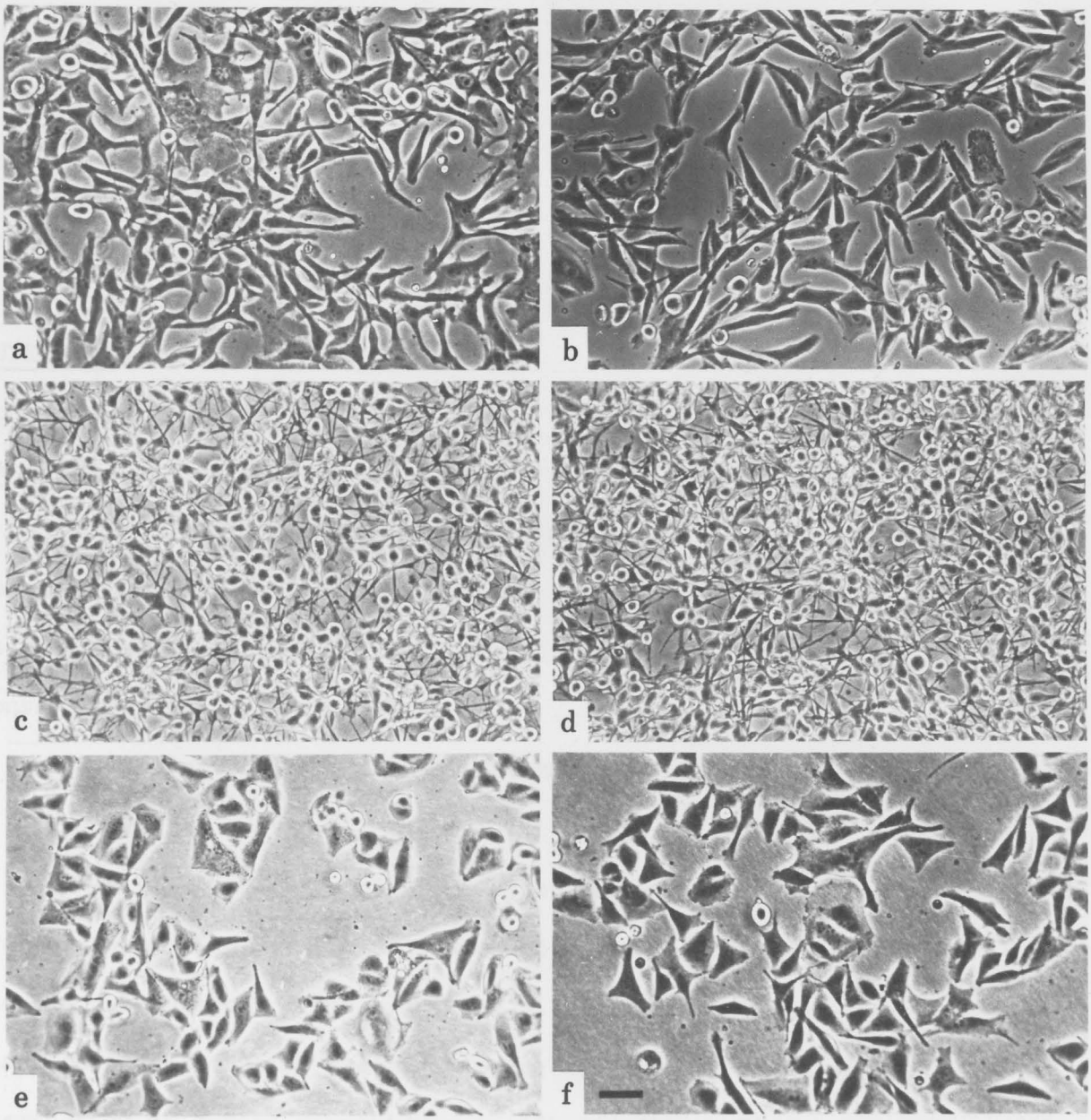


FIGURE 6.10

FIGURE 6.11

Morphology of melanoma cell lines before and after passage through nude mice.

The microphotographs display the phase contrast microscopic appearances of cells growing as adherent monolayers, before and after passage through nude mice. The protocol used for establishing the tumours and subsequent monolayers was as described in Chapter 2. The tumours are as follows: a & b) UCT-Mel 4a; c & d) UCT-Mel 4b; e & f) UCT-Mel 5; g & h) UCT-Mel 7.

In all cases, the photographs on the left (a, c, e and g) represent the morphological appearance of cells maintained *in vitro*, while the photographs on the right (b, d, f and h) represent the appearance of cells after passage through nude mice. Note the contamination by murine fibroblasts in (d) and (h). The scale marker in (h) represents 50 μm. All photographs are at the same magnification.

FIGURE 6.11

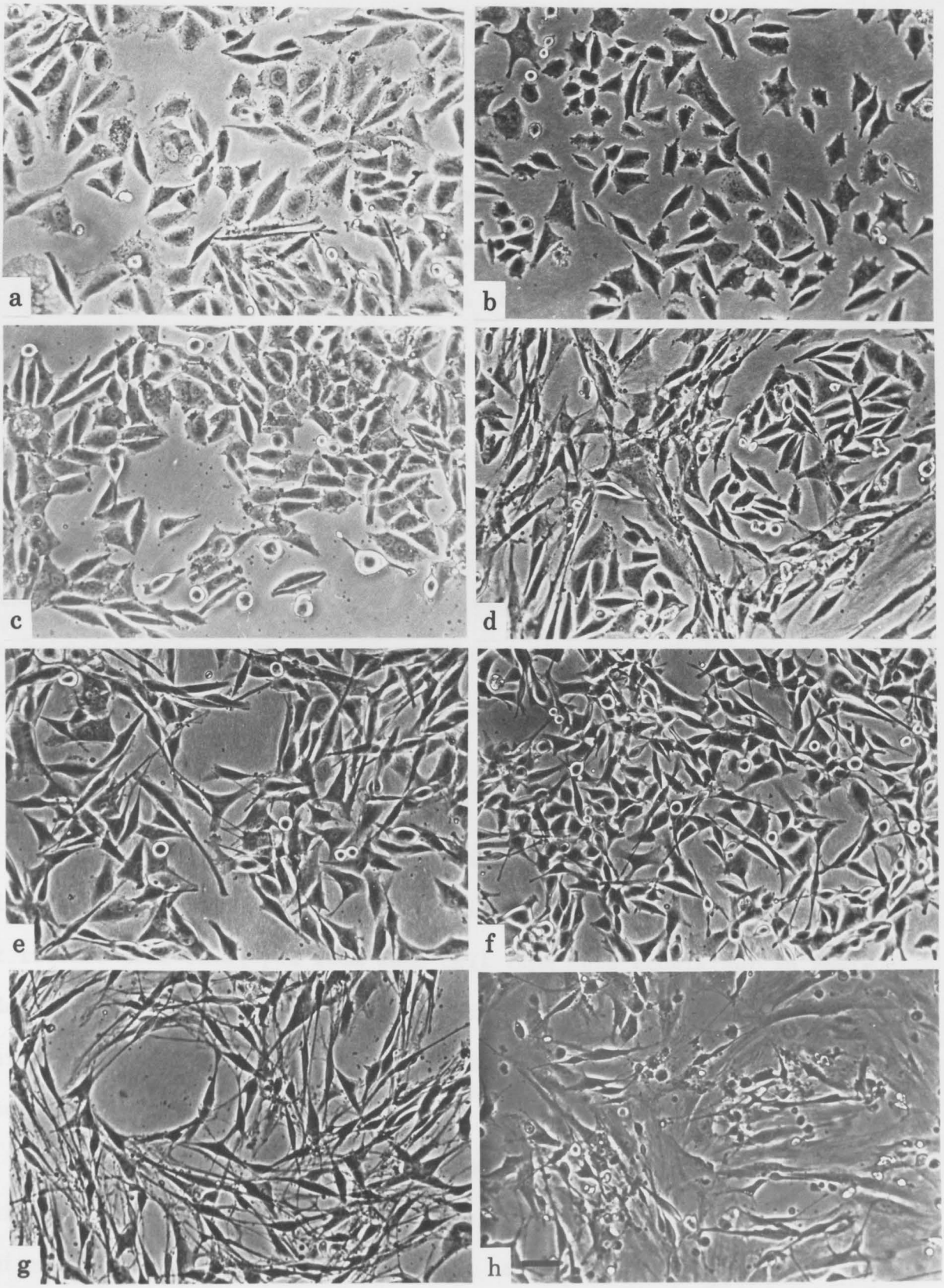


FIGURE 6.11

with mouse fibroblasts was undetectable (Fig. 6.10). It was fairly easy to distinguish melanoma cells from fibroblasts under phase contrast microscopy and thus to monitor reasonably accurately the extent to which cultures included mouse fibroblasts from one passage to the next. In 4 cases (UCT-Mel 1, 2, 3 and 4a) the tumour tissue grew free of fibroblasts from the beginning. In 2 instances (UCT-Mel 4b and 7) I was unable, even with selective passaging procedures such as differential trypsinization and differential plating, to produce fibroblast-free cultures by the 5th passage.

In one case I observed that the extent to which cultures were contaminated with fibroblasts was influenced by the manner in which the culture was prepared. Thus cultures prepared from UCT-Mel 5 cells that were released mechanically were completely free of fibroblasts, whereas cells released by enzymatic dispersion initially contained a large number of contaminating mouse fibroblasts, but these had disappeared by passage No. 3.

Although I did not have sufficient numbers to pronounce definitively on this matter, I had the distinct impression in all 6 cases, that tumours that developed from each cell line were consistent in their tendency to attract or support proliferation of host fibroblast cells. Generally speaking this correlated with the fibrous tissue content of the tumours as illustrated by a silver stain for reticulin (Fig. 6.12).

The two pigmenting melanoma lines (UCT-Mel 1 and 2) continued to pigment *in vitro* after passaging through the mice. None of the nonpigmenting lines acquired the tendency to pigment.

The results of experiments to study the effects of xenografting or plating efficiency and anchorage independent growth are summarized in Table 6.7. These experiments were done only on UCT-Mel 1 and 2. The

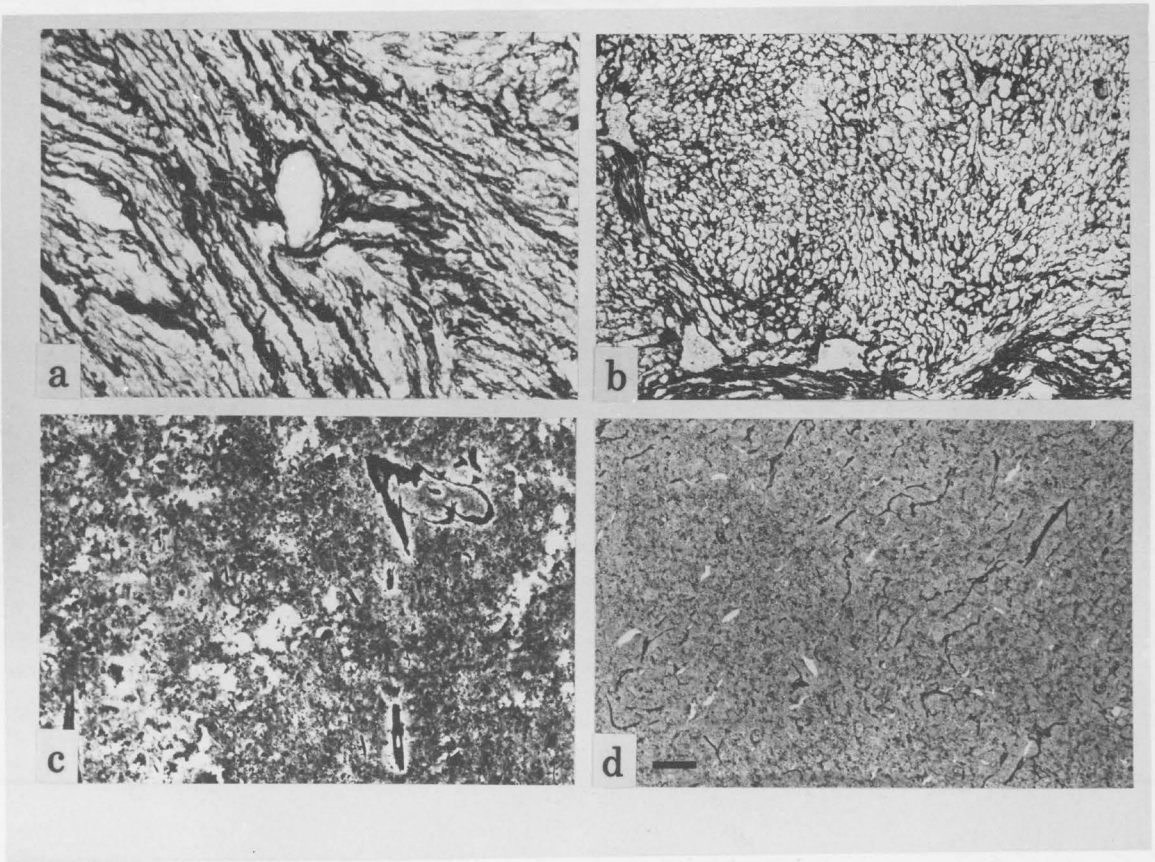


FIGURE 6.12

Melanomas excised from nude mice and stained for reticulin.

