THE DETECTION OF OCCULT METASTATIC DISEASE IN PATIENTS WITH CUTANEOUS MELANOMA

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DECLARATION

I, **Gideon S. Hanekom**, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or any part of it has been, is being, or is to submitted for another degree in this or any other University.

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March 1999

AAN

MY BEMINDE VROU

THERESHA

EN MY FAMILIE

The most important thing in life

is not the triumph but the struggle.

The essential thing is not to have conquered

but to have fought well.

Author unknown

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LIST OF ABBREVIATIONS

2c-tPA	two-chain tPA
ABTS	2,2' azino-di (3-ethyl-benzthiazoline) sulfonic acid
AI	additivity index
AM	advanced melanoma
BM	bone marrow
BSA	bovine serum albumin
cDNA	complementary DNA
CM	conditioned medium
CMCs	circulating melanoma cells
CTCs	circulating tumor cells
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
DFP	diisopropylfluorophosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylene-diamine-tetra acetate
ELISA	enzyme-linked immunosorbent assay
dNTP	deoxynucleotide triphosphate
FCS	fetal calf serum
Fg	fibrinogen
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HAT-medium	hypoxanthine-aminopterin-thymidine medium
HUCS	human umbilical cord serum
IHC	immunohistochemistry
LDH	lactate dehydrogenase
LN	lymph node
mαPAI1/C2, etc.	mouse anti-PAI1 Mab, hybridoma clone 2, etc.
Mab	monoclonal antibody
MIA	melanoma-inhibiting activity
MMLV-RTase	Moloney-murine leukemia virus reverse transcriptase
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MW	molecular weight

nPAI1	native PAI1
OD	optical density
Pab	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PAP	peroxidase anti-peroxidase complex
PAs	plasminogen activators
PAI1	plasminogen activator inhibitor type 1
PB	peripheral blood
PBL	peripheral blood lymphocytes
PBS	phosphate-buffered saline
PFP	platelet-free plasma
PFU	plaque forming units
Plg	plasminogen
PM	primary melanoma
PMSF	phenylmethyl sulfonylfluoride
PNPP	p-nitrophenyl phosphate
PVDF	Immobilon-P polyvinylidene difluoride
RαPAI1	rabbit anti-PAI1 polyclonal antibody
RNase	ribonuclease
rPAI1	recombinant PAI1
RPMI	Roswell Park Memorial Institute 1640 medium
RP10	RPMI medium plus 10% fetal calf serum
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SAM	sheep anti-mouse IgG
SDS	sodium dodecyl sulfate
Sf9	Spodoptera frugiperda insect cells, strain 9
sICAM-1	soluble intercellular adhesion molecule 1
sVCAM-1	soluble vascular adhesion molecule 1
SLNs	sentinel lymph nodes
SMB	somatomedin B domain
TBS	tris-buffered saline
TE	tris-EDTA
TEMED	N,N,N,N'-tetramethylene diamine
tPA	tissue plasminogen activator
TRP-1 and -2	tyrosinase-related proteins 1 and 2

TST	tris-saline-tween
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
VN	vitronectin
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

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ABSTRACT

The ability to identify melanoma patients with progressive disease is central to efficient management. The challenge therefore, is to develop prognostic markers and techniques which will allow the identification of those patients whom, at the time of primary tumor diagnosis, already have micrometastases (occult or clinically undetectable metastases).

The use of the reverse transcription-PCR (RT-PCR) technique for the detection of circulating melanoma cells (CMCs) is potentially a powerful tool for identifying those patients at risk for developing metastases. The first aim of this study was to develop a more sensitive, reproducible, cost effective and clinically applicable assay and to eliminate the problem of false positives. A combined RT-PCR assay for tyrosinase mRNA, a marker specific for melanoma cells, was developed and tested. It was shown that the assay can reproducibly detect a single, viable melanoma cell in 10-15 ml of peripheral blood. Furthermore, a simple but effective procedure was developed to prevent carry-over contamination. It was found that the chance of obtaining normal melanocyte contamination with the needle prick during blood sampling was only 2% and that illegitimate transcription does not contribute to sporadic false positives.

The second aim of this study was to determine whether the early detection of CMCs is of any clinical value to monitor melanoma progression. Peripheral blood samples from 143 patients with primary melanoma (PM) were analysed by RT-PCR for the presence of tyrosinase mRNA. Seven percent (10/143) of the patients with PM had detectable CMCs. The percentage of PCR-positive patients was higher for stage II patients (9.0%) compared to stage I (5.3%) but the difference was not significant. A significantly higher percentage ($P < 0.05$) PCR-positive patients were found to have tumors greater than 1.5 mm thick and with ulceration present. Although this finding supports the notion that tumor thickness and ulceration are the two most significant prognostic factors, it was not possible at this stage, to link this directly to a poor prognosis since the majority of the PCR-positive patients have not yet (within four years) developed metastatic disease. However, the data does indicate that cells from tumors greater than 1.5 mm thick and with ulceration have a greater propensity to enter the circulation but that these cells do not necessarily have the ability to establish metastases. The results suggest that the detection rate of 9% for patients with stage II disease is much lower than would be expected, since 23.9% (16/67) of the stage II patients subsequently developed metastases. Of these 16 patients, only one was PCR-positive, one week before the metastases became clinically evident. Thus, the current technique fails to predict the likelihood of developing metastatic disease ($P = 0.3485$). The other nine PCR-positive patients had not yet developed metastases after a median follow-up period of

four years. It is concluded that the current technique for the detection of CMCs is of limited clinical value to predict the likelihood of metastasis in patients with PM. It is suggested that other anatomic compartments, such as sentinel lymph nodes, should be explored for the identification of patients at risk for developing metastases.

The third aim of this study was to determine whether high or low plasma levels and/or activity of plasminogen activator inhibitor type 1 (PAI1) correlate with the presence of metastatic disease in patients with melanoma. PAI1 is considered to be the main regulator of fibrinolytic activity in blood and has been identified as a key enzyme in the metastasis and vascularization of solid tumors. A unique enzyme-linked immunosorbant assay was developed to measure both the total amount of PAI1 in plasma as well as the active fraction of the inhibitor. This novel assay was then used to analyse and compare the plasmatic PAI1 levels and activity of a group of patients with advanced melanoma (AM) with a group of patients with primary disease and a control population. There was no statistical difference in the total plasmatic PAI1 levels between the controls and patients with PM and AM ($P = 0.6199$). In contrast, there was a significant difference in the active fraction of PAI1 between the controls and patients with PM or AM ($P = 0.0076$). A value of less than 44% active PAI1 was shown to be clinically meaningful by linear discriminant analysis. This means that a melanoma patient with a plasmatic PAI1 activity value less than 44% will have a 50% chance of harbouring metastases. Of the patients with PM, 19% had PAI1 activity values less than 44%, which strongly supports further investigations to determine whether plasmatic PAI1 activity levels might be predictive of metastatic disease. The false positive rate was 2.6%. It is speculated that this reduction in the active fraction of PAI1 for patients with AM might be attributed to tumor-derived tissue plasminogen activator and/or other melanoma-derived proteases or factors.

The last section of this study describes several monoclonal antibodies (Mabs) that were developed against PAI1 in order to obtain useful reagents to study the regulatory functions of PAI1 in the metastasis and vascularization of solid tumors. The baculovirus expression system was used to express human PAI1 in insect cells and the crude infected cell population was used as the immunogen in mice. This approach was followed since the *Escherichia coli*-derived recombinant molecule elicited a poor immune response. A unique panel of anti-PAI1 Mabs was developed that were characterized with regard to their use for immunoblotting, immunofluorescence and immunocytochemistry. One of these antibodies blocked the binding of PAI1 to vitronectin and inhibited the activity of the inhibitor. Finally, two of these Mabs turned out to be extremely valuable and were used to develop a novel microtiter plate assay for measuring the active fraction of PAI1 in biological fluids by making use of Mabs against different epitopes of PAI1.

SECTION A

CHAPTER 1: LITERATURE REVIEW
MOLECULAR DETECTION OF CIRCULATING MELANOMA CELLS

1.1 Introduction

Cutaneous melanoma is a potentially lethal tumor that arises from epidermal melanocyte transformation. The main factor involved in the etiology of melanoma is environmental exposure to ultraviolet B-range (280-320 nm) irradiation (reviewed by Koh, 1991) although genetic components have also been implicated (reviewed by Chin *et al.*, 1998; Kraehn *et al.*, 1995). Data from the United States show a consistent 6% annual increase in the incidence of melanoma and a 2% annual increase in mortality since the 1950's (reviewed by Rigel, 1997). It has been speculated that the depletion of the earth's ozone layer and the consequent increase in UV radiation may contribute to the increased incidence of melanoma (Fitzpatrick, 1996; Moan & Dahlback, 1992). The lifetime risk for developing melanoma in the United States has increased from 1 in 250 in 1980 to 1 in 87 in 1996 and is estimated to be 1 in 75 by the year 2000. In Cape Town (South Africa) the overall incidence is 24.4 per 100 000 per annum which is almost as high as in Australia (Saxe *et al.*, 1998). These alarming figures emphasise the need for more effective prevention, detection and treatment strategies.

The thickness of the primary tumor is currently the most important parameter for predicting the metastatic phenotype (reviewed by Garrison & Nathanson, 1996). The 5-year survival rate for patients with thin tumors (less than 1.5 mm) is 85%, whereas for patients with thick tumors (greater than 4 mm), it is only 46% (Morton *et al.*, 1993). Various other clinico-pathological parameters have been implicated as prognostic factors for melanoma namely, in descending order of significance, ulceration, anatomical site of the primary tumor and the pathological stage (reviewed by Balch *et al.*, 1992; Beahrs *et al.*, 1992). However, these factors do not provide the clinician with sufficient prognostic information, especially during the advanced stages of the disease. Patients with thin tumors may nevertheless die of melanoma and patients with thick tumors may experience long-term disease-free survival. Those patients with tumors of intermediate thickness ($1.5 \geq \leq 4.0$) present the greatest prognostic challenge since surgical excision will cure some while others will die of metastates in spite of complete excision of the primary tumor.

Current techniques for the detection of metastatic disease rely on clinical manifestations (reviewed by Rigel *et al.*, 1986; Romero *et al.*, 1994), imaging studies (reviewed by Berman & Reintgen, 1993; Neal & Hoskin, 1997) and serum marker assays (reviewed by Provost *et al.*, 1997; Weiss *et al.*, 1995). The success of these techniques is dependant on a critical minimum tumor mass. The

clinical reality reveals that widespread systemic metastasis may be well underway long before the tumor is detectable with these techniques (Crowley & Seigler, 1992). The challenge therefore, is to develop prognostic markers and techniques which will allow the identification of those patients whom, at the time of primary tumor diagnosis, already have micrometastases. Such markers and techniques will have the potential to identify those patients at risk for developing widespread macrometastatic disease. It will also justify more expensive adjuvant therapies and more intensive surveillance. Most importantly, it will permit therapeutic intervention (surgical, radiation and/or systemic) prior to the appearance of macrometastases. The development of a successful melanoma vaccine (reviewed by Pardoll, 1998; Sakai *et al.*, 1997) would benefit from techniques which could identify micrometastases, since it is unlikely that immunotherapy will have much success in patients with a large tumor burden (McNamara *et al.*, 1998; Staveley *et al.*, 1998). In addition, the economic implications of new treatment modalities such as tumor vaccines and chemotherapy, are likely to dictate that they be offered primarily to those patients at high risk.

The ability to identify patients with progressive disease is central to efficient management. This section of the thesis reviews the literature with regard to the use of reverse transcription-polymerase chain reaction (RT-PCR) technology for the detection of micrometastatic disease (occult or clinically undetectable metastases) in patients with primary melanoma (PM). The clinical value of applying this technology on tissue from different secondary sites, including peripheral blood (PB), bone marrow (BM) and lymph nodes (LNs), will be discussed.

1.2 The detection of metastatic cells in peripheral blood (PB)

The detection of circulating tumor cells (CTCs) in PB using RT-PCR technology was initially described for hematopoietic malignancies (Kawasaki *et al.*, 1988). The detection of CTCs has been associated with poor outcome in malignant lymphoma (Gribben *et al.*, 1994) and in chronic myelogenous leukemia (Roth *et al.*, 1992). The use of PCR technology for the detection of CTCs has been recently reviewed for various carcinomas, including colorectal, abdominal, urinary tract, prostate and breast cancers (Raj *et al.*, 1998) as well as melanoma (Jung *et al.*, 1996; Keilholz, 1998; Pelkey *et al.*, 1996).

In general, the blood vessels and the lymphatics are the main routes whereby tumor cells metastasize to distant organs (Hart & Saini, 1992; Morton *et al.*, 1993). The detection of circulating melanoma cells (CMCs) by the amplification of tyrosinase mRNA was initially proposed by Smith *et al.* (1991). In view of the fact that RT-PCR is so highly specific and sensitive, the detection of CMCs by RT-PCR has the potential for being superior to conventional serum marker assays (Provost *et al.*, 1997) for the prediction of metastatic disease. This is because serum markers are dependant on a critical minimum tumor mass and not necessarily the metastatic potential of the melanoma cells.

Tyrosinase, the key enzyme in melanin biosynthesis, catalyzes the hydroxylation of tyrosine to 3,4-dihydroxy-phenylalanine (DOPA) and ultimately to DOPAquinone. It is one of the most specific markers of melanocytic differentiation and is only expressed by melanocytes, Schwann cells and melanoma cells (Battyani *et al.*, 1993). The detection of CMCs by RT-PCR relies on the fact that melanocytes and Schwann cells are not normally present in the blood, that peripheral blood lymphocytes (PBL) do not express tyrosinase and that the majority of melanoma tumors express tyrosinase. The procedure involves 30 PCR amplification rounds with external oligonucleotide primer pairs, followed by another 30 amplification rounds with internal primer pairs (Smith *et al.*, 1991). A sensitivity of one melanoma cell (most often SK-Mel-28 melanoma cell line) in a background of 10^6 (Kunter *et al.*, 1996) to 10^7 (Glaser *et al.*, 1997) PBL is obtained with this protocol. The PCR primers are placed within different exons of the tyrosinase gene to eliminate the amplification of contaminating genomic DNA in the RNA sample. Van der Velde-Zimmermann *et al.* (1996) reported a modified pair of internal primers to improve the specificity of the assay.

The initial experiments on the detection of CMCs by RT-PCR for tyrosinase mRNA raised somewhat false hopes for using the technique to predict metastatic disease. The detection rates varied between 17% (Brossart *et al.*, 1993) and 91% (Hoon *et al.*, 1995) for patients with stage II melanoma, between 35% (Brossart *et al.*, 1993) and 86% (Hoon *et al.*, 1995) with stage III melanoma and between 50% (Battayani *et al.*, 1995) and almost 100% (Brossart *et al.*, 1993; Hoon *et al.*, 1995; Mellado *et al.*, 1996) with stage IV melanoma. In contrast, other research groups reported that the assay was of limited or no clinical value in the prediction of metastatic disease. Their detection rates were considerably lower than reported earlier and varied between 0% (Reinhold *et al.*, 1997) and 13% (Farthmann *et al.*, 1998) for patients with stage II melanoma, between 0% (Reinhold *et al.*, 1997) and 17% (Farthmann *et al.*, 1998) with stage III melanoma and between 13% (Pittman *et al.*, 1996) and 44% (Farthmann *et al.*, 1998) with stage IV melanoma.

Several factors could have contributed to these highly contradictory results. First, the number of patients studied within each stage was often too low and could have biased the results. For example, Hoon *et al.* (1995) reported 10 of 11 (91%) stage II patients to be positive for CMCs, compared to 6 of 46 (13%) stage II patients as reported by Farthmann *et al.* (1998).

Secondly, the assay technique used by the different investigators differed substantially. This has resulted in different sensitivities (varying from one to ten melanoma cells per 10 ml blood) and emphasizes the need for quality standards between laboratories (Keilholz *et al.*, 1998).

Thirdly, unrecognized carry-over PCR contamination might be the main culprit in producing these contradictory results. A high level of contamination will raise the baseline detection rate and mask

the true results. The introduction of effective procedures to prevent carry-over contamination should be of high priority, since the generation of false positive tests could result in confusing and potentially harmful information. Carry-over contamination which is often associated with nested-PCR reactions, seems to be a major stumbling block for successful clinical application. To reduce the contamination risk, Pittmann *et al.* (1996) developed a single 50-cycle PCR reaction which was as sensitive as the previously described nested-PCR method (Smith *et al.*, 1991). This was also the rationale of a 40-cycle PCR-ECL system recently described (O' Connell *et al.*, 1998). General guidelines to limit the contamination risk have been reviewed extensively elsewhere (Belak & Ballagi-Pordany, 1993; Kwok & Higuchi, 1989; Sarkar & Sommer, 1990; Verkaik *et al.*, 1997; Walder *et al.*, 1993).

A fourth possible reason for the contradictory results is the phenomenon of illegitimate transcription - the fact that any gene may be transcribed at a very low level in any cell type (Chelly *et al.*, 1989). This implies that a positive RT-PCR reaction could be the result of very low-level ubiquitous tyrosinase transcription from PBL. In fact, Smith *et al.* (1991) have suggested that increasing the number of nested-PCR cycles from 30 to 40 could contribute to false positive results. Sufficient evidence for this phenomenon has been obtained from a study where RT-PCR for CK19 mRNA was used to detect breast cancer micrometastases in lymph nodes (Schoenfeld *et al.*, 1994). It was concluded that the optimal cut-off point to distinguish involved nodes from metastasis-free nodes remained at the level of 40 PCR cycles when compared to a two-stage amplification (total of 60 cycles) with nested primers.

A fifth possible reason for the contradictory results from different studies is that there might also be false positives arising from the existence of melanocyte-specific pseudogenes (Oetting *et al.*, 1993; Takeda *et al.*, 1991). The tyrosinase pseudogene contains sequences that correspond to exons 4 and 5 of the tyrosinase gene (Giebel *et al.*, 1991). However, the oligonucleotides used for amplification of tyrosinase mRNA overlap exons 1, 2 and 3 of the tyrosinase gene (Smith *et al.*, 1991) and theoretically could therefore not create a false positive result. The possibility of false GAPDH signals as a result of the presence of GAPDH pseudogenes (Keilholz, 1998; Willhauck *et al.*, 1998) could also contribute to discrepant results. If the PCR is generated off pseudogene genomic DNA, this would give a false impression of good RNA quality, leading to incorrect deductions regarding the tyrosinase detection rate. The omission of reverse transcriptase from a control RT-PCR reaction should determine the contribution of pseudogenes in creating false positive results.

The last possible cause of false positives could be contamination with normal melanocytes obtained from a skin plug during blood sampling. To circumvent this problem it was suggested that

the first vacutainer containing a few millilitres of blood should be discarded to avoid skin plug contamination when venipuncture is performed (Foss *et al.*, 1995).

Besides the potential value of the RT-PCR assay to predict metastasis, the quantitation of CMCs has also been proposed as a possible means to monitor the efficacy of immunotherapy in patients with AM (Brossart *et al.*, 1995). This was conducted by comparing the amplified tyrosinase signal with a corresponding signal of diluted SK-Mel-28 cells. In 50 patients with AM, the strength of the amplified tyrosinase signal in PB correlated with the tumor burden. In patients that responded to immunotherapy a statistically significant decrease of the tyrosinase signal was observed, whereas in patients with stable disease the signal was unchanged. However, the results obtained with this approach were based on the assumption that an equivalent level of tyrosinase expression in tumor cells (*in vivo*) compared to tested SK-Mel-28 cells (*in vitro*) rather than a precise number of an individual patient's tumor cells. As a result of this study, different assay systems for the quantitation of CMCs have been proposed. In one such system, a competitive RT-PCR assay for tyrosinase mRNA was developed with heterologous DNA as an internal standard (Curry *et al.*, 1996). In another, an electrochemiluminescence (ECL) system was developed to verify the specificity and to improve quantitation of tyrosinase PCR product in PB (O' Connell *et al.*, 1998). This system involves the use of rTth (Juhasz *et al.*, 1996) for reverse transcription and PCR, followed by hybridization of the biotinylated PCR product to a tris (2,2'-bipyridine) ruthenium (II) (TBR) chelate-labeled oligonucleotide probe. The resulting hybrid is captured on magnetic beads for activation of the TBR and quantitation by ECL. A sensitivity of one to ten melanoma cells in a background of 10^7 B231 breast cancer cells was obtained with a single 40-cycle PCR reaction. In their pilot study, they could detect tyrosinase mRNA in the PB from 7 of 16 melanoma patients, whereas none of the 5 normal controls were positive.

A number of investigators have attempted to correlate the presence of CMCs to histological criteria, but the results from these studies reveal a number of contradictions. Whereas some groups found that the presence of CMCs seemed to correlate with the clinical stage (Curry *et al.*, 1998; Farthmann *et al.*, 1998; Mellado *et al.*, 1996) and the thickness of the primary tumor (Curry *et al.*, 1998; Farthmann *et al.*, 1998), other groups found no correlation between the presence of CMCs and the clinical stage (Ghossein *et al.*, 1998) or the thickness of the primary tumor (Hoon *et al.*, 1995; Mellado *et al.*, 1996). In a recent comprehensive study by Farthmann *et al.* (1998), CMCs were detected in 13% (6/46) of patients with stage II disease, 17% (7/41) with stage III disease and 44% (16/36) with stage IV disease. Positive results were obtained more frequently in (a) men compared with women, (b) patients with thick primary melanomas (> 4 mm, 38%) compared with those with thinner tumors (1.1 to 4 mm, 22%; ≤ 1 mm, 5%), and (c) patients with non-classifiable (38%), nodular (34%), and occult primary melanomas (30%) compared with those with acrolentiginous (17%), superficial spreading (9%), or lentigo maligna melanoma (0%).

In terms of the predictive value of the assay, Curry *et al.* (1998) assessed the value of tyrosinase and Mart-1 as mRNA markers for the detection of CMCs in 276 stages I, II and III melanoma patients. The presence of CMCs was assessed in relation to multiple time periods before and after surgical removal of the primary tumor. Fourteen percent of the patients who were negative before surgery became positive in the 3 months after surgery. From this finding it was concluded that the surgical manipulation itself may cause shedding of melanoma cells into the circulation. Sixty-five percent of the patients who were positive before surgery became negative in tests conducted in the first 3 months after surgery. The tests were most frequently positive in the first 3 months after surgery and declined in frequency thereafter. It was therefore concluded that tests conducted in the first 3 months after surgery may have the best chance of detecting patients who might subsequently develop recurrent disease. Of the 276 patients with stages I, II and III disease studied, 123 were followed up for 18 months of which 47 (38.2%) recurred. Of these with recurrent disease, 66% were positive within 3 months of surgery so that 34% of the recurrences were not predicted by the tests. Assays for tyrosinase alone predicted recurrence in 51% of patients and Mart-1 alone in 21%. For patients with stage III disease, the tests were positive in 73% of those with a recurrence. The false-negative results led the authors to question the reliability of the assay for selection of patients for adjuvant therapy. Approximately 35% of 76 patients who had not had a recurrence, had positive tests in samples taken within 3 months of surgery and more than 60% in tests conducted over the 12-month period. However, it was too early to assess whether these represent false-positive results. It was therefore concluded that it seems most likely that a large proportion of patients with PM in whom CMCs are detected, is the result of sporadic shedding of melanoma cells into the circulation.

In this regard, it is imperative to understand that various factors play essential roles in the successful establishment of metastases (reviewed by Fidler, 1996). These include, the capability of melanoma cells to survive the flow forces of the blood (Fidler, 1990), their proteolytic capacity to cross anatomic barriers (reviewed by Andreasen *et al.*, 1997; Mignatti & Rifkin, 1993), expression of cell adhesion molecules (reviewed by Albelda, 1993; Pignatelli & Stamp, 1995), increased cell motility (reviewed by Danen *et al.*, 1995), growth and colonization potential (reviewed by Fidler, 1990; Halaban, 1996), induction of angiogenesis (reviewed by Fidler & Ellis, 1994; Hanahan & Folkman, 1996) and the host immune response (reviewed by Cella *et al.*, 1997; Shresta *et al.*, 1998). In addition, animal studies have demonstrated that between 10^2 to 10^6 tumor cells need to be introduced into the circulation to obtain a 50-100% chance of forming a single metastasis and that the formation of tumor emboli is an equally important factor promoting the formation of metastases (Glaves, 1983). Animal studies also indicate that less than 0.01% of circulating tumor cells eventually survive to produce metastases (Liotta & Stetler-Stevenson, 1991). Thus, although the presence of CMCs seems to correlate with the clinical stage and the thickness of the primary tumor (Curry *et al.*, 1998; Farthmann *et al.*, 1998), its value to predict metastatic spread before

clinically detectable metastases are evident, remains elusive and needs to be assessed more extensively.

1.3 The detection of metastatic cells in bone marrow (BM)

The BM, like the lymph nodes (LNs), could act as clearing sites for normal and tumorigenic cells (Raj *et al.*, 1998). Brossart *el al.* (1994) made the first attempt to analyse PB samples and BM aspirates from 28 patients with AM for the presence of metastatic cells prior to and after therapy with interferon alpha-2b and interleukin-2. All 28 patients, including those with no clinical evidence of disease after treatment, were RT-PCR positivite for tyrosinase mRNA in their blood and BMs, prior to and after immunotherapy. Recently, a more comprehensive study evaluated the prognostic value of the RT-PCR assay for the detection of metastatic melanoma cells in PB and BMs of patients with stages II to IV melanoma (Ghossein *et al.*, 1998). Overall, 19% of the 123 patients had tyrosinase mRNA in their blood and/or BMs. The PCR-positivity was slightly higher for BMs (16.5%) than for PB (12%) and neither correlated with the clinical stage. Within stage II, BM PCR-positive patients had a shorter median survival than BM PCR-negative patients. However, BM PCR-positivity was not predictive of overall survival in the whole population studied. In contrast, blood PCR-positivity showed a statistically significant correlation with decreased overall survival in stages II and III, but was not a predictor of overall survival in stage IV. Multivariate analysis showed that blood PCR-positivity was independent of clinical stage in predicting overall survival. The PCR-positivity for stage II patients was twice as high for BM aspirates (25%) as for PB (12.5%). Thus it seems, that BM aspirates are more useful than small blood samples for predicting metastasis in patients with PM.

1.4 The detection of metastatic cells in lymph nodes (LNs)

The LNs may act as clearing sites for normal and tumorigenic cells. Therefore, the presence of micrometastases in LNs may signal the functional effectiveness of the immune system in filtering and clearing the CTCs (Raj *et al.*, 1998). The presence of clinically evident LN metastases is currently the most powerful prognostic factor for predicting survival (Drepper *et al.*, 1993; Reintgen *et al.*, 1983). If metastases are present, the 5-year survival rate decreases by 50% and other variables based on the primary tumor are of little prognostic value. Approximately 20% of clinically node-negative patients harbour micrometastases in regional draining lymph nodes (Reintgen *et al.*, 1994). These patients will have an 85% risk of harbouring micrometastases at distant visceral sites, which will most likely develop into fatal metastases. Thus, the molecular detection of micrometastases in LNs seems to be potentially useful in the clinical setting.

Until now, routine examination of LNs has been done using standard histopathology with hematoxylin-eosin staining and/or immunohistochemistry (IHC) with S100 and HMB45 antibodies (Cochran *et al.*, 1988). However, IHC (especially serial sectioning) is a time-consuming and

expensive technique, prone to human errors, subject to individual interpretation and is not particularly sensitive. In addition, standard clinical and pathological evaluations of the regional LNs underestimate the number of patients with micrometastases by 25-30% (reviewed by Cochran, 1997). Wang *et al.* (1994) investigated the use of the RT-PCR technique for the detection of micrometastases in LN biopsies. They analysed the LNs of 29 patients with intermediate thickness PM that underwent elective LN dissections, by standard pathological staining and RT-PCR for tyrosinase mRNA. Thirty eight percent of the samples were pathologically positive and 66% were RT-PCR positive and these included all of the pathologically positive samples, so that the false-negative rate was zero (Wang *et al.*, 1994). However, elective LN dissection is a controversial procedure because the majority of dissected LNs are reported to be tumor free (Drepper *et al.*, 1993). Morton *et al.* (1992) introduced an intra-operative mapping procedure to identify the sentinel lymph node (SLN). The SLN is the first node in the lymphatic basin that drains the primary tumor and is known to be the first possible site of metastatic disease (Morton *et al.*, 1992). The rationale is that if the SLN is negative for metastatic cells, then the remainder of the nodes are also expected to be negative and the patient will most likely be free of metastatic disease (Godellas *et al.*, 1995; Reintgen *et al.*, 1994). Therefore, only those patients with positive SLNs would require a complete LN dissection, sparing those with negative SLNs the morbidity associated with this procedure. A 98% success rate has now been obtained for the SLN mapping technique by using a combination of a vital blue dye and ^{99m}Technetium-sulfur colloid (reviewed by Brady & Coit, 1997; Karakousis *et al.*, 1997; Leong *et al.*, 1997; Morton, 1997; Reintgen, 1997; Ross, 1997; Thompson, 1997).

In a pilot study (Van der Velde-Zimmermann *et al.*, 1996), 28 LNs derived from 4 patients after lymphadenectomy and 12 patients after SLN resection, were evaluated for micrometastases using RT-PCR for tyrosinase mRNA. Immunohistochemistry revealed positivity in 10 LNs from the 4 patients with lymphadenectomy. RT-PCR analysis detected an additional 6 positive SLNs which were otherwise histologically negative. The 5 LNs from patients with other diseases, were RT-PCR negative. In a more recent study (Joseph *et al.*, 1997), the SLNs of 583 patients, having primary melanoma but no evidence of regional or systemic disease, were analysed for micrometastases over a 4-year period. Micrometastases was detected in the SLNs of 64 patients (11%). Of these, 73.8% was identified by routine histological staining, 7.8% with serial sectioning and the additional 18.4% with IHC. SLNs from a subset of 29 patients were analysed with RT-PCR for tyrosinase mRNA and compared with routine histology. Of these patients, 38% of the SLNs were histologically positive compared to a 66% RT-PCR positivity, which included all pathologically positive samples. In a similar study among 48 patients negative by immunohistochemical assessment, 21 (44%) had nodal micrometastases using RT-PCR analysis for tyrosinase mRNA (Blaheta *et al.*, 1998). All 68 LNs from 46 non-melanoma patients serving as negative controls, were tyrosinase RT-PCR negative. Additional support for the detection of micrometastases in SLNs by molecular analysis

was recently obtained (Shivers *et al.*, 1998). They also show a recurrence rate among 13% of the patients that were upstaged by RT-PCR analysis after a relative short average follow-up period of 28 months.