Histological sections of representative tumours were stained for reticulin (Gordon and Sweet, 1936). The tumours are as follows:

a) UCT-Mel 7, showing abundant reticulin arranged in bundles or around individual cells;

b) UCT-Mel 4b, showing plentiful reticulin arranged in a fine reticular pattern;

c) UCT-Mel 1, showing mainly perivascular staining;

d) UCT-Mel 2, showing very weak staining. Scale marker = 60 μm.

UCT-Mel 7 and 4b consistently yielded high numbers of fibroblasts on re-establishment of the tumours *in vitro*, while UCT-Mel 1 and 2 yielded fibroblast-free cultures.

TABLE 6.7

EFFECT OF PASSAGE THROUGH NUDE MICE ON PLATING EFFICIENCY AND ANCHORAGE-INDEPENDENT GROWTH OF MELANOMA CELLS[a]

Cell Line[b]		Plating efficiency[c] %		Growth in Soft Agar[c] (% Colonies)	
		Cells maintained *in vitro*	Cells from tumour	Cells maintained *in vitro*	Cells from tumour
UCT-Mel 1	(118')(5 wk)	100.0	100.0(0)	57.4	n.d
UCT-Mel 2	(82')(7 wk)	n.d	n.d	57.6	25.1(3)
"	(82')(8 wk)	n.d	n.d	78.9	55.1(3)
"	(82')(8 wk)	n.d	n.d	78.9	55.1(3)
"	(82')(8 wk)	84.0	84.8(3)	78.9	55.0(3)
"	(82')(16 wk)	84.0	90.4(0)	n.d	n.d
"	(96')(11 wk)	100.0	100.0(0)	n.d	n.d

(a) n.d = not determined

(b) Primed values in parenthesis indicate passage number at which cells were injected into nude mice. Comparisons were made between control cells from the same passage which were maintained in culture until tumours were removed and re-established *in vitro*. The length of time in weeks that elapsed between injection and the final comparison is given by the second figure in parenthesis.

(c) Plating efficiencies and colony forming ability in agar were measured on viable cells (as established by trypan blue exclusion).
Numbers in parenthesis indicate the number of days that elapsed between disaggregation and plating of the mouse tumour and the performance of the plating efficiency assay. Values of zero signify assays that were done on disaggregated cells obtained directly from excised tumours.

plating efficiencies were unaltered but, as observed with UCT-Mel 2, growth in agar of viable cells isolated from the tumour or cultures established from the tumour was approximately 63% of that observed in cultures maintained *in vitro*.

As judged by inspection of the cultures before trypsinizing them for the soft agar experiments, this diminution in cloning efficiency could not be attributed to fibroblast contamination of the cultures.

The release of plasminogen activator by cells established in culture from the tumours was either lower than, or similar to, that exhibited by the cultures that had not been passaged in nude mice. In 3 cases UCT-Mel 1, 3 and 5) this was consistent over 5 passages (Fig. 6.13a, c and e). With UCT-Mel 5 cells released by trypsin, the rate of PA release rose as fibroblast contamination diminished with passage of the culture (Fig. 6.13e). In 3 instances (UCT-Mel 1, 2 and 4a) the rate of PA secretion rose with serial passage in a manner that could not be correlated with fibroblast content of the cultures (Fig. 6.13a, b and d). Cultures of UCT-Mel 4b and 7 established from mouse tumours released very little PA. Since these cultures were consistently and heavily contaminated with murine fibroblasts throughout all passages studied, I was unable to draw any conclusions from these studies.

It is of interest to note that in the one case where a metastatic tumour was observed (UCT-Mel 1) PA released by cultures established from the metastasis in the lung released PA at a rate that was 4 times greater than that observed with cultures established from either the primary tumour in the same mouse or a tumour derived from the same cell line in another mouse (Fig. 6.13a). In addition, it should be noted that the rate of PA release by the metastatic culture 7.9 u/10^6 cells/24 hr) was significantly higher than the average rate of PA secretion (4.3 u/10^6 cells/24 hr) by the cells which had been maintained *in vitro*.

FIGURE 6.13

Effect of passage through nude mice on plasminogen activator synthesis and secretion.

The above composite figure depicts the rate of plasminogen activator synthesis and secretion by melanoma cells as a function of cell doublings after removal from the nude mice. In each case, cells were injected subcutaneously into the nude mice. When the resulting tumour had reached what was judged to be a suitable size it was removed, disaggregated and replated. Each point represents the average result of duplicate assays of PA released into serum-free medium over a 24 hr period. Cells were examined daily and passaged when confluent.

PA was measured in nude mouse tumour-derived cultures of A) UCT-Mel 1; B) UCT-Mel 2; C) UCT-Mel 3; D) UCT-Mel 4a; E) UCT-Mel 5. In each case, the cell cultures from which harvest fluids were obtained are indicated as follows:

Original inoculum	Method of cell release	Symbol
1×10^6	Mechanical	▲– – –▲
1×10^6	Enzymatic	◆– – –◆
5×10^6	Mechanical	▲·····▲
5×10^6	Enzymatic	■——■
5×10^6	Enzymatic	▼·····▼

Fig. (A) included a culture of cells released mechanically from the melanoma-infiltrated lung of the mouse in which metastasis of UCT-Mel 1 was found (◆·····◆).

The mean for the rates of PA release by control cells (i.e. corresponding cells that were not injected into the nude mice) is indicated by the arrow in each figure. The means and ranges are shown to the right of the figures.

Note the relative high rate of PA release by cells derived from a metastatic deposit (Fig. 6.13A,◆·····◆). In all other cases the rate of cellular PA release immediately after removal from the nude mouse was low. In some cases (A, C and E), the rate of PA release remained consistently low, while in other cases, it rose with serial passage to approximate control levels (B and D).

FIGURE 6.13

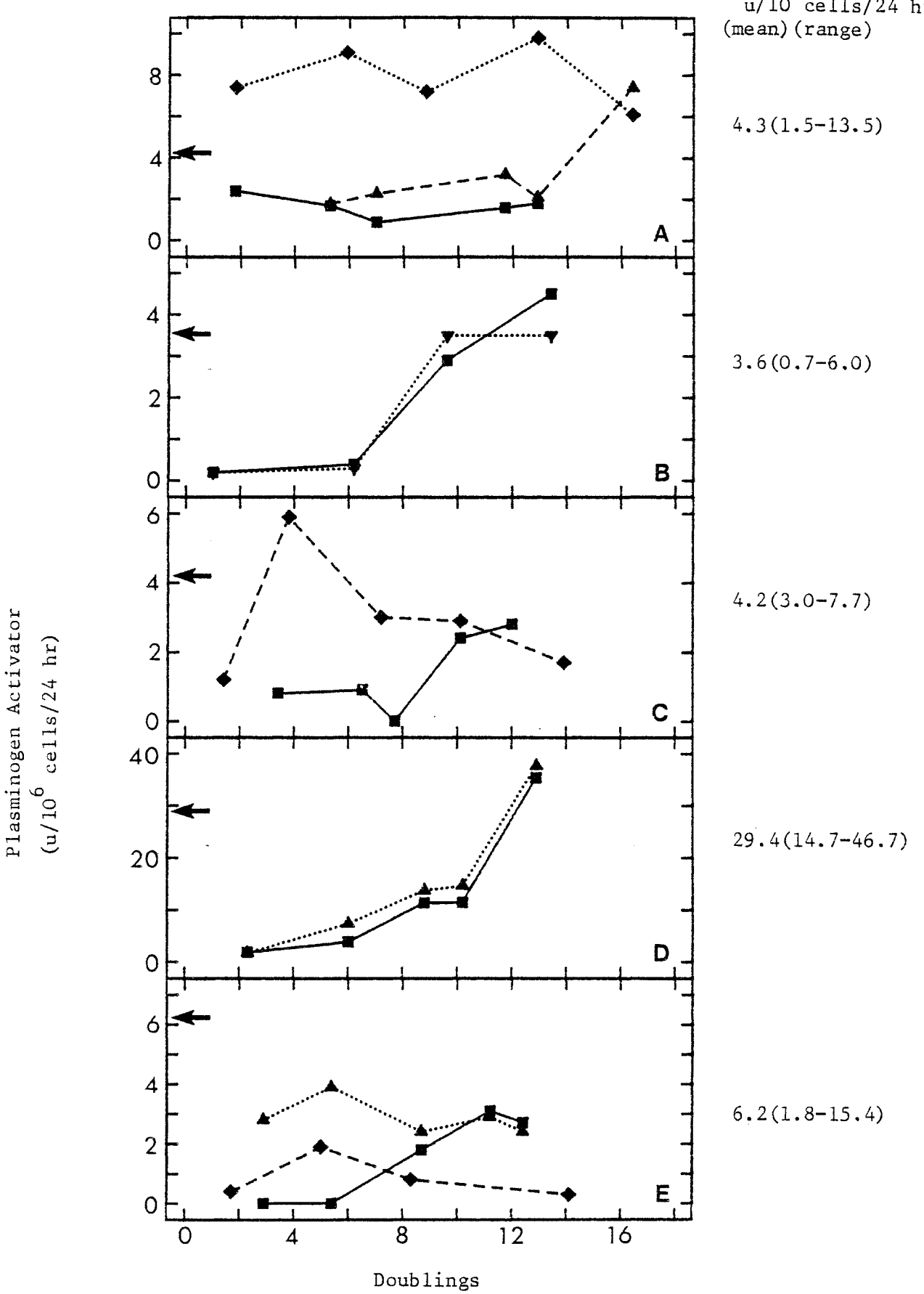

Release of PA
by Control cells
u/10^6 cells/24 hr
(mean) (range)
4.3(1.5-13.5)
3.6(0.7-6.0)
4.2(3.0-7.7)
29.4(14.7-46.7)
6.2(1.8-15.4)
A
B
C
D
E
Plasminogen Activator
(u/10^6 cells/24 hr)
Doublings
0
4
8
12
16
20
40

FIGURE 6.13

Measurements of intracellular PA content on samples of tumour tissue gave values that were lower by a factor of 10 to 100 than those observed with the corresponding cell lines from which the cultures were derived. The only exception to this general rule was seen in the case of UCT-Mel 2 where cellular PA content measured directly in tumour tissue approximated that found in the cell line in some cases (Table 6.8).

Effect of Co-inoculation of Skin Fibroblasts on the Growth of UCT-Mel 2 Cells in Nude Mice

Fibroblast feeder layers are frequently used to provide growth factors for tumours cultured *in vitro* and for this reason alone, it was of interest to examine the effect of administering fibroblasts together with melanoma cells, to the nude mice. This experiment was of particular interest since I had shown that fibroblasts were able to inactivate plasminogen activator in medium covering melanoma cell cultures. If the extracellular presence of this enzyme correlated with the increased proliferative potential that one associates with neoplasia (Reich, 1978) I might have expected co-inoculation of fibroblasts to have inhibited growth *in vivo*. Accordingly, human adult skin fibroblasts were co-injected with UCT-Mel 2 cells subcutaneously into nude mice and *in vivo* tumour growth was monitored.

The results of these experiments are summarized in Table 6.9 and Figs 6.14 and 6.15 where melanoma cell inocula ranging from 10^3 to 10^6 were injected either alone or together with 2.5×10^5 to 10^6 skin fibroblast cells. In all cases the total inoculum volume was 0.1 ml.

Striking effects of co-injection of fibroblasts were observed in two respects. Firstly, when small melanoma cell inocula were used (10^3 - 10^5 cells), fibroblasts increased the number of successful xenografts. Secondly, fibroblast co-inoculation invariably shortened the

TABLE 6.8

INTRACELLULAR PLASMINOGEN ACTIVATOR IN NUDE MOUSE TUMOUR EXTRACTS

Cell Line	No. of melanoma cells inoculated	Intracellular PA (per mg protein) Tumour	Cells cultured *in vitro*[a] Mean (range) (n)
UCT-Mel 1	5×10^6	0.284	13.1(1.0-46.5) (98)
"	5×10^6	0.392	
"	5×10^6	0.330	
UCT-Mel 2	10^5	2.675	3.5(1.6-6.5) (4)
"	10^5	3.345	
"	10^6	0.541	
UCT-Mel 3	10^6	0.204	2.5(0.8-7.0) (4)
UCT-Mel 4a	5×10^6	0.206	18.1(16.2-20.0) (2)
UCT-Mel 4b	10^6	1.006	9.9(5.6-14.1) (2)
"	5×10^6	0.657	
UCT-Mel 5	5×10^6	0.974	10.3(3.1-17.5) (2)

(a) Data in this column represent averages and ranges for the results of intracellular PA assays on cells cultured *in vitro*. The value in the second set of parenthesis is the number of samples analysed.

TABLE 6.9

EFFECT OF CO-INOCULATION OF SKIN FIBROBLASTS ON THE GROWTH OF UCT-MEL 2 CELLS IN NUDE MICE

No. of cells inoculated		No.of tumours/ No. of mice	Latency Period (a) (days)	Doubling Time
UCT-Mel 2	Skin fibroblasts			
10^3	-	0/2	-	-
10^3	10^6	2/2	52	4.7
10^4	-	1/2	63	3.7
10^4	10^6	1/2	41	2.7
10^5	-	2/6	51;91 (b)	3.9;4.7 (b)
10^5	10^6	4/4	22;30 (c)	5.9
10^6	-	4/4	31	4.2
10^6	2.5×10^5	2/2	25	4.2
10^6	10^6	2/2	14	4.7

(a) The latency period in this case was taken as the time at which the tumour volume reached the arbitrarily defined size of 50 cu mm. (See text).

(b) Individual results are presented when these differed by more than 20%. In all other cases, the results presented represent the means observed for each experimental group.

(c) Duplicate mice were injected in two separate experiments. The results presented represent the means observed in each experiment.

FIGURE 6.14

Effect of fibroblasts on growth of melanomas in the nude mice.

Nude mice were inoculated subcutaneously with melanoma cells either alone, or mixed with viable adult skin fibroblasts. Inocula were prepared as summarized in the following table.

Inoculum

UCT-Mel 2	Adult skin fibroblasts	Symbol
10^5	0	△——△
10^5	10^6	▽·····▽
10^6	0	▲——▲
10^6	2.5×10^5	■—·—■
10^6	10^6	◆– –◆

Tumour volume, calculated as the product of three axial measurements, is shown as a function of time after inoculation for each case. When 5×10^6 fibroblasts were injected alone no tumours were observed after 205 days.

Note that at both melanoma cell inoculum sizes, co-injection of fibroblasts displaced the growth curve to the left, indicating a shortening of the latent period for tumours to become visible, without significant effect on the growth rate of established tumours. This effect of the fibroblasts was dose-related when 10^6 melanoma cells were injected.

FIGURE 6.14

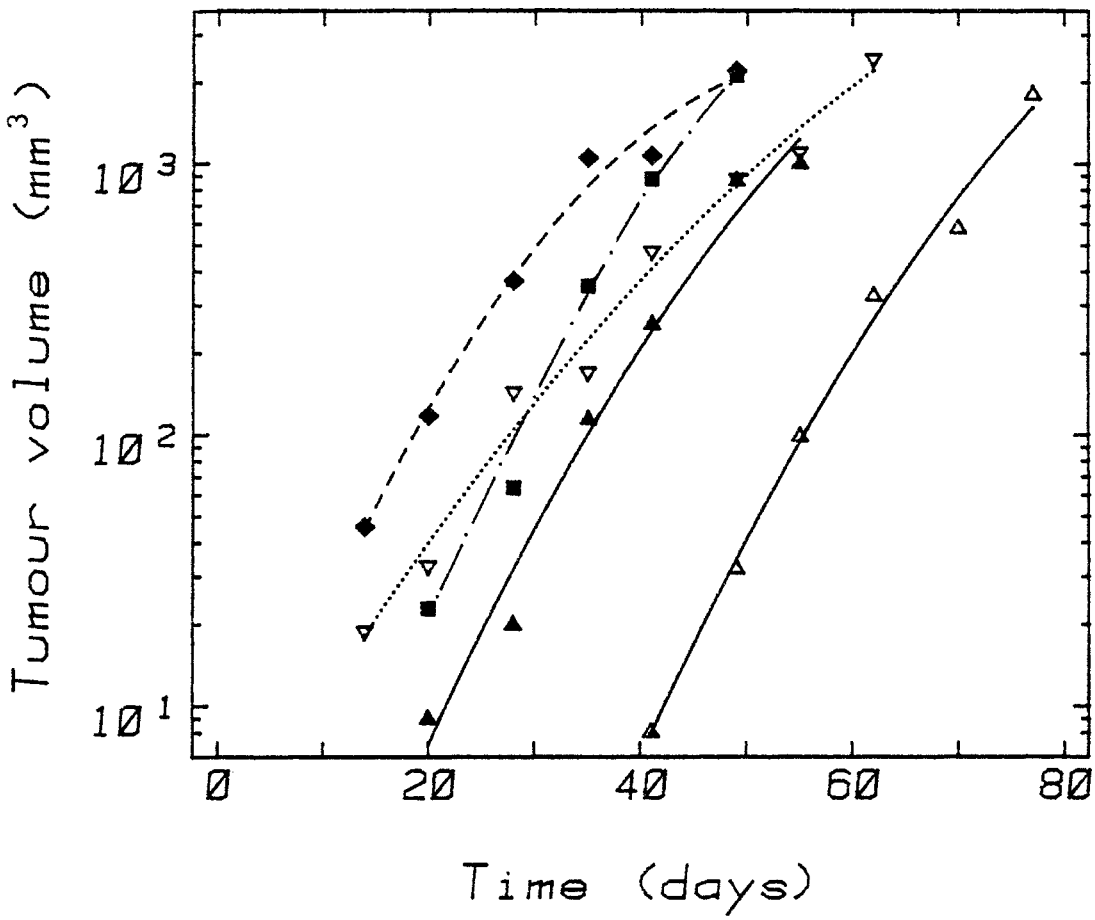


FIGURE 6.14

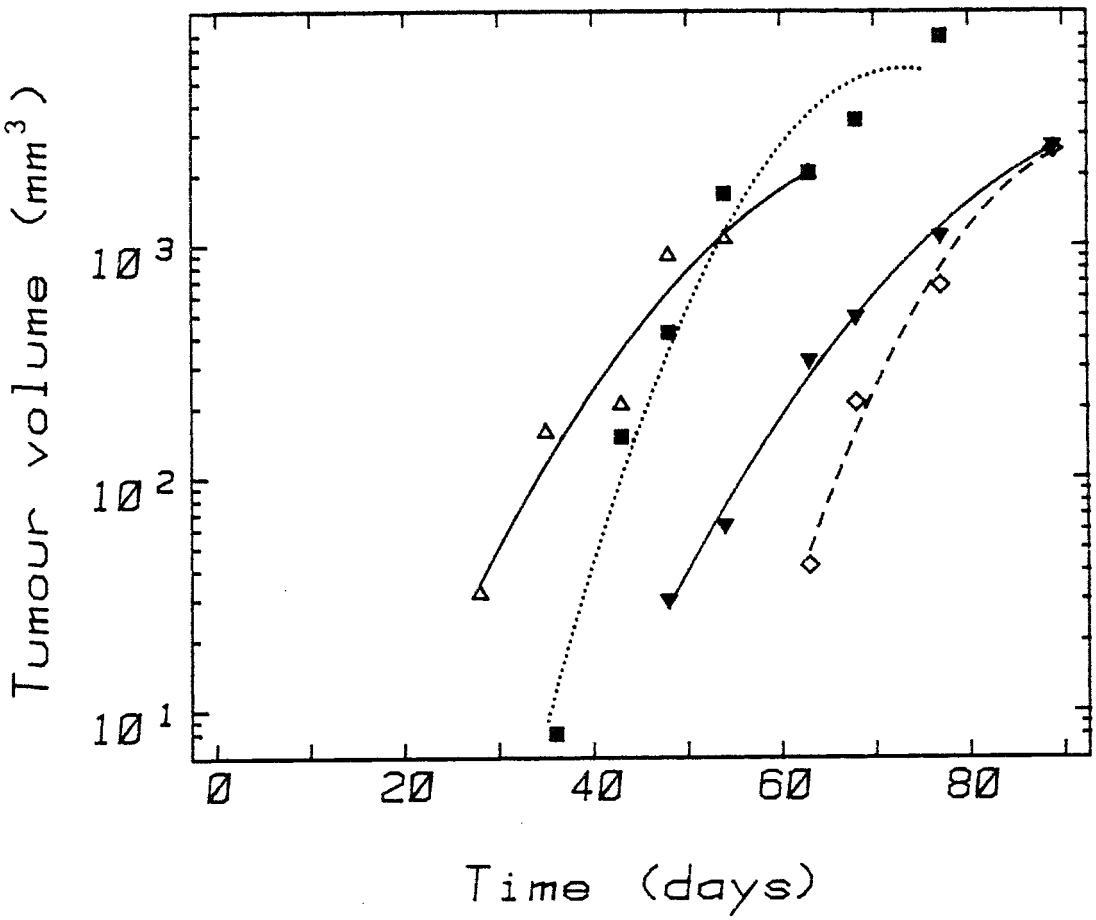


FIGURE 6.15

Effect of fibroblasts on growth of melanomas in the nude mice.

Nude mice were inoculated subcutaneously with melanoma cells either alone or mixed with viable adult skin fibroblasts. Inocula were prepared as summarized in the following table:

Inoculum UCT-Mel 2	Adult skin fibroblasts	Symbol
10^3	0	no growth
10^3	10^6	▼——▼
10^4	0	◇- - -◇
10^4	10^6	■·····■
10^5	0	no growth
10^5	10^6	△——△

Tumour volume, calculated as the product of 3 axial measurements, is shown as a function of time after inoculation for each case.

The tumour take rate at low cell inocula was low and inconsistent (see Table 6.9). Note, however that inocula of 10^3 and 10^5 UCT-Mel 2 cells (in this experiment) resulted in tumours only when cells were coinjected with fibroblasts. Co-inoculation of fibroblasts with 10^4 melanoma cells displaced the growth curve to the left without affecting the growth rate of the tumours.

latency period. It is of interest, however, to note that fibroblasts had no significant effect on the doubling times of tumours or upon their pigmentation (Tables 6.9 and 6.4).

In order to accommodate the difficulties associated with graphic analysis of semi-log plots (i.e. the absence of a "zero" ordinate value to define the precise time at which the tumour was first visible) and the fact that it proved to be impractical to examine the mice at intervals more frequent than one week, I have resorted to the following analytical technique to define the "latency period".

Firstly, by inspection, points on the growth curve that depicted exponential growth were selected. A least mean squares regression line was fitted to these points and the parameters so obtained (i.e. the slope and the intercept) were used to obtain, by interpolation, the time taken for the tumour to reach an arbitrarily defined volume of 50 cu. mm.

The data in Table 6.9 and Fig. 6.15 which best illustrate the effects of fibroblast co-injection are those derived from the inoculation of lower melanoma cell numbers. When 10^3 UCT-Mel 2 cells were injected alone, no tumours resulted and the inoculation of 10^5 melanoma cells into 6 mice gave rise to only 2 tumours. However, the co-inoculation of 10^6 skin fibroblasts with these cell numbers gave rise to tumours in all the mice tested. In all cases, co-injection of fibroblasts had the effect of shortening the latency period as shown by the length of time taken for the tumour to reach the arbitrary volume of 50 cu. mm.