Peripheral support for the SLN-mapping technique and the use of RT-PCR analysis to improve disease staging and the detection rate of micrometastases was recently provided by Gershenwald *et al.* (1998). Of 243 patients with histologically negative SLNs, 27 (11%) developed nodal and/or distant metastases after a median follow-up time of 35 months. Ten of these patients developed nodal recurrences in the previously mapped basins which were histologically negative. Detailed analysis of the SLNs by serial sectioning demonstrated evidence of occult metastases in 80% of the cases (Gershenwald *et al.*, 1998). In addition, since node dissection offers increased survival for patients with node metastases only, Cascinelli *et al.* (1998) support SLN biopsy as a tool to identify patients with occult node metastases, who could then undergo node dissection (Cascinelli *et al.*, 1998).

Thus, SLN mapping and RT-PCR analysis are potentially powerful tools to identify micrometastatic disease in patients with PM that are otherwise clinically free of metastases. Patients with a positive SLN would then be eligible for a complete LN dissection and interferon alpha-2b therapy. In combination with adjuvant therapy, this strategy has the potential to provide more accurate staging for the melanoma patient, at lower costs for the health care system and a lower morbidity for the patient (Joseph *et al.*, 1997). However, tyrosinase transcripts have been reported to be present in normal LNs, possibly from Schwann cell origin (Battyani *et al.*, 1993) although a recent and more thorough study reported the controversy (Blaheta *et al.*, 1998). Therefore, large clinical studies with appropriate and sufficient negative controls and with additional melanocytic and/or tumor-specific markers, must be pursued before clinical application.

1.5 General and specific aims of study

As described in the literature review, the early detection of CMCs in patients with PM using molecular techniques, is potentially a powerful tool for identifying those patients at risk for developing metastases. The original method, as described by Smith *et al.* (1991), is based on the amplification of the mRNA for tyrosinase, an enzyme specific to melanocytes. Unfortunately, the assay is laborious, expensive, not reproducible and not sensitive enough. The extraction of RNA from large quantities of blood (more than 1 ml) with potent, but costly, RNase inhibitory reagents such as guanidine isothiocyanate, and the separation of reverse transcriptase and PCR reactions in separate tubes, makes it an extremely laborious and expensive assay. In addition, the problem of false positives, either due to contamination with amplicons (Foss *et al.*, 1995), illegitimate transcription (Chelly *et al.*, 1989; Smith *et al.*, 1991) or contamination with normal melanocytes

obtained by the needle prick during blood collection (Foss *et al.*, 1995), are potential stumbling blocks for successful clinical application.

The broad aim of the present study was to develop a more sensitive, reproducible, cost effective and clinically applicable assay and to eliminate the problem of false positives. The second broad aim was to make use of this assay to test blood samples from patients with PM and to evaluate the results in relation to various clinical and histological criteria.

The specific aims of the study were:

- to eliminate the possibility that the needle prick during blood collection could result in false positives due to contamination of the blood sample with normal skin melanocytes. A large number of controls were screened to determine the false positive rate.
- to address the problem of illegitimate transcription. Blood samples from controls were subjected to increasing numbers of first-round and second-round (nested) PCR cycles and analysed for the presence of false positives.
- to develop a simple and effective procedure to prevent carry-over contamination.
- to determine whether the early detection of CMCs will allow the identification of patients at risk for developing metastases. A large number of patients that presented with PM were screened for the presence of CMCs (using the "in-house" standardized RT-PCR assay for tyrosinase mRNA) and the findings were correlated with the outcome of the disease.
- to established whether there was a correlation between the presence of CMCs and various histological criteria, including tumor thickness, ulceration and Clark's level of invasion.
- to establish whether blood sampling at different time points would improve the detection rate. Multiple blood samples were collected with intervals from two to twelve months and analysed in terms of number of times tested.

CHAPTER 2: MATERIALS AND METHODS

2.1 Tissue culture

The melanoma cell lines UCT-Mel-1 to 6 were established in the Department of Clinical Science and Immunology from patient tumors as described before (Hoal-Van Helden *et al.*, 1986). The human melanoma cell lines SK-Mel-1 and SK-Mel-28 were obtained from the American Type Culture Collection. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 U/ml penicillin and 20 µg/ml streptomycin at 37°C under 5% CO_2/95% air and 90% humidity (see A.1 – A.5 for details). UCT-Mel-1 and -2, and SK-Mel-1 and -28 were classified as pigmented cell lines, while UCT-Mel-3, -4, -5 and -6 were classified as non-pigmented cell lines according to the visual detection of pigment.

2.2 Sample preparation

Duplicate 10-15 ml samples of heparinized blood from a normal control, were combined with 100, 10 or one human melanoma cell(s) from the different pigmented or non-pigmented cell lines. The introduction of one melanoma cell to the blood sample was achieved by plating approximately two cells per well of a microtiter plate. The cells were allowed to settle for one hour at 37°C. Each well was viewed with phase contrast microscopy (100x) and those containing a single, viable cell were marked and the cell transferred with a Pasteur pipette to the blood sample. The well was then viewed again to confirm that the cell had been transferred.

2.3 RNA extraction

Efficient erythrocyte lysis of each 10-15 ml blood sample was obtained by the addition of 35-40 ml cold TE-buffer (10 mM Tris, pH 7.5 and 1 mM EDTA, pH 8) for 3-5 min on ice. The lymphocytes (+/- melanoma cells) were centrifuged at 700 g for 5 min and the total RNA was extracted from the cell pellet according to a rapid extraction method described before (Xie & Rothblum, 1991). The average yield per 10 ml blood was 30 µg total RNA and the $OD_{260/280}$ ratio was >1.5 and <1.8. Each sample was stored in 5 µg aliquots in 10 µl diethyl pyrocarbonate (DEPC)-treated water (under mineral oil) at -80°C. Samples were thawed only once for RT-PCR. (see A.6 – A.11 for details)

2.4 RT-PCR

All RNA samples were incubated at 90°C for 5 mins and then snap-cooled on ice to eliminate mRNA secondary structure. Combined RT-PCR was achieved by adding 40 µl RT-PCR mix containing 1x PCR-buffer (10 mM Tris, pH 8.3 and 50 mM KCl), 1.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (dNTP, Boeringer and Mannheim, Germany), 30 pmol sense (HTYR1) and anti-sense (HTYR2) outer primers for human tyrosinase, 20 U RNase inhibitor

(Boeringer and Mannheim, Germany), 10 U Moloney-murine leukemia virus reverse transcriptase (MMLV-RTase, Superscript II RNase H$^-$ from Gibco, Grand Island, NY) and 2.5 U Thermoprime^{PLUS} DNA polymerase (Advanced Biotechnologies, Surrey, UK). Synthesis of complimentary DNA (cDNA) was completed at 37°C for one hour. This was directly followed by 30 PCR cycles with an initial denaturation at 94°C for 60 sec. All subsequent cycles were carried out at 94°C for 15 sec (denaturation), 55°C for 30 sec (annealing) and 72°C for 30 sec (extention). The PCR was performed with a water-based cycler fitted with a 3000 Watt element. The second round of amplification was carried out by adding 5 µl of a 1/100 dilution (opening only one tube at a time) from the first 30 cycles of amplification to 20 µl PCR mix containing 1x PCR-buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 30 pmol sense (HTYR3) and anti-sense (HTYR4) nested primers for human tyrosinase and 1 U Thermoprime^{PLUS} DNA polymerase as final concentrations. The previous step was carried out by using aerosol resistant tips (Molecular Bio-Products, USA) only. Thirty PCR cycles were performed as described above, except that the initial extended denaturation was omitted. The final reaction product (207 bp fragment) was visualized on a 1.5% agarose gel with ethidium bromide (0.5 µg/ml) staining (see A.12 – A.14 for details).

The integrity of the RNA of each sample was verified by RT-PCR with primers (Battyani *et al.*, 1993) for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The RT-PCR mix (40 µl), as described above for the first-round of amplification but with sense and anti-sense GAPDH primers, was added to 1 µg total RNA in 10 µl DEPC-treated water and subjected to cDNA synthesis and 30 PCR cycles as described above for the first PCR profile.

2.5 Oligonucleotide primers

The outer and nested primers for human tyrosinase were synthesized by the department of Biochemistry (University of Cape Town) using an automated process. The sequences were as previously described (Smith *et al.*, 1991). They included:
outer sense HTYR1 (5' TTGGCAGATTGTCTGTAGCC 3');
outer anti-sense HTYR2 (5' AGGCATTGTGCATGCTGCTT 3');
inner sense HTYR3 (5' GTCTTTATGCAATGGAACGC 3') and
inner anti-sense HTYR4 (5' GCTATCCCAGTAAGTGGACT 3') primers.
The primers for human GAPDH were a modification of those as described before (Battyani *et al.*, 1993). They included the sense GAPDH (5' ACGGATTTGGTCGTATTGGG 3') and anti-sense GAPDH (5' AGGGATGATGTTCTGGAGAG 3') primers. GAPDH was the choice for the internal control since it is expressed in almost all types of cells and tissues (Battyani *et al.*, 1993; Taylor & Heasman, 1994).

2.6 Laboratory precautions

Gloves were worn at all times, disposable tubes were used where possible, a separate set of Gilson pipettes were designated to each separate area, all solutions were treated with 0.01% DEPC or prepared with DEPC-treated water and all chemicals were of the finest grade available. Blood samples were processed for erythrocyte lysis in a vertical laminar flow in 50 ml conical polypropylene disposable tubes. The tubes with the lymphocyte pellets were then transferred to a separate "RNase-free" area for the extraction of total RNA. The preparation of RT-PCR reaction mixtures were carried out in a separate room fitted with a glass cabinet and ultra violet sterilization. After the first 30 PCR cycles, the reaction products were cooled to 4°C and the diluted products were then transferred to the nested reaction mix in a separate room fitted with a fume hood. The final PCR products were cooled to 4°C and analysed by electrophoresis in a separate room also fitted with a fume hood.

2.7 Patients

A total of 410 blood samples from 181 melanoma patients attending the Groote Schuur Hospital Melanoma Clinic were examined for the presence of metastatic cells. Control blood samples were collected from 50 South Africans with no history of skin cancer. The study was approved by the Ethics and Research Committee of the University of Cape Town. Written and informed consent was obtained from all patients before they entered into the study.

The clinical staging was based on the TNM classification system as agreed upon by the International Union Against Cancer and the American Joint Committee on Cancer (reviewed by Garrison & Nathanson, 1996). There were 10 patients with stage 0 (in situ melanomas), 76 patients with stage I (pT1 and pT2), 67 patients with stage II (pT3 and pT4), 10 patients with stage III (regional nodal involvement), 12 patients with stage IV (distant metastasis) and 6 patients with uveal melanoma. Standard histological criteria were recorded, including: Location of primary and secondary tumors, number of lesions per site, morphological type, tumor length and width, Breslow's tumor thickness, Clark's level of invasion, ulceration, pigmentation, evidence of regression, presence of satellites, invasion of vascular and/or lymphatic spaces, symptoms suggestive of metastasis and evidence of metastasis on the chest radiograph.

2.8 Blood preparation

Blood samples were drawn at the time of primary tumor diagnosis and thereafter, if possible, at two to 12 month intervals. Ten to 15 ml samples of heparinized blood were collected from each patient. The first few milliliters of each blood sample was discarded to avoid possible contamination by normal skin melanocytes. Blood samples were processed within 2 to 3 hours after collection. The RNA extraction, yield and RT-PCR procedures were as described and all neccesary steps were taken to prevent carry-over contamination. For each reaction, 5 μg of total RNA was analysed in a

50 µl combined RT-PCR reaction mix, followed by the second round nested PCR. A negative (normal blood RNA) and a positive control (RNA from normal blood containing one SK-Mel-28 melanoma cell) were included with each batch of samples to verify a contamination-free environment and a faultless RT-PCR operating system. The integrity of the RNA for each sample was verified by RT-PCR with primers for GAPDH as described before (see 2.4 and 2.5).

2.9 Statistical analysis

Fisher's exact test for 2 x 2 tables was used to assess the significance ($P < 0.05$) between RT-PCR results (coded as positive or negative) and disease stage (stage I versus stage II), tumor thickness ($\leq$ 1.5 mm versus > 1.5 mm), ulceration (present versus absent), Clark's level of vertical invasion (Clark IV versus Clark V) and metastases (present versus absent). Pearson's Chi-analysis was used to assess the significance between disease stage (I versus II) and metastases (present versus absent). All data analyses were performed with the Epi Info Version 6.01 software (USD, Inc., Stone Mountain, GA).

CHAPTER 3

3.1 TECHNICAL RESULTS AND DISCUSSION

3.1.1 Sensitivity and reproducibility

The primary aim of the first part of this study was to develop a sensitive, reproducible, cost effective and clinically applicable RT-PCR assay for tyrosinase mRNA. Duplicate 10-15 ml blood samples from controls were combined with 100, 10 or one melanoma cell(s) from different pigmented or non-pigmented melanoma cell lines. The RT-PCR assay enabled the detection of seven out of eight melanoma cell lines but with different degrees of sensitivity (Figure 3.1, 207 bp fragment). All four of the pigmented melanoma cell lines, SK-Mel-1 and -28, UCT-Mel-1 and -2 (Figure 3.1A and 3.1B), as well as one of the non-pigmented cell lines UCT-Mel-3 (Figure 3.1C), could be detected at one cell per 10-15 ml blood. This is equivalent to one melanoma cell in a background of 1 to 5 x 10^7 lymphocytes. A lower level of sensitivity was obtained with the non-pigmented cell lines, UCT-Mel-4, -5 and -6 (Figure 3.1C and 3.1D). This is most likely the result of reduced tyrosinase mRNA levels in these cell lines. Only 100 and 10 UCT-Mel-5 cells and 100 UCT-Mel-6 cells could be detected per blood sample. UCT-Mel-4 was completely negative at 100 cells per blood sample. Similar results were obtained in two independent experiments.

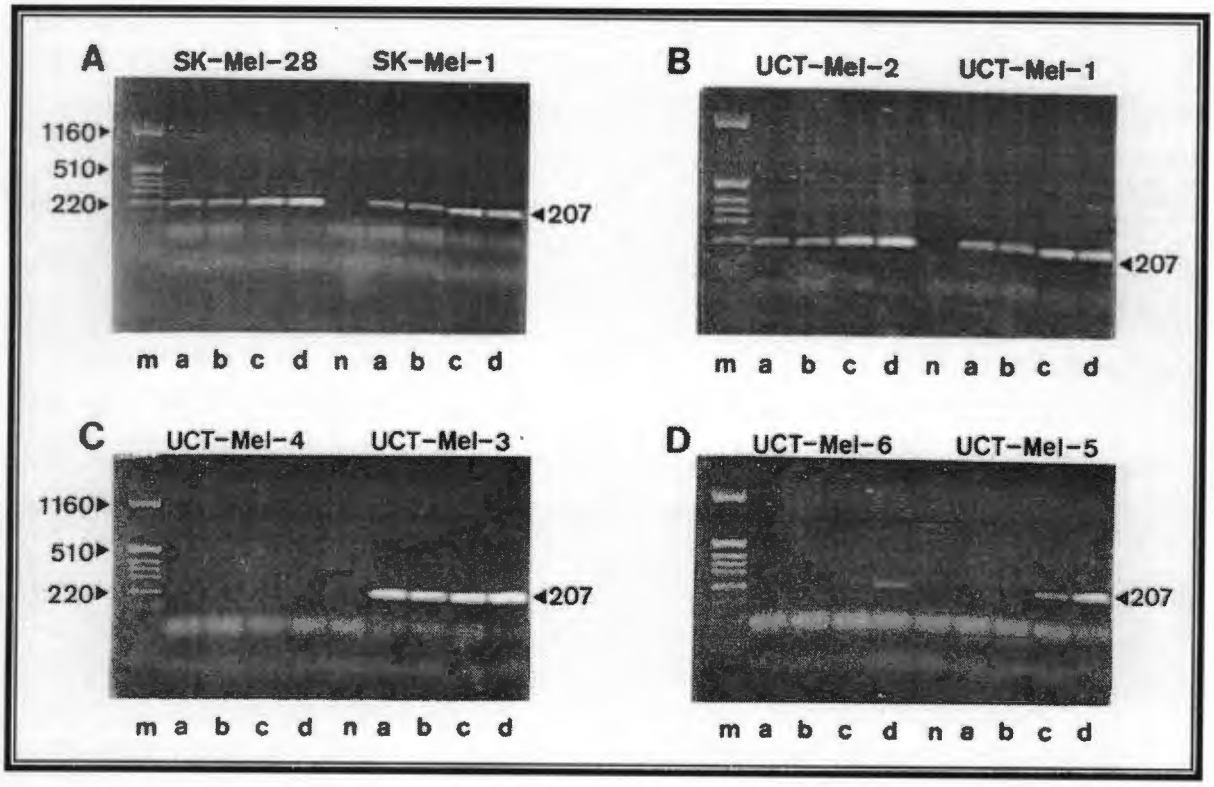


Figure 3.1. Combined RT-PCR for tyrosinase mRNA of eight melanoma cell lines. **A,** SK-Mel-1 and SK-Mel-28. **B,** UCT-Mel-1 and UCT-Mel-2. **C,** UCT-Mel-3 and UCT-Mel-4. **D,** UCT-Mel-5 and UCT-Mel-6. **Lane m,** plasmid pKT279 digested with Hinf I. **Lane a and b,** one melanoma cell per 10-15 ml blood. **Lane c,** ten melanoma cells per 10-15 ml blood. **Lane d,** one hundred melanoma cells per 10-15 ml blood. **Lane n,** control blood sample containing no melanoma cells. Each sample tested positive for the presence of intact RNA using combined RT-PCR for GAPDH (results not shown).

These results suggest that this assay is superior to previously described methods in the following respects: First, a single melanoma cell could be detected in 10-15 ml blood, while previous publications (Brossart *et al.*, 1993; Foss *et al.*, 1995; Kunter *et al.*, 1996; Mellado *et al.*, 1996; Pittman *et al.*, 1996; Smith *et al.*, 1991) reported a sensitivity of 2 to 10 SK-Mel-28 cells in 10 ml blood. Secondly, the assay is highly reproducible, since a single, viable melanoma cell could be detected in duplicate blood samples in five out of eight melanoma cell lines tested. Thirdly, efficient erythrocyte lysis eliminates the use of large volumes of RNA extraction buffer and it would appear that the lysis step does not significantly reduce the survival of the melanoma cells in the blood samples. The assay is therefore more economical and clinically applicable since less reagents and consumables are used. Fourthly, rapid total RNA extraction with multiple sample processing (up to 12 samples) are easily performed within 2-3 hours. Lastly, combining reverse transcription and PCR in one reaction tube simplifies the technique, minimizes the unnecessary handling of samples and is more economical since less reagents and consumables are used.

The results obtained with a number of thermocyclers suggest that PCR profiles should be optimized for every individual thermocycler. In general, shorter denaturation times, will extend the half-life of the DNA polymerase and consequently increase the product yield. The RoboCycler of Stratagene was found to be not suitable for obtaining a sensitive RT-PCR assay for tyrosinase. Water-cyclers produce a temperature gradient with cooling and heating of the water. The annealing efficiency of primer pairs with different melting temperatures will be favoured by the temperature gradient and therefore produce greater sensitivity. In contrast, the RoboCycler of Stratagene, which operates with static heat-blocks and a robot arm, transfer samples quickly from one temperature to another. The shorter temperature gradient generated by the RoboCycler most likely reduces the efficiency of primer annealing.

3.1.2 Contamination with normal melanocytes

One of the specific aims of the present study was to investigate the possibility that the needle prick during blood collection could result in the contamination of blood samples with normal melanocytes. Fifty controls were screened for the presence of false tyrosinase signals. One of the 50 controls was positive for tyrosinase mRNA. Subsequent blood samples from this control was negative for tyrosinase mRNA and no clinical evidence of the disease could be found. The results therefore indicate a 2% chance of obtaining normal melanocyte contamination among all skin categories. This 2% risk factor could most likely be eliminated by rejecting the first few milliliters of blood taken from the patient.

To confirm that the above failure to detect false positives was not due to poor quality RNA, each normal control sample was also subjected to combined RT-PCR for GAPDH. Figure 3.2 represents nine of the 50 controls. A specific 600 bp fragment for GAPDH was obtained for each

control after cDNA synthesis and 30 cycles of PCR. These results therefore indicate that the rapid total RNA extraction method used in this study (Xie & Rothblum, 1991) is sufficient to obtain good quality, intact RNA for RT-PCR.

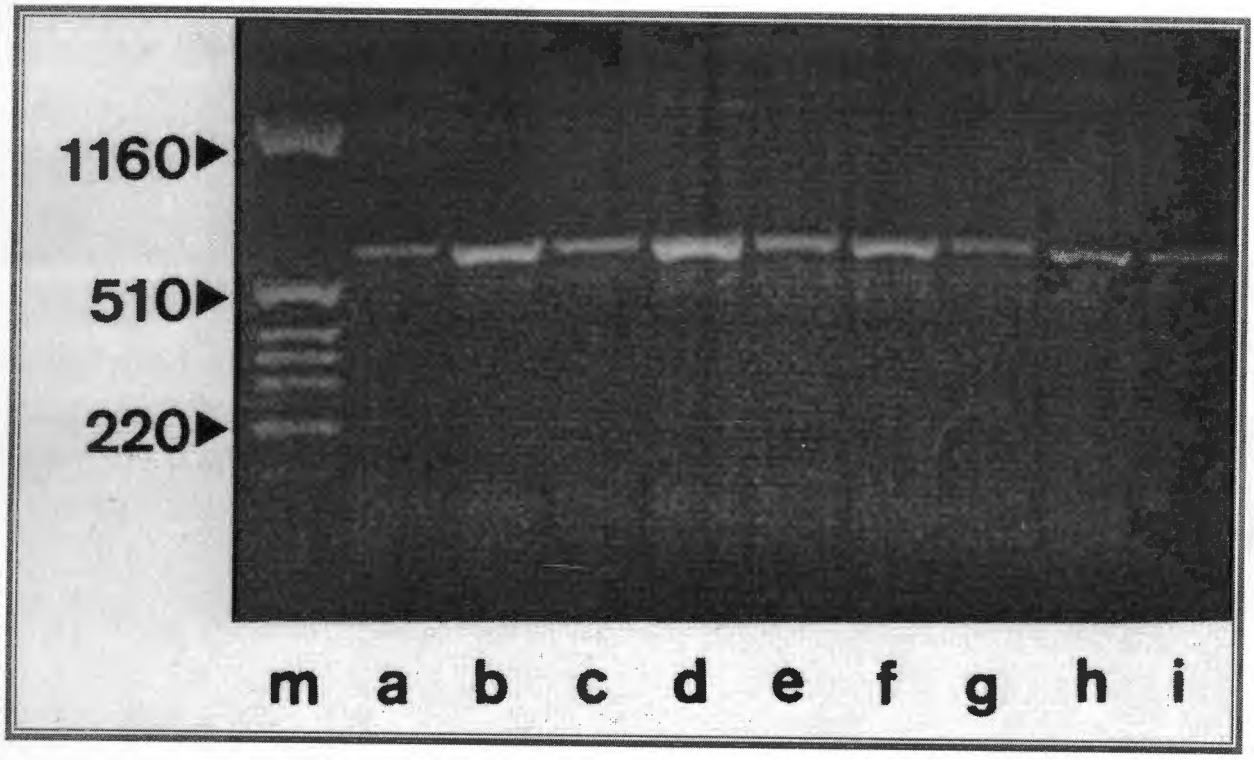


Figure 3.2. Combined RT-PCR for GAPDH mRNA. Fourty-nine of the 50 controls were negative for tyrosinase mRNA, indicating that there was a 2% chance of obtaining normal melanocytes contamination during blood sampling (results not shown). Therefore each control was tested for the presence of intact RNA by combined RT-PCR for GAPDH (600 bp fragment) of which nine **(lanes a-i)** are shown here. **Lane m,** plasmid pKT279 digested with Hinf I.

3.1.3 Illegitimate transcription

Because of the phenomenon of illegitimate transcription (Chelly *et al.*, 1989; Smith *et al.*, 1991) that is, that any gene may be transcribed at a very low level in any cell type, it is possible that a positive RT-PCR reaction could be the result of very low-level ubiquitous tyrosinase transcription from the different blood cell types. In order to investigate this phenomenon, RNA from ten controls (10-15 ml blood samples) were subjected to 30 or 40 first-round PCR cycles followed by either 30 or 40 second-round (nested) PCR cycles. Figure 3.3 demonstrates that every control was still negative after 30 or 40 first-round and 30 or 40 second-round PCR cycles. A gradual increase in the non-specific background was obtained after 40 cycles, but no specific product was generated. The positive control generated a specific tyrosinase product (207 bp fragment) with a slight increase in the yield after 40 nested PCR cycles (Figure 3.3.A, lane a). It was therefore concluded that the phenomenon of illegitimate transcription mentioned by Smith *et al.* (1991) obtained after 40 nested PCR cycles for tyrosinase, was most likely the consequence of carry-over contamination resulting from long-term amplicon accumulation in the laboratory atmosphere.

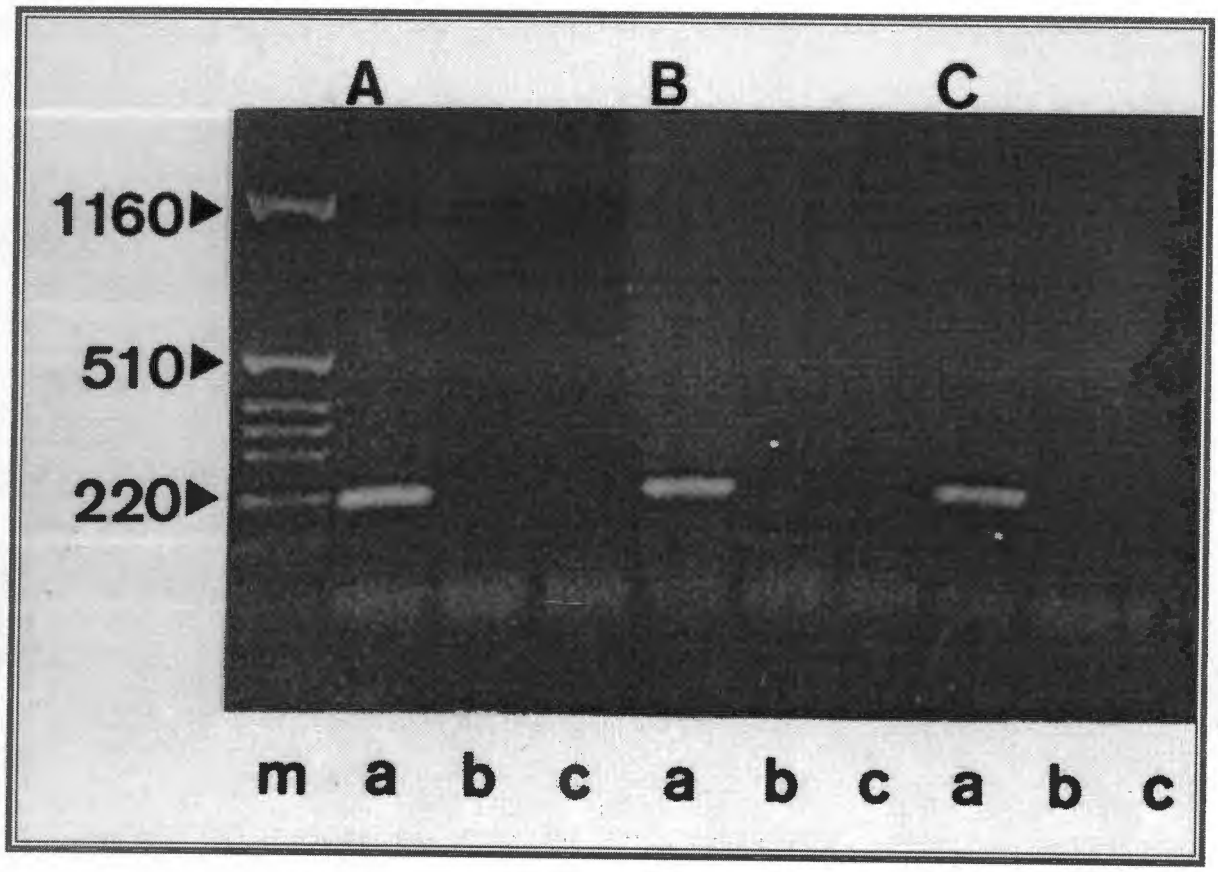


Figure 3.3. Combined RT-PCR for tyrosinase mRNA of ten controls to test for illegitimate transcription. **A,** forty first-round and 40 second-round PCR cycles. **B,** forty first-round and 30 second-round PCR cycles. **C,** thirty first-round and 40 second-round PCR cycles. **Lane m,** plasmid pKT279 digested with Hinf I. **Lane a,** ten to 15 ml blood combined with a single SK-Mel-28 cell as a positive control. **Lane b and c,** two of the ten controls.

3.1.4 Carry-over contamination

Various methods and tools have been tested to prevent and to eliminate false positive results that can arise from carry-over contamination (Belak & Ballagi-Pordany, 1993; Kwok & Higuchi, 1989; Sarkar & Sommer, 1990; Walder *et al.*, 1993). It was found that the pre-cooling of the PCR reaction product and the use of fume hoods, before and after the nested PCR reaction, will induce condensation of volatile amplicons and will prevent the release of amplicons in the laboratory atmosphere, respectively. In addition, the use of aerosol-resistant tips before the nested PCR reaction and application of the general precautions as outlined in *Materials and Methods* (see 2.6) will prevent carry-over contamination. Not a single false positive in more than 200 reactions was obtained after applying strict contamination precautions as described and by following the optimized PCR profile. However, some false positives was obtained when the general precautions were not applied. Therefore, it is imperative to include a negative and a positive control with each batch of patient samples to verify a contamination-free environment and a faultless RT-PCR operating system.