The injection of 5×10^6 fibroblasts alone produced no tumour growth after a period of 205 days.

The fibroblasts exerted their effects directly, as no stimulation of tumour growth was obtained when fibroblasts were injected at an adjacent area. Similarly no effect was obtained when fibroblasts were injected 6 days prior to the injection of melanoma cells. In this experiment cells were injected at the same site.

DISCUSSION

In the work reported in this chapter I have been able to demonstrate conclusively that cell lines established from metastatic deposits of malignant melanoma tissue in humans will grow as tumours in nude mice. The fact, of course, that nude mice will support the growth of human tumour xenografts is well-known, and others have had no difficulty in establishing human melanomas as tumours in these animals (Giovanella et al, 1972, 1976; Fogh et al, 1977; Aubert et al, 1976, 1980).

In my series, 6 of the 7 cell lines grew without difficulty, provided the inoculum size was adequate. The one case (UCT-Mel 6), in which the cells did not form tumours in the nude mice despite inoculation in doses as high as 10^7 cells, was interesting inasmuch as these cells were morphologically slightly different from other melanoma cells and they released a PA that was immunochemically similar to urokinase. This is the only instance I know of where a melanoma cell line in culture has released UK-type activator and not the tissue-type PA (Wilson et al, 1980; Rijken and Collen, 1981; Vetterlein et al, 1979).

This cell line together with the justification for regarding it as a melanoma cell line has been discussed in Chapters 2 and 3, and I can do no more at this stage than record its failure to grow in nude mice as yet another anomalous attribute.

The literature reports success in establishing melanomas as viable xenografts in nude mice with success rates ranging from 58% (Giovanella et al, 1974) to 100% (Fogh et al, 1977). The interesting point has been made that metastatic tumours are more likely to succeed in nude mice than primary tumours (Fogh et al, 1980).

The two criteria of tumorigenicity which normally correlate well

are growth in soft agar and growth in the nude mouse (Freedman and Shin, 1974). In this series, a similar correlation was observed in all cases with the exception of the cells lines derived from one patient (UCT-Mel 4a and 4b) which gave rise to rapidly growing tumours in nude mice but resisted all my attempts to grow them in soft agar. Other workers (Marshall et al, 1977; Creasey et al, 1979) have noted instances where growth in the nude mouse and in soft agar were not associated and it has been suggested (Hastings and Franks, 1981) that this may reflect specific chromosomal abnormalities. To the best of my knowledge no satisfactory explanation is as yet available for such discrepancies and I have no data or speculations to contribute to this problem.

The kinetics of tumour growth in vivo.

I have attempted in this work to quantitate the kinetics of tumour growth in nude mice and although this study has been confined to a relatively small number of animals, I think that my results are worthy of comment in several respects.

Firstly, although relatively crude, the method that I used to estimate tumour volume in the live animal proved to be reasonably reliable in that it gave results that correlated well with the mass of the excised tumour (Fig. 6.16). Using this method I was able to record growth rates and "latency periods" for the different tumours that varied rather widely from one cell line to the next. The variation that I observed was in all respects similar to that reported by others for the latency periods and growth rate of human melanomas in nude mice (Fogh et al, 1977; Aubert et al, 1976, 1980; Giovanella et al, 1972, 1973, 1974).

All tumours showed a period of exponential *in vivo* growth from which a doubling time could be calculated. This *in vivo* doubling time was invariably longer than the corresponding doubling time observed *in vitro*.

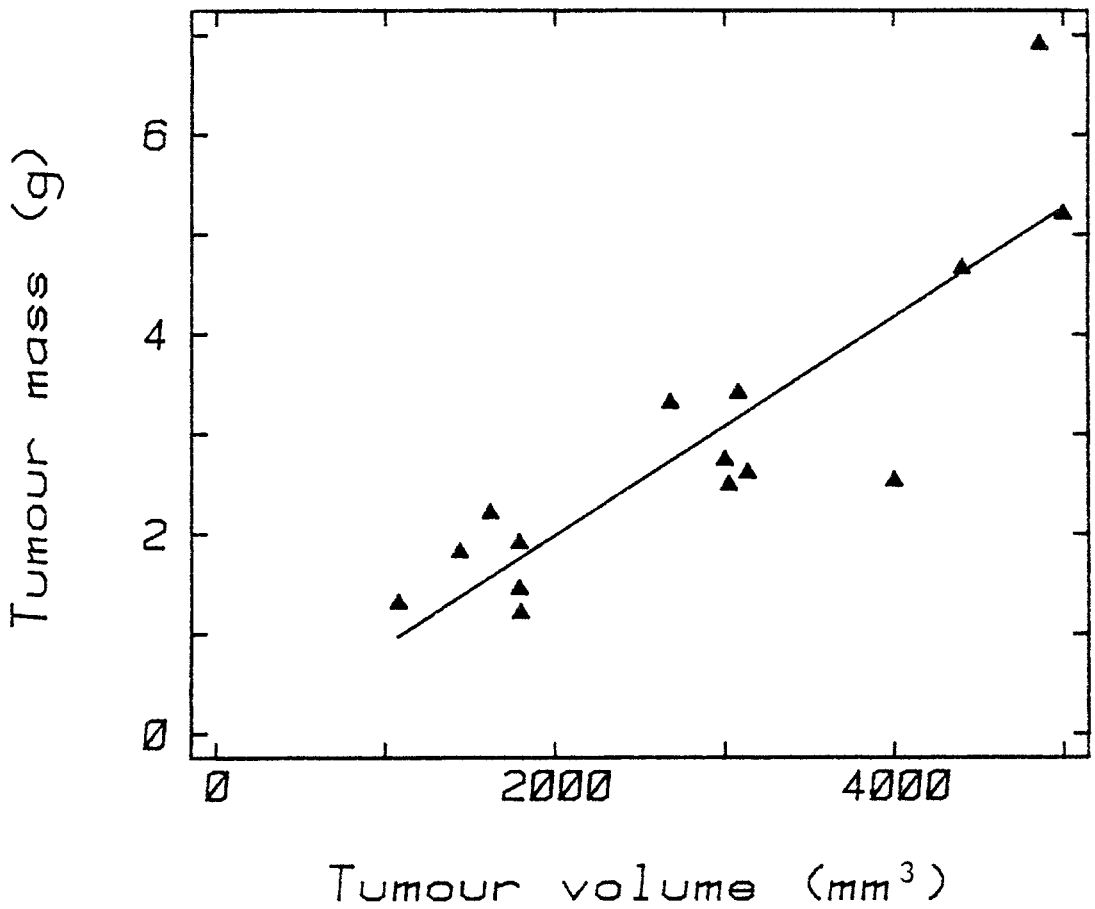


FIGURE 6.16

Direct relationship between tumour mass and estimated tumour volume.

The estimated tumour volume, derived from the product of 3 axial measurements of the tumour in the live nude mouse, was plotted against the tumour mass obtained when these tumours had been removed from the mouse. Note the approximately linear relationship between these two parameters, with a correlation coefficient of r = 0.87.

Furthermore, there was no correlation between the rate of cell division in tissue culture and the rate of tumour growth *in vivo* (Fig. 6.2).

These discrepancies may be explained by the fact that the doubling time for tumour volume is probably a poor indicator of cellular proliferation rate since, at any given time, the size of the tumour is governed by factors that cause cell loss - such as necrosis, exfoliation, metastasis or terminal differentiation - or factors that contribute to tumour size, such as host stromal response and vascularity. Where experiments have been done to measure cellular generation times in human tumours, these were found to be of the order of 3-4 days (Shirakawa et al, 1970). Human tumours, however, increase in volume *in vivo* with doubling times of the order of 2-16 weeks (Nathanson et al, 1967b). Although to the best of my knowledge, measurements of tumour generation time and volume doubling time have not been done on the same tumour *in vivo*, it is of interest to note that volume growth rates *in vivo* in the nude mouse were more rapid than those usually encountered in humans and were closer to the cellular generation times. From this one may infer that the nude mouse provides an environment for growth of human tumours that is nutritionally or immunologically more favourable than that which exists in the human host.

Secondly, at any given time after injection of the melanoma cells the size of the tumour could be directly related to the size of the original inoculum (Table 6.3 and Figs 6.3 and 6.4). This phenomenon has been observed in passing by others (Giovanella et al, 1973, 1974; Kyriazis et al, 1978; Helson et al, 1975) who made no mention of whether inoculum size affected latency or doubling time.

Following inoculation of tumour cells there was invariably a period of latency before the tumour became visible and measurable. This

was evident in the current study and has been well documented by Giovanella et al (1973, 1974) and Aubert et al (1980). The reason for this latent or lag period is not known. It may represent the time needed for sufficient cell divisions to take place for the tumour to become evident or it may be analogous to the lag phase seen in a typical sigmoidal growth curve *in vitro* before logarithmic growth starts. If so, it probably represents the adaptation of the cells to different conditions and perhaps their slow multiplication to a certain threshold level after which logarithmic growth can occur. This "latent" period may also reflect the growth of the avascular tumour, as exponential growth begins only when the tumour becomes vascularized, which normally occurs at a tumour diameter of 1-2 mm (Folkman, 1974).

Whatever the reason, the length of the latency period was clearly important in determining tumour volume at a particular time after inoculation and was inversely related to the inoculum size. The second factor determining tumour volume would be the innate tumour growth rate as measured by the tumour volume doubling time. The effect of inoculum size on this parameter was less certain. In most cases, tumour growth during the exponential phase was unrelated to the tumour inoculum size.

In two cases, however, (UCT-Mel 4b and 5; Table 6.2) the tumour grew more rapidly when the inoculum size was increased. If this observation could be confirmed, it would imply that the initial growth advantage conferred by a larger inoculum was sustained. The implications for a clinical relationship between tumour burden and growth rate are obvious and further investigation of this phenomenon would clearly be warranted by the relevance that it has to the natural history of tumour growth *in vivo*.

Thirdly, it is of interest to note that the inverse correlation that one might have expected between differentiation, as judged by the degree of pigmentation, and growth rate *in vivo* was not observed. In fact

the opposite was seen. UCT-Mel 1 and 2, the most heavily pigmented tumours, grew most rapidly. Aubert et al (1980) observed a somewhat similar phenomenon when they noted that with metastatic melanomas, pigmented cell lines grew more rapidly than nonpigmented cells. This was not the case with primary tumours. Giovanella et al (1976) and Fogh et al (1980) found no correlation between pigmentation of melanoma cell lines in tissue culture and their tumorigenicity in nude mice. If nothing else, these observations illustrate the fact that in melanomas, pigmentation, although it may be taken as evidence of differentiation, cannot be taken to imply *terminal* differentiation. The pigmented cell is every bit as capable of dividing as the nonpigmented cell (Kitano and Hu, 1970).

Comparisons between tumours in the nude mice and the original tumour in the human host.

Despite prolonged passage *in vitro*, a number of the cell lines produced, when injected into nude mice, tumours that closely resembled in histological appearances, the tumours that were seen in the original patient. This implied that the cells are destined not only to display cellular characteristics which are constant but also to interact with each other in such a manner as to produce patterns of association that must be mediated by molecular mechanisms that are as yet undefined.

As mentioned earlier, similarities were not always observed. In one case (UCT-Mel 4a and 4b) the original tumour was deeply pigmented, but *in vitro* culture selected for a less differentiated amelanotic line which preserved these characteristics in the nude mouse.

Reports in the literature recording observations similar to my own with respect to similarities between tumours in the nude mice and the original patient are to be found in papers by several workers (Giovanella

and Stehlin, 1974; Ozello et al, 1974; Sharkey et al, 1978).

One respect in which tumours in the nude mice appeared to differ in their behaviour from those in the original patient related to their metastatic potential. All of the cell lines were established from metastatic tumours in humans whereas, in the nude mice, only three unequivocal cases of metastatic spread were observed. In a sense, this is scarcely surprising since it is well known that most tumours in the nude mouse grow as well-circumscribed nodules at the site of inoculation without invasion of adjacent tissue. Metastatic spread of human tumours in nude mice is very rarely seen (Sharkey et al, 1978; Shimosato et al, 1976; Sharkey and Fogh, 1979). It may be that inhibition of spread is related to natural killer cell activity in the nude mouse (Herberman, 1978; Herberman and Holden, 1978; Minato et al, 1979) and that when anti-interferon antiserum promotes the occurrence of distant secondaries it does so by interfering with the action of these cells (Reid et al, 1981).

One of the great potential advantages of the nude mouse as a host for human tumours is the opportunity that it offers for the study of metastatic spread and the fact that this is so seldom seen is in many ways frustrating. Ueyama et al (1978) and Tseng et al (1980) have made an important contribution to this problem by showing that the likelihood of observing metastases can be increased if the primary subcutaneous tumour is removed before it causes death of the animal by local complications. Micrometastases that occurred before excision are thus given time to develop. The mice, in my series, in which metastatic spread was seen, were treated in this way.

The effects of passage through the nude mouse on the in vitro characteristics of human melanoma cells.

Experimental approaches to the study of the cellular biology of human neoplasia that involve *in vitro* culture techniques carry the inevitable uncertainty regarding cellular characteristics *in vitro* as faithful indicators of the neoplastic phenotype *in vivo*. There is no doubt that the environment provided by cell culture conditions is far from physiological and very different from that provided by the tissues of the host. One might reasonably expect, therefore, that the transition from *in vivo* to *in vitro* circumstances (or the reverse), would select for cells particularly suited to survive or benefit from the change.

In order to investigate the effects that such drastic modifications in growth conditions might have, xenografts in nude mice were examined for similarities or differences when compared with corresponding cells that had been maintained *in vitro* and had not been subjected to *in vivo* passage.

Generally speaking, passage through the nude mouse had little if any effect upon the phenotypic characteristics of the cells as judged by their morphology (Figs. 6.10 and 6.11) or their tendency to pigment. In the two cases in which I performed the experiment (Table 6.7) the plating efficiency was similarly unaffected.

There were, however, a number of respects in which consistent differences were found between nude mouse-passaged cells and their corresponding *in vitro* controls.

Firstly, I noted that the different lines tended to emerge from the nude mouse with varying degrees of murine fibroblastic contamination. The experience of others (Owen,1980: Giovanella et al, 1973; and Tveit et al, 1980b) with human and animal melanomas growing in nude mice has been essentially similar. Kyriazis et al (1978) and Tveit et al (1980a) observed varying numbers of mouse stromal cells contaminating other human

carcinoma cell cultures derived from nude mouse xenografts. Although I have insufficient data to allow a definite conclusion in this regard, the tendency towards fibroblastic contamination appeared to be a consistent attribute of each cell line. Thus, UCT-Mel 1 and UCT-Mel 2, for example, invariably emerged from the mouse without fibroblastic contamination. UCT-Mel 4b and 7, in 2/2 cases each, showed heavy fibroblastic contamination that persisted to the 5th passage *in vitro*, despite my attempts to select against transfer of murine fibroblasts. If this observation could be confirmed, it would carry the rather interesting implication that each line differed innately in its ability to induce a desmoplastic response. This, in turn, might mean that cells differed in their ability to release fibroblast growth factors, fibroblast chemoattractants or substances that influenced the relative ability of fibroblasts to survive explantation and *in vitro* passage. The situation in cases where heavy fibroblast contamination was observed may be analogous to that described by Todaro et al (1980) when they reported the existence of a "transforming growth factor" in the harvest fluids of human melanoma cells. Whatever the cause, these observations emphasize the potential of the malanoma-nude mouse system for the study of host-tumour cell relationships as these are expressed in fibrous tissue responses.

Secondly, the rate of synthesis and release of plasminogen activator by nude mouse-passaged cells was lower by up to 18-fold than that observed with cells maintained *in vitro*. The same was true for intracellular PA measured in tumour cells and the cultured cells from which the tumours were derived.

This fall in PA synthesis may, in certain instances, have been apparent rather than real and due to the presence of contaminating murine fibroblasts (cf. Chapter 5). I feel that this is unlikely, however, since a 2-fold decrease in PA was consistently evident with UCT-Mel 1,

where murine fibroblast contamination was not observed. In other cases where fewer experiments were performed, a similar lack of correlation between visible fibroblast contamination and depression of PA synthesis was noted.

It may also be argued that exposure to hormonal and other influences obtaining *in vivo* may have continued to exert an effect on early passage cultures after re-establishment *in vitro*. The fact that PA release is an inducible cellular phenomenon that is, in many cases, exquisitely sensitive to modulation by such influences, would support this interpretation. It seems, however, unlikely that *in vivo* effects could have persisted in some instances for more than 15 doublings and 5 passages *in vitro*. Some form of selection may therefore have operated in this regard so that high rates of PA release correlated with vigorous *in vitro* growth whereas the enzyme was suppressed in cells selected to proliferate *in vivo*. In this context it is of particular interest that the culture established from the metastatic deposit of UCT-Mel 1 produced PA at a rate that was equal to or higher than that of the *in vitro*-maintained cells. This may indicate that, in the malignant cell, metastatic potential, vigorous *in vitro* proliferation and PA release are concomitant attributes that could be mechanistically related. Unfortunately, the incidence of metastases in this series of experiments was low with the result that I have been unable to perform the experiments to evaluate this suggestion. It is, however, in accord with the observation made by Ossowski and Reich (1980b), who observed a correlation between PA secretion and metastatic potential in human epidermoid carcinoma cells cultured on the chick chorioallantoic membrane.

Thirdly, in the experiments performed with UCT-Mel 2, I noted a consistent decrease in the ability of the cells to form colonies in soft agar following passage through the nude mice. Experiments showing

a similar decrease in anchorage independent growth of cells taken directly from human melanoma xenografts in nude mice have been reported by Tveit et al (1980b). It is also well established that malignant melanoma cells isolated directly from tumour tissue obtained from patients have a very low cloning efficiency in soft agar (Asano and Riglar, 1981; Hamburger and Salmon, 1977; Courtenay and Mills, 1978), whereas melanoma cell lines characteristically show high anchorage-independent growth. Reports on this phenomenon have been largely descriptive with no serious attempts to explain the fact that human melanoma cells grow as clones in soft agar with an efficiency that is directly related to the length of time and the number of *in vitro* passages they are distant from the primary tumour.

My own inclination is to attribute this phenomenon to the fact that cells present in solid tumours growing *in vivo*, unlike their counterparts growing *in vitro*, comprise a relatively large proportion that have lost the ability for self-renewal. Whether this in turn represents a state of terminal differentiation with G-1 arrest, the effects of relative ischaemia due to the tumour having outgrown its blood supply, or the subtle effects of hormones, intimate cellular contact, or other environmental factors that obtain *in vivo* but not *in vitro*, is a matter for speculation. In any event, it was not due to the fact that cells released from the tumours were dead. They excluded trypan blue and the cloning experiments were performed only on cells that had survived three days in culture as apparently healthy adherent monolayers. Should the above notion prove to be correct, the loss of a certain measure of capacity for continued exponential growth *in vivo* would explain the longer doubling times noted with tumour growing in nude mice relative to the same cells growing *in vitro*.

Finally, as found by other workers (Dodson et al, 1981; Hastings and Franks, 1981; Marshall et al, 1977; Stiles et al 1976c) there appeared to be little correlation between growth in soft agar and tumorigenicity

in the nude mouse. Although the three cell lines which had the highest cloning efficiency in soft agar (UCT-Mel 1, 2 and 3) also grew most rapidly in the nude mouse, two cell lines with far lower colony-forming ability in soft agar (UCT-Mel 5 and 7) gave rise to tumours which did not grow a great deal slower. Three of the cell lines did not exhibit anchorage independence and one of these (UCT-Mel 6) was also unable to grow in the nude mouse. The other two lines, however (UCT-Mel 4a and 4b) were tumorigenic in the nude mice. It is clear that the property lacking in UCT-Mel 4a and 4b which prevents them from proliferating in soft agar nevertheless proved to be no handicap in the true test of tumorigenicity *in vivo*.

Effects of co-injection of fibroblasts on the growth of melanoma cells in vivo.

When, as described in Chapter 5, I first noted a dramatic inhibitory effect of fibroblast co-cultivation on PA release by melanoma cells *in vitro*, I was led to the initial conclusion that the adult human skin fibroblasts suppressed melanoma cell activator release by some active mechanism involving either cell-cell contact or the secretion of some inhibitory compound.

My results, and the interpretation I put upon them at the time, were given special relevance by two reports that appeared in the literature. In the first, Silagi (1976) showed that tumorigenicity and PA release correlated closely in mouse melanoma cells treated with bromodeoxyuridine. The second set of papers (Kyner et al, 1978; Newcomb et al, 1978) was concerned with two cloned sublines of the mouse B16 melanoma. One of these was tumorigenic and released large amounts of PA; the other did not give rise to tumours in syngeneic animals and released undetectable amounts of PA. When these two sublines were co-cultivated, the one inhibited the

release of PA by the other and, when they were co-injected into susceptible mice, the non-tumorigenic cells inhibited formation of tumours by the tumorigenic subline.

On the basis of these observations, I predicted that co-injection of fibroblasts and human melanoma cells into nude mice should *inhibit* tumour formation. As it happened, the opposite was observed. Co-injection of fibroblasts promoted tumour formation *in vivo*. This effect was evident in two respects. Firstly, tumorigenic inocula gave rise to tumours with a very much shorter latent period when fibroblasts were present in the inoculum. Secondly, marginally or subtumorigenic inocula gave rise to tumours when fibroblasts were present. The fibroblasts did not appear to influence tumour size by any effect upon the doubling time. Other workers (Stiles et al, 1976a; Sager and Kovac, 1978) have observed the effect of co-injection of fibroblasts in improving the tumour-forming efficiency of low cell inocula, but have not commented on the decreased latency period I observed with inocula that were normally tumorigenic.

Although as yet I have no satisfactory explanation for this supportive effect of fibroblasts, it is of interest for several reasons. In the first instance, it provides an *in vivo* counterpart to the well-known effect of fibroblasts as "feeder" cells in tissue culture, where they will frequently support the growth of tumour cells present at a concentration that would ordinarily be too sparse to permit their proliferation. Furthermore, there is now increasing evidence to indicate that fibroblasts release somatomedins (Atkison et al, 1980; Clemmons et al, 1981) and possibly other growth factors that have a role in the recently described autocrine or paracrine hormonal systems suggested by Sirbasku (1981). The apparent requirement for close proximity of fibroblasts and co-injected melanoma cells is illustrated by the failure of the fibroblasts to enhance tumour formation if they were injected simultaneously but at different sites

or at the same site but at different times.

Finally, the effects of fibroblasts on tumour growth in the nude mouse are sufficiently dramatic and reproducible for them to provide an excellent model system for studying the manner in which mesenchymal cells support the growth of adjacent tumours of ectodermal origin.

APPENDIX

A.1 Cell culture

Cells were cultured in a Hotpack CO_2 incubator (Hotpack Corporation, Philadelphia, PA, U.S.A) at 37°C in a humid atmosphere containing 5% CO_2 in air.

The culture medium used was either DB or RPMI, supplemented with 300 µg penicillin/ml, 200 µg streptomycin sulphate/ml, 10 µg tylocine/ml, and 10% heat-inactivated (56°C; 30 min) FCS.

Cultures were passaged by incubating the cells briefly in 0.25% trypsin in TD, as described in detail in Chapter 2 (Materials and Methods). Cell stocks were maintained in liquid nitrogen and prepared by controlled freezing in medium containing 10% DMSO (Chapter 2).

All solutions used in the culture of cells were sterilized by filtration through a 0.45 µ Millipore filter.

Adult human skin fibroblasts

Skin fibroblasts were obtained from fragments of tissue removed from patients at the time of skin grafting or from skin removed from renal transplant donors. Tissue fragments were placed in DB supplemented with 2% FCS and processed as rapidly as possible.