3.1.5 Reverse transcriptase activity

The efficiency of reverse transcription was tested by titrating the concentration of MMLV-RTase from 80 to 5 Units (two-fold dilutions) in a 50 µl reaction (results not shown). As little as 5 Units of the Superscript II RNase H^- MMLV-RTase was sufficient to obtain complete reverse transcription after one hour at 37°C. A ratio of four parts RTase (10 Units) to one part DNA polymerase (2.5 Units) was found to be the most effective combination. The RTase from Gibco was chosen among other MMLV-RTases, since it carries a point mutation to remove endogeneous RNase H activity which doesn't affect the reverse transcriptase activity. In addition, it was found to be the most cost effective RTase, since 10x more units of RTase had to be used with those enzymes which carry the deletion to remove endogeneous RNase H activity.

3.2 CLINICAL RESULTS

3.2.1 Correlation between the presence of CMCs and clinical stage

It was first determined whether there was a correlation between clinical stage of disease and the presence of CMCs. Overall, 11 of 181 patients (6.0%) had detectable melanoma cells in their blood as measured by RT-PCR for tyrosinase mRNA. This included four patients (5.3%) with stage I disease, six (9.0%) with stage II disease and one (16.7%) with uveal melanoma (Figure 3.4). The difference between the number of PCR-positive patients for stages I and II was not statistically significant (P = 0.2958). Surprisingly, none of the 22 stage III and IV patients were PCR-positive. Negative and positive controls confirmed a contamination-free environment and faultless RT-PCR reactions respectively. RNA integrity was confirmed in each sample with GAPDH amplification.

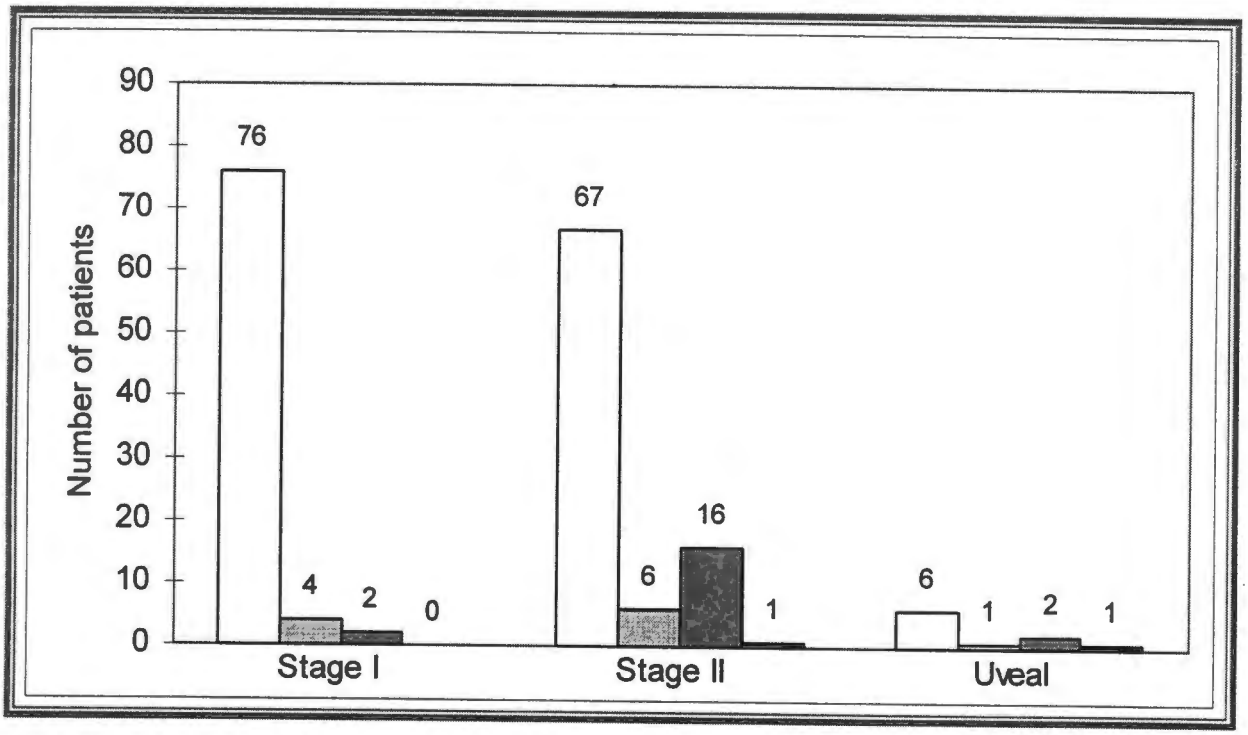


Figure 3.4. RT-PCR results in relation to clinical stage and metastases.
(□) The number of patients studied within each stage; (▩) the number of PCR-positive patients within each stage (P = 0.2958 for stages I and II); (■) the number of patients that subsequently developed metastatic disease (P = 0.0004 for stages I and II); (■) the number of patients that tested PCR-positive in relation to those that subsequently developed metastatic disease.

3.2.2 Correlation between the presence of CMCs and metastases

Correlations between patients with PM and those that subsequently developed metastases (regional nodal and systemic) were carried out (Figure 3.4). It was found that two (2.6%) of the stage I patients, 16 (23.9%) of the stage II patients and two (33.3%) of the uveal melanoma patients subsequently developed metastatic disease (P = 0.0004 for stage I versus stage II). The mean intervals between primary tumor diagnosis and clinically detectable disease were as follows:

Twenty-eight months for the two stage I patients, 13 months for 15 of the 16 stage II patients (with the exception of one stage II patient which had an interval of six years) and four months for the two uveal melanoma patients. Surprisingly, only one (tumor thickness = 5 mm, Clark = IV and ulcerated) of the 16 stage II patients and one of the two uveal melanoma patients were PCR-positive before clinically detectable metastases became evident. Both these patients had visceral metastases, implying haematogenous rather than lymphatic metastasis. Both patients (Table 3.2: patients no's 9 and 6) tested positive shortly before (one week and three months respectively) the metastases became clinically evident and both died of metastases within three months after the positive PCR tests were obtained. There was no correlation between the PCR-positive patients and the development of metastases (P = 0.3485). Each of the other nine PCR-positive patients have been followed-up for a median period of 4 years without clinical evidence of metastases to date (Table 3.2).

3.2.3 Correlation between the presence of CMCs and histological parameters

It was next determined whether the presence of CMCs correlated with any histological parameters. Correlation to tumor thickness (Figure 3.5) revealed that a higher percentage of PCR-positive patients (11.9%) had tumors greater than 1.5 mm thick compared to 3.3% with tumors less than 1.5 mm thick. The difference was statistically significant (P = 0.0442). This correlates with our finding (Figure 3.1) that a significantly higher percentage (23.9%, P = 0.0004) of stage II patients (tumor thickness > 1.5 mm) subsequently developed metastases compared to only 2.6% of stage I patients (tumor thickness < 1.5 mm).

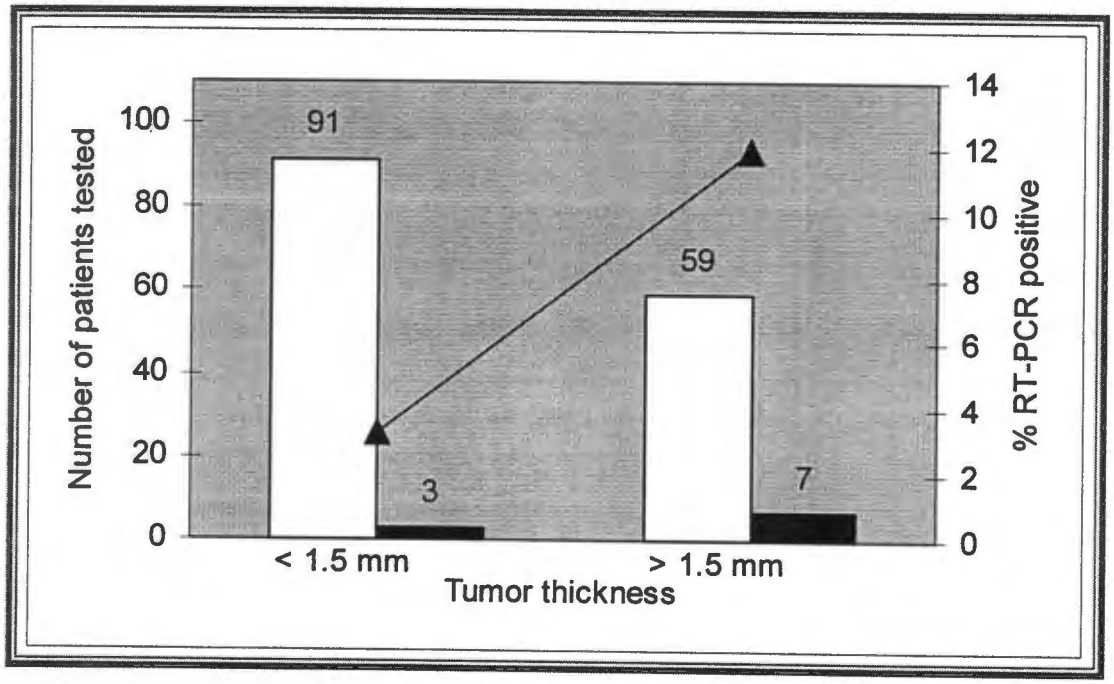


Figure 3.5. Correlation between PCR-positive patients and Breslow's tumor thickness. (□) The number of patients studied for the two main tumor thickness categories; (■) The number of PCR-positive patients for each category with the line graph (-▲-) representing the percentages. The difference between the two tumor thickness categories was statistically significant (P = 0.0442).

The percentage of PCR-positive patients was approximately 5% for Clark's levels II, III and IV with a higher frequency (21.4%) for Clark's level V (Figure 3.6). The difference between Clark's levels IV and V approached significance (P = 0.06). A statistically significant relationship (P = 0.0252) was found between patients with ulcerated tumors and the presence of CMCs (Table 3.1). Fifty percent (5/10) of the PCR-positive patients with cutaneous melanoma had ulcerated tumors compared to only 19.4% of the total population studied. All five patients with ulcerated tumors had tumor thicknesses greater than 1.5 mm. There was no correlation between PCR-positive patients and other histological parameters such as the presence of satellites, invasion of vascular and/or lymphatic spaces, signs of regression, symptoms suggestive of metastasis or the presence of metastases on chest radiographs.

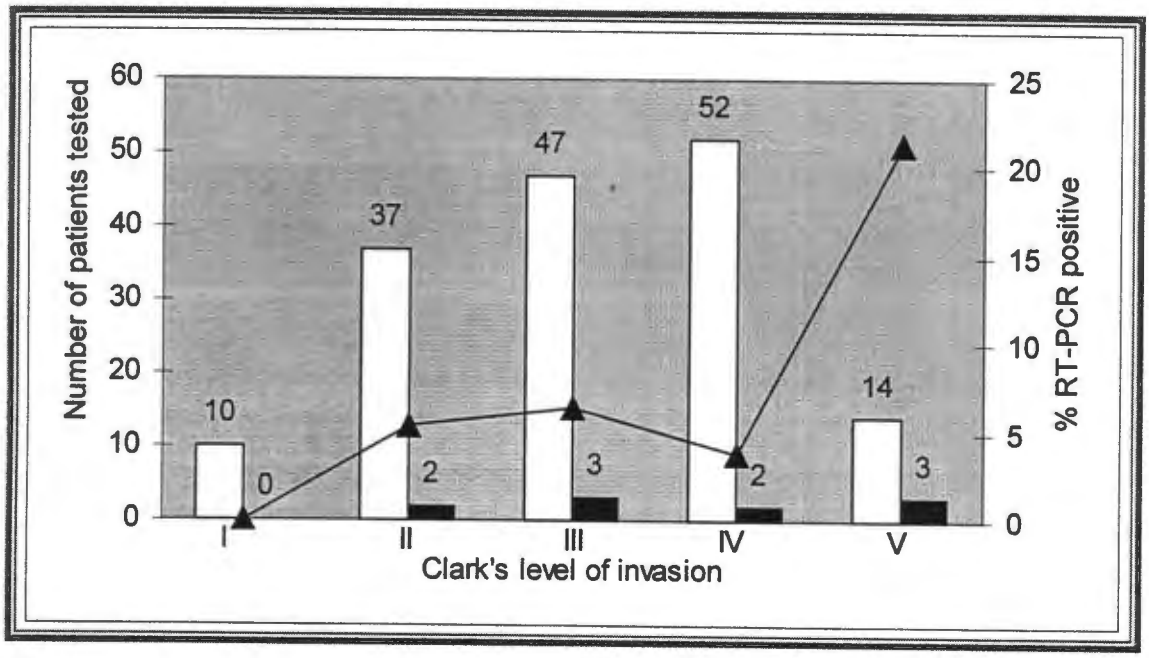


Figure 3.6. Correlation between PCR-positive patients and Clark's level of invasion. (□) The number of patients studied for each level of invasion; (■) The number of PCR-positive patients for each level of invasion with the line graph (-▲-) representing the percentages. The difference between Clark's level IV and V approached significance (P = 0.06).

Table 3.1. RT-PCR results in relation to histological criteria.

Histological criteria	[1]PCR-positive	[2]Group totals
Ulceration	5 (14.7)	34 (19.4)
Satellites	1 (25.0)	4 (2.3)
Invasion*	1 (9.1)	11 (6.3)
Regression	2 (7.1)	28 (16.0)
Total	10	175

1 The PCR-positive patients as a percentage of the group totals given in brackets.
2 The group totals as a percentage of the total population given in brackets.
* Invasion of vascular and/or lymphatic spaces.

3.2.4 Sample analysis

The final aim was to establish whether blood sampling at different time points would improve the detection rate. Fifty percent (91/181) of the total population was tested once, 23% (41/181) twice and 27% (49/181) three to seven times. The intervals between tests for patients that underwent multiple testing, was two to twelve months. Six PCR-positive patients were tested only once, two were tested twice and three were tested four to five times (Table 3.2). Each of these patients tested positive only once. For example, patient number 10 tested negative at the time that the primary tumor was diagnosed (T^1). Two months later the patient tested positive (T^2) but three times subsequently again tested negative (T^3-T^5). The majority of the 18 stage I and II patients that subsequently developed metastatic disease were tested at least three times with 3 to 6 month intervals, yet only one of these (patient no. 9) was PCR-positive. Thus, the data supports the general view that the shedding of melanoma cells into the circulation is sporadic and that the detection rate could be improved with sampling at different time points.

Table 3.2. RT-PCR results and temporal sample analysis

N°	T^1	In^1	T^2	In^2	T^3	In^3	T^4	In^4	T^5	IS	SS	In^+	D	C	FP
1	+									II			N		47
2	+									I			N		46
3	+									I			N		45
4	+									II			Y	HF	11
5	+									I			N		27
6	+									U	IV	3.0	Y	MM	3
7	+	9	-							I			N		45
8	-	2	+							II			N		41
9	-	2	-	2	-	2	+			II	IV	0.2	Y	MM	3
10	-	2	+	2	-	3	-	2	-	II			N		46
11	+	4	-	11	-	2	-	3	-	II			N		40

Abbreviations: **N°**, patient number; **T^1, T^2,** etc., RT-PCR test one, two, etc.; **In^1, In^2,** etc., interval one, two, etc. in months between tests; **IS,** initial clinical stage; **SS,** sub-sequent clinical stage; **In^+,** interval in months between first positive RT-PCR test and clinical detectable metastases; **D,** died; **C,** cause of death; **FP,** follow-up period in months after a positive RT-PCR test had been obtained; **+,** RT-PCR positive; **-,** RT-PCR negative; **U,** uveal melanoma; **N,** no; **Y,** yes; **HF,** heart failure; **MM,** metastatic melanoma.

CHAPTER 4: CLINICAL DISCUSSION

In this study, a combined RT-PCR assay for tyrosinase mRNA was developed and tested. It was shown that the assay can reproducibly detect a single, viable melanoma cell in 10-15 ml of peripheral blood. The assay was then used to test blood samples from patients with melanoma (primarily stages I and II) and the results were evaluated in relation to various clinical and histological criteria. The results confirm the recent doubts raised by other investigators that the current technique for the detection of CMCs by RT-PCR is of limited or no clinical value for monitoring melanoma progression (Farthmann *et al.*, 1998; Glaser *et al.*, 1997; Reinhold *et al.*, 1997). It was found that 7% (10/143) of the patients with PM had detectable melanoma cells in their blood (Figure 3.4). As would be predicted, the percentage of PCR-positive patients was higher for stage II patients (9.0%) than for stage I patients (5.3%) but the difference was not statistically significant (P = 0.2958). The detection rate of 9% observed for patients with stage II disease is in concordance with two recent groups who reported detection rates of 12-13% (Farthmann *et al.*, 1998; Ghossein *et al.*, 1998). However, none of the stage III and IV patients were PCR-positive. There are a number of possible explanations for this. First, it is possible that the number of stage III and IV patients studied, was simply too low. Alternatively, it was known that one third of the stage III and IV patients underwent chemotherapy at the time of blood sampling and it is possible that effective chemotherapy could have contributed to a more stabilized disease and thus a lower detection rate.

The data in this study supports the notion that tumor thickness and ulceration are the two most significant prognostic factors for predicting the likelyhood of developing metastases. A significantly higher percentage PCR-positive patients (±12%) was found to have tumors greater than 1.5 mm thick (Figure 3.5) compared to 3.3% with tumors less than 1.5 mm thick (P = 0.0442). Furthermore, 50% of the PCR-positive patients had ulcerated tumors (Table 3.1) compared to 19.4% of the total population studied (P = 0.0252). It should be cautioned that although a higher percentage PCR-positive patients had ulcerated tumors greater than 1.5 mm thick, it was not possible at this stage, to link this directly to a poor prognosis since the majority of the PCR-positive patients have not yet (within four years) developed metastatic disease. However, the data does indicate that cells from tumors greater than 1.5 mm thick and with ulceration have a greater propensity to enter the circulation but that these cells do not necessarily have the ability to establish metastases. It was also found that a higher percentage (21.4%) of Clark's level V patients were PCR-positive compared to patients with Clark's levels IV (3.8%) with a P value (0.06) approaching significance (Figure 3.6). This indicates that the shedding of tumor cells into the circulation is associated with a deeper tumor penetration (vertical growth) and supports the observations that a deeper tumor penetration correlates with a poorer prognosis (reviewed by Garrison & Nathanson, 1996).

The results suggest that the detection rate of 9% for patients with stage II disease is much lower than would be expected, since 23.9% (16/67) of the stage II patients subsequently developed metastases (Figure 3.4). Of these 16 patients, only one was PCR-positive. Thus, the current technique fails to predict the likelihood of developing metastatic disease (P = 0.3485). None of the PM patients received any form of therapy (besides surgery of the primary tumor) which might have influenced the low detection rate observed. The risk of recurrence after a 4 year follow-up period for stage I patients (tumors < 1.5 mm thick) is approximately 3-6% and for stage II patients with intermediate thickness melanoma (1.5 > < 4 mm) the risk is 12-20% (Delaunay, 1992). Recurrence rates of 2.6 and 23.9% were obtained for the stage I and II patients respectively after a median follow-up period of 4 years. It is therefore clear, at least for the stage I patients, that follow-up periods longer than 4 years will be necessary to establish whether the presence of CMCs will ultimately result in metastatic disease.

The presence of tumor cells in the circulation will not necessarily result in the establishment of secondary tumors, as most circulating tumor cells die rapidly and the establishment of metastases is an inefficient process involving multiple factors (reviewed by Fidler, 1996). Animal studies indicate that less than 0.01% of circulating tumor cells eventually survive to produce metastases (Glaves, 1983; Liotta & Stetler-Stevenson, 1991). Thus, this study support this animal model observation. The strong correlation between the presence of CMCs and important prognostic factors (ie. tumor thickness and ulceration) but not between CMCs and metastasis is not necessarily a contradiction. It is most likely that this lack of correlation is indicative of a subpopulation of metastatic cells from the primary tumor that has the enzymatic capability of entering the circulation but which do not have the ability to survive the immune surveillance system, nor extravasate into target organ interstitium, proliferate or induce angiogenesis at secondary sites. In contrast to the findings of this study, Ghossein *et al.* (1998) reported that blood PCR-positivity was associated with a significantly decreased overall survival for stage II patients and Curry *et al.* (1998) reported that 66% of recurrences were predicted by PCR analysis in the early postoperative period.

To address the question of whether multiple sampling would increase the positive detection rate, the RT-PCR results were examined in terms of number of times tested. Fifty percent of the total population studied underwent a single test of which six tested positive (Table 3.2: patient numbers 1-6) and the other 50% underwent two or more tests of which five patients tested positive only once (Table 3.2: patient numbers 7-11). This suggests that sampling at different time points will increase the detection rate, as was suggested previously (Battayani *et al.*, 1995; Pittman *et al.*, 1996) and that some of the PCR-negative patients could in fact be positive if they were tested more than once. However, of the 18 PM patients that developed metastases, only one was PCR-positive even though these patients underwent multiple tests at different time points. This strongly

supports the concept of sporadic shedding of melanoma cells into the circulation and that the number of cells that circulate at any given moment is very low. Thus, sampling the large blood compartment seems to have severe limitations: First, the time of blood sampling may not coincide with the shedding process, leading to sampling errors. Secondly, sampling small blood volumes (10-20 ml) from the large blood pool seems to be statistically insufficient for obtaining a positive RT-PCR test due to the low numbers of CMCs. It was suspected that in addition to sampling at different time points, multiple sampling at each time point will also increase the detection rate. However, this was not tested. Lastly, the presence of CMCs will not necessarily be indicative of metastasis for reasons as discussed previously.

In conclusion, the results indicate that the current technique for the detection of CMCs is of limited clinical value for the identification of patients with PM at risk for developing metastases. However, the presence of CMCs does seem to correlate with the clinical stage (Curry *et al.*, 1998; Farthmann *et al.*, 1998; Mellado *et al.*, 1996) and the tumor thickness (Curry *et al.*, 1998; Farthmann *et al.*, 1998) although some groups found no correlation with the clinical stage (Ghossein *et al.*, 1998) or the tumor thickness (Hoon *et al.*, 1995; Mellado *et al.*, 1996). In agreement with Verkaik *et al.* (1997) it is advised that clinical decisions should not be based on the presence of circulating tumor cells in small blood volumes. It is suggested that other options should be explored to predict the presence of micrometastates. One option is to enrich melanoma cells from larger blood volumes (ie. 50 to 500 ml for PM patients) with immunomagnetic technology (Hardingham *et al.*, 1993), although this option might not be practical or feasible. Another option is to make use of a multimarker approach to compensate for the heterogeneity of tumor cells or lack of expression of tyrosinase. Various mRNA markers, such as p97, Muc-18 and Mage-3 (Hoon *et al.*, 1995) β-human chorionic gonadotropin (Doi *et al.*, 1996) Pmel17/gp100, Mart-1/Melan-A, Trp-1 and Trp-2 (Sarantou *et al.*, 1997) and more recently β1→4-N-acetylgalactosaminyl-transferase (Kuo *et al.*, 1998) and ganglioside GM3 (Zhang *et al.*, 1998) are potentially suitable markers for the detection of CMCs. However, Curry *et al.* (1998) reported that gp100, p97, and Muc-18 were not suitable markers because the mRNAs were detected in normal blood samples. They also provided evidence that the use of Mart-1 in combination with tyrosinase allows the detection of CMCs in a larger percentage of patients and increases the potential utility of the assay by reducing the false-negative rate.

Other options that should be explored to predict the presence of micrometastases, is to make use of biopsies from anatomic compartments other than blood, such as bone marrow (Brossart *et al.*, 1994), lymph nodes (Schwurzer-Voit *et al.*, 1996; Van der Velde-Zimmermann *et al.*, 1996; Wang *et al.*, 1994) and subcutaneous fat of the primary tumor (Proebstle *et al.*, 1996). The sampling error associated with the detection of CMCs, will then be obviated. The only comprehensive study (to the best knowledge of the investigator) on the use of bone marrow aspirates for the prediction of

metastatic disease was reported by Ghossein *et al.* (1998) who found that the PCR-positivity for stage II patients was twice as high for bone marrow aspirates (25%) as for blood (12.5%). This suggests that sampling the smaller bone marrow compartment is more informative than sampling the larger blood compartment. However, it is the opinion of the investigator that the detection of micrometastases in sentinel lymph nodes (SLNs) with RT-PCR analysis will most likely prove to be of more clinical value since the SLN (identified by radio-guided surgery; reviewed by Brady & Coit, 1997) is the first node in the lymphatic basin to receive metastases from the primary tumor (Gershenwald *et al.*, 1998; Reintgen *et al.*, 1994). Several recent reports (Blaheta *et al.*, 1998; Goydos *et al.*, 1998; Joseph *et al.*, 1997; Shivers *et al.*, 1998) indicate that the SLNs of patients with PM that were negative by immunohistochemical assessment, could be upstaged by approximately 50% using RT-PCR analysis for tyrosinase alone. Moreover, Shivers *et al.* (1998) have shown a recurrence rate amongst 13% of the patients that were upstaged by RT-PCR analysis after a relative short average follow-up period of 28 months. The SLN mapping technique together with a multimarker/RT-PCR approach could therefore prove to be a more powerful tool to predict metastatic disease.

FUTURE CONSIDERATIONS

A very challenging and potentially viable research prospect would be to use RT-PCR analysis for the identification of SLN micrometastases in patients with PM and to offer these patients a potentially effective immunotherapy approach. The rationale is that patients with a small metastatic tumor burden are more likely to respond to immunotherapy since dormant micrometastases are more susceptible to cell-mediated immunological attack (McNamara *et al.*, 1998; Staveley *et al.*, 1998). Amongst the various immunotherapy strategies that have been investigated, dendritic cell (DC)-based immunotherapy is currently the most promising (reviewed by Pardoll, 1998). DCs are potent antigen presenting cells (APCs) which play a central role in the induction of an antigen-specific cytotoxic T-lymphocyte (CTL) response. APCs express costimulatory molecules (eg. B7) in response to certain inflammatory cytokines which promote T-cell activation. It was recently demonstrated that the interaction of mouse DCs with mouse melanoma or lung tumor cells will result in effective CTL-mediated tumor-specific lytic activity, either by conferring sufficient APC function to tumor cells for T-cell activation or by facilitating the delivery of tumor antigens to DCs for processing and presentation (Celluzzi & Falo, 1998). Mice immunised with irradiated DC-B16 tumor cell aggregates were completely protected from lethal challenge with B16 tumor cells. Mice with established tumors demonstrated complete regression when challenged with the immunogen and demonstrated long-lasting antitumor immunity.

Thus, DC-melanoma cell aggregates seem to be potentially effective immunogens that could be used to eradicate dormant micrometastases before the onset of tumor vascularization and progression to macrometastatic disease. The tumor cells of a patient that is positive for

micrometastases (using SLN biopsy and RT-PCR analysis) could be cultured and selected from the primary tumor or from a portion of the SLN (Reinhold *et al.*, 1997; Thompson & Ioli, 1997). The patients DCs could be expanded from blood precursors with the addition of GM-CSF, SCF, Flt-3 ligand, TGF-α and TGF-β (Soligo *et al.*, 1998). Once melanoma cells and DCs have been established successfully *in vitro* from a patient with proven SLN micrometastases, such a patient becomes eligible for autologous immunotherapy. The patients DCs could then be co-cultured with its own melanoma cells for 24 hrs and irradiated (Celluzzi & Falo, 1998). The immunogen (irradiated DC-melanoma cell aggregate) could then be given to the patient during the early stages of tumor progression.

&&&&&&&&&&&&&&&&&&&&

SECTION B

CHAPTER 5: LITERATURE REVIEW
SERUM MARKERS FOR CUTANEOUS MELANOMA

5.1 Introduction

The dramatic improvements in the survival of patients with cutaneous melanoma over the past decades are mainly the result of earlier diagnosis and surgical intervention of primary disease (Halpern & Schuchter, 1997). The ability to predict micrometastatic disease is therefore of utmost clinical importance for making rational therapeutic decisions. Although a number of clinico-pathological parameters (reviewed by Cochran, 1997; Garrison & Nathanson, 1996) have been implicated as prognostic factors for melanoma, little progress has been made in the identification of reliable serum markers for monitoring the progression of PM or AM (Guo *et al.*, 1995; Provost *et al.*, 1997; Weiss *et al.*, 1995).

The detection of substances secreted or shed by tumor cells into the PB may have value in the diagnosis of cancer. In addition, it could also be used to monitor the progression from localised to metastatic disease and the response to therapy in patients with metastatic disease or the possibility of recurrence. Substances secreted into the PB by the normal host tissue in response to invading malignant cells could possibly also serve as prognostic markers. A brief evaluation of serum markers and their potential use for monitoring tumor progression and response to therapy follows:

5.2 Liver function tests

The liver function tests are usually used to obtain prognostic information (reviewed by Provost *et al.*, 1997; Weiss *et al.*, 1995) and to monitor treatment (Bedikian *et al.*, 1995; Huang *et al.*, 1990; Miller *et al.*, 1989) in patients with AM. These tests include the determination of aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase, lactate dehydrogenase (LDH), alkaline phosphatase, creatine kinase, total bilirubin and direct bilirubin levels in serum. In addition, the serum levels of creatinine and urea nitrogen may be used to monitor renal function (Huang *et al.*, 1990). A survey (Provost *et al.*, 1997) amongst 30 experienced physicians indicated that the majority of them routinely order chest X-rays with or without liver function tests (mostly LDH) for stages I, II and III and during follow-up for stages IB, II and III, and baseline computed tomographic or magnetic resonance imaging scans of the chest, abdomen, pelvis and brain for stage III patients. It was concluded from this survey that complete liver function tests and blood cell counts are justified only for patients with clinically established metastases. The value of the liver function tests as indicators of melanoma progression is controversial. In 12.5% (11/88) of stage II

patients, a persistent increase in serum LDH was the first indicator of recurrent visceral metastatic disease (Finck *et al.*, 1983), but others reported that routine liver function tests have no (Kersey *et al.*, 1985) or limited value (Weiss *et al.*, 1995) in the prediction of metastatic disease. The pre-treatment values of LDH and albumin, dichotomized as high and low, have been reported to be independent prognostic factors for survival in patients with AM (Sirott *et al.*, 1993). Because LDH is metabolized at a fairly constant rate, elevated levels are believed to represent tumor cell turnover (growth and necrosis) and tumor burden but it does not necessarily indicate tumor involvement of the liver as reported initially (Garg *et al.*, 1979). In this study, the median survival was 11.8 months in patients in whom both the LDH and serum albumin were normal versus 3.6 months in patients with a low albumin and a LDH that was greater than or equal to two times the upper limit of normal. Elevated LDH levels have been found to be an independent prognostic parameter for response to treatment and survival in patients with AM treated with interferon-α and interleukin-2 (Keilholz *et al.*, 1996). A more recent study indicates that by using only serum LDH and disease site as prognostic parameters, patients with both favorable factors had a median survival time of 12 months, whereas patients with neither had a median survival time of 5 months (Eton *et al.*, 1998).

5.3 Melanin-related metabolites

The use of melanin-related metabolites to monitor the progression of melanoma were reported to be of some promise. In particular, serum levels of 5-S-cysteinyldopa seem to reflect melanoma progression better than urine levels, whereas the serum or urine levels of 6-hydroxy-5-methoxyindole-2-carboxylic acid was of little value (Horikoshi *et al.*, 1994). It was proposed that the measurement of tyrosinase activity in the serum of melanoma patients could be a possible progression marker (Sonesson *et al.*, 1995b). However the method, which measures stereospecific dopa oxidation, was found to be of little value and was troubled by seasonal variation in tyrosinase activity. The mean tyrosinase activity values in 30 normal individuals was 0.4 $\pm$0.2 nkatal/l and for 10 patients with AM the mean value was 3.1 nkatal/l (Sonesson *et al.*, 1995a).

5.4 Soluble adhesion molecules

Elevated serum levels of adhesion molecules may be indicative of an enhanced host cell-mediated immune response to a primary tumor or may reflect a passive shedding of adhesion molecules by tumor cells (Harning *et al.*, 1991; Miller *et al.*, 1997). Adhesion molecules that have been studied in melanoma as possible progression markers, include soluble isoforms of intercellular adhesion molecule 1 (ICAM-1, CD54), vascular cell adhesion molecule 1 (VCAM-1, CD106) and E-selectin (CD62e). A number of studies have suggested a positive correlation between melanoma progression and elevated serum levels of soluble ICAM-1 (Altomonte *et al.*, 1992; Denton *et al.*, 1992; Harning *et al.*, 1991; Miller *et al.*, 1997; Viac *et al.*, 1993) although not all studies agree with this finding (Bosserhoff *et al.*, 1997; Kageshita *et al.*, 1993; Kageshita *et al.*, 1992). A more recent study (Franzke *et al.*, 1998) compared the pre-treatment serum levels of sVCAM-1, sICAM-1, sE-

selectin and LDH to determine their prognostic value in 97 patients with AM. Multivariate analysis showed that elevated serum levels of sVCAM-1 and LDH were dominant independent prognostic variables and correlated with poor outcome in metastatic melanoma, while sICAM-1 and sE-selectin were of no prognostic value. Combining elevated sVCAM-1 and LDH serum levels, identified a subgroup of patients at high risk, all of who died of metastatic disease within one year.

5.5 S100 protein

S100 protein has received considerable attention as a possible prognostic serum marker for melanoma (Bosserhoff *et al.*, 1997; Fagnart *et al.*, 1988; Guo *et al.*, 1995; Hansson *et al.*, 1997; von Schoultz *et al.*, 1996). It is a 21 kD acidic, intracellular calcium-binding protein originally isolated from the central nervous system (Dannies & Levine, 1969; Moore, 1965). Although the biological functions have not been elucidated, it has been shown to affect the assembly and disassembly of microtubules (Baudier *et al.*, 1982; Isobe & Okuyama, 1978). S100 is a dimeric protein composed of α- (10.4 kD) and β- (10.5 kD) subunits, with the possible combinations αα, αβ and ββ (reviewed by Donato, 1991; Kligman & Hilt, 1988). High concentrations of the β-subunit are present in glial and Schwann cells of the central nervous system (Takahashi *et al.*, 1984) but have also been found in epidermal Langerhan's cells (Nakajima *et al.*, 1982). Melanocytes, macrophages and monocytes contain only the α-subunit (Nakajima *et al.*, 1982; Takahashi *et al.*, 1984).

Polyclonal and monoclonal antibodies against protein S100 and in conjunction with HMB-45 antibodies (Chiamenti *et al.*, 1996; Walts *et al.*, 1988) are now being used routinely in the histopathological diagnosis of melanoma and its differential diagnosis from other solid tumors (Carrel & Rimoldi, 1993; Cho *et al.*, 1990; Gatter *et al.*, 1985). The α- and β-subunits are rarely found in ordinary nevi, with dysplastic nevi expressing only the α-subunit. The expression of the β-subunit is strongly associated with vertical growth and invasiveness of superficial spreading melanomas. The majority of nodular melanomas express both subunits. It was therefore suggested that the expression of the β-subunit might be related to vertical progression (Cho *et al.*, 1990; Paul *et al.*, 1987; Smoller, 1991).

The levels of protein S100 in serum and cerobrospinal fluid have been shown to increase after damage to the central nervous system and is now being used to monitor brain damage in stroke patients (Aurell *et al.*, 1989; Persson *et al.*, 1987). Fagnart *et al.* (1988) initially suggested that the serum levels of protein S100 might be a useful marker to monitor melanoma progression. The clinical significance of serum S100 was demonstrated by Guo *et al.* (1994) using an immunoradiometric assay, which measures the β-subunit. They found that a persistent rise in serum S100 indicated progression of the disease, whereas a decline indicated response to therapy. A sensitivity of 1.3% for stages I and II patients versus 41.3% for stages III and IV patients

indicated clearly that serum S100 levels was of no value for the early detection of metastatic disease. In addition, serum S100 showed a better discrimination between patients with distant metastases and those without it, than neurone-specific enolase serum levels (41.3% versus 23.9% respectively). Similar studies (Karnell *et al.*, 1997; von Schoultz *et al.*, 1996) show a strong association between overall survival rate and serum S100β levels. The observed/expected death ratio was markedly increased with increasing S100β levels. These data strongly suggest that serum S100β is an independent prognostic marker that may be useful in identifying high-risk cases and to monitor response to therapy in patients with AM.

5.6 Melanoma-inhibiting activity (MIA)

MIA has been identified initially as an 11kD soluble protein secreted from melanoma cell lines *in vitro* (Apfel *et al.*, 1992; Bogdahn *et al.*, 1989). More recently, it has been identified independently by a differential display approach and has been referred to as CD-RAP (Dietz & Sandell, 1996). It is speculated that the function of this growth-regulatory protein is to slow tumor progression in vivo by an autocrine mechanism (Graeven & Herlyh, 1992; Weilbach *et al.*, 1990). High MIA mRNA levels have been detected in almost 100% of malignant melanoma biopsies and at low levels from benign melanocytic nevi but not from normal skin melanocytes (Bosserhoff *et al.*, 1996; van Groningen *et al.*, 1995). Bosserhoff *et al.* (1997) investigated whether MIA was a clinically useful progression marker in patients with melanoma. They reported enhanced MIA serum levels in 13 and 23% of patients with stage I and II disease, respectively, and in 100% with stage III or IV disease. MIA was the most sensitive marker when compared with S100 and soluble ICAM-1 serum levels in these patients. Response to therapy in stage IV disease correlated with changes in MIA serum levels. Thirty-two of 350 stage I and II patients developed enhanced MIA serum levels. Fifteen of the 32 patients developed metastases at the time of a raised MIA serum level and one presented with metastatic disease 6 months later. None of the patients with normal MIA serum levels developed metastases during a 6-12 month follow-up period. An extended follow-up period will be necessary to determine whether the 16 patients who developed enhanced MIA serum values, but had no clinical signs of disease progression, will develop metastases at a later stage.

5.7 Plasminogen activators and inhibitors

The plasminogen activator (PA) cascade plays a fundamental role in tumor cell migration and invasion processes which require the coordinated and temporal regulation of a series of adhesive, proteolytic and migratory events (reviewed by Andreasen *et al.*, 1997; Blasi, 1997). High levels of urokinase plasminogen activator (uPA) and its receptor (uPAR) (Duffy, 1996; Duffy *et al.*, 1998), but paradoxically also plasminogen activator inhibitor type 1 (PAI1), have been correlated with a poor prognosis for a variety of solid tumors (reviewed by Pappot *et al.*, 1995). The regulatory role of PAI1 in uPAR-mediated cell adhesion and release may explain why high tumor PAI1 levels correlate with a poor prognosis for many cancers (Deng *et al.*, 1996a). In support of this model, de

Vries *et al.* (1994) demonstrated by immunohistochemistry and *in situ* hybridization that uPA, uPAR, PAI1 and PAI2 could not be detected in benign and in early stages of PM but appeared frequently in advanced PM and melanoma metastasis lesions (de Vries *et al.*, 1994). Convincing experimental evidence for the role of host-produced PAI1 in cancer cell invasion and angiogenesis was recently provided by Bajou et al. (1998). PAI1-deficient mice failed to support local invasion and tumor vascularization of transplanted malignant keratinocytes. When this PAI1 deficiency was circumvented by intravenous injection of a replication-defective adenoviral vector expressing human PAI1, invasion and associated angiogenesis were restored (Bajou *et al.*, 1998).

Although the high levels of tumor PAI1 in breast (Grondahl-Hansen *et al.*, 1993; Janicke *et al.*, 1994; Kim *et al.*, 1998; Knoop *et al.*, 1998), lung (Pappot *et al.*, 1997; Pedersen *et al.*, 1994), gastric (Allgayer *et al.*, 1997; Nekarda *et al.*, 1994), colorectal (Sier *et al.*, 1994) and ovarian cancers (Chambers *et al.*, 1998; Kuhn *et al.*, 1994) appear to be independent predictors of short survival, the use of tumor extracts to obtain prognostic information is usually not clinically practicable. It has been suggested that plasma PAI1 levels may provide the same prognostic information as when measuring tumor PAI1 (reviewed by Pappot *et al.*, 1995). Some patients with ovarian cancer have been shown to have raised plasma levels of PAI1 (Casslen *et al.*, 1994). In patients with colorectal cancer (Nielsen *et al.*, 1998), high preoperative plasma concentrations of PAI1 have been associated with a shorter survival and increasing severity of the disease and in gastric cancer, the plasma levels of PAI1 were significantly higher than in benign gastric disease (Ho *et al.*, 1998). In breast cancer patients, plasmatic PAI activity was significantly higher for patients with metastasis compared to patients whose cancer did not recur (von Tempelhoff *et al.*, 1997).

5.8 Specific aims of study

Despite the correlation between high tumor or plasma levels of PAI1 and a poor prognosis in patients with various solid tumors, the role of PAI1 in cancer progression and invasion is still poorly understood. In this section of the thesis, the specific aim was to investigate whether high or low plasma levels and/or activity of PAI1 correlate with the presence of metastatic disease in patients with melanoma. To the best knowledge of the investigator, the plasma levels or activity of PAI1 for patients with AM has not previously been reported. A unique enzyme-linked immunosorbent assay (ELISA) was developed to measure both the total amount of PAI1 in plasma as well as the active fraction of the inhibitor. (The production of these antibodies and their uses are fully described in section C of this thesis.) This novel Mab-based immunofunctional assay was then used to analyse and compare the plasmatic PAI1 levels and activity of a group of patients with AM (stages III and IV disease) with a group of patients with primary disease (stages I and II) and a control population.

CHAPTER 6: MATERIALS AND METHODS

6.1 Reagents

Latent and active *Escherichia coli*-derived human recombinant PAI1 (rPAI1) was obtained and prepared as described in section C of this thesis (see 10.1). The concentration of latent and active rPAI1 was determined with the BCA protein assay (Pierce Chemical Company, Rockford, USA) using the 60°C protocol and with bovine serum albumin (BSA) as a standard according to the instructions of the manufacturer. The rabbit anti-PAI1 polyclononal antibody (RαPAI1 Pab, see 10.2) and the mouse anti-PAI1 monoclonal antibodies, clones 6 and 11 (mαPAI1/C6 and –C11 Mabs, see 10.4, 11.4 and 11.11) were prepared as described in section C of this thesis.

6.2 Calibration curves for PAI1

A 96-well polypropylene MaxiSorb ELISA plate (NUNC A/S, Roskilde, Denmark) was coated with the RαPAI1 Pab at 50 μg/ml in PBS for 16 hours at 4°C and then blocked with 0.1% BSA in PBS for 30 mins at RT. The reagents used by the ELISA procedure were added to the plates at 50 μl per well (unless otherwise stated) and all steps were followed by five washes with TST-buffer (see A.27 for details) except for the last wash which was done ten times. All incubation steps were for 30 mins at RT (except if mentioned differently). Calibration curves were constructed with latent or active rPAI1 as follows: The rPAI1, prepared by serial dilutions from 12 ng/ml down to 2 ng/ml in blocking solution, was added to the plate for 1 hour at RT. The mαPAI1/C6 and –C11 Mabs were tested for their ability to detect the bound latent or active PAI1 and were added at 1 μg/ml. The bound antibody was detected with goat anti-mouse IgG conjugated with alkaline phosphatase (Dako, Glostrup, Denmark, diluted 1 in 2000). p-Nitrophenyl phosphate (PNPP, 100 μl per well) was used as a substrate for alkaline phosphatase (see A.57 for details). The plate was incubated for 6 hrs at RT and the absorbance was read at 405 nm.

6.3 Patients

Blood samples were collected from 39 melanoma patients attending the Melanoma Clinics of Groote Schuur and Kingsbury Hospitals in Cape Town. There were 21 patients with PM (7 stage I and 14 stage II patients) and 18 patients with AM (10 stage III and 8 stage IV patients). The clinical staging was based on the TNM classification system as agreed upon by the International Union Against Cancer and the American Joint Committee on Cancer. There were 38 controls, which included South Africans working in the Groote Schuur Hospital and University of Cape Town academic institutions. The controls were selected on the basis of no history of skin cancers and cardiovascular diseases. The study was approved by the Ethics and Research Committee of the University of Cape Town. Written and informed consent was obtained from all patients before they entered into the study.

6.4 Sample preparation and PAI1 measurements

Blood from control and melanoma patients was collected from a steadily flowing venipuncture between 2:00 and 4:00 p.m. into 0.1 volume of 0.1 M sodium citrate at pH 4.5 (Declerck *et al.*, 1988a; Kruithof *et al.*, 1987). Blood samples for control and patient groups were collected within the same time period to minimise the effect of diurnal variation in plasma PAI1 (Andreotti *et al.*, 1988; Juhan-Vague *et al.*, 1992; Macy *et al.*, 1993). The first 5 ml of blood was discarded, as was any tube that collected non-free-flowing blood or plasma contaminated with erythrocytes. The blood were mixed three times by inversion and kept on ice. The tubes were centrifuged at 700 g for 10 mins at RT within one to two hours of collection. These restrictions imposed on blood collection were carried out at all times to minimise the release of platelet-containing PAI1 into the plasma (Macy *et al.*, 1993). Platelet-free plasma (PFP) was prepared by filtering the citrated-plasma through a 0.45 μm acetate membrane filter, fitted with a 1.0 μm borosilicate glass prefilter (Micron Separations Inc., Westborough, USA). The PFP was stored in suitable aliquots at -70°C.
PFP samples were thawed only once and were diluted 1/2 to 1/80 in 0.1%BSA/PBS for PAI1 measurements. ELISA's were carried out as described in 6.2 above. The plasmatic PAI1 levels for the samples were estimated from the calibration curves using the linear function, $y = mx + c$. Two measurements were carried out for each sample (in duplicate): The first measurement (with mαPAI1/C11) is used to calculate the total amount of PAI1 (latent plus active PAI1). The second measurement (with mαPAI1/C6) is used to calculate the amount of latent PAI1. The two measurements are then subtracted from each other to obtain the amount of active PAI1. The percentage active PAI1 is then calculated as (ng/ml active PAI1) / (ng/ml total PAI1) x 100.

6.5 Statistical analysis

Since the variances were not homogeneous, differences between controls and patients with PM and AM were tested with the Kruskal-Wallis ANOVA test using the Statistica Version 5.1 software (StatSoft, Inc., Tulsa, OK). A p-value less than 0.05 was considered as being statistically significant. The Linear Discriminant Function test was used to determine the cut off values for the active fraction of plasmatic PAI1 between groups using the StatGraph Version 6.0 software (Manugistics, Inc.).

CHAPTER 7: RESULTS

7.1 Calibration curves for PAI-1

Calibration curves for active and latent PAI1 were constructed for each of the Mabs mαPAI1/C6 and -C11 in order to quantitate active and latent PAI1 in plasma samples (see *Materials and Methods*, 6.2). Figure 7.1 illustrates the calibration curves obtained for both mαPAI1/C6 and -C11. The mαPAI1/C11 allows the quantitation of free latent (open square) and active (solid square) PAI1 with an equal affinity. In contrast, mαPAI1/C6 showed a high affinity for latent (open triangle) PAI1 but failed to detect active (solid triangle) PAI1 within the concentration range of the standard curve. Thus, mαPAI1/C6 allows the quantitation of active PAI1 by subtracting the amount of latent PAI1 (detected by mαPAI1/C6) from the amount of total PAI1 (detected by mαPAI1/C11). The lower limit of sensitivity for the assay was 2 ng/ml PAI1 in blocking buffer.

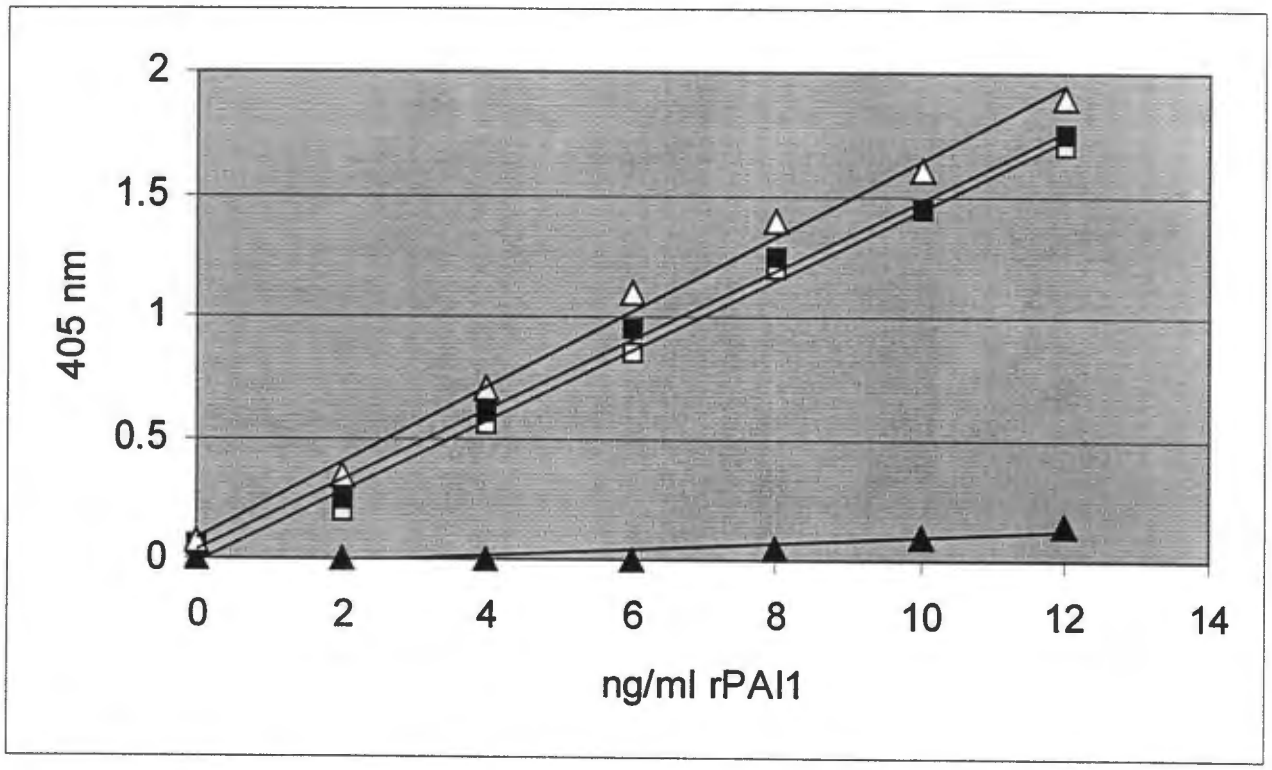


Figure 7.1. Calibration curves for PAI1.
Decreasing concentrations of latent (open symbols) or active (solid symbols) rPAI1 from 12 ng/ml down to 2 ng/ml were used to construct calibration curves. The mαPAI1/C11 (squares) detected latent (□) and active (■) PAI1 with equal affinity. The mαPAI1/C6 (triangles) showed a high affinity for latent (Δ) PAI1 but failed to detect active (▲) PAI1 within the concentration range of the standard curve. The background 405 nm readings were less than 0.7 after 6 hours at RT and were subtracted from each reading to construct standard curves ($r > 0.98$). Each value represents the mean from duplicate readings.

7.2 PAI1 quantity and activity for controls and patients with melanoma

The total plasmatic PAI1 levels for patients with PM and AM were compared with the controls to determine whether there are any statistical differences between the three groups. The data are summarised in Table 7.1 (see also Appendixes 58 to 60 for raw data). The mean concentration of total PAI1 was 57 ±66 ng/ml for the controls (n = 38), 139 ±295 ng/ml for patients with PM (n = 21) and 88 ±124 ng/ml for patients with AM (n = 18). There were two PM patients (see A.59, patient numbers 13 and 15) and one AM patient (see A.60, patient number 1) with total PAI1 values greater than 500 ng/ml . These outliers have contributed significantly to the large standard deviations of 295 and 124 ng/ml that were obtained for PM and AM patients respectively. However, the differences in total PAI1 between the three groups were not statistically significant (P = 0.6199) with the Kruskal-Wallis ANOVA test (Figure 7.2).

In the following analysis, the active fraction of plasmatic PAI1 (percentage active PAI1) for patients with PM and AM were compared with the controls to determine whether there are any statistical differences between the three groups. The data are summarised in Table 7.1 (see also A.58 – A.60 for raw data). The mean percentage of active PAI1 was 62 ±10.5% for the controls, 55 ±12.4% for patients with PM and 46 ±15.1% for patients with AM. The differences in the percentage active PAI1 between the three groups was statistically significant (P = 0.0076) with the Kruskal-Wallis ANOVA test (Figure 7.3). The difference between the control and AM groups was highly significant (P = 0.0042). Surprisingly, the difference between the control and PM groups was also significant – although borderline (P = 0.0488), while the difference between PM and AM groups was not statistically significant (P = 0.2556). Overall, there was a definite order of linearity between the three groups with a correlation coefficient of 0.49 (P < 0.0005) using multiple regression analysis.

TABLE 7.1. Number of samples, median and range of plasmatic PAI1 levels.

Patients	n	Total PAI1 (ng/ml)		Active PAI1 (%)	
		Median	Mean (range)	Median	Mean (range)
Controls	38	34	57 (10-326)	64	62 (38-84)
PM	21	43	139 (15-1257)	55	*55 (28-76)
AM	18	39	88 (9-528)	44	*46 (23-73)

* Differs significantly from controls.

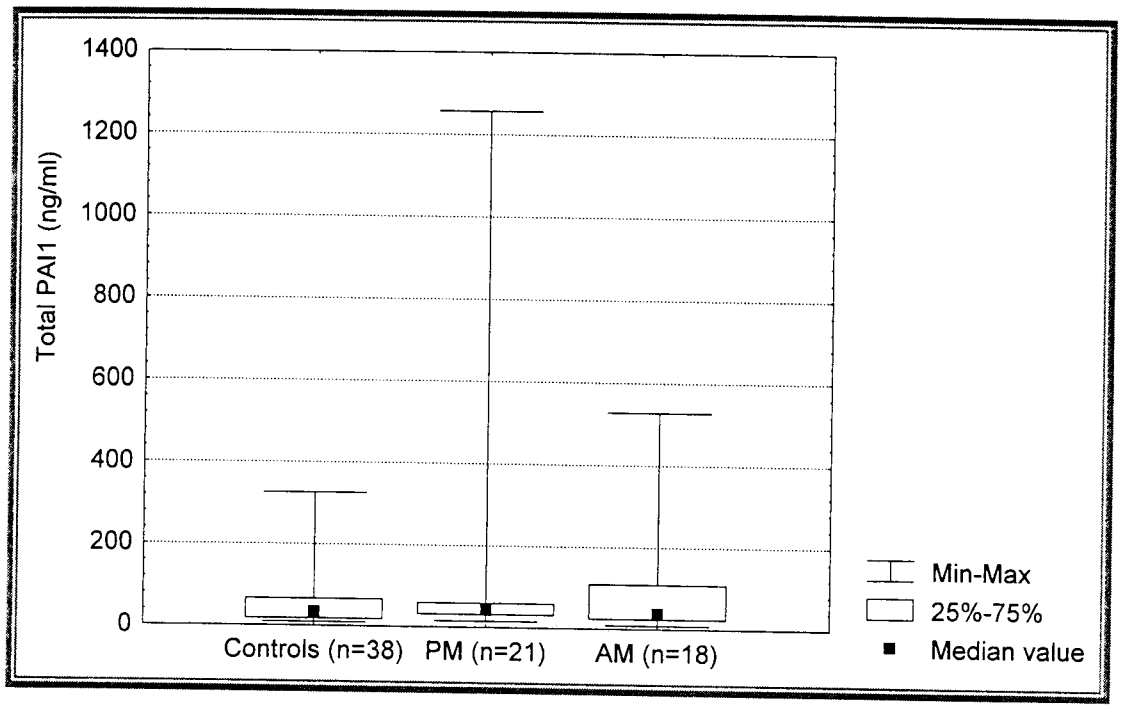


Figure 7.2. Box plots of total PAI1 in controls versus patients with PM and AM. The boxes encompass the 25th to 75th percentiles of the data with the median values (34, 43 and 39 ng/ml respectively) as the solid squares within the boxes.

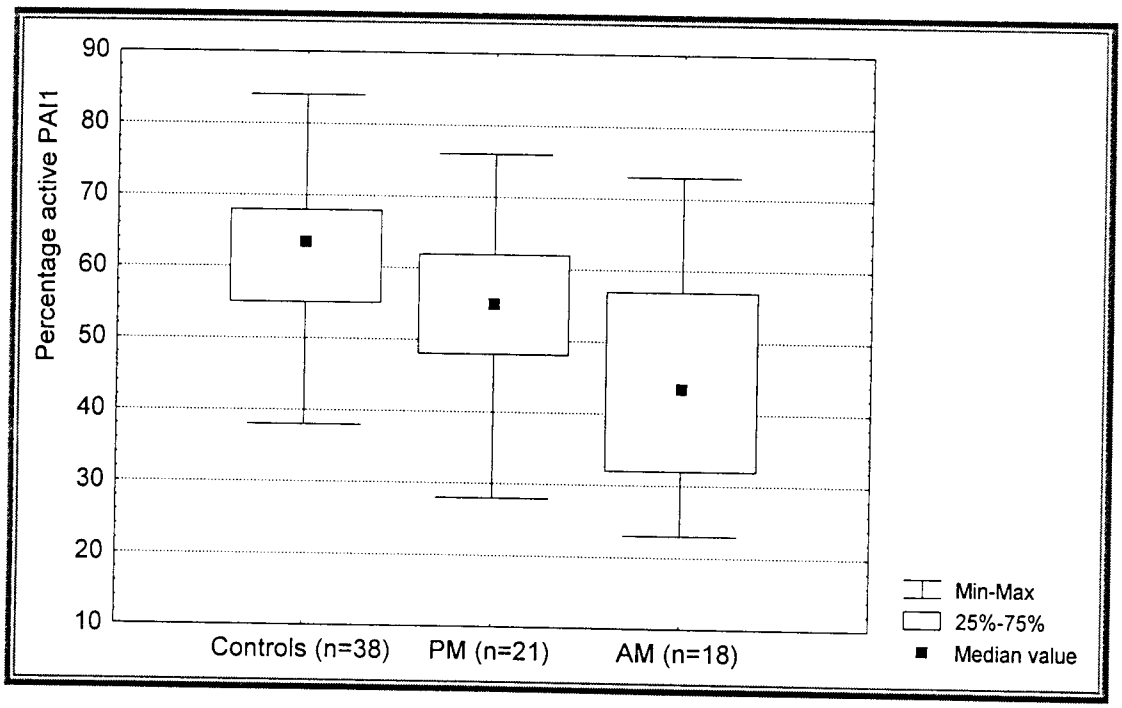


Figure 7.3. Box plots of percentage active PAI1 in controls versus patients with PM and AM. The boxes encompass the 25th to 75th percentiles of the data with the median values (64, 55 and 44% respectively) as the solid squares within the boxes.

7.3 Linear discriminant function analysis

The data were analysed according to the linear discriminant function in order to determine a clinically meaningful value for the percentage active PAI1 in plasma. The centroid values for the controls, PM and AM patients were 0.4820, -0.1378 and –0.8787 respectively. The midpoints between these centroid values were substituted into the linear discriminant function equation (0.0818 x % active PAI1 – 4.6106) to obtain the relevant cut off values. The cut off values for the controls was greater than 61% active PAI1, for the PM patients it was between 44 and 61% and for the AM patients it was less than 44%. A value of less than 44% active PAI1 was considered to be clinically meaningful. The data are summarised in Table 7.2.

For the controls, 55.3% (21/38) had active plasmatic PAI1 values greater than 61%, 42.1% (16/38) had values between 44 and 61% whereas only 2.6% (1/38) had values smaller than 44%. This represents a false positive rate of less than 3%. For patients with PM, there was a shift in the active fraction of plasmatic PAI1 predominantly (52.4%) towards the 44 to 61% range. For patients with AM, there was a shift in the active fraction of plasmatic PAI1 predominantly (50%) towards the less than 44% range. Overall, 2.6% (1/38) of the controls, 19% (4/21) of the patients with PM and 50% (9/18) of the patients with AM had values less than 44% active PAI1.

Table 7.2. Summary of the linear discriminant function analysis.

Groups	Percentage active PAI1			
	> 61	44 ≥ ≤ 61	< 44	Total
CC	21 (55.3)*	16 (42.1)	1 (2.6)	38
PM	6 (28.6)	11 (52.4)	4 (19.0)	21
AM	4 (22.2)	5 (27.8)	9 (50.0)	18
Total	31	32	14	77

* Percentage of the total in brackets.