The tissue was cut into 1 mm fragments with a scalpel blade. Care was taken not to compress the tissue or to allow the fragments to become dry. Approximately 20-30 fragments were placed on a 60 mm petri dish in a small volume of medium such that the fragments were kept moist but remained attached to the dish. Twelve to 18 hours later, when all viable fragments had adhered to the dish, an additional 5 ml of medium was added. Epithelial cell outgrowth was observed after 1-5 days and

this was followed by the appearance of fibroblasts. Once confluence had been reached (2-6 weeks), cells were passaged with 0.25% trypsin containing 0.02% EDTA and reseeded. Epithelial cells did not adhere following passage by trypsinization and pure fibroblast cultures were obtained. These were stored in liquid nitrogen and thawed and replated as required.

Foreskin fibroblasts

Foreskins obtained from neonatal circumcision were placed in DB supplemented with 2% FCS and processed as described for adult skin.

Human embryo cultures

Human embryo skin, heart and lung were obtained from a 16 week old human embryo and kidney from a 17 week old embryo.

Skin was processed as described for adult skin.

Heart, kidney and lung were minced finely with scissors and the mince was incubated with gentle agitation at 37°C with 0.25% trypsin containing 0.02% EDTA. After 30 minutes, the tissue fragments were allowed to settle at 1 x g for 5 minutes and the supernatant was removed and the cells were pelleted by centrifugation at 350g for 5 min. The cell pellet was resuspended in DB containing 10% FCS and seeded on plastic tissue culture dishes. Fresh trypsin-EDTA was added to the remaining fragments and the process was repeated until all tissue fragments had been digested. In many instances, following trypsinization a gelatinous mass was obtained. This gelatinous matrix was dissolved following incubation with 10 µg/ml of DNAase at 37°C for 5 min.

A.2 Measurement of cell growth

Cell growth was measured as follows, unless otherwise stated. Cells were seeded at a low density (usually 1 x 10^5 cells per 35 mm dish) in the specified medium which contained 10% FCS. Cell monolayers from duplicate dishes were trypsinized at 48 hr intervals unless otherwise stated and cells were counted in a Coulter Counter.

The effect of various agents on cell growth was determined by adding the agent at the indicated concentration 24 hr after seeding the cells. Medium was replaced at 48 hr intervals.

A.3 Cell morphology

Morphological changes in the cells were observed and recorded with the aid of a Nikon phase-contrast microscope with camera attachment.

A.4 Chromosome analysis

Ten µg colchicine per ml was added to exponentially growing cell cultures for a 2 hr period. Nonadherent cells were added to the trypsinized monolayer and the cells were sedimented at 350g for 5 min. The cells were resuspended in 0.07M KCl and incubated for 15 min at room temperature and centrifuged as described above. Approximately 0.3 ml acetic acid:methanol (1:3) was added to the cell pellet, care being taken not to disturb the pellet. After 10-15 min the pellet was resuspended, and fixative was gradually added, with gentle mixing, to a final volume of 5-10 ml. Following centrifugation, the pellet was resuspended in 0.1 to 0.3 ml fixative (depending on the cell concentration). Two drops of this

solution were allowed to fall from a pasteur pipette held 20 cm above pre-chilled (-20°C) clean glass slides. Slides were heat-dried (65°C for 1 min) and stained with 2% Giemsa stain for 5 min.

A.5 Growth in soft agar

Cells were tested for anchorage independent growth by the method of MacPherson (1973). A 7 ml feeder layer of 0.5% Difco Bacto Agar in RPMI 1640 medium containing 10% FCS, was added to 60 mm petri dishes and allowed to solidify at room temperature. Cells were seeded at 200 to 5000 cells per 60 mm tissue culture dish, in a solution of 0.33% agar in medium, in a total volume of 1.5 ml. After the agar had solidified the dishes were transferred to a 37°C incubator. One day after seeding, cultures were examined microscopically to ensure that a single cell suspension had been obtained. Cell colonies were scored after 14 days incubation.

A.6 Plating efficiency

The methods used for measuring plating efficiency are described in detail in Chapter 2 (Materials and Methods).

A.7 Mycoplasma contamination

The method used to test for mycoplasma contamination was that described by Chen (1977). This involved staining cultures with a DNA-

specific fluorescent stain.

Cells were cultured for 7 days in medium from which tylocine had been omitted. Cells were then fixed with acetic acid:methanol (1:3) and stained with bisbenzimide fluorochrome (Hoechst No. 33258) at 0.5 µg/ml in Hank's balanced salt solution for 30 min. The cell layer was well rinsed in deionized water and mounted in buffer containing 0.02M citric acid, 0.06M disodium phosphate and 50% glycerol, pH 5.5. Cultures were examined with a Leitz fluorescence microscope.

A.8 Collection and storage of harvest fluids

Secretion of PA by the cells was measured in serum-free harvest fluids. Serum was removed as it contains many protease inhibitors (Heimburger, 1975). The culture medium was aspirated and the cultures were rinsed three times with 2 ml of warm serum-free medium. Serum-free medium was then added (1 ml per 35 mm dish; 2 ml per 60 mm dish) and the cultures incubated for 24 hr , unless otherwise stated. After this time the harvest fluid was collected and PIF-BSA (see A.19) was added to a final concentration of 0.4 mg/ml. The harvest fluid was centrifuged at 350 g for 5 min to remove nonadherent cells and cell debris and stored at -20^{o}C until assay.

When experiments involved investigating the effects of various compounds on PA secretion, the compound was added, at the indicated concentration, to the harvest fluid. None of the compounds tested had any direct effects on the PA assay.

A.9 Preparation of cell lysates

Adherent cells on 60 mm dishes were washed 3 times with 3 ml of ice-cold PBS. Two ml of PBS was added to the monolayer which was removed with a teflon policeman, and transferred to a plastic centrifuge tube. The culture dishes were rinsed with 2 ml of ice-cold PBS, which was pooled with the original suspension. The cells were centrifuged at 350 g for 5 min at 4°C. The cell pellet was washed once with PBS, drained and lysed by the addition of 100 µl of 0.5% Triton X-100 in water per 10^6 cells. The lysate was incubated on ice for 1 hr, then frozen and stored at -20°C.

A.10 Addition of compounds to cells cultured *in vitro*

All steroid hormones and retinoid derivatives were dissolved in absolute ethanol to give a final concentration of 10^{-2}M and stored at -20°C. Retinoid solutions were protected from light and fresh stock solutions were prepared at weekly intervals. TPA was dissolved in absolute ethanol and stored at -20°C at a concentration of 400 µg/ml. αMSH was dissolved in 70% ethanol to give a final concentration of 5×10^{-3}M. Dibutyryl cAMP was dissolved in medium immediately prior to addition to the cultures.

Dilutions were made immediately prior to use in medium containing 10% FCS or in serum free medium as stated in the text. Unless otherwise stated hormones and compounds were added to cells in the presence of FCS for 48 hr. Cells were then incubated for an additional 24 hr in serum-free medium - the "harvest fluid", which also contained the hormone or compound at the indicated concentration.

A.11 The measurement of macromolecular synthesis

After an 8 hr pulse with L-^{3}H-leucine (5 µCi/ml), ^{3}H-uridine (5 µCi/ml; 1 µg/ml) or ^{3}H-thymidine (5 µCi/ml; 1 µg/ml) the cultures (on 35 mm petri dishes) were washed 3 times with 2 ml PBS and then incubated with 1 ml of 0.25% trypsin containing 0.02% EDTA. Cell concentration was determined in a haemocytometer. The culture dishes were placed on ice, and 1 ml of ice-cold 10% TCA was added. The dishes were kept at 4°C for 30 min. The TCA precipitate was collected on a Whatman GFC filter and washed with 40 ml of ice-cold 5% TCA. The filter was dried overnight in a hot oven and radioactivity measured following the addition of 5 ml of Instagel in a Packard liquid scintillation spectrometer.

A.12 Protein determination

Protein was determined fluorimetrically (Böplen et al, 1973) using BSA as a standard.

Cell lysates in 0.5% Triton X-100 were vortexed vigorously to obtain a homogeneous suspension. Aliquots of each sample were diluted in 0.2M borate buffer, pH 9.2 to give a final volume of 800 µl and assayed in duplicate or triplicate. Control samples contained (1) 800 µl borate buffer alone and (2) 0.5% Triton X-100 diluted similarly to the cell lysates. All dilutions were made in plastic tubes (Falcon 2038) or well rinsed neutral glass fluorimeter tubes.

Standard tubes were prepared containing 1-15 µg BSA in 800 µl borate buffer.

Fluram (Hoffman-La Roche, Basle, Switzerland) was dissolved in acetone to give a final concentration of 0.25 mg/ml. 200 µl Fluram

solution was added to the sample, the mixture was rapidly mixed and the fluorescence measured on a Perkin-Elmer spectrofluorimeter (excitation at 390 nm, emission at 475 nm).

All samples were assayed in duplicate.

A.13 Plasminogen activator assay

Plasminogen activator was assayed as described by Wilson and Dowdle (1978). Briefly, the assay relies on the measurement of plasminogen-dependent release of soluble, radioactive fibrin degradation peptides from an insoluble substrate of ^{125}I-labelled fibrin adhering to the bottom surface of a plastic well.

^{125}I fibrin-coated Linbro multi-well plates were prepared with purified bovine fibrinogen as described by Strickland and Beers (1976) with the modification that each well contained 120 000 to 130 000 cpm which could be solubilized by trypsin. 30 µg fibrinogen was added per well and the plates were dried at 37°C for 3 days before use. The plates were stored at room temperature.

^{125}I fibrinogen was converted to fibrin by the addition of Eagles Minimal Essential medium containing 5% FCS from which the plasminogen had been removed by the method of Deutsch and Mertz (1970). After incubation at 37°C for 2 hr , the wells were washed twice with PBS, pH 7.4 and once with 0.1M Tris-HCl, pH 8.1.

For the PA assay, each well contained 2 µg of purified human plasminogen and 80 µg of PIF-BSA (see A.19) in a final volume of 300 µl of 0.1M Tris-HCl, pH 8.1. Ten and 20 µl aliquots of harvest fluid were added to initiate the reaction.

Intracellular PA was assayed similarly and aliquots of the cell lysate containing 0.8 to 6 µg of protein in 10 µl of 0.5% Triton X-100 were added to each well.

Control wells contained (1) 300 µl trypsin (to give the total radioactivity present in each well); (2) 20 µl of sample without plasminogen (to detect plasminogen-independent proteolysis) and (3) plasminogen without sample (to detect background lysis due to plasmin contamination of the plasminogen solution). Standard wells included in each assay contained UK in 12 doubling decrements, usually commencing with 0.2 Ploug units per well. The UK was diluted in the same medium used for the harvest fluid collection.

The Linbro multi-well plates were incubated at 37°C in a humid atmosphere. Fibrinolysis was monitored by measuring the solubilized radioactivity in 50 µl aliquots, at three separate time points.

For each time point, the UK standard curve was plotted and sample values falling on the linear range of the standard curve were determined and expressed as UK units per assay well.

The average value from 10 µl and 20 µl aliquots of harvest fluid at each time point was determined.

A.14 Molecular weight and antigenicity of melanoma plasminogen activator

A.14.1 Rabbit anti-human urokinase antibody

Rabbits were immunized as previously described (Wilson et al, 1980). An initial subcutaneous injection of 1250 Ploug units of purified human UK dissolved in 0.5 ml of 0.1M Tris-HCl, pH 7.4 and emulsified with an equal volume of complete Freund's adjuvant was given. After 6 weeks, animals

were boosted by intramuscular injection with a similar quantity of UK in incomplete Freund's adjuvant and bled 2 weeks later.

The crude immune serum was passed through a lysine-Sepharose affinity column (Deutsch and Mertz, 1970), precipitated by the addition of 40% ammonium sulphate, passed through a diethylaminoethyl-cellulose column, and treated with DFP as described in the preparation of plasminogen (see A.18). These procedures resulted in an IgG antibody fraction that was free of plasmin, plasminogen, plasminogen activators, and protease inhibitors, and the final product gave a single precipitin line in Ouchterlony double diffusion against UK.

Irrelevant rabbit IgG for use as a control in inhibition assays was similarly prepared from a rabbit immunized according to the same protocol with ovalbumin. This IgG preparation did not react with UK.