CHAPTER 8: DISCUSSION

PAI1 is considered to be the main regulator of fibrinolytic activity in blood (reviewed by Loskutoff *et al.*, 1989). Elevated plasma levels and/or activity of PAI1 have been implicated in a large number of disease conditions. These include, an increased risk for cardiovascular diseases (reviewed by Vaughan, 1998), obesity and insulin resistance (Juhan-Vague & Alessi, 1997), acute inflammatory syndrome (D' Angelo *et al.*, 1985), survival in cancer patients (Ho *et al.*, 1998; Nielsen *et al.*, 1998), aging (Aillaud *et al.*, 1986), pregnancy (Kruithof *et al.*, 1987), rheumatoid arthritis (Kopeikina *et al.*, 1997), pancreatitis and liver diseases (Juhan-Vague *et al.*, 1984). It has been suggested that PAI1 is an acute-phase reactant since plasmatic PAI1 levels frequently increases after major surgery (Aillaud *et al.*, 1985), in response to acute myocardial infarction (Almer & Ohlin, 1987) and with severe trauma (Kluft *et al.*, 1985). Deficiency of PAI1 is a rare phenomenon associated with significant clinical bleeding. Only a single patient with complete deficiency of PAI1 has been reported and is associated with life-threatening haemorrhage (reviewed by Eitzman & Ginsburg, 1997).

Due to this wide clinical relevance of PAI1, a number of anti-PAI1 Mabs have been developed for measuring it in plasma (Declerck *et al.*, 1988a; MacGregor & Booth, 1988; Nielsen *et al.*, 1986). However, at least four molecular forms (active, latent, substrate and cleaved) of PAI1 can occur in blood (reviewed by Pappot *et al.*, 1995) which makes it difficult to obtain an accurate measure of the total molecular concentration of plasmatic PAI1. Of the several immunoassays that have been developed to measure plasmatic PAI1, the *Innotest* PAI1 antigen assay appears to be the only assay which can detect all molecular forms of plasmatic PAI1 to a similar degree (Meijer *et al.*, 1994). By convention, PAI activity is measured with functional assays which are based on the neutralisation of tPA added to samples and measurement of residual tPA activity on fibrin plates (Chmielewska & Wiman, 1986) or with spectrophotometric methods (Eriksson *et al.*, 1988). A Mab-based immunofunctional assay, based on the measurement of PAI1/tPA complexes, has been described for human PAI1 (Declerck *et al.*, 1988b) and recently for rat PAI1 (Ngo *et al.*, 1998). To the best knowledge of the investigator these are the only two alternative assays for PAI activity. However, there are several limitations to these functional assays: For example, interference by competing protease inhibitors, the non-parallelism of calibration curves in buffer and in plasma and the fact that functional assays cannot distinguish between the different PAIs. These factors have undoubtedly contributed significantly to the variability in results reported between different laboratories (Samama *et al.*, 1985; Speiser *et al.*, 1986). To overcome these limitations, a novel Mab-based immunofunctional assay specific for PAI1 activity was developed.

This novel immunoassay allows the user to quantitate the total plasmatic PAI1 level (latent plus active) as well as the active fraction of the inhibitor. The latter is obtained by subtracting the

amount of latent PAI1 (quantified with mαPAI1/C6) from the amount of total PAI1 (quantified with mαPAI1/C11). A possible way to explain the principle of the ELISA is illustrated in Figure 8.1. A RαPAI1 Pab is used to capture latent and active PAI1 from the biological sample on a microtiter plate. The mαPAI1/C11 would allow the quantitation of latent and active PAI1 since the recognisable epitope (▲) is available for binding with both molecular forms captured on the plate. The results show that this Mab detects both molecular forms with an equal affinity across the dilution range of the calibration curve (see Figure 7.1). The mαPAI1/C6 would allow the quantitation of only latent PAI1 since the recognisable epitope (◗) would only be available for binding with the latent molecule captured on the plate. The epitope is most likely hidden when the active molecule, due to its conformational change, is captured on the plate. The fact that mαPAI1/C11 detects both molecular forms with an equal affinity across the dilution range of the calibration curve is important, since it will ensure an accurate estimation of the active fraction of PAI1 after subtracting the quantity for latent PAI1 obtained with mαPAI1/C6. It is also important to note that although mαPAI1/C6 detects only latent PAI1 on the plate, it does in fact bind to active PAI1 in the fluid phase since it inhibits the activity of PAI1 in the ^{125}I-fibrin plate assay (see Section C, 11.10 and Figure 11.9 for details).

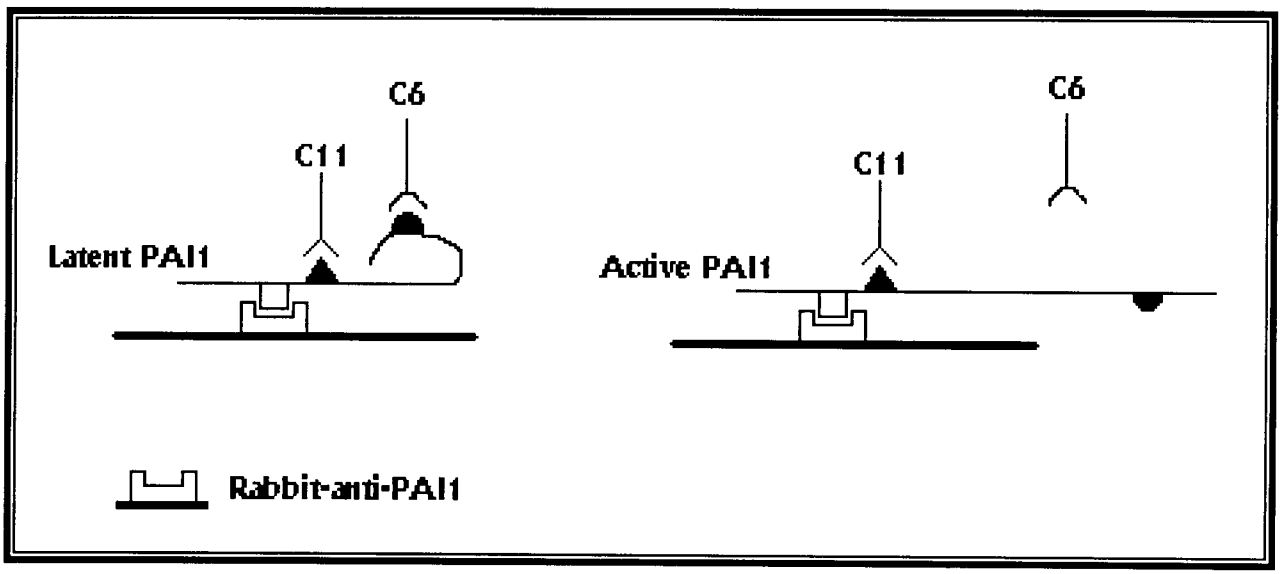


Figure 8.1. Hypothetical principle of the PAI1 ELISA.

Besides the free latent and active PAI1 found in blood, it is also present in complex with tPA, uPA and VN (reviewed by Loskutoff *et al.*, 1989). In terms of the total molecular concentration of PAI1, it would be of value to determine whether this novel assay is also able to detect the above mentioned complexes of PAI1. However, no definite investigations were undertaken to determine this. In spite of this, there is some peripheral evidence that suggests that mαPAI1/C11 is most likely also able to quantitate PA/PAI1 complexes. For example, the western blotting results

presented in section C of this thesis (see 11.5 and Figure 11.4) reveals two bands (lane 5) which might correspond to tPA/PAI1 and uPA/PAI1 complexes. Additional support was obtained from a modified PAI1 ELISA procedure that was carried out as follows: tPA/PAI1 complexes were generated and captured on a microtiter plate with the RαPAI1 Pab. Both mαPAI1/C11 and a mouse anti-tPA Mab recognised the tPA/PAI1 complexes on the plate (data not shown). However, these experiments do not provide conclusive evidence that the assay can detect PA/PAI1 complexes since the presence of free PAI1 could not be ruled out in the latter experiment. There is also some peripheral evidence to suggest that mαPAI1/C11 is most likely also able to quantitate VN/PAI1 complexes. For example, the results presented in section C of this thesis (see 11.9 and Figure 11.8) show that the Mab can detect VN/PAI1 complexes bound on a microtiter plate. Since mαPAI1/C6 blocks the binding of PAI1 to VN, it may be assumed that this Mab is not able to detect VN/PAI1 complexes in this assay. Overall, it can be concluded that mαPAI1/C11 can measure free latent and active PAI1 and most likely also PA/PAI1 and VN/PAI1 complexes. In contrast, mαPAI1/C6 seems to measure only free latent PAI1. However, future investigations will address these aspects more thoroughly.

In order to measure plasmatic PAI1, it is essential to remove the platelet-fraction which constitutes 92% of the blood PAI1 (Macy *et al.*, 1993). Failure to remove the platelets will result in a significant rise in plasmatic PAI1 and consequent false values. Until now investigators have made use of centrifugation to remove the platelet fraction from the blood. A minimum centrifugation speed of 30 000 g has been reported to be essential in removing the platelets effectively from the blood (Macy *et al.*, 1993). In order to simplify the technique, additional options were explored. It was found that membrane (1.0 μm followed by 0.45 μm) filtration is an effective alternative to remove the platelets, which are approximately 2-4 μm in diameter (Hutton, 1989), from plasma. Three control samples were analysed before and after cryopreservation for total PAI1 to test the efficiency of the filtering procedure (results not shown). The rationale being that freezing and thawing of the sample will release any platelet-PAI1 due to platelet lysis (Macy *et al.*, 1993). Since there were no differences in the PAI1 levels of the samples before and after cryopreservation, it was concluded that the filtering procedure was effective in removing the platelets from the plasma.

In order to verify the reliability of the PAI1 antigen levels obtained for the controls with this novel PAI1 assay, data from other investigators were compared with the findings from this study. Most investigators have reported a wide variation in the PAI1 antigen levels for normal human plasma which varied from nearly undetectable levels to values greater than 200 ng/ml (reviewed by Vaughan, 1998). The majority of normal individuals have PAI1 antigen levels that vary between 0 and 60 ng/ml, with a mean level around 20 ng/ml (Declerck *et al.*, 1988a). In this study, the mean concentration of total PAI1 for the controls was 57 ±66 ng/ml (mean age of 41 years). The median value, which is more representative of the normal range than the mean, was 34 ng/ml. When

population and/or assay differences (eg. calibration curves for PAI1) are taken into account, it may be concluded that the mean, median and range for plasmatic PAI1 obtained with this novel assay is reliable and is a true reflection of the normal population studied. There were only two outliers (greater than 200 ng/ml) amongst the 38 controls (see A.58, patient numbers 15 and 28). To ensure that these high PAI1 levels were not due to technical errors, one of the patients (number 28) was tested three times. Since approximately the same PAI1 value was obtained with all three samples, it could be concluded that the elevated PAI1 levels of these two control individuals is not due to experimental error or artefacts but rather due to some unknown pathological condition.

In order to verify the ability of the assay to quantitate the active fraction of PAI1 in plasma, data from other investigators were compared with the findings from this study. Previous studies have shown that approximately two thirds of plasmatic PAI1 is in the active conformation (Booth *et al.*, 1988; Declerck *et al.*, 1988a). In this study, a mean value of 62 ±10.5% for the active fraction of PAI1 was observed for the controls (median was 64%). Thus, it was concluded that the values obtained with this novel assay are reliable and is representative of the active fraction of plasmatic PAI1 for a normal population. It was important to ensure that the blood collection and storing procedures used in this study would not contribute to artefacts in determining the active fraction of plasmatic PAI1. To test this, three control samples were collected as described in *Materials and Methods* (6.4) and were analysed after various time points (0–3 hours). There were no differences in the active fraction of PAI1 between the different time points when samples were stored at 4°C (results not shown). Thus, it was concluded that processing the patient samples within 1–2 hours of blood collection provided reliable and reproducible measurements.

The primary aim of this study was to determine whether high or low plasma levels and/or activity of PAI1 correlates with the presence of metastatic disease in patients with melanoma. It is important to note that all comparative analyses excluded gender as a parameter, since there was no statistical difference in plasmatic PAI1 antigen or activity levels between male and female control subjects (results not shown). The results of this study did not reveal a statistical difference in total plasmatic PAI1 levels between the controls and patients with PM or AM (see Figure 7.2). In order to interpret these results, it is important to note that a number of factors have been shown to stimulate PAI1 synthesis by endothelial cells (reviewed by Loskutoff *et al.*, 1989). These include growth factors like transforming growth factor beta (TGFβ), basic fibroblast growth factor (bFGF), interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα). The involvement of steroids in stimulating PAI1 biosynthesis have also been described. It could therefore be concluded that the above mentioned factors that are known to upregulate PAI1 biosynthesis are unlikely to contribute significantly to an alteration in the fibrinolytic balance of the blood for the patients in this study. In contrast to the findings of this study, a rise in the plasmatic PAI1 level has been reported previously for patients with advanced ovarian (Casslen *et al.*, 1994), colorectal (Nielsen *et al.*,

1998) and gastric cancer (Ho *et al.*, 1998) and a rise in plasmatic PAI activity (total inhibitory activity) has also been described for patients with advanced breast cancer (von Tempelhoff *et al.*, 1997). However, at this stage it is unclear why the plasmatic PAI1 levels for patients with AM was not significantly elevated compared to the controls, as reported for the above mentioned carcinomas. It could be that elevated plasmatic PAI1 levels is a late event in the disease progression and that it is strongly linked to liver metastases (Leiper *et al.*, 1994). The data of this study supports this theory, since none of the eight stage IV patients had any clinical evidence of liver metastases.

Although there was no difference in plasmatic PAI1 levels between the controls and patients with PM or AM, there was a significant difference (P = 0.0042) in the percentage active PAI1 (see Figure 7.3). The data for the active fraction of plasmatic PAI1 was subsequently analysed by the linear discriminant function to determine a clinically meaningful value for the percentage active PAI1. A cut off value of less than 44% active PAI1 was obtained: This means, that a melanoma patient with a plasmatic PAI1 activity value of less than 44% will have a 50% chance of harbouring metastases (see Table 7.2). A false positive rate of 2.6% was obtained with this analysis. Only 4 out of 18 (22.2%) patients with AM had PAI1 activity values greater than 61% and another five (27.8%) had values between 44 and 61%. This may indicate a more stabilised or less aggressive disease amongst the other 50% of the patients with AM. Of the nine patients with AM having a PAI1 activity value of less than 44%, 6/10 were stage III and 4/8 were stage IV patients. There were four (19%) patients with PM having PAI1 activity values of less than 44%. Of these four patients, 1/7 were stage I and 3/14 were stage II patients. It is therefore possible that the plasmatic PAI1 activity level could be predictive of metastatic disease for patients with PM. A follow-up period of three to five years should be sufficient to determine whether these four patients will ultimately develop metastases (Delaunay, 1992). The active fraction of PAI1 for patients with PM differed also significantly (although borderline, P = 0.0488) from the controls. The data therefore strongly supports further investigations to determine whether plasmatic PAI1 activity levels might be predictive of metastatic disease.

In conclusion, in this study it was found that the plasmatic PAI1 levels for patients with AM was not significantly elevated compared to the controls, but a significant reduction in the active fraction of plasmatic PAI1 was found. This last section of the discussion attempts to explain the biological basis of this reduction of the active fraction of PAI1 in PM and AM patients. It is possible that certain individuals with PM are genetically predisposed to develop metastases. Lower levels of PAI1 activity would provide a biological basis for such a predisposition. However, if this was the case then one would predict that in a normal population one would find some patients with levels of PAI1 activity as low as those patients with AM. The data of this study does not support this theory: The lowest percentage active PAI1 for the controls was 38% (which is an outlier). Seven of the

nine patient with AM (see A.60) had percentage active PAI1 below 38%. This means that there is no overlap between the lowest range of the controls and the lowest range of patients with AM (see Figure 7.3). Thus, from this it can be deduced that there is not a genetic predisposition for lower levels of PAI1 activity in the normal population. Instead, it suggests a causative link between the development of metastases and a reduction in the active fraction of plasmatic PAI1.

There are a number of factors that have the potential to influence the overall fibrinolytic activity through their effects on PAI1 (reviewed by Edelberg *et al.*, 1994; Loskutoff *et al.*, 1989) and these are summarized in Figure 8.2. The first group of factors includes molecules, like vitronectin (VN), that stabilize plasma PAI1 against spontaneous inactivation. The second group of factors includes a number of agents that may inactivate PAI1 directly, including oxidants liberated by activated inflammatory cells and proteases. There are two classes of proteases that have the potential to inactivate PAI1: Those that inactivate it through the formation of an enzyme-inhibitor complex, for example PAs and activated protein C and those that directly cleave PAI1 as a substrate, for example thrombin and neutrophil elastase. The third group of factors includes molecules that may convert latent PAI1 into its active conformation, for example negatively charged phospholipids on cell surfaces (eg. platelets) that could provide the site for activation of latent PAI1. This is unlikely, since in this study a reduction in the active fraction of PAI1 was observed and not an increase.

The reduction in the active fraction of plasmatic PAI1 in patients with AM could possibly be explained in the light of the above mentioned information. The first possibility is that there could be a functional defect with VN or factors that might interfere with the binding of VN to PAI1. This would ultimately affect PAI1 activity since VN is known to stabilize the activity of PAI1. It has been shown that heparin (an endothelial matrix component like VN) can block the interaction between VN and PAI1 and that this competition between heparin and VN may decrease the overall PAI1 inhibitory capacity due to displacement of the inhibitor from VN (Preissner *et al.*, 1990). It is possible that elevated proteolytic activity in the blood could cause a release of endothelial matrix-bound heparin into the blood. The elevated heparin levels may compete with VN for binding to PAI1, thus destabilizing the activity of PAI1. Plasmin, in turn, could proteolytically digest VN, destroying its PAI1- and heparin-binding capacity (Sane *et al.*, 1991).

The second option, that is, that there are agents that may inactivate PAI1, seems to be an equally possible option. Tissue PA is the most likely candidate from all the proteases mentioned, since it is commonly upregulated in melanoma tumors (Hoal-Van Helden *et al.*, 1986) and it plays a key role in the metastasis of melanoma (reviewed by de Vries *et al.*, 1996). It is possible that the presence of melanoma metastases (regional or systemic) could result in elevated plasmatic tPA antigen and/or activity levels. Since PAI1 is known to be the main regulator of fibrinolytic activity in blood, it

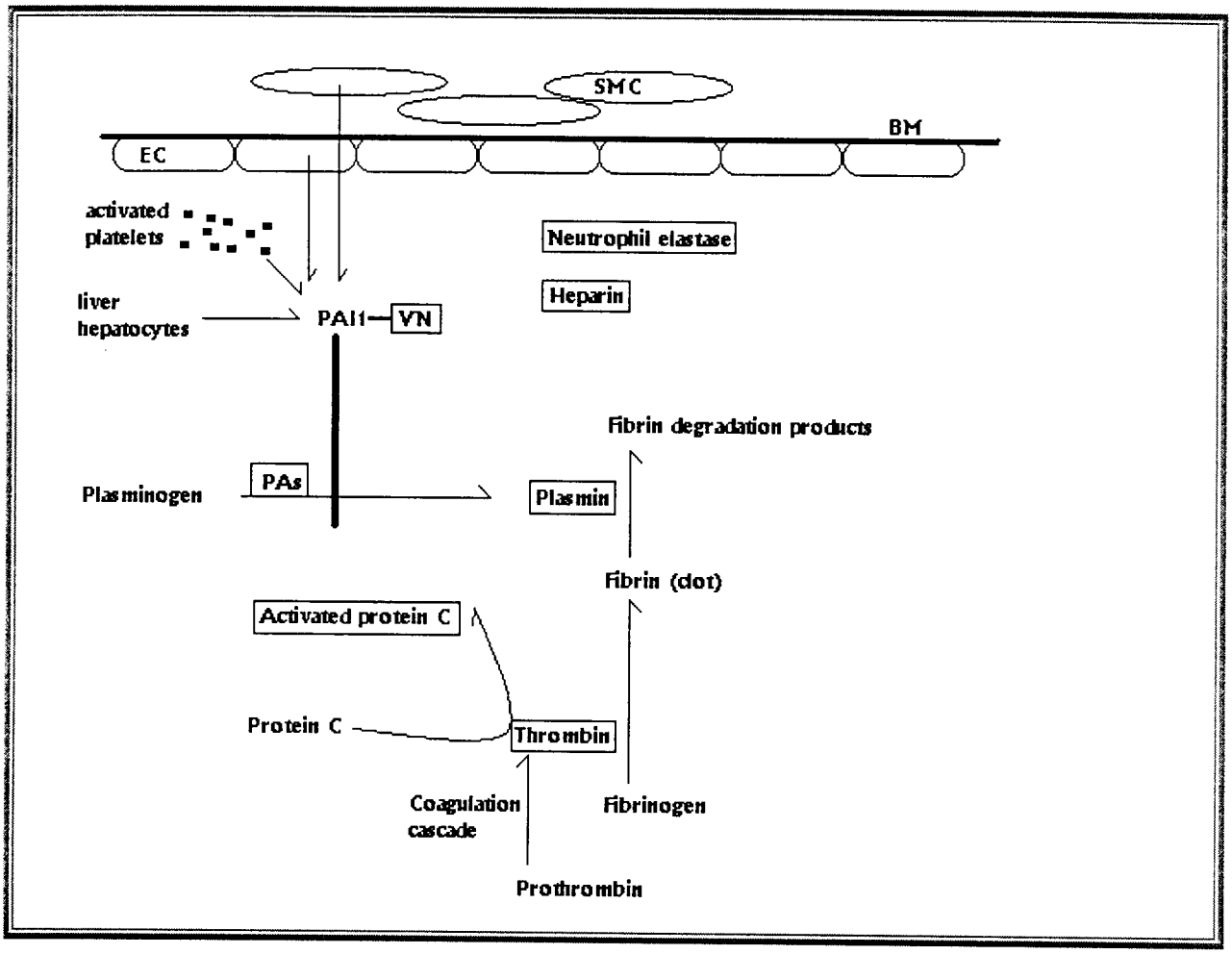


Figure 8.2. A model of the factors involve in the regulation of PAI1 activity.
EC, endothelial cell; **SMC**, vascular smooth muscle cell; **BM**, basement membrane; **PAs**, plasminogen activators; **PAI1**, plasminogen activator inhibitor type 1; **VN**, vitronectin. The factors that might play a role in the modulation of PAI1 activity have been boxed.

can be speculated that the tumor-derived tPA will bind to the free or VN-bound active plasmatic PAI1. This could result in a reduction of the active fraction of plasmatic PAI1 due to tPA/PAI1 complex formation. Another candidate could be tumor-derived neutrophil elastase which has been shown to inactivate PAI1 by proteolytic cleavage (Urano *et al.*, 1996; Wu *et al.*, 1995). Similarly, thrombin has been shown to efficiently inactivate PAI1 in the presence of either VN or heparin, unless a sufficient excess of the inhibitor is present (van Meijer *et al.*, 1997a; van Meijer *et al.*, 1997b). Thrombin, in turn, can activate protein C which in turn can inactivate PAI1 by complex formation (Sakata *et al.*, 1985). In addition, PAI1 may also bind to fibrin to inhibit tPA-mediated fibrin dissolution (Reilly & Hutzelmann, 1992). However, the role of the fibrin clot in altering the specificity of PAI1 is poorly understood. The end effect of the above mentioned proteases will be an increase in the overall fibrinolytic activity since more plasmin is generated. It should be possible to test these hypotheses by monitoring the antigen and/or activity levels of these candidate factors.

In summary, this study shows for the first time that there is a reduction in the active fraction of plasmatic PAI1 in patients with AM and that a value of less than 44% seems to be indicative of

metastatic melanoma. Furthermore, it is speculated that this reduction might be attributed to tumor-derived tPA and/or other melanoma-derived proteases or factors which may disturb the fibrinolytic balance of the blood and which might reflect the progression of the disease towards a more severe condition. As mentioned earlier, it is possible that elevated plasmatic PAI1 levels could be a late event in melanoma progression and that this is strongly linked to liver metastases. In contrast, it seems that a reduction in the active fraction of plasmatic PAI1 could be an early event in melanoma progression that is related to an alteration of the fibrinolytic balance brought about by the proliferation and metastasis of melanoma. It is generally accepted that the ability of serum markers to predict metastatic disease is dependent on a critical minimum tumor mass. It would therefore be of enormous value to determine to what extent the active fraction of plasmatic PAI1 could be used to monitor the progression of primary and/or metastatic melanoma.

FUTURE CONSIDERATIONS

There are several technical issues that need to be addressed before larger clinical studies are pursued. Immunoassays that make use of Pabs are often troubled with calibration problems and suffer from a lack of reproducibility and availability of reagents. It would therefore be imperative to develop a mouse or rat Mab to PAI1, which could replace the rabbit anti-PAI1 Pab that was used as a capture antibody in this study. This would allow the development of a standardised assay that could be used by other investigators. Furthermore, it would be advisable to calibrate the rPAI1 standard by amino acid analyses or to obtain an international standard for PAI1. Another aspect to consider is to make use of calibration curves that were constructed in PAI1-depleted, pooled normal human plasma (Declerck *et al.*, 1988a). This would ensure more accurate PAI1 measurements since the plasma components might interfere with the linearity of the calibration curves when plasma dilutions smaller than one in ten are used. Special attention should be given to determine the ability of the assay to measure with accuracy the active fraction of PAI1 in plasma.

Once these technical issues have been addressed thoroughly, a larger number of melanoma patients with primary and advanced disease should be studied. It would be interesting to follow-up patients with AM who have plasmatic PAI1 activity values less than 44%. Will the prognosis of these patients be worse? Conversely, will the prognosis of patients diagnosed with regional or systemic metastases and with normal plasmatic PAI1 activity values be better? Moreover, could plasmatic PAI1 activity be used as a predictor of metastasis for patients diagnosed with PM? Long follow-up periods (3-5 years) would be necessary to determine whether patients with PM that develop plasmatic PAI1 activity values less than 44%, would be indicative of metastatic progression. Finally, factors that might cause false positives (eg. medication) is another important aspect to investigate.

SECTION C

CHAPTER 9: THE DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1

9.1 Introduction

Tumor cell invasion and metastasis depends on the ability of tumor cells to degrade and to migrate through connective tissue barriers by modulating cell-to-cell or cell-to-matrix adhesive interactions (reviewed by Andreasen *et al.*, 1997; Chen, 1992; Mignatti & Rifkin, 1993). The plasminogen-plasmin system (reviewed by Kwaan, 1992; Plow *et al.*, 1995) is prominently involved in these processes. The activation of plasminogen (Plg) yields the important extracellular proteinase, plasmin, which may degrade specific extracellular matrix (ECM) components directly or indirectly by the activation of metalloproteinases and growth factors (reviewed by Keski-Oja *et al.*, 1991; Mignatti & Rifkin, 1993). The generation of plasmin is strictly controlled by the action of the plasminogen activators (PAs). Of the two types, tissue plasminogen activator (tPA) has a predominant fibrinolytic function (Edelberg *et al.*, 1991), while urokinase (uPA) is believed to be principally responsible for extracellular proteolysis (reviewed by Blasi, 1993). The activity of the PAs is regulated at the transcriptional level by hormones, cytokines and growth factors (reviewed by Keski-Oja *et al.*, 1991; Laiho & Keski-Oja, 1989), at the processing level by plasmin, which converts the single-chain form to the proteolytically active two-chain form (de Serrano & Castellino, 1990) and at the functional level by inhibitors (Potempa *et al.*, 1994), of which PAI1 is considered to be the most important (reviewed by Loskutoff *et al.*, 1989; Reilly *et al.*, 1994). Plg and uPA bind to specific receptors and thereby direct plasmin activity to the migrating tumor cell surface (reviewed by Andreasen *et al.*, 1997; Bu *et al.*, 1994; Plow *et al.*, 1995).

Active two-chain uPA and its single-chain pro-enzyme bind with high specificity to the glycosylphosphatidylinositol (GPI)-anchored cell surface receptor, uPAR (Ploug *et al.*, 1991). This interaction alters the conformation of the uPAR (Ploug *et al.*, 1994), thereby increasing its affinity for the somatomedin B (SMB) domain (Seiffert *et al.*, 1994) of vitronectin (VN) in the ECM. The induction of the uPAR promotes cell adhesion through its interaction with VN and facilitates cell migration and invasion by localizing uPA to the cell surface. Active PAI1 binds with a higher affinity to the SMB domain of VN (Deng *et al.*, 1996b) than uPAR and competes with the receptor for binding to SMB (Deng *et al.*, 1996a). This interaction detaches U937 myeloid cells from their VN substratum and appears to occur independently of its ability to function as a protease inhibitor (Waltz *et al.*, 1997). An excess uPA will reverse the anti-adhesive effect of PAI1. Stahl and Mueller (1997) have demonstrated that PAI1 can stimulate M24met melanoma cell migration on

VN, presumably by inhibiting uPA/uPAR-mediated cell adhesion to VN and thereby releasing the inhibition of cell migration induced by uPA (Stahl & Mueller, 1994; Stahl & Mueller, 1997).

Solid tumors are typically composed of transformed cancer cells which display some form of genetic instability and normal stromal cells (eg. fibroblasts, inflammatory and endothelial cells) which are thought to be genetically stable but which may become activated during tumor progression to produce proteases, inhibitors and other regulatory factors (reviewed by Edwards & Murphy, 1998; Hanahan, 1998; Lochter *et al.*, 1997). In several human cancers only stromal cells produce PAI1 (eg. colon adenocarcinomas; Pyke *et al.*, 1991) while in others only cancer cells make PAI1 (eg. squamous cell carcinomas of the skin; Sappino *et al.*, 1991). The presence of ECM-associated PAI1 inside the tumor could serve to protect neovascularized tissues from the uPA-mediated proteolysis the tumor imposes on itself. PAI1 might also be of importance for reimplantion of circulating tumor cells, since formation of new stroma at the metastatic site would require the blocking of uPA-mediated degradation of ECM-components (Pappot *et al.*, 1995).

The uPA/uPAR/Plg system may provide surface-associated protease activity to promote tumor cell invasion and simultaneously promote tumor cell adhesion through the interaction of cell-bound uPA with matrix-bound VN (reviewed by Blasi, 1997). Considerable evidence indicates that PAI1 is the molecular switch that controls tumor cell adhesion and migration through the uPA/uPAR system (Deng *et al.*, 1996a). PAI1 acts as a cell detachment factor by competing with the uPAR for binding to VN (Stahl & Mueller, 1997; Waltz *et al.*, 1997). These findings may explain why increased tumor levels of PAI1 correlate with a poor prognosis (Allgayer *et al.*, 1997; Foekens *et al.*, 1994; Kim *et al.*, 1998; Knoop *et al.*, 1998; Sier *et al.*, 1994) and why PAI1-deficient mice show reduced invasion and vascularization of malignant keratinocytes compared with wild-type mice (Bajou *et al.*, 1998).

9.2 Specific aim of study

The specific aim of this study was to develop Mabs against a variety of diverse epitopes of PAI1 in an attempt to obtain useful reagents to study the role of PAI1 in the metastasis and vascularization of human melanoma. Such antibodies would for example, be useful reagents for studying the interaction of PAI1 with ECM and PA components, for blocking its activity in tissue culture experiments, for immunoblotting, for immunofluorescence or immunocytochemistry and for the measurement of PAI1 in biological fluids. In a previous attempt to produce murine Mabs against *Escherichia coli*-derived rPAI1, it was found that the compound was a poor immunogen (C. Heussen-Schemmer: personal communication). This was possibly due to inappropriate or inadequate post-translational modification of the molecule. However, it was then decided to investigate the baculovirus expression system (Webb & Summers, 1990) as a means of expressing PAI1 in insect

cells and to follow then the recommendations of de Boer *et al.* (1992) using the crude infected cell population as the immunogen (de Boer *et al.*, 1992).

This section of the thesis describes the success that was obtained with this approach and the characteristics of the Mabs that it provided. During the course of this study it became clear to the investigator that studies concerning the role of PAI1 in tumor cell invasion and vascularization will fall outside the main theme of this thesis. Therefore, the potential value of these Mabs in the study of metastasis was not pursued but will most likely form part of future studies. However, two of the Mabs that were obtained with this approach turned out to be extremely valuable and were used in the study described in section B of this thesis.

CHAPTER 10: MATERIALS AND METHODS

10.1 Cell lines and reagents

The complementary DNA (cDNA) for human PAI1 (clone PAIB6, 1.9 kb) was obtained as a gift from Dr. David Ginsburg from the Department of Internal Medicine and Human Genetics, Howard Hughes Medical Institute, Medical School of the University of Michigan. The pBlueBac-2 vector, wild-type *Autographa californica* multiple nuclear polyhedrosis virus (WT-AcMNPV) DNA and *Spodoptera frugiperda* insect cells (Sf9 strain) were purchased from Invitrogen Corporation (San Diego, CA). Sf9 cells were maintained as a monolayer in TNMFH medium supplemented with 10% FCS and 50 μg/ml gentamycin sulphate at 27°C under 100% air and 90% humidity (see A.15 – A.16 for details). Suspension cultures were grown under the same conditions in Erlenmeyer tissue culture flasks (Corning, Acton, MA) on a horizontal platform with orbital shaking.

The PAI1 ELISA kit was obtained from American Diagnostica Inc. (New York, NY). *Escherichia coli*-derived human recombinant PAI1 (rPAI1) in the latent form was obtained as a gift from Dr. E.J. Goldsmith from the Department of Biochemistry, University of Texas, South-West Medical Centre, Dallas. Latent rPAI1 was activated with 4M guanidine.HCl for 30 mins at 37°C and then dialysed extensively at 4°C against PBS (Hekman & Loskutoff, 1985). The activated PAI1 was stored at -80°C and thawed once for experiments. Recombinant tPA was a generous gift from Dr. O. Safriel, Technifin, South Africa. The enzyme was converted to the two-chain form by treatment with plasmin linked to Sepharose 4B (see A.56 for details). The SP-2 and PA-1 mouse myeloma cell lines and the HT1080 fibrosarcoma cell line were purchased from the American Type Culture Collection. The cells were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 20 μg/ml streptomycin at 37°C under 5% CO_2/95% air and 90% humidity (see A.20 for details).

10.2 Production of polyclonal antibodies to PAI1

The polyclonal antibodies to PAI1 were prepared by Dr. Christa Heussen-Schemmer previously from the Department of Clinical Science and Immunology at the University of Cape Town. Briefly, two New Zealand rabbits each received 100 μg *E. coli.*-derived rPAI1 in Complete Freund's Adjuvant (Difco) subcutaneously (s.c.) at several sites. Subsequent immunizations were performed on days 30, 38, 88 and 255 with 50 μg rPAI1 in Incomplete Freund's Adjuvant. High titre anti-sera were pooled and shown to be specific by ELISA and immunoblotting (results not shown). The antibodies were purified by caprylic acid and ammoniumsulphate (Reik *et al.*, 1987) precipitations (see A.33 for details).

10.3 Expression of PAI1 in insect cells

I am greatly indebted to Mrs. Debby Clarke who has carried out the cloning of the PAI1 cDNA into the baculovirus vector and subsequent plaque purifications. Briefly, the 1.46 kb coding sequence for the mature PAI1 protein (Ginsburg *et al.*, 1986) was removed from the vector pUC13 by digestion with EcoR-I and Bgl-II, blunt-ended and attached to Nhe-I linkers for insertion into the pBlueBac-2 vector (Figure 10.1). This construct was cloned and amplified in Top10f competent bacterial cells (after verification of the correct orientation by Pst-I and Bam-HI restriction analysis) and used to co-transfect Sf9 cells with WT-AcMNPV DNA (Figure 10.2). Recombinant plaques were selected for expression of beta-galactosidase after one round of plaque purification and screened by dot blotting with a digoxigenin-dUTP labelled PAI1 cDNA probe to verify stable insertion of PAI1. A positive plaque was subjected to four rounds of plaque purification until no occlusion bodies were visible and until the plaque was PCR-negative for WT virus contamination. PAI1 recombinant virus stock was prepared and the virus titre (plaque forming units per ml) was calculated. Monolayer and suspension cultures were infected with the PAI1 recombinant virus at a multiplicity of infection (MOI) of three. Aliquots of the conditioned medium (CM) were removed every 24 hours and quantitated for PAI1 antigen using a commercial PAI1 ELISA. PAI1 levels of more than 10 μg/ml was measured after 4 days in culture. Details of the procedures used are described elsewhere (Summers & Smith, 1987; Webb & Summers, 1990).

10.4 Monoclonal antibody production

The titre of the PAI1 recombinant virus stock preparation was determined by a standard protocol (see A.17 for details). A suspension culture of Sf9 cells (see A.15 for details) was infected with the purified PAI1 recombinant virus at a MOI of five (see A.18 for details). After 3 days in culture a sample of cells was removed and multi-well slides (Veenstra & Dowdle, 1992) were prepared for immunofluorescence (see A.45 for details) to confirm the expression of PAI1 using the anti-PAI1 Mab supplied by American Diagnostica Inc. as the primary antibody (approximately 10 μg/ml) and fluorescein-conjugated goat anti-mouse IgG (Cappel, 50 μg/ml) as the secondary antibody. Intraperitoneal (i.p.) injections of 4-6 x 10^6 PAI1 expressing Sf9 cells in PBS were used to immunize Balb/c female mice. Three immunizations were carried out at two week intervals. Two weeks after the second immunization, the sera (see A.19 for details) were tested for anti-PAI1 antibodies using a capture ELISA as described below under *Screening assay*. A final booster was given three days before the fusion. The spleen cells from an immune mouse were fused with SP2 mouse myeloma cells (see A.20-A.26 for details) according to published methods (Harlow & Lane, 1988; Pontecorvo, 1975). The resulting hybridomas were screened with the capture ELISA as described below under *Screening assay* (see 10.5) and cloned once by limiting dilution.

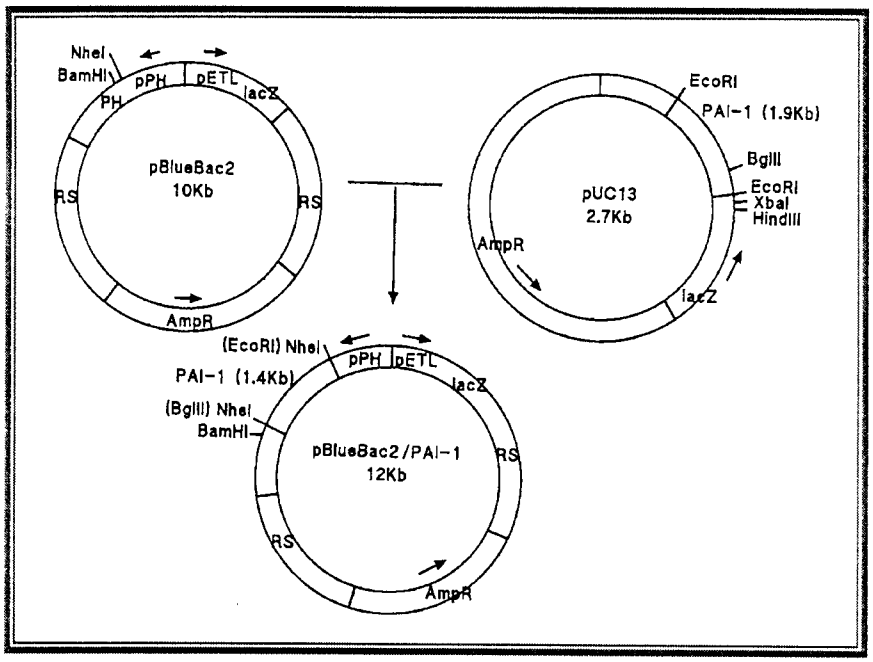


Figure 10.1. Construction of pBlueBac2/PAI1 vector. **AmpR,** ampicillin resistance; **lacZ,** beta-galactosidase; **RS,** recombination sequences; **pPH,** polyhedrin promotor; **pETL,** ETL promotor.

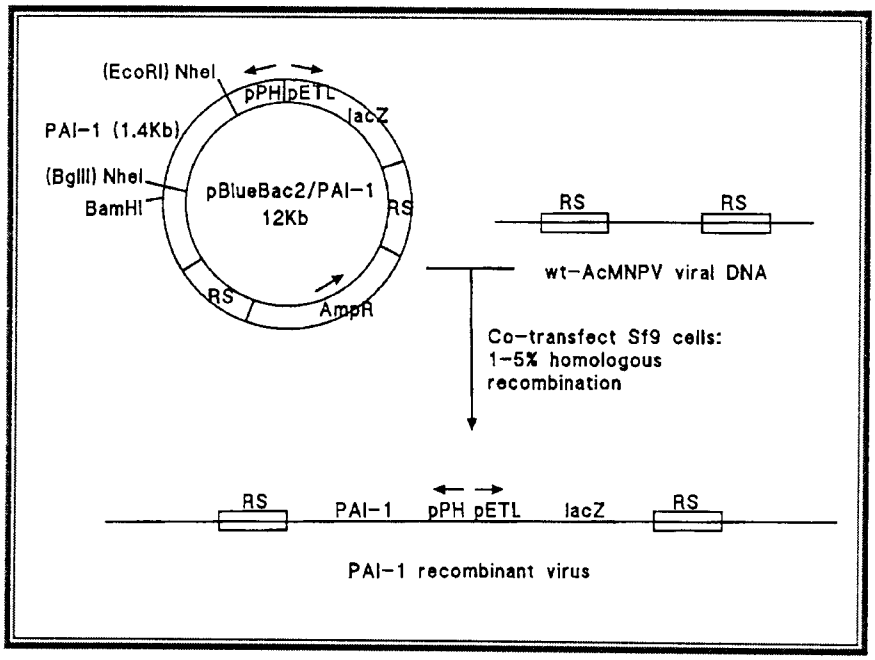


Figure 10.2. Construction of PAI1 recombinant baculovirus. **AmpR,** ampicillin resistance; **lacZ,** beta-galactosidase; **RS,** recombination sequences; **pPH,** polyhedrin promotor; **pETL,** ETL promotor.

10.5 Screening assay

Coating an antigen directly on an ELISA plate for screening purposes could result in a preferential orientation of the antigen on the plastic surface. Consequently, some epitopes of the antigen might be hidden and would not be available for antibody binding. The use of a polyclonal antibody will minimise this problem by capturing the antigen in different spatial orientations on the ELISA plate. A round-bottom polyvinylchloride (PVC) ELISA plate (Dynatech) was coated with rabbit anti-PAI1 (RαPAI1, immunoglobulin fraction) at 20 μg/ml in PBS for 2 hours at room temparature (RT). The reagents used by all the ELISA procedures were added to the plates at 50 μl per well (unless otherwise stated) and all steps were followed with five washes TST-buffer (see A.27 for details) except for the last wash which was done ten times. All incubation steps were for 30 mins at RT (except if mentioned differently). The plate was blocked with 0.1% bovine serum albumin (BSA) in PBS. This was followed by 1 μg/ml latent rPAI1 in blocking solution and then the antibody-containing hybridoma CM (100 μl/well) to duplicate wells. An anti-PAI1 Mab from American Diagnostica was included as a positive control. Mouse anti-beta galactosidase Mab (see A.28 for details), normal mouse serum and sheep serum were included as negative controls. Bound antibody was revealed with the peroxidase anti-peroxidase (PAP) method (De Blas & Cherwinski, 1983). The sheep anti-mouse IgG antibody (SAM) and the PAP were prepared (see A.28-A.29 for details) as described previously (Heussen-Schemmer *et al.*, 1993). 2,2-Azino-di (3-ethyl-benzthiazoline) sulphonic acid (ABTS) was used as a substrate (see A.30 for details). The absorbance at 405 nm was read after 15 mins on an Organon Teknika Microwell ELISA Plate Reader.

10.6 Additivity assay

The epitope specificity of 20 selected anti-PAI1 clones was determined by a standard additivity assay (Friguet *et al.*, 1985). Saturating amounts of each antibody alone, and in all possible paired combinations, were added to duplicate wells (50 μl each) of an ELISA plate prepared with RαPAI1 capture antibody and 1 μg/ml rPAI1. This concentration of PAI1 gave a 405 nm reading of approximately 1.0 after 15 mins of substrate addition. The total amount of bound antibody was determined by the PAP method as described under *Screening assay* (see 10.5). The additivity index (AI) for each combination of antibodies was calculated as

$[A_{1+2} - (A_1+A_2)/2] / [(A_1+A2)/2] \times 100$ where A_{1+2} = 50 μl of Mab1 + 50 μl of Mab2,

A_1 = 50 μl of Mab1 + 50 μl of blocking solution and A_2 = 50 μl of Mab2 + 50 μl blocking solution. Index values of greater than 40% were considered significant. Hybridoma clones with significant index values were selected and cloned once more by limiting dilution.

10.7 Isotyping and purification of antibodies

Serum-free hybridoma CM from the selected clones were used to determine the isotype of the antibodies by a standard ELISA procedure (see A.31 for details; Coligan *et al.*, 1994a). The Mabs

from the selected clones were purified from ascites fluid (see A.32 for details) or from concentrated serum- and protein-free hybridoma CM (Hybrimax, Sigma) by caprylic acid and ammonium sulphate precipitations (see A.33 for details; Reik *et al.*, 1987). The purity and the molecular weight of each antibody preparation was verified on a 10% polyacrylamide gel under reducing conditions (see A.34-A.41 for details; Laemmli, 1970).

10.8 Preparation of extra- and intracellular and matrix-associated PAI1

HT1080 cells (Laug *et al.*, 1992) were used to prepare PAI1 from different components of the cell culture for immunoblotting. The cells were plated at $5x10^5$ cells per 100 mm dish and were allowed to grow for 48 hrs. Serum-free 24-hour CM was collected in 0.1% BSA, filtered through a 0.45 μm syringe filter and stored at -80°C as a source of extracellular (secreted) native PAI1 (nPAI1). The cells were dislodged from the culture dish with versene and lysed for 30 mins on ice at $5x10^6$ cells/ml lysis buffer (PBS containing 0.5% NP-40, 1 mM phenylmethyl sulfonylfluoride [PMSF, Sigma], 2 mM EDTA). The cell nuclei were removed by centrifugation (700 g, 10 mins) and the supernatant was stored at -80°C as a source of intracellular nPAI1. The cell-matrix attached to the culture dish was washed with cold PBS containing 0.5% Triton X100, 1 mM PMSF and 2 mM EDTA for 5 mins at 4°C to lyse remaining cells. It was then rinsed twice with cold TST-buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.1% Tween-80) and solubilized with 500 μl 1x SDS sample buffer (see A.36 for details; Mimuro *et al.*, 1987). The viscous ECM preparation was stored at -80°C as a source of matrix-associated nPAI1.

10.9 Immunoblotting

PAI1-containing samples were electrophoresed (100 V/cm; 2 hours; 4°C) in a 10% polyacrylamide gel (Laemmli, 1970) without boiling the samples and under non-reducing conditions (see A.34 – A.41 for details). The separated proteins were electro-blotted onto Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore) using a standard electro-blotting procedure (see A.42–A.43 for details). A cocktail of the selected Mabs was used as the primary antibody (5 μg/ml each in PBS containing 0.5% BSA). A biotinylated rabbit anti-mouse Ig (Dako) was used as a secondary antibody and was followed with peroxidase-conjugated streptavidin (Dako). Luminol was used as a chemiluminescent substrate (Boehringer Mannheim) to detect peroxidase activity after a 30 second exposure to Cronex-4 X-ray film (see A.44 for details).

10.10 Immunofluorescence and immunocytochemistry

HT1080 cells were grown to semi-confluency on glass slides fitted with 8-well (7x7 mm) tissue culture chambers (Lab-Tek). The cells were washed with PBS, fixed with 50% methanol / 50% acetone for 90 secs and then transferred to TBS-buffer (50 mM Tris.HCl pH 7.6, 100 mM NaCl) for 5 mins. After blocking with 0.1% BSA in PBS, each well was incubated for one hour with a

selected Mab (20 ug/ml). The bound antibody was detected with a fluorescein-conjugated goat anti-mouse IgG (Cappel, diluted 1/400). Cell nuclei were counterstained with ethidium bromide and viewed under a fluorescent microscope using a blue excitation filter (see A.45-A.46 for details; Veenstra & Dowdle, 1992). For immunocytochemistry a similar protocol was used, with 19 parts methanol / 19 parts acetone / 1 part formaldehyde (40%) for 90 secs as the fixative and biotinylated rabbit anti-mouse IgG (Dako, diluted 1/500) as the secondary antibody. Bound biotin was detected with peroxidase-conjugated streptavidin (Dako, diluted 1/1000) and peroxidase activity with the insoluble chromogenic substrate diaminobenzidine (see A.47 for details). The nuclei were counterstained for 5 mins with 1% haematoxylin (in 100% ethanol) and the cells were examined microscopically for the presence of brown precipitate.

10.11 Matrix-associated PAI1 ELISA

A flat-bottom 96-well tissue culture plate (Cell-Cult) was seeded with $2x10^4$ HT1080 cells per well and allowed to grow for 48 hours. In the steps to follow, all reagents were carefully added with a Digital Multichannel Pipette (Labsystems for Flow Laboratories) and drained carefully by inverting the plate on an absorbent cloth. The plate was washed once with PBS before dislodging the cells with versene for 15 mins at 37°C. Residual cells on the cell matrix were removed by washing the plate with cold PBS containing 0.5% Triton X100, 1 mM PMSF and 2 mM EDTA. The plate was washed once with PBS and twice with TST-buffer and then blocked with 0.1% BSA in PBS. Serial ten-fold dilutions were made for each selected Mab, starting at 10 μg/ml and then incubated with the HT1080 cell matrices. The SAM, PAP and substrate were added as described under *Screening assay* (see 10.5). The absorbance at 405 nm was read after 20 mins.

10.12 Matrix-binding inhibition ELISA

In part 1 of this experiment, the results of the *Matrix-associated PAI1 ELISA* was mimicked, by substituting the endogenous matrix-accociated PAI1 with exogenous active rPAI1. The experimental design was essentially as described under *Matrix-associated PAI1 ELISA* (10.11). Before proceeding with the blocking, endogenous nPAI1 was depleted from the cell matrix with glycine-buffer pH 2.5 (200 μl/well) with two incubations at 37°C for 30 mins each (Mimuro *et al.*, 1987). The plate was washed once with glycine-buffer; twice with TST-buffer and blocked as before. Active rPAI1 was incubated at 2 μg/ml with the PAI1-depleted cell matrices. Serial two-fold dilutions for mαPAI1/C2, -C6 or -C14, starting at 20 μg/ml, were added to duplicate wells. The SAM, PAP and substrate were added as described under *Screening assay* (10.5). The absorbance at 405 nm was read after 20 mins. Negative controls were included for each Mab by substituting active rPAI1 with latent rPAI1.

In part 2 of this experiment, PAI1-depleted HT1080 cell matrices were prepared as described above. Active rPAI1 at 1 μg/ml was incubated in the fluid phase with serial two-fold dilutions of

mαPAI1/C2, -C6 or -C14, starting at 1000 μg/ml. The antibody-antigen complex (100 μl) was then incubated with the PAI1-depleted cell matrices. The wells were washed with TST-buffer and matrix-bound PAI1 was detected with mαPAI1/C2 or -C11 at 1 μg/ml. The SAM, PAP and substrate were added as described under *Screening assay* (10.5). The absorbance at 405 nm was read after 20 mins.

10.13 Vitronectin-binding inhibition ELISA

A flat-bottom 96-well ELISA plate (Nunc, MaxiSorb) was coated with 2 μg/ml human vitronectin (VN, Sigma) in PBS for 1 hr at RT and then blocked with 0.1% BSA in PBS. Active rPAI1 at 1 μg/ml was incubated in the fluid phase with serial two-fold dilutions of mαPAI1/C2, -C6 or -C14, starting at 100 μg/ml. The antibody-antigen complex (100 μl) was then incubated with the VN-coated wells. The wells were washed with TST-buffer and VN-bound PAI1 was detected with mαPAI1/C2 or -C11 at 1 μg/ml. The bound antibody was detected with goat anti-mouse IgG conjugated with alkaline phosphatase (Dako, diluted 1 in 2000). PNPP (100 μl per well) was used as a substrate for alkaline phosphatase (see A.57 for details). The plate was incubated for 1 hr at 37°C and the absorbance was read at 405 nm.

10.14 Activity inhibition assay

Fifty microliter active rPAI1 at 0.2 μg/ml (in RPMI medium supplemented with 3% plasminogen-depleted FCS) was incubated for 15 mins at RT with 50 μl 10-fold dilutions of each selected Mab, starting at 200 μg/ml. An anti-beta galactosidase Mab (IgG1) was included as a negative control. The antibody-antigen complex was incubated with an equal volume of 1 IU/ml 2c-tPA (see A.56 for details) for 30 mins at RT. The residual PA activity of this mixture was measured as the rate of plasminogen-dependent release of soluble radioactive fibrin degradation peptides from insoluble ^{125}Iodine-labelled fibrin adsorbed to Linbro multiwell plates (see A.48-A.53 for details; Wilson & Dowdle, 1978).

10.15 Recognition of latent and active PAI1 by ELISA

A 96-well polypropylene ELISA plate (MaxiSorb, Nunc) was coated with RαPAI1 at 50 μg/ml in PBS for 2 hrs at RT and then blocked with 0.1% BSA in PBS. This was followed by 100 ng/ml latent or active rPAI1 in blocking solution. The plate was washed with TST-buffer before adding mαPAI1/C2, -C6, -C11, -C12 and -C14 at 1 μg/ml. The bound antibody was detected with goat anti-mouse IgG conjugated with alkaline phosphatase (Dako, diluted 1 in 2000). PNPP (100 μl per well) was used as a substrate for alkaline phosphatase. The plate was incubated for 1 hr at 37°C and the absorbance was read at 405 nm.

CHAPTER 11: RESULTS AND DISCUSSION

11.1 The expression of rPAI1 in Sf9 insect cells

The cDNA for PAI1 was cloned into the pBlueBacII vector as was described in *Materials and Methods* (10.3) and placed under the control of the polyhedrin promotor. This results in the inactivation of the polyhedrin gene. Sf9 insect cells were then co-transfected with the vector and wild type baculovirus. This results in homologous recombination (in 1-5% of cases) between the recombination sequences of the vector and the virus. The resulting recombinant virus expressed human PAI1 instead of the polyhedrin protein. Putative recombinant plaques were selected by overlaying a monolayer cell culture with an agarose-medium containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) as a substrate for beta-galactosidase. PAI1 recombinant plaques expressed simultaneously beta-galactosidase through the ETL promotor (Figure 11.1.A and B).

11.2 Determination of PAI1 recombinant virus titre

In order to prepare an insect cell suspension culture expressing human PAI1 for immunization, it was first necessary to determine the titre of the recombinant virus preparation. A standard protocol was followed as described in *appendix 17*. The results are presented in Figure 11.2. In this example the virus titre (plaque forming units per ml) was calculated as $(1/10^{-6} \times 21 \times 1/2) = 1.05 \times 10^{7}$.

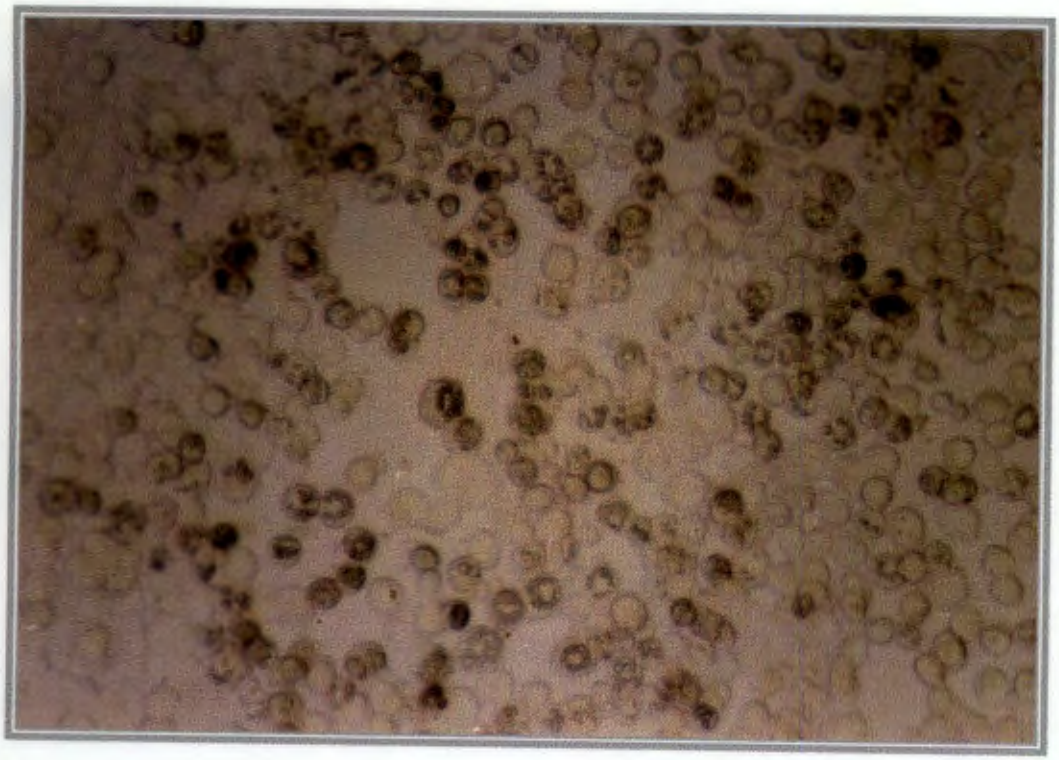
11.3 Confirmation of PAI1 synthesis by insect cells for immunization

Before proceeding with immunization, it was necessary to confirm the synthesis of PAI1 by insect cells. This was done with immunofluorescence using a commercial anti-PAI1 Mab (Figure 11.3). A strong immunofluorescent signal for PAI1 was detected in the cytoplasm of the infected cells. A suspension culture positive for PAI1 synthesis was used to immunize balb/c mice as described in *Materials and Methods* (10.4).

11.4 Epitope analysis

The additivity index (AI) is based upon the principle that two Mabs against two different epitopes of the same antigen will give a higher optical density reading than each of the antibodies alone, providing that there is no steric hindrance involved and that antigen sites are saturated by the antibodies. Five hybridoma clones were selected from the 20 clones since they had AI values greater than 40%. These were named mαPAI1/C2, -C6, -C11, -C12 and -C14. All five Mabs were of the sub-class IgG1. The AI values of the negative controls were less than 10%.

(A)



(B)

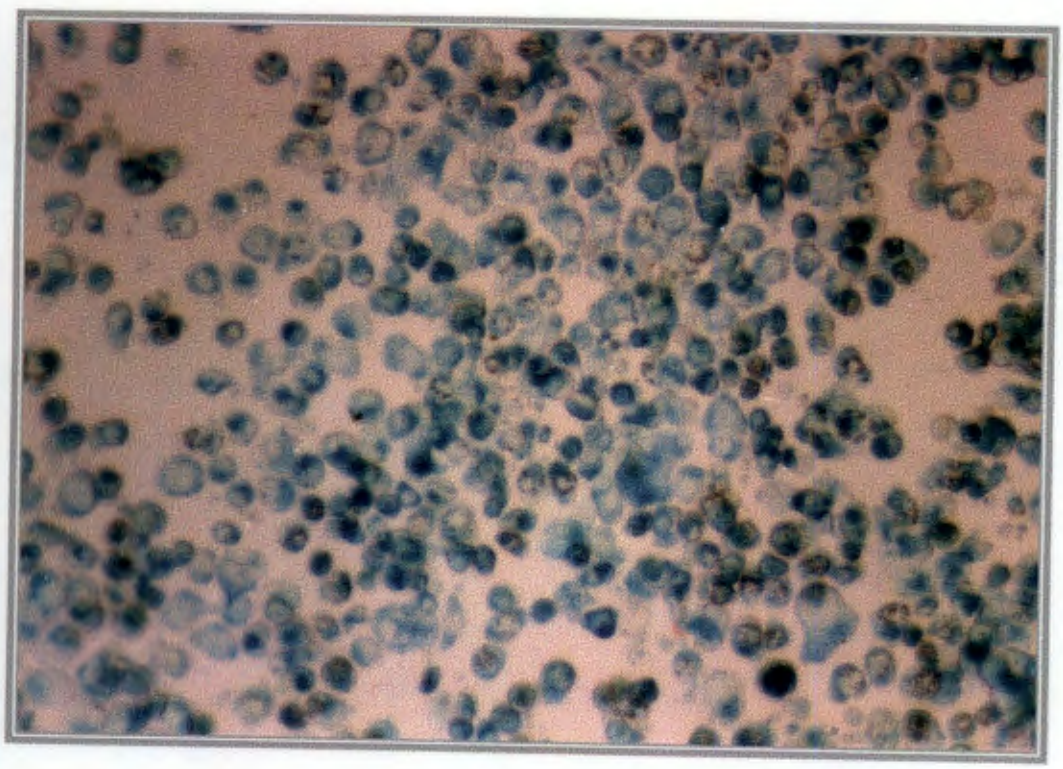
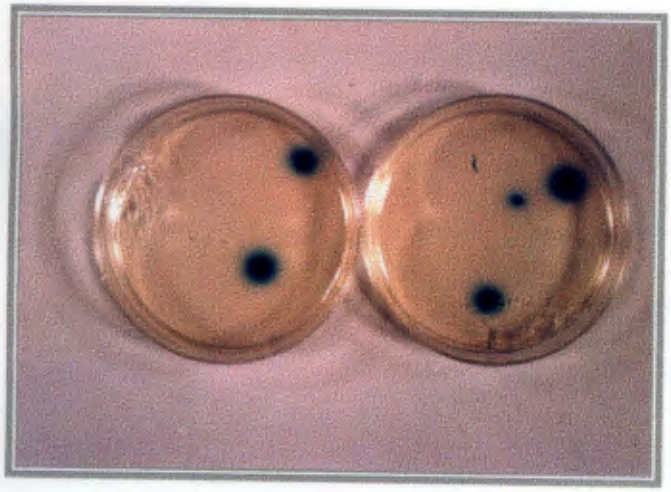
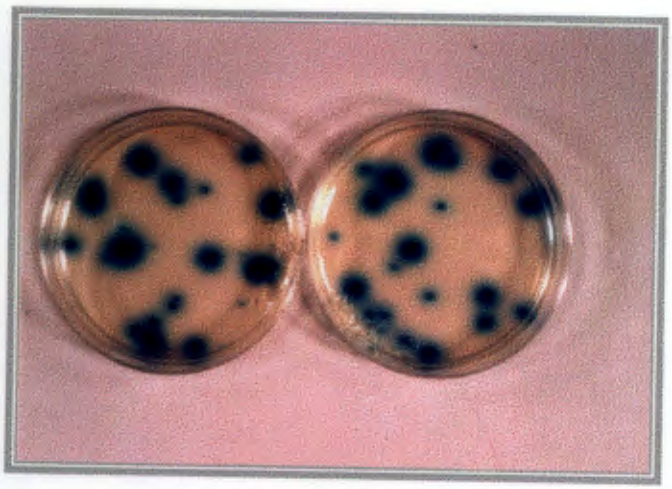


Figure 11.1. The expression of rPAI1 in Sf9 insect cells.
A, Wild type viral plaques (40x magnification) with occlusion bodies in the cells containing the virus which is embedded in the polyhedrin protein. **B,** Recombinant viral plaques (40x magnification) expressing human PAI1 and beta-galactosidase (blue cells).
The photographs for Figure 11.1 are courtesy of Mrs. Debby Clarke.