A.14.2 Rabbit anti-melanoma plasminogen activator antibody

The PA secreted by the Bowes human melanoma cell line (RPMI-7272, obtained from Dr. E. Reich, Rockefeller University, New York) was purified in this laboratory according to the method of Danø et al (1980). Briefly, serum-free culture fluid collected from the melanoma cells was applied to a column of benzamidine CH-sepharose CL-4b. The PA adsorbed to this column was eluted with 0.5M arginine in acetate buffer, pH 5.5, and fractions containing PA activity were pooled, precipitated with 10% TCA, redissolved in SDS sample buffer, and electrophoresed in 11% SDS-polyacrylamide slab gels.

The protein band corresponding with a band of PA activity was located by electrophoresing undenatured PA in an adjacent track. The corresponding protein band in the track containing the TCA precipitate was removed from the gel, homogenized in a polytron homogenizer and suspended in 1 ml of PBS.

Rabbits were immunized by subcutaneous injection of 0.5 ml of the polyacrylamide-PA homogenate emulsified with an equal volume of Freunds complete adjuvant. After 3 weeks a booster consisting of 0.5 ml homogenate mixed with an equal volume of incomplete adjuvant was given subcutaneously. The rabbits were bled at 2-3 weekly intervals thereafter. Further booster injections were given at 2 monthly intervals.

The immune serum was processed as described for the preparation of rabbit anti-human UK antibody. An IgG fraction which specifically inhibited melanoma plasminogen activator was obtained.

A.14.3 Enzyme inhibition assay with anti-UK and anti-melanoma PA IgG

Serial 2-fold dilutions of immune or irrelevant IgG were made in 0.1M Tris HCl, pH 8.1 in 10 x 75 mm plastic test tubes to give a final volume of 50 µl per tube and PIF-BSA was added to give a final concentration of 1.3 mg/ml. Fifty µl samples of harvest fluid (diluted to contain approximately 0.2 Ploug units per ml) were added to each tube of a series of IgG dilutions, and the tubes were incubated at $4^{\circ}C$ for 1 hr. An aliquot of the tube contents was then assayed for residual plasminogen activator activity in the ^{125}I-fibrin plate assay.

A.14.4 Polyacrylamide gel electrophoresis and zymography of plasminogen activators

The procedures followed were as described by Granelli-Piperno and Reich (1978) and Wilson et al (1980).

Plasminogen activators present in harvest fluid were separated by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The SDS was removed after electrophoresis by incubation of the gel in a solution of nonionic detergent. The gel was then layered onto a second indicator gel consisting of fibrin, plasminogen and agar. After incubation at $37^{\circ}C$, the

position of proteases in the SDS-gel could be determined by the corresponding zone of lysis in the fibrin gel. These bands were seen as clear areas of lysis in the opaque background under dark-ground illumination.

(a) Polyacrylamide gel electrophoresis

Buffers used were as follows:

Running gel buffer: 0.4% SDS in 1.5M Tris-HCl, pH 8.8;

Stacking gel buffer: 0.4% SDS in 0.5M Tris-HCl, pH 6.8;

Sample buffer: 2.3% SDS, 10% glycerol in 0.0625M Tris-HCl, pH 6.8;

Reservoir buffer: 0.1% SDS in 0.025M Tris-HCl, 0.192M glycine, pH 8.3.

Seven x 8 cm slab gels of 11% acrylamide with stacking gels of 4% acrylamide were used. The final concentration of SDS in the gel was 0.1%. Catalysts added were Temed (0.05% in running gel and stacking gel) and ammonium persulphate (0.05% in running gel and 0.1% in stacking gel). Samples contained a final concentration of 1.2% SDS.

Electrophoresis was performed at constant current (8mA) until the dye front reached the bottom of the gel (approximately 3 hr).

Following electrophoresis, the track containing molecular weight markers was removed and stained. The remainder of the gel was washed for 1 hr with gentle shaking, in a solution of 2.5% Triton X-100 in H_2O. The gel was then rinsed well in distilled water, dipped briefly into 0.1M Tris-HCl buffer, pH 8.1, drained and layered on the fibrin-plasminogen-agar indicator gel.

(b) Fibrin-plasminogen-agar gel

The fibrin-plasminogen-agar gels were cast between two clean glass slabs, held 0.8 mm apart by thin wires. The entire assembly was clamped together and warmed in a 45°C incubator.

The gel solution contained 1.25% agar, 5 µg/ml of purified

human plasminogen, 2 mg/ml of purified bovine fibrinogen and 0.06 units/ml of thrombin in Tris-HCl pH 8.1.

The agar was added to water and boiled for 15 minutes, then transferred to a 45°C water bath. The solutions of fibrinogen, plasminogen and thrombin were kept at 37°C, mixed and this solution added rapidly to the agar. The final mixture was immediately pipetted between the two pre-warmed glass slabs, care being taken to avoid air bubbles. The mixture was allowed to solidify at room temperature, then kept at 4°C in a humid chamber until needed.

The SDS gel was layered onto the indicator gel, incubated at 37°C in a humid chamber and removed at intervals for viewing and photography under dark-ground illumination. Bands of PA activity could be seen as clear lysis zones in the cloudy background provided by the unlysed fibrin. Failure of fibrinolysis to occur in plasminogen-free indicator gel slabs was used as the criterion for defining the enzymes as plasminogen activators.

The fibrin gel was preserved by staining for 10 min with a solution of 0.1% amido black in 70% methanol and 10% acetic acid, and was destained in 70% methanol, 10% acetic acid.

Combined electrophoretic and immunochemical procedures were used to identify individual electrophoresed enzyme bands. These procedures are described in detail in Chapter 3 (Materials and Methods).

A.15 Tyrosinase assay

A sensitive fluorometric assay was used (Adachi and Halprin, 1967). This assay depends on the hydroxylation of tyrosine to DOPA by tyrosinase. The DOPA is oxidized by potassium ferricyanide and the fluorophore of DOPA thus obtained is rearranged in alkali to form a strongly fluorescent tri-

hydroxyindole (Bertler et al, 1958). Measuring the trihydroxyindole thus formed, provides a very sensitive method for assaying tyrosinase. Cell lysates were prepared as described (see A.9) and used as the source of enzyme. The concentration of Triton X-100 present was found to have no effect on this assay (Fig. A.15a).

The reaction mixture consisted of 0.75 mM L-tyrosine; 100 µM L-DOPA; 0.75 mM ascorbic acid; 40 mM phosphate buffer, pH 6.9 and 10 µl of the enzyme in a total volume of 50 µl. The ascorbic acid and DOPA solutions were prepared fresh daily.

Solutions were added to tubes in an ice water and salt bath at $0^{o}C$ and transferred to a $37^{o}C$ water bath for the appropriate incubation period (usually 30 minutes). The reaction was terminated by transferring the tubes to an ice water and salt bath.

The DOPA formed during the $37^{o}C$ incubation period was measured at room temperature as follows:- 5 µl of the mixture was transferred into 900 µl of 10 mM phosphate buffer, pH 6.5, containing 0.0025% zinc sulphate in a neutral glass fluorimeter tube. After mixing, 10 µl of 0.25% potassium ferricyanide was added and the contents of the tube mixed immediately on a vortex mixer. After exactly 2 minutes this oxidation step was inhibited by the addition of 100 µl of a mixture of 5N NaOH and 2% ascorbic acid solution, 9:1 (v/v), followed by mixing.

The fluorescence was measured in a Perkin-Elmer spectrofluorimeter (excitation at 360 nm, emission at 490 nm) after 5 min and within 30 min of terminating the reaction.

All samples were assayed in duplicate.

Blanks consisted of cell lysate in a reaction mixture lacking tyrosine and DOPA. Standards consisted of (1) the complete reaction mixture lacking cell lysate, (2) the complete reaction mixture containing

FIGURE A.15

Tyrosinase assay.

(a) Doubling dilutions of UCT-Mel 1 cell lysate in water (▲——▲) or 0.5% Triton X-100 (■---■) produced similar decrements in the amount of DOPA detected after a 30 min incubation period. Note that this decrease was linear, and that the presence of 0.5% Triton in the cell lysate had no effect on the enzyme activity in the assay.

(b) Ten µl aliquots of UCT-Mel 1 cell lysate were incubated in the reaction mixture described (Appendix A.15) for the times indicated. The amount of DOPA produced at the end of this time was found to increase linearly between 5 and 25 min. Maximal amounts of DOPA were evident at 30-40 min and 30 min was chosen as a convenient assay period.

FIGURE A.15

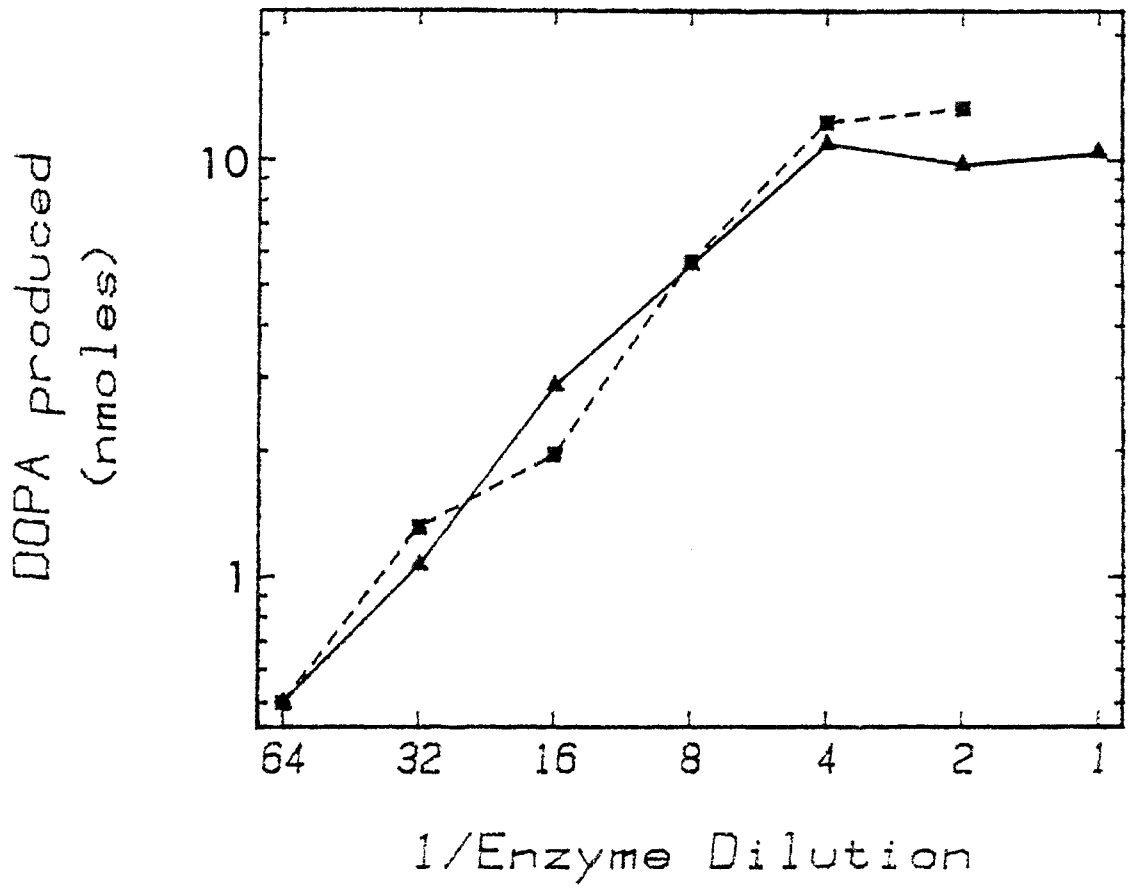


FIGURE A.15(a)