(A)



(B)



(C)

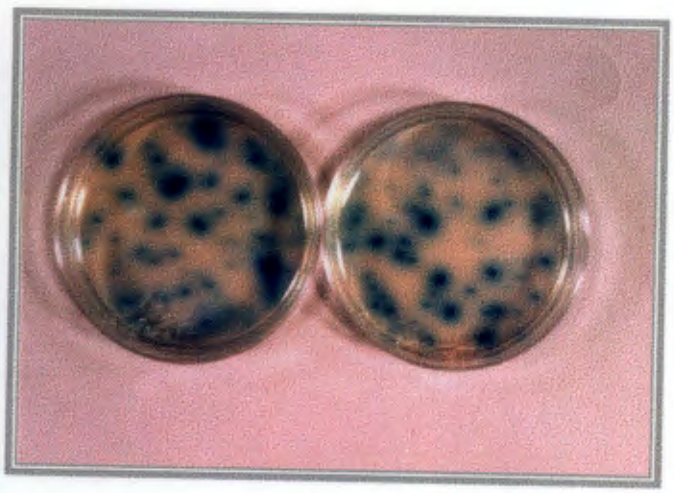
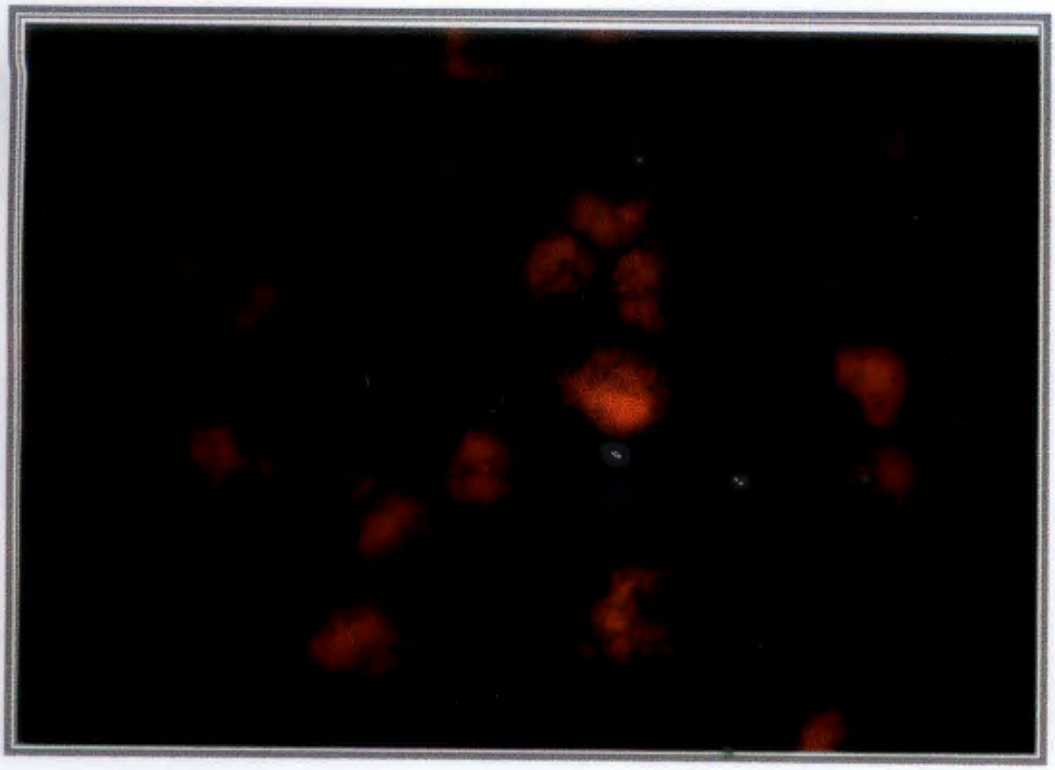


Figure 11.2. Determination of PAI1 recombinant virus titre.
Duplicate monolayer Sf9 cultures were infected with the PAI1 recombinant baculovirus using 10-fold dilutions of the virus stock and overlayed with an agarose-medium containing X-gal as a substrate for beta-galactosidase. The blue plaques that formed on the plate with the 10^{-6} virus dilution were counted in order to calculate the virus titre. **(A)** 10^{-7}, **(B)** 10^{-6} and **(C)** 10^{-5} virus dilutions.

(A)



(B)

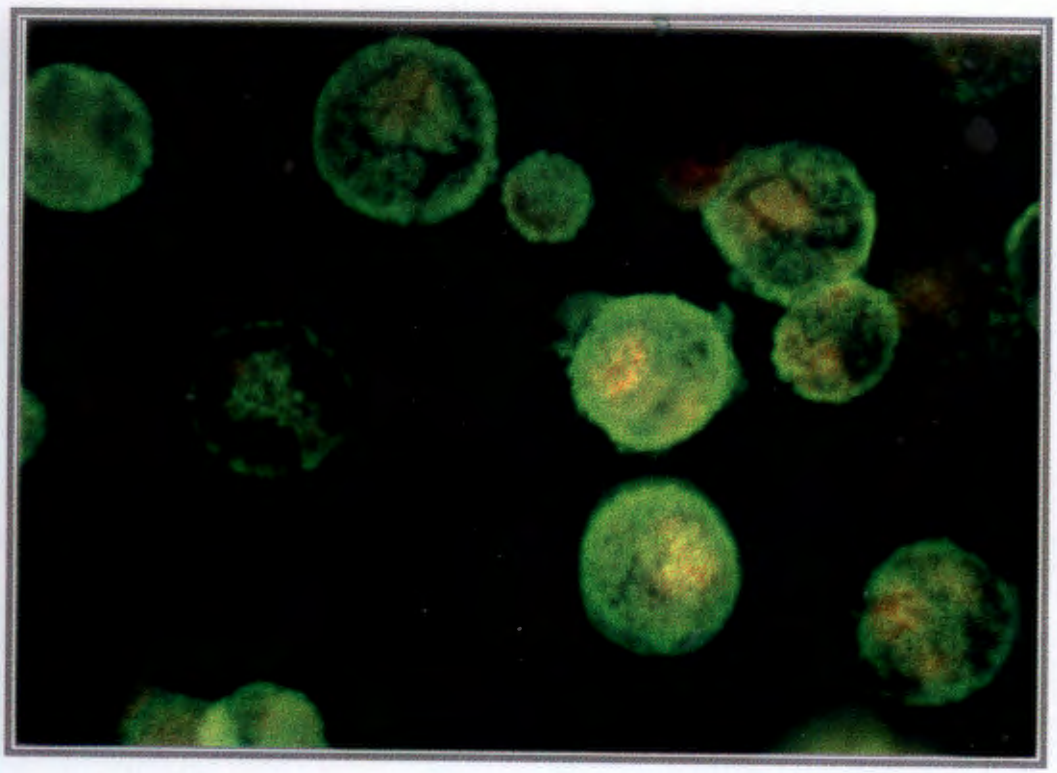


Figure 11.3. Immunofluorescence of a Sf9 suspension culture infected with the PAI1 recombinant baculovirus (400x magnification). **A,** Normal mouse immunoglobulin as the negative control showing background fluorescence. **B,** Mouse anti-PAI1 Mab (American Diagnostica) showing the expression of human PAI1 by insect cells. A fluorescein-labelled goat anti-mouse was used as the secondary antibody. The nuclei were counterstained with ethidium bromide.

11.5 Immunoblotting

In order to evaluate whether any of these Mabs would be suitable for immunoblotting, each of the antibodies was tested for its ability to detect PAI1 from different tissue compartments. These included PAI1 from the intracellular (cytoplasmic) and extracellular (secreted) compartments as well as ECM-bound PAI1. All fractions were prepared from the human fibrosarcoma cell line, HT1080. The PAI1 from the HT1080 cells was compared with the PAI1 secreted by the insect cells (see *Materials and Methods*, 10.8).

The western blotting results, using the mαPAI1/C11 Mab, are presented in Figure 11.4. A molecular weight (MW) of 45 kD was obtained for recombinant PAI1 (rPAI1, lane 1) and corresponds to the expected size as estimated from the 379 amino acid sequence. A 52 kD glycosylated native PAI1 (nPAI1) was detected in mammalian cell HT1080 conditioned medium (CM, lane 5) compared to a 48 kD glycosylated rPAI1 in insect cell CM expressing human PAI1 (Sf9/PAI1, lane 2). This clearly indicates differences in the glycosylation of human PAI1 by insect cells compared to human cells. The detection of PAI1 in Sf9/PAI1 CM confirms that PAI1 is secreted by the infected insect cells. Non-glycosylated PAI1 (45 kD faint band in lane 5) was also present in the HT1080 CM and might be explained by cell lysis over the 24 hour period of CM collection, since non-glycosylated forms are not expected to be secreted. Two additional bands were observed in HT1080 CM (lane 5): these bands are most likely tPA/PAI1 (127 kD) and uPA/PAI1 (106 kD) complexes, since (a) the MW's of the complexes correspond to published data (Laug *et al.*, 1992), (b) HT1080 cells are known to secrete both forms of PA and (c) the bands disappear when electrophoresis is performed at RT [It has been shown that prolonged incubation of SDS (present during electrophoresis) with PA/PAI1 complexes will result in their dissociation in a time- and dose-dependent manner (Gaussem *et al.*, 1993)]. Antibodies against tPA and uPA should clarify with certainty the nature of these two bands.

The HT1080 ECM-preparation (lane 7) reveals a doublet in the 52-55 kD range. The 55 kD band is most likely intact, active PAI1 while the 52 kD band is latent PAI1 which, owing to a conformational change runs slightly ahead. It is unlikely that the 52 kD band is inactive PAI1 since the 52 kD band is the major fraction of PAI1 found in HT1080 CM (lane 5). This PAI1 from the CM can be activated with guanidine.HCl and thus confirms that it is the latent form. Alternatively, the 55kD band could be active PAI1 bound to a fragment of a cell matrix component, for example a fragment from vitronectin (VN). Only glycosylated latent PAI1 was observed intracellularly (lane 8) - presumably having accumulated in the Golgi-apparatus. The two high MW bands (lane 8) were the result of non-specific streptavidin binding as detected on a control blot (results not shown). Lanes

3 and 6 were shorter exposures of 10 seconds each to confirm that the heavy bands in lanes 2 and 5 were not doublets.

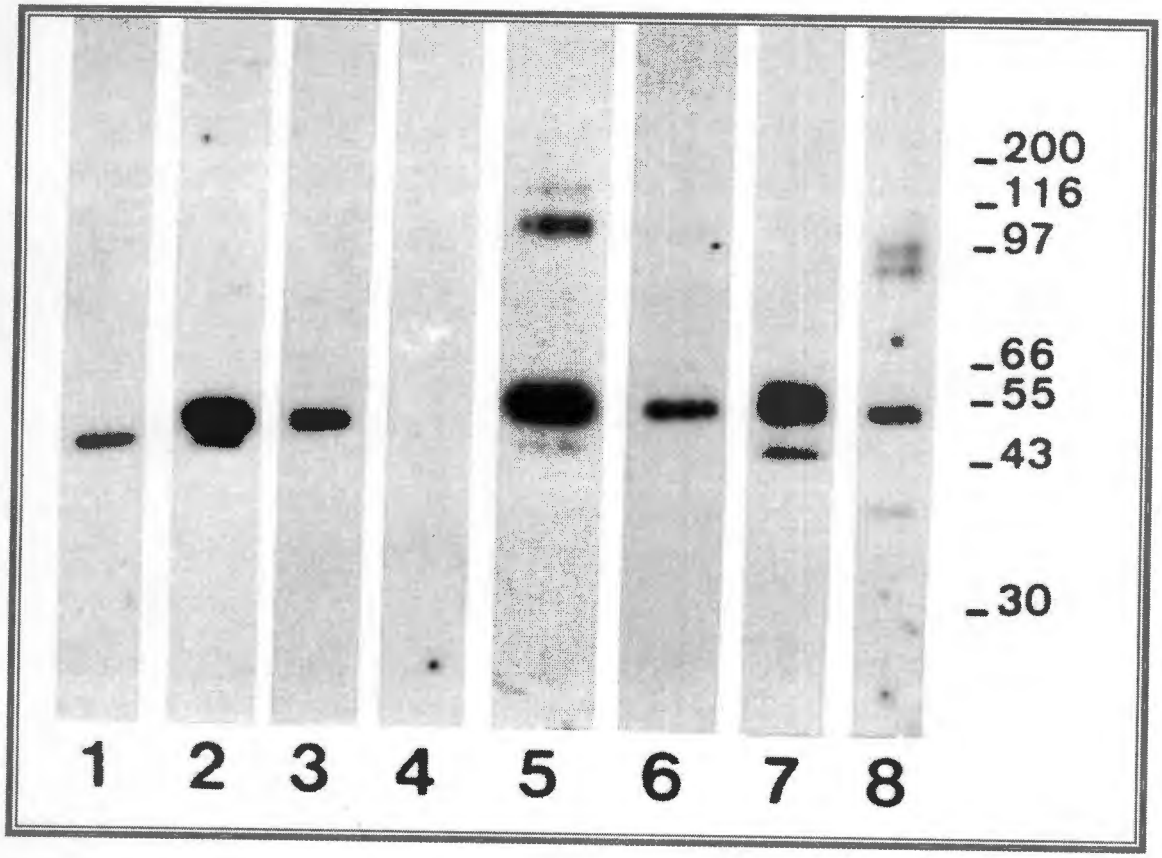


Figure 11.4. Western blotting with mαPAI1/C11 Mab.
Lane 1, rPAI1 (45 kD) from E.coli; **Lane 2**, rPAI1 from Sf9/PAI1 CM; **Lane 3**, the same as lane 2 but a shorter exposure of 10 secs; **Lane 4**, WT-baculovirus CM as a negative control; **Lane 5**, nPAI1 from HT1080 CM; **Lane 6**, the same as lane 5 but a shorter exposure of 10 secs; **Lane 7**, nPAI1 from HT1080 ECM; **Lane 8**, nPAI1 from HT1080 cell lysate. Molecular weight markers are given in kilodaltons.

Each individual Mab was similarly tested for its use in immunoblotting (results not shown). It was concluded that mαPAI1/C11 was the most suitable antibody since it was the only one that reacted with both free and PA-complexed forms of PAI1. The mαPAI1/C6 was found not to be suitable for Western blotting.

11.6 Immunofluorescence and immunocytochemistry

To assess the suitability of the Mabs for immunofluorescence and immunocytochemistry, each of the antibodies was tested as described in *Materials and Methods* (10.10). Strong fluorescence was obtained with mαPAI1/C11 when methanol/acetone fixation was used (Figure 11.5.A). A considerable amount of fluorescence was observed intracellularly as well as on the cell matrix where PAI1 is deposited in a "carpet-like" manner. Weaker fluorescence was obtained with mαPAI1/C2, -C12 and -C14 while mαPAI1/C6 was completely negative. A similar profile was

obtained with immunocytochemistry using methanol/acetone/formaldehyde fixation (Figure 11.5.B).

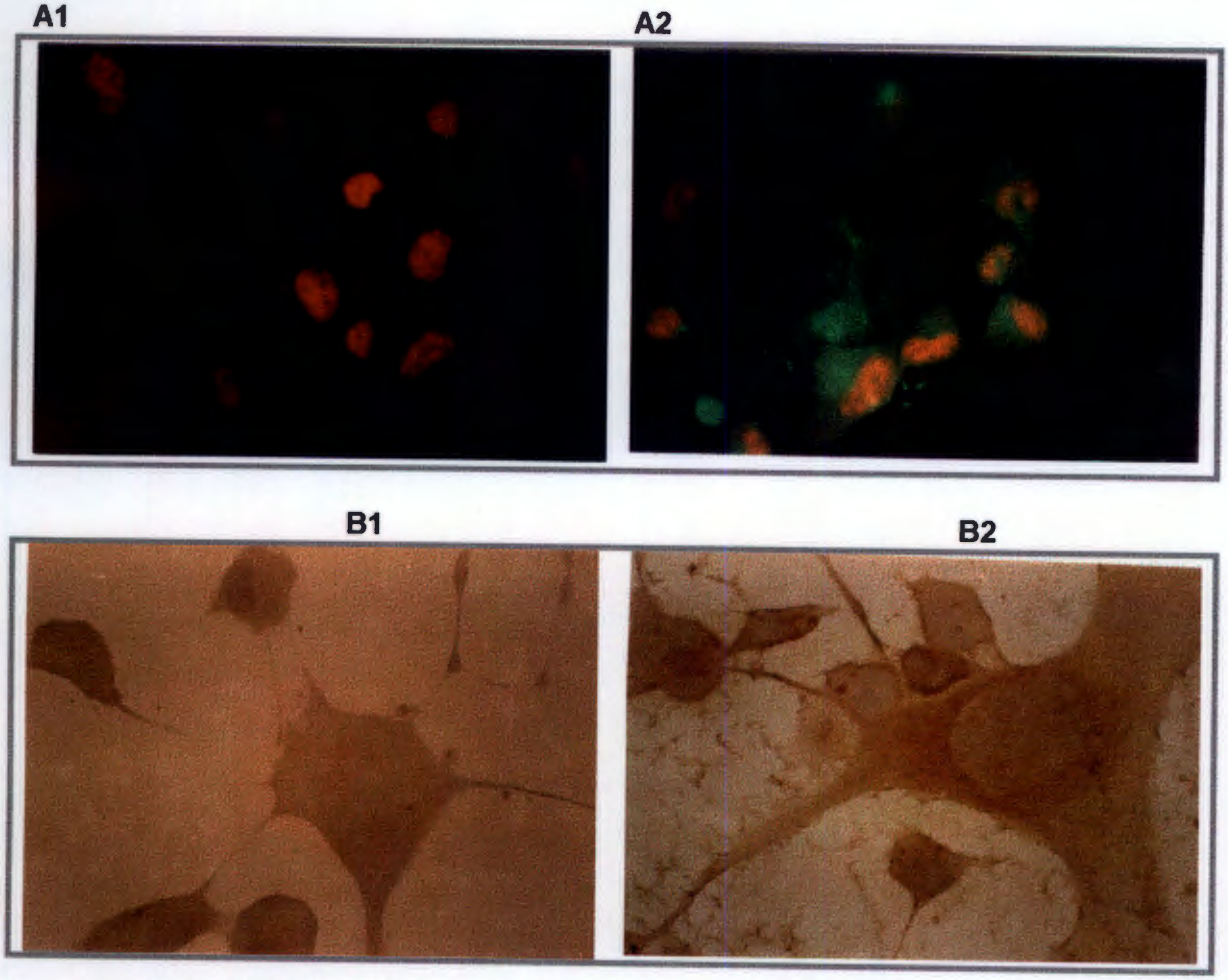


Figure 11.5. A, Immunofluorescence (400x magnification) and **B,** immunocytochemistry (800x magnification) on HT1080 cells. **1,** Mouse anti-beta-galactosidase Mab (IgG1) as the negative control for background staining. **2,** MαPAI1/C11. A fluorescein-conjugated goat anti-mouse IgG was used as the secondary antibody for immunofluorescence. The nuclei were counterstained with ethidium bromide. A biotinylated rabbit anti-mouse IgG was used as the secondary antibody for immunocytochemistry. The biotin was detected with peroxidase-conjugated streptavidin. The nuclei were counterstained with haematoxylin.

11.7 Binding of Mabs to matrix-associated PAI1

PAI1 is a secreted protein whose activity is stabilized by binding to VN, an adhesive protein present in plasma, platelets and the ECM (Declerck *et al.*, 1988a; Lawrence *et al.*, 1994). It was therefore of interest to identify those Mabs which would recognize matrix-accociated PAI1. Cell matrices were prepared by culturing of HT1080 cells on microtiter plates and the Mabs were tested as described in *Materials and Methods* (10.11). MαPAI1/C2 (squares) and -C11 (triangles) detected matrix-associated PAI1 with high affinity (Figure 11.6). MαPAI1/C12 (diamonds) and -C14 (crosses) gave weaker signals. MαPAI1/C6 (circles) failed to detect matrix-associated PAI1, indicating that this Mab most likely binds to the matrix-binding epitope of PAI1.

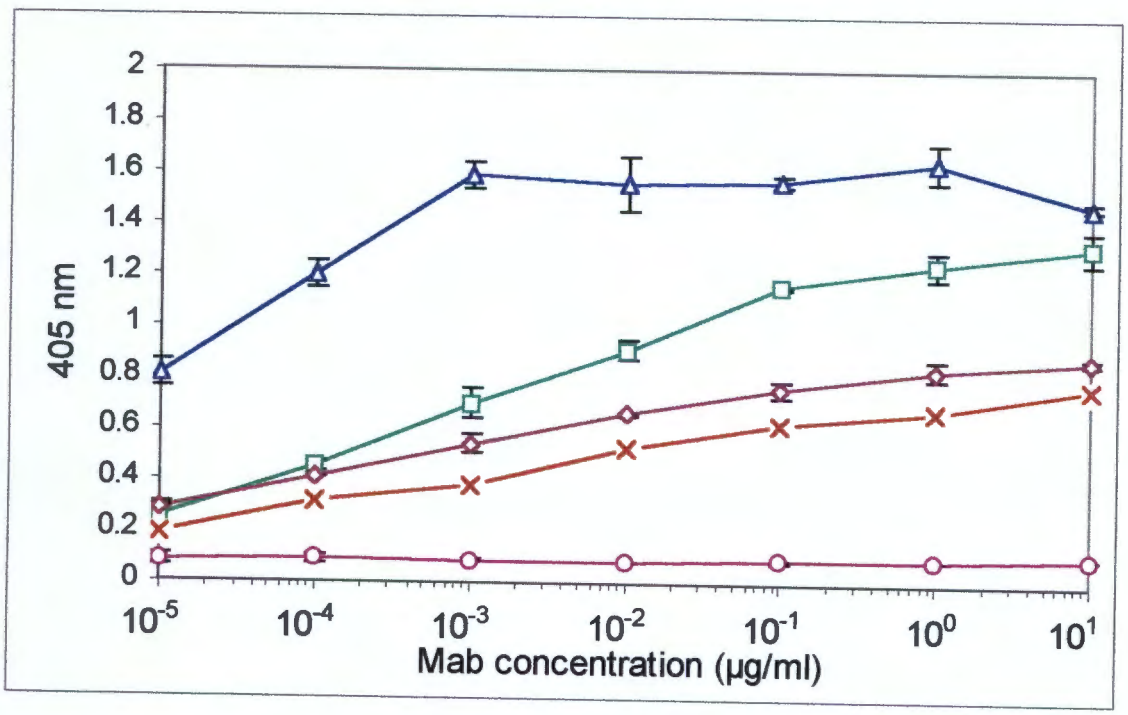


Figure 11.6. The recognition of endogenous matrix-associated PAI1 by selected Mabs. Decreasing concentrations of mαPAI1/C2 (□), -C6 (○), -C11 (Δ), -C12 (◊) and -C14 (×) were incubated with HT1080 cell matrices starting with 10 µg/ml Mab. The mαPAI1/C6 failed to detect matrix-associated PAI1 which indicated that this Mab might bind to the matrix-binding epitope of PAI1. The absorbance at 405 nm was plotted against the logarithm of antibody concentration. The results are representative of two independent experiments and are expressed as the mean ± SDM of duplicate determinations.

11.8 A Mab against the matrix-binding epitope of PAI1

Before it was possible to test whether mαPAI1/C6 is indeed against the matrix binding epitope of PAI1, it was necessary to mimic the results of the previous experiment by replacing the endogenous PAI1 with exogenous active rPAI1. The rationale being that this would clarify whether active rPAI1 will bind to the PAI1-depleted (acid buffer treatment) cell matrices. When endogenous PAI1 was depleted from HT1080 cell matrices and replaced with exogenous active rPAI1 (see *Materials and Methods*, 10.12), similar results (as portrayed in Figure 11.6) were obtained (Figure 11.7a). As expected, the results were negative when active rPAI1 was replaced with latent rPAI1 since it is not able to bind to the cell-matrix (results not shown). The next step was to test the ability of each of the Mabs to block the binding of PAI1 to the cell matrices. Figure 11.7b illustrates that complete inhibition of PAI1 binding to cell matrices was obtained when PAI1 was pre-incubated with a 100-fold excess of mαPAI1/C6 (squares). None of the other PAI1 antibodies inhibited PAI1 binding to the cell matrix. This confirms that mαPAI1/C6 most likely does bind to the VN-binding epitope of PAI1.

11.9 A Mab against the VN-binding epitope of PAI1

To confirm that mαPAI1/C6 will block the binding of PAI1 to VN and not to another matrix component, the Mabs were tested for their ability to block the binding of PAI1 to VN immobilized on a microtiter plate. Figure 11.8 illustrates that complete inhibition of PAI1 binding to VN was observed when the inhibitor was pre-incubated with a 100-fold excess of mαPAI1/C6 (squares). MαPAI1/C14 (triangles) moderately inhibited the binding of active rPAI1 to VN. None of the other PAI1 antibodies inhibited PAI1 binding to VN as shown with mαPAI1/C2 (diamonds). This indicates that mαPAI1/C6 either binds to the VN-binding epitope of PAI1 or in the vicinity of the epitope bringing about an inhibitory effect through steric-hindrance. It was recently shown that both the somatomedin B (SMB) domain at the N-terminus and the cluster of basic amino acids at the C-terminal moiety of VN contributes to PAI1 binding (Gechtman *et al.*, 1997). MαPAI1/C6 most likely binds, in close proximity to the domain of PAI1 that interacts with the SMB domain of VN, since the immobilization of VN to plastic surfaces renders its cluster of basic amino acids inaccessible for binding of PAI1. The ability of the antibody to effectively block the binding of active PAI1 to immobilized VN suggests an inhibitory effect through steric hindrance since the antibody also binds to latent PAI1 (see 11.11).

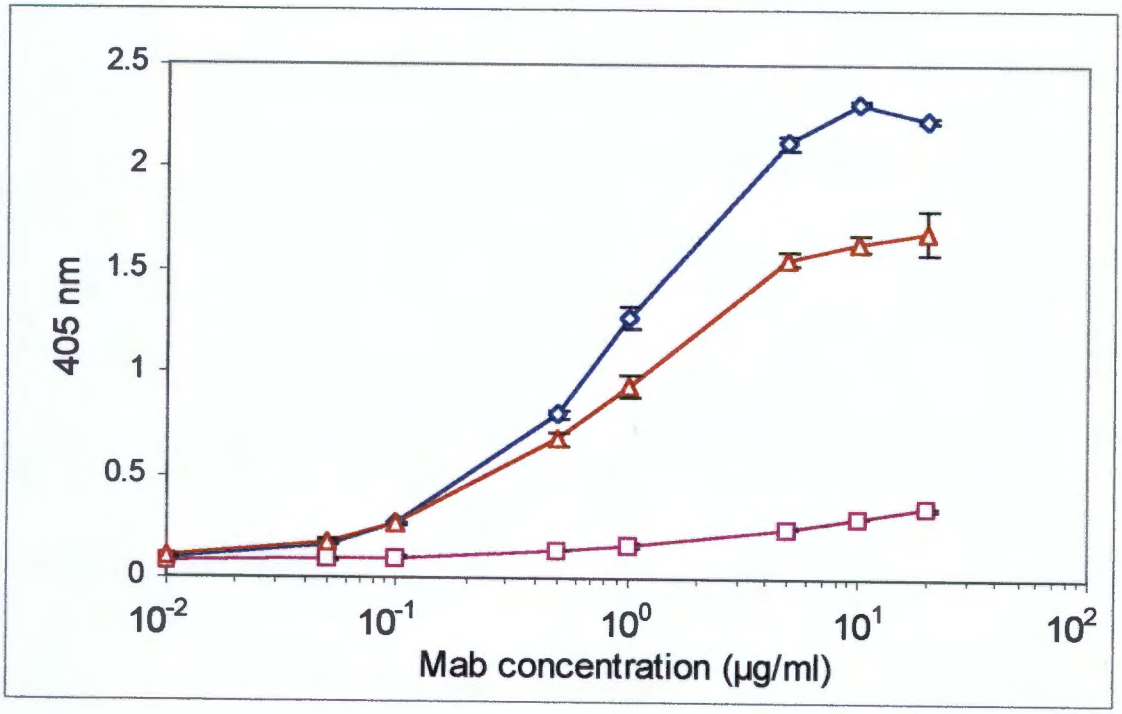


Fig.11.7a. The recognition of exogenous matrix-associated PAI1 by selected Mabs when added to PAI1-depleted matrices. Decreasing concentrations of mαPAI1/C2 (◊), -C6 (□) and -C14 (Δ) were incubated with HT1080 cell matrices (starting with 20 µg/ml Mab) of which the endogenous PAI1 was replaced with exogenous active rPAI1. Again, as seen in Figure 11.6, mαPAI1/C6 failed to detect matrix-associated rPAI1. The absorbance at 405 nm was plotted against the logarithm of antibody concentration. The results are representative of two independent experiments and are expressed as the mean ± SDM of duplicate determinations.