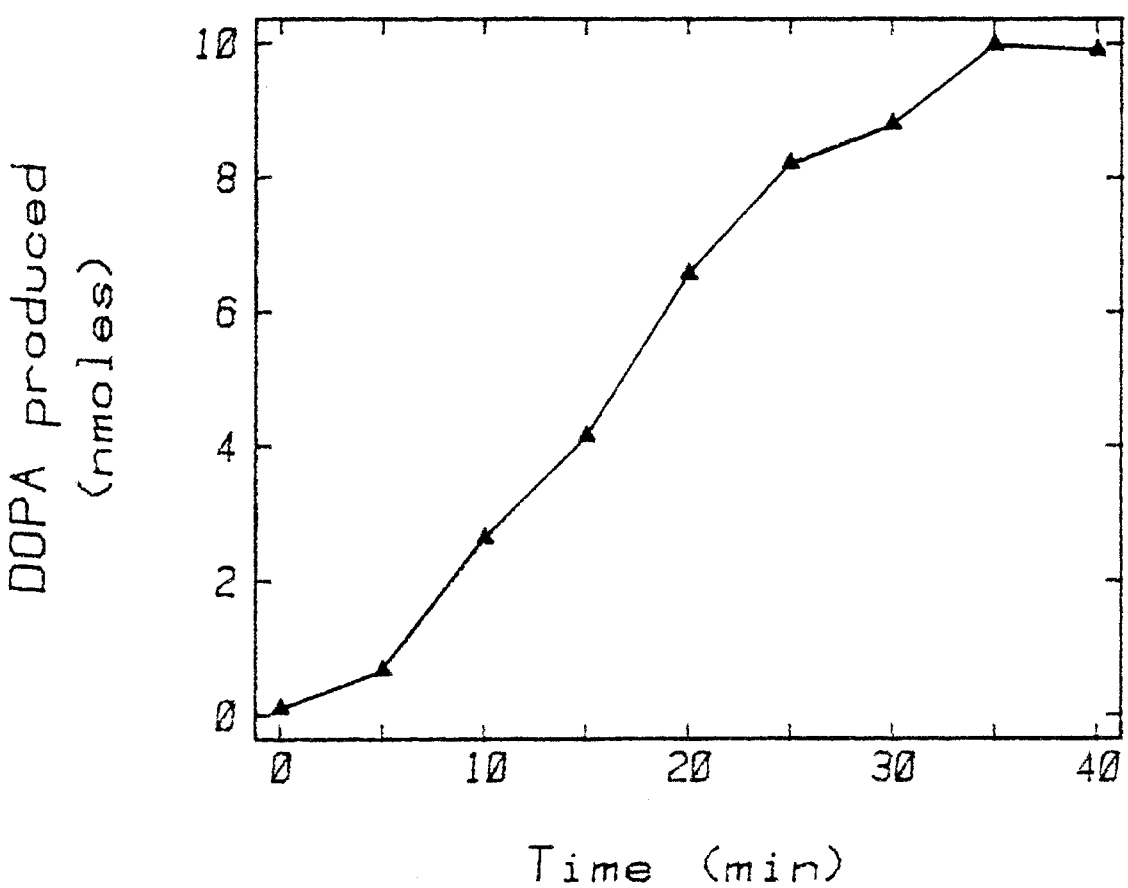


FIGURE A.15(b)

boiled lysate (100°C for 5 minutes) instead of native lysate, and (3) the complete reaction mixture kept at 0°C for the same period of time as the samples incubated at 37°C. The amount of fluorescence produced by the DOPA in the standards was used to calculate the amount of DOPA (in nmoles) generated by the enzymatic conversion of tyrosine to DOPA.

Under the conditions described, the amount of DOPA produced was linear with enzyme concentration (Fig. A.15a) and over a time period of 30 min (Fig. A.15b). Dilutions of the most active and least active samples were included to ensure that the enzyme activity recorded was within the linear range of the assay. One unit of tyrosinase was defined as that amount of enzyme which generated 1 nmole DOPA in 30 min.

The potassium ferricyanide and ascorbic acid solutions were prepared fresh daily.

A.16 Lactate dehydrogenase assay

Lactate dehydrogenase was assayed in cell lysates according to the method of Kornberg (1955).

The reaction mixture consisted of 333.3 µM sodium pyruvate and 66.7 µM nicotinamide adenine dinucleotide (reduced) in 0.03M sodium phosphate buffer, pH 7.4 at room temperature. This mixture was placed in a cuvette in a Unicam SP 1800 ultraviolet spectrophotometer. At time 0, a 33.3 µl aliquot of cell lysate in 0.5% Triton X-100 was added to the reaction mixture (to give a final volume of 1 ml), mixed rapidly and the change in absorbance at 340 nm recorded for 3-4 min. The reaction was measured against a solution consisting of the reaction mixture and 0.5% Triton X-100 in place of the sample.

Sodium pyruvate and NADH solutions were made fresh daily.

Results were calculated according to the formula:

$$\text{units/mg protein} = \frac{\text{Absorbance/min}}{6.2 \times \text{mg protein/ml reaction mixture}}$$

A.17 Urokinase

Purified human UK was obtained in the form of a lyophilized powder from Mochida Pharmaceutical Co. Ltd., Tokyo, Japan. The UK was dissolved in 0.1M Tris-HCl, pH 8.1, containing 8 mg/ml PIF-BSA to give a stock standard solution of 2000 Ploug units/ml. This solution was stored in aliquots at -80°C and each aliquot was thawed and used once only. Each new batch of UK was standardized in the PA assay against the previous batch.

A.18 Plasminogen

Plasminogen was isolated from human serum according to the method of Deutsch and Mertz (1970). Briefly, filtered human serum was passed through a lysine sepharose affinity column at 4°C. Adsorbed plasminogen was eluted with a solution containing 0.2M 6-aminocaproic acid in 0.1M potassium phosphate buffer, pH 7.2, and dialysed against PBS. To inactivate traces of contaminating plasmin, the plasminogen was made 10 mM with respect to DFP and incubated at 40°C for 2 hours. Unreacted DFP was removed by extensive dialysis against 0.1M Tris-HCl, pH 8.1. The plasminogen was stored at -20°C.

A.19 Protease and inhibitor free bovine serum albumin

Bovine serum albumin (British Drug Houses Ltd., Poole, England) was dissolved in water to give a solution of 20 mg/ml. Acid-labile protease inhibitors were removed by adjusting this solution to pH 3.0 with 0.1M HCl and incubating at room temperature for 2 hours. The solution was then neutralized with 0.1M NaOH and protease activity was

removed by treatment with DFP, as described in the preparation of plasminogen (A.18). The PIF-BSA was stored at -20°C.

A.20 Fibrinogen

Bovine fibrinogen (Fraction 1, Sigma) was precipitated with ammonium sulphate (Laki, 1951), resuspended in 0.6M NaCl, and then precipitated with 7% ethanol in the presence of lysine (Mosesson, 1962). The precipitate was collected by centrifugation, resuspended in 0.6M NaCl and the ethanol precipitation step was repeated. The final precipitate was dissolved in 0.6M NaCl and dialysed against PBS. Labelling of the fibrinogen with ^{125}I was performed by the method of Helmkamp et al (1960). Briefly, 20 mg of purified human fibrinogen was iodinated with 10 mCi ^{125}I, using a 3M excess of iodine monochloride.

A.21 Histological appearances of human melanomas in nude mice

The pathologist was requested to examine the tumours removed from the nude mice and to review their histological appearances in comparison with those of the original tumours as they were removed from the patients.

I am grateful to Dr. J.A.H. Campbell for the following reports which I refer to in Chapter 6.

UCT-Mel 1

Histological examination of these tumours showed a morphology similar to that of the original biopsy (Fig. 6.8a) namely a moderate to heavily pigmented epithelioid-like malignant melanoma with prominent

eosinophilic nuclei and frequent mitoses. Giant tumour cells were occasionally noted. The morphology, pigmentation and presence of giant cells all correlate with the appearance of the cell line *in vitro* (Fig. 6.10a). In all 4 tumours removed from the nude mice, numerous small, and occasionally large necrotic areas were present.

UCT-Mel 2

The original tumour as removed from the patient showed areas of both pigmentation and nonpigmentation. A similar pattern was observed in the tumours derived from the nude mice, where small areas of pigmentation were frequently found in macroscopically nonpigmented tumours. A few tumours derived from UCT-Mel 2 cells injected into mice were deeply pigmented. The majority however appeared to be either nonpigmented or exhibited "speckled" pigmentation (Table 6.4).

Histological examination demonstrated in most cases a lightly pigmented malignant melanoma composed of moderate sized pleomorphic epithelioid type cells with amphophilic cytoplasm, similar in appearance to the original tumour (Fig. 6.8b). The giant cell formation and extensive pigmentation of the original tumour were not noted in the grafted tumours, however. Almost all the cells in the grafted tumours contained large nucleoli, and mitoses were very numerous, as found in the original tumour. The grafted tumours frequently showed numerous small areas of necrosis, with the pigmentation being heavier in these necrobiotic areas. The features of pigmentation, morphology and absence of giant cells found in the grafted tumours reflects the situation *in vitro* (Fig. 6.10c).

UCT-Mel 3

These tumours appeared to be pigmented, although UCT-Mel 3 does not pigment *in vitro*. However, it was found that the dark colour of the tumour was due to the large number of erythrocytes present in the tumour tissue.

Histological examination revealed, in both cases, tumours which correlated well with the original (Fig. 6.8c) and with the appearance of the cells *in vitro* (Fig. 6.10e), the appearance being that of a nonpigmented malignant melanoma of epithelioid-like cells. These cells had prominent nucleoli and were often in mitosis.

UCT-Mel 4a and 4b

The original tumour from which both these lines were derived had the histological appearance of areas of deep pigmentation and the morphology of the cells had strong epithelioid characteristics. In culture, both lines retained features of the epithelioid morphology, but lost the ability to produce pigment. The tumours removed from the nude mice were non-pigmented and both cell lines gave rise to tumours with the histological appearance of undifferentiated malignant melanomas (Fig. 6.8d and e). There was, however, a similarity to those parts of the original tumour which were more undifferentiated.

UCT-Mel 5

Both the original tumour and the xenograft in the mouse comprised a mixture of spindle-shaped and epithelioid cells. The relative proportions in which they were present, and their structural organization, however, differed. In the tumour removed from the patient, epithelioid cells were sequestered into "nests" or "islands" surrounded by prominent whorls of

spindle cells. In the xenograft, the spindle cells were far less prominent and appeared as isolated cells distributed randomly amongst the epithelioid cells. The epithelioid cell element in the grafted tumour had the same lack of pigment, numerous mitoses and prominent nucleoli seen in the original tumour (Fig. 6.8f). Areas of necrosis of moderate size were noted with moderate frequency.

UCT-Mel 7

Histologically, both tumours were very similar to the original human tumour (Fig. 6.8g). The tumours derived from the mouse showed cells in a herring-bone pattern and both spindle-celled and epithelioid in character, the latter suggesting malignant melanoma more than fibrosarcoma. The epithelioid cells in particular had ample cytoplasm, and pigmentation and tumour necrosis were not features.

The lack of pigment was as expected from the *in vitro* cell culture. *In vitro*, however, only spindle-shaped cells were evident (Fig. 6.11g). These also grew in a herring-bone pattern.

REAGENTS

Reagents were obtained from the following sources:-

Cell culture media from Grand Island Biological Co., Grand Island, N.Y.; foetal calf serum (FCS) from the State Vaccine Laboratories; plastic tissue culture ware from Falcon Plastics Ltd., Oxnard, Calif.; Linbro multiwell plates (Cat. No. FB-16-24-TC) from Flow Laboratories Ltd., Irvine, Scotland; sodium ^{125}I (carrier-free), ^{3}H-uridine, ^{3}H-thymidine and ^{3}H-leucine from the Radiochemical Centre, Amersham, Bucks., England; collagenase from Worthington Biochemical Corporation, Freehold, N.J.; trypsin from Difco Laboratories, Detroit, Mich.; molecular weight marker proteins, Pharmacia Fine Chemicals, Uppsala, Sweden, comprising phosphorylase b (M.W. 94 000), albumin (M.W. 67 000), ovalbumin (M.W. 43 000), carbonic anhydrase (M.W. 30 000), trypsin inhibitor (M.W. 20 100) and α-lactalbumin (M.W. 14 400); tetradecanoylphorbol acetate (TPA) from Dr. P. Borchert, University of Minnesota, Minneapolis, Minn.; actinomycin D from Aldrich Chemical Co., Milwaukee, Wis. The following reagents were obtained from the Sigma Chemical Co., St. Louis, Mo:- retinoids; dexamethasone; β-estradiol; progesterone; androstan (testosterone); α-MSH; cycloheximide; mitomycin C; dBcAMP; DFP.

All other reagents were of analytical grade.

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