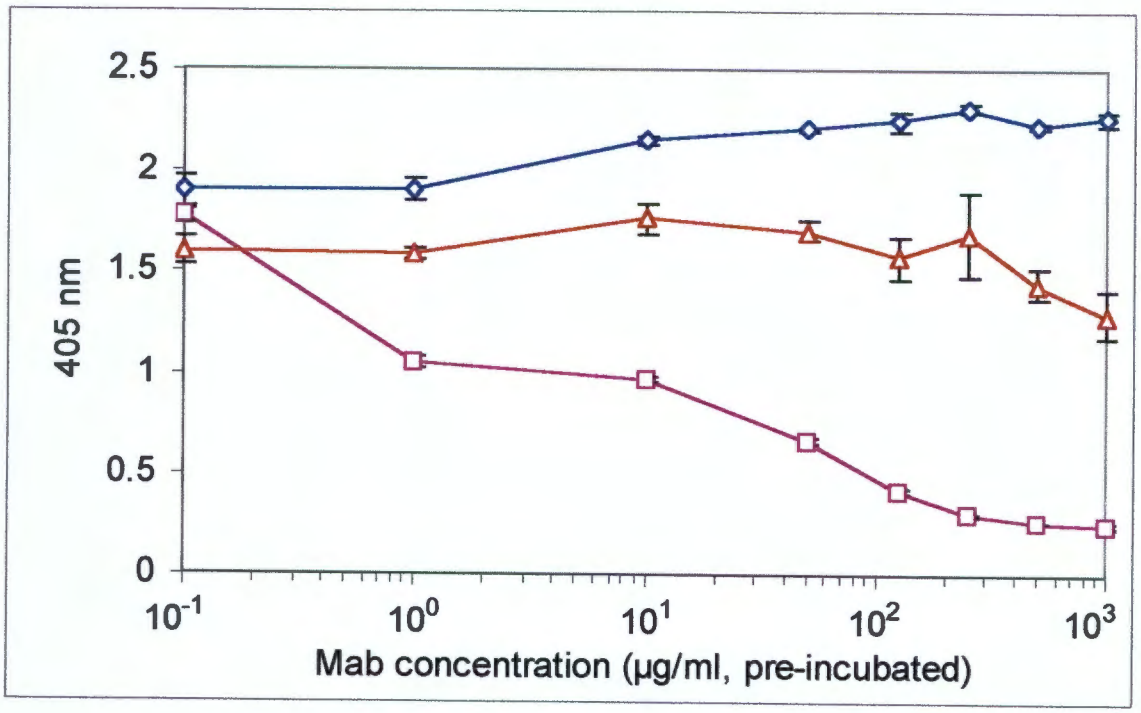


Fig.11.7b. Inhibition of PAI1 binding to HT1080 cell matrices by mαPAI1/C6. Decreasing concentrations of mαPAI1/C2 (◊), -C6 (□) and -C14 (Δ), starting at 1000 µg/ml, were pre-incubated with active rPAI1 (1 µg/ml) in the fluid phase. The antibody-antigen complex was then incubated with the PAI1-depleted HT1080 cell matrices. Matrix-bound rPAI1 was detected with mαPAI1/C2 or -C11 (1 µg/ml). A 100-fold excess of mαPAI1/C6 completely inhibited the binding of active rPAI1 to the HT1080 cell matrix. The absorbance at 405 nm was plotted against the logarithm of antibody concentration. The results are representative of two independent experiments and are expressed as the mean ± SDM of duplicate determinations.

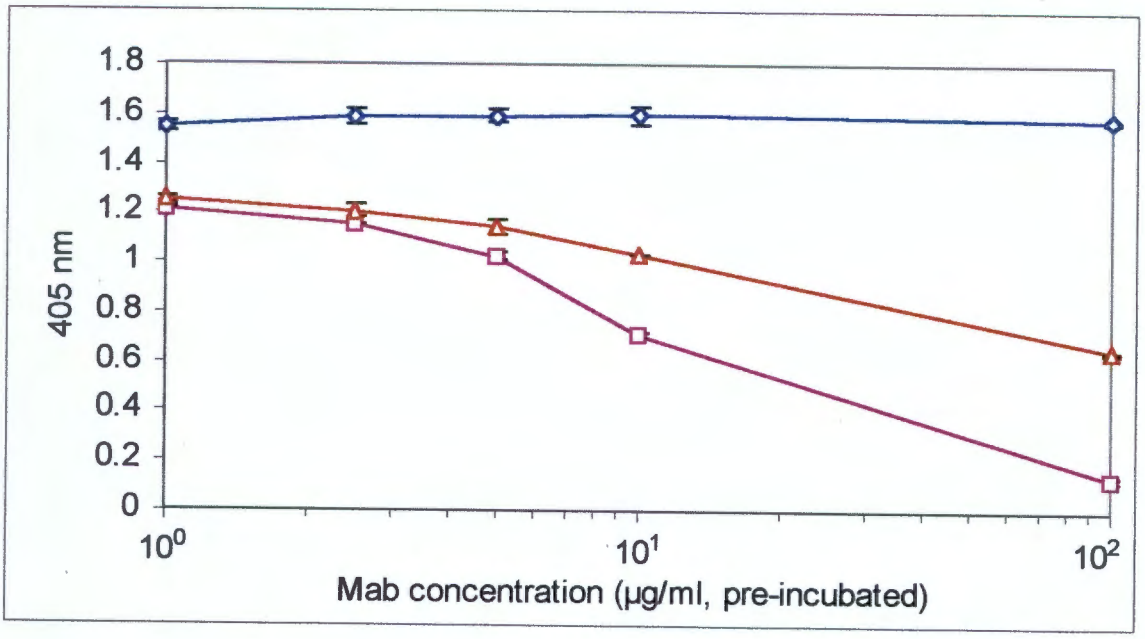


Figure 11.8. Inhibition of PAI1 binding to VN by mαPAI1/C6.
Decreasing concentrations of mαPAI1/C2 (◊), -C6 (□) and -C14 (Δ), starting at 100 µg/ml, were pre-incubated with active rPAI1 (1 µg/ml) in the fluid phase. The antibody-antigen complex was then incubated with VN coated on a microtiter plate. VN-bound rPAI1 was detected with mαPAI1/C2 or -C11 (1 µg/ml). A 100-fold excess of mαPAI1/C6 completely inhibit the binding of active rPAI1 to VN. The absorbance at 405 nm was plotted against the logarithm of antibody concentration. The results are representative of two independent experiments and are expressed as the mean ± SDM of duplicate determinations.

11.10 A Mab which inhibits PAI1 activity

To establish whether any of the Mabs inhibit the activity of PAI1, they were tested with a standard ^{125}Iodine-fibrin plate assay as described in *Materials and Methods* (10.14). The concentration of 0.05 μg/ml active rPAI1 used in the assay resulted in 18% residual PA activity which was considered as the background activity (Figure 11.9). PAI1 activity was significantly inhibited (78%) with mαPAI1/C6 at 1000-times excess (bar C6). Lower concentrations of antibody were not inhibitory. None of the other Mabs inhibited PAI1 activity significantly (bars C2, C11, C12 and C14). The negative control (1000-times excess anti-beta galactosidase Mab) did not inhibit PAI1 activity. These results therefore demonstrate that mαPAI1/C6 not only blocks the binding of PAI1 to VN but also significantly inhibits the activity of PAI1.

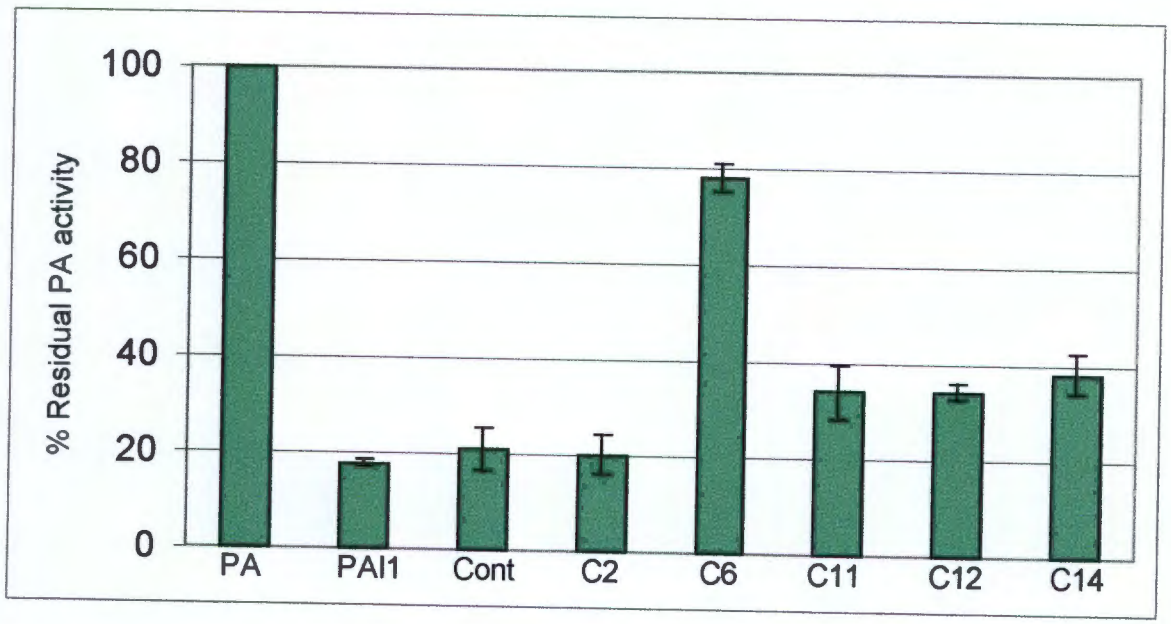


Figure 11.9. Inhibition of PAI1 activity with selected Mabs. The ability of each selected Mab to inhibit PAI1 activity was measured with the ^{125}I-fibrin plate assay and the results are presented as percentage residual PA activity. **PA**, 0.5 IU/ml 2c-tPA; **PAI1**, 0.05 μg/ml active rPAI1; **Cont**, Negative control with 1000-times excess anti-beta galactosidase Mab; **C2**, **C6**, **C11**, **C12**, **C14**, Individual anti-PAI1 Mabs (1000-times excess). The results are representative of two independent experiments and are expressed as the mean ± SDM of duplicate determinations.

11.11 Recognition of latent and active PAI1 by ELISA

To establish whether any of the Mabs would recognize latent and/or active rPAI1 captured on a microtiter plate with a rabbit anti-PAI1 Pab, they were tested with an ELISA procedure as described in *Materials and Methods* (10.15). MαPAI1/C11, -C12 and -C14 recognized latent and active rPAI1 with approximately equal affinities (Figure 11.10). The affinity of mαPAI1/C2 was two-fold greater for latent PAI1 than for the active molecule. MαPAI1/C6 detected only latent PAI1 with an affinity similar to that of mαPAI1/C11. The absorbance value for the active molecule was the equivalent of background levels. It was concluded that mαPAI1/C6 and –C11 might allow the quantitation of the active fraction of PAI1 in biological fluids by subtracting the amount of latent PAI1 (detected with mαPAI1/C6) from the amount of total PAI1 (detected with mαPAI1/C11).

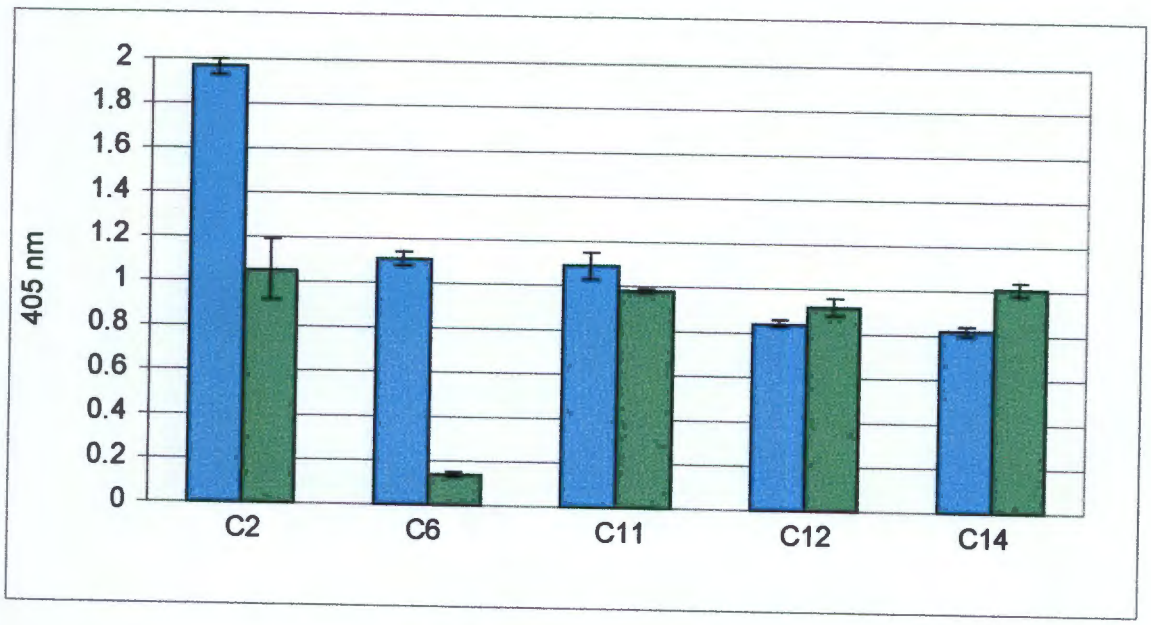


Figure 11.10. The recognition of latent (■) and active PAI1 (■) by selected Mabs.
A rabbit anti-PAI1 Pab was used to capture latent or active rPAI1 on a MaxiSorb ELISA plate. Each of the selected Mabs, ie. mαPAI1/C2, -C6, -C11, -C12 and -C14 was tested for the ability to recognize bound latent or active PAI1. The results are representative of two independent experiments and are expressed as the mean ± SDM of duplicate determinations.

CHAPTER 12: SUMMARY AND FUTURE CONSIDERATIONS

This section of the thesis describes several anti-PAI1 Mabs that were developed by making use of baculovirus-expressed rPAI1 as the immunogen in order to study the regulatory roles of this inhibitor in cancer progression and invasion. Factors contributing to the success of this approach may include the effective post-translation modification of the recombinant molecule and the secretion of a more soluble form of rPAI1 compared to *E. coli*-derived recombinant molecules which are often troubled by insolubility. Five hybridoma clones with additivity index values greater than 40% were selected from 20 clones. Their individual characteristics are presented in Table 12.1. The data indicate that the five selected Mabs are against different epitopes of PAI1, except for mαPAI1/C12 and –C14 which are most likely against the same epitope of PAI1. The high affinity mαPAI1/C11 is suitable for immunoblotting, immunofluorescence and immunocytochemistry. This indicates that the epitope is resistant to denaturation by SDS, methanol, acetone and formaldehyde. MαPAI1/C2 and –C11 can be used in ELISA assays for the detection of matrix-associated PAI1. MαPAI1/C6 should be suitable for tissue culture experiments to block the binding of PAI1 to the ECM (thus destabilizing the activity of PAI1) and for the simultaneous inhibition of PAI1 activity when added in excess quantities.

Table 12.1 Summary of individual characteristics of selected Mabs

Mab characteristic	C2	C6	C11	C12	C14
Immunoblotting	+	-	++	+	+
Immunofluorescence	+	-	++	+	+
Immunocytochemistry	+	-	++	+	+
Recognition of ECM-associated PAI1	++	-	++	+	+
Inhibition of PAI1 binding to VN	-	++	-	-	-
Inhibition of PAI1 activity	-	+	-	-	-
Recognition of latent PAI1 with ELISA	++	+	+	+	+
Recognition of active PAI1 with ELISA	+	-	+	+	+

(++) Strong reaction, (+) Weaker reaction, (-) Negative reaction

In addition, mαPAI1/C11 allows the quantitation of latent and active PAI1 with an equal affinity on a microtiter plate. In contrast, mαPAI1/C6 can only detect latent PAI1 on the microtiter plate but with a similar affinity as mαPAI1/C11 has for latent and active PAI1. This allows the quantitation of the active fraction of PAI1 in biological fluids by subtracting the amount of latent PAI1 (detected with mαPAI1/C6) from the amount of total PAI1 (detected with mαPAI1/C11). The application of this approach is described and discussed in section B of this thesis.

FUTURE CONSIDERATIONS

It is now eminently clear that PAI1 has regulatory functions in the metastasis and vascularization of solid tumors. Increased levels of PAI1 in metastases as compared to the primary tumor, support this hypothesis (Sier *et al.*, 1994). In concordance, Fletcher and Dowdle (1994) describe the presence of PAI1 in adrenal and lung metastases of the highly metastatic melanoma cell line UCT-Mel-3 in athymic mice, while the parental cell line had no detectable PAI1 (Fletcher & Dowdle, 1994). This suggests the existence of PAI1-producing melanoma cells within the parental cell line which may preferentially metastazise. The UCT-Mel-3 cell line and the two PAI1-producing clones that were obtained from adrenal and lung metastases in athymic mice could be an ideal model system for studying the role of PAI1 in tumor cell invasion and vascularization using *in vitro* transwell culture systems. As mentioned before, PAI1 acts as a cell detachment factor (Deng *et al.*, 1996a) by competing with the uPAR for binding to VN (Figure 12.1). Theoretically, the PAI1-producing clones should have higher metastatic potentials than the parental cell line in a transwell culture system with VN supplied as a cell substratum. It might be possible to use the mαPAI1/C6 Mab in this type of experimental design to block the binding of PAI1 to VN and to inhibit its activity. This may cause the PAI1-producing clones to become more adhesive to the VN substratum and ultimately to become less invasive.

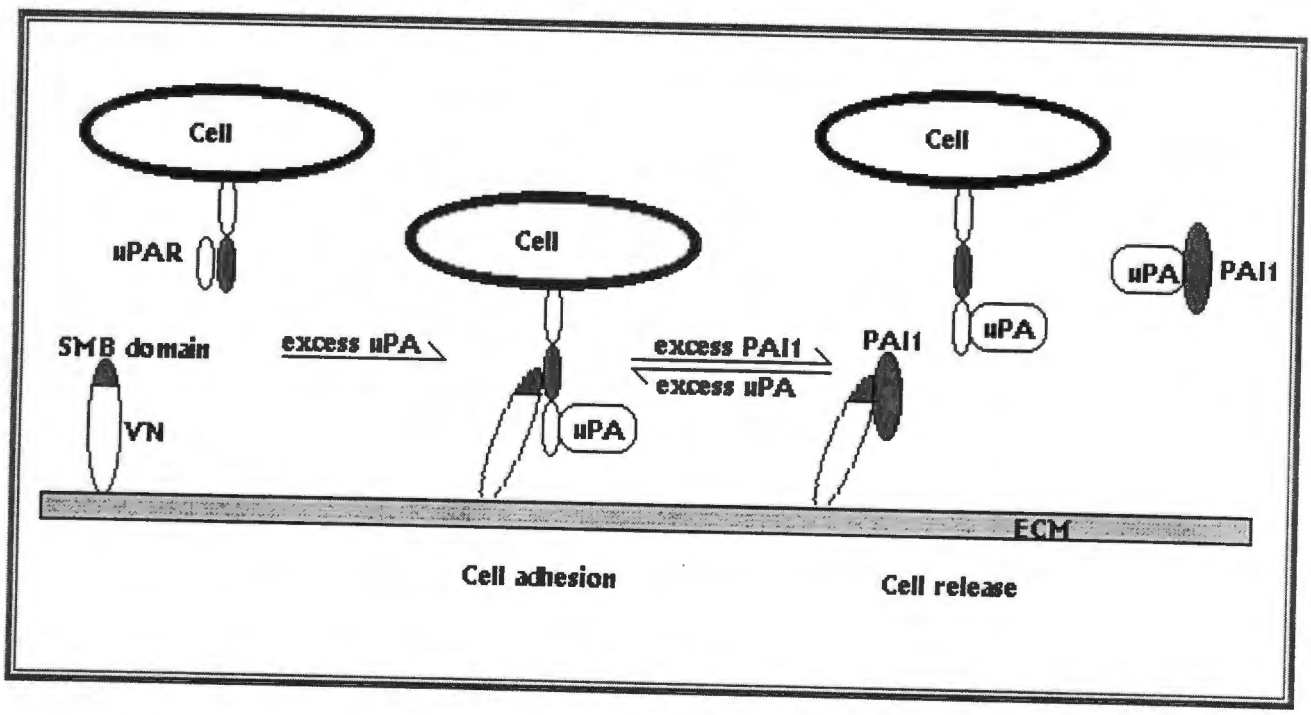


Figure 12.1. A model for the regulation of uPAR dependant cell adhesion and release by PAI1 and uPA. Adapted from Deng *et al.* (1996).

The above mentioned model system could also possibly be used to explore the role of PAI1 in tumor vascularization. It has been shown that the angiogenic inhibitor, angiostatin (O' Reilly *et al.*, 1994), can be generated from plasmin in HT1080 fibrosarcoma cells by reduction and proteolysis (Stathakis *et al.*, 1997). Physiologically relevant concentrations of reduced glutathione fulfill the role

of the reductase. In another study is was demonstrated that human prostate carcinoma cells can convert plasminogen to angiostatin (Gately *et al.*, 1997). In this system, plasminogen is first converted to active plasmin by uPA, and in the presence of a free sulfhydryl donor, plasmin serves as both the substrate and enzyme for the generation of angiostatin (Figure 12.2). It would be interesting to explore whether UCT-Mel-3 cells are able to generate angiostatin from plasminogen since it is known that these cells secrete tPA (Hoal-Van Helden *et al.*, 1986).

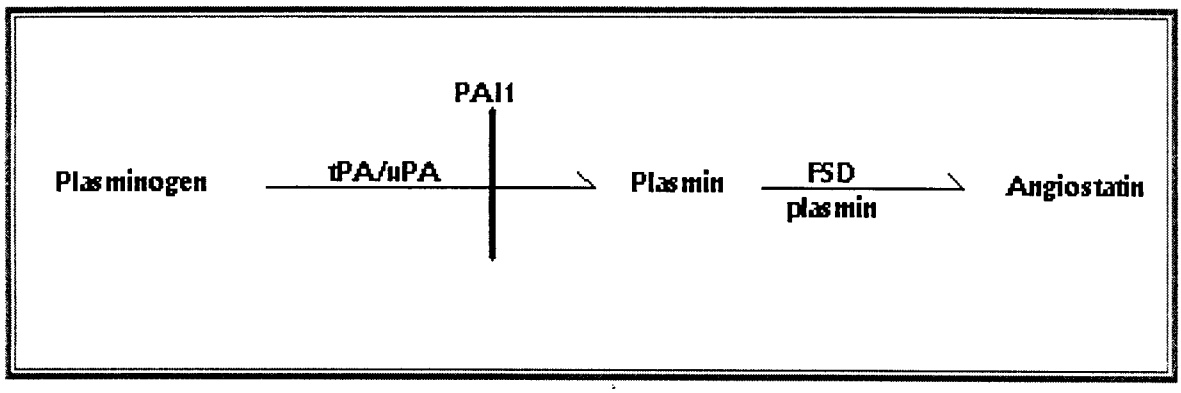


Figure 12.2. A model for the generation of angiostatin from plasminogen by tPA or uPA and free sulfhydryl donors (FSDs).

The UCT-Mel-3 parental cell line and its PAI1-producing clones could provide an interesting model system to study the role of PAI1 in tumor vascularization, provided that the parental cells are able to generate angiostatin from plasminogen (possibly via tPA and a reductase). The cells should theoretically inhibit endothelial cell proliferation if they are able to generate angiostatin. In contrast, the PAI1-producing clones should theoretically allow endothelial cell proliferation since PAI1 will block tPA activity and consequently less angiostatin will be generated by these cells. It might also be possible to use the mαPAI1/C6 Mab to block the binding of PAI1 to VN and to inhibit its activity, thereby promoting angiostatin generation and consequently inhibit endothelial cell proliferation.

In conclusion, it is clear that the Mabs produced in the present study will be most helpful in answering various questions related to PAI1 and its interaction with other molecular components. In addition, as shown in section B of this thesis, these Mabs provide invaluable tools for carrying out clinical studies related to melanoma progression.

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APPENDIX: METHODS AND SOLUTIONS

A.1 Routine culturing of melanoma cells

Melanoma cell lines were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS, State Vaccine Institute, Cape Town), 50 U/ml penicillin (Novo Nordisk) and 20 µg/ml streptomycin (Gibco) at 37°C under 5% CO_2/95% air and 90% humidity. The complete growth medium is abbreviated as RP10 medium. Sub-culture the cells as follows: Wash the cultures once with PBS (see A.4 for details) and dislodge the cells with versene-buffer (see A.5 for details) for 10 mins at 37°C. Centrifuge the cells at 700 g for 5 mins at RT. Resuspend the cell pellet in fresh RP10 medium and plate $1/10^{th}$ of the original amount of cells on a 100 mm tissue culture dish (Falcon). Maintain frozen cell stocks in RP10 medium plus 10% dimethylsulphoxide (DMSO) at -80°C or in liquid nitrogen (-196°C). Thaw the cells for 5 mins at 37°C and transfer quickly to 10 ml RP10 medium in order to dilute out the DMSO. Centrifuge for 5 mins at 700 g. Resuspend the cell pellet in fresh RP10 medium and culture as described above.

A.2 RPMI 1640 tissue culture medium

Dissolve 52.0 g RPMI 1640 powder medium (Sigma) in 4000 ml double distilled water (ddH_2O). Add 10.0 g $NaHCO_3$ (BDH), 2.5 x 10^5 Units penicillin (50 U/ml) and 0.1 g streptomycin sulphate (20 µg/ml). Adjust the pH to 7.1 with 1 M HCl and make up to 5000 ml with ddH_2O. Sterilize the solution through a 0.45 µm filter and store in 400 ml aliquots at 4°C. The pH should be approximately 7.4.

A.3 Complement-inactivated fetal calf serum (FCS)

Heat the FCS for 30 mins at 56°C to inactivate complement factors. Pre-filter the serum (Millipore type AP) and sterilize through a 0.45 µm filter. Store in 50 ml aliquots at -20°C.

A.4 10x Phosphate-buffered saline (PBS)

Dissolve 80.0 g NaCl (1.37 M), 2.0 g KCl (0.03 M), 14.4 g Na_2HPO_4 (0.08 M) and 2.4 g KH_2PO_4 (0.01 M) in ddH_2O and make up to 1000 ml. Sterilize the solution through a 0.45 µm filter and store at RT. Dilute the buffer 1 in 10 as a working solution. The pH should be approximately 7.4.

A.5 10x Versene-buffer

Dissolve 40.0 g NaCl, 1.3 g Na_2EDTA, 1.0 g KCl, 5.7 g $Na_2HPO_4.2H_2O$ and 1.0 g KH_2PO_4 in ddH_2O and make up to 500 ml. Sterilize the solution through a 0.45 µm filter and store at RT. Dilute the buffer 1 in 10 as a working solution. The pH should be approximately 7.4.

A.6 RNA extraction

Add 1.5 ml cold solution A (freshly prepared) to the PBL pellet (1-5 x 10^7 cells) after erythrocyte lysis with TE-buffer. Apply repetitive pipetting to ensure complete cell lysis and dissociation of nucleoprotein complexes. Transfer the solution to a sterile 2.2 ml polypropylene tube containing 200 µl chloroform. Vortex the sample for 30 secs. Separate the solution into three phases at 12 000 g for 15 mins at 4°C. Transfer the upper aqueous phase (0.7 ml) to a sterile 1.5 ml polypropylene tube containing 0.7 ml isopropanol. Precipitate the RNA overnight at -20°C. Centrifuge at 12 000 g for 15 mins at 4°C and pour off the supernatant. Add 1 ml 75% ethanol to the RNA pellet and vortex for 15 secs. Centrifuge at 12 000 g for 5 mins at 4°C and aspirate the supernatant. Do not allow the RNA pellet to dry completely. Dissolve the RNA in 60 µl DEPC-treated water. Use repetitive pipetting to aid solubility. Store in 10 µl aliquots under two drops of mineral oil at -80°C. An average yield of 30 µg total RNA (≡ 0.3 µg mRNA) is obtained from 10 ml whole blood.

$OD_{260/280}$ ratio of 1.8 – 2.0 for pure RNA.

Ratio < 1.8 is protein contamination.

Ratio > 2.0 is phenol contamination.

OD_{260} / 25 x dilution factor = µg/µl total RNA.

A.7 Solution B

Prepare a 4 M guanidinium thiocyanate (Merck) solution by dissolving 25 g in 45 ml dd-H_2O at 65°C. Add 1.67 ml 0.75 M sodium citrate (pH 7) and make up to 50 ml with ddH_2O. Sterilize the solution through a 0.45 µm filter while warm. Store at 4°C for up to one year. The pH should be approximately 5.5.

A.8 Water-saturated phenol

Melt crystallized phenol (Merck, 99.5% pure) at 65°C (should be a clear solution). Cool down and add 0.04% 8-hydroxyquinoline (AnalaR). Add an equal volume of DEPC-treated water (pH 5.5) and tumble for 5 mins at RT. Allow the phases to separate overnight at 4°C. Store protected from light at 4°C for up to 3 months.

A.9 2 M Sodium Acetate (pH 4)

Dissolve 3.3 g sodium acetate (Merck, anhydrous) in 6.5 ml DEPC-treated water. Add 12 ml acetic acid (UniLab, 99.5% pure). The pH should be approximately 4.0.

A.10 DEPC-treated dd-H_2O

Add 100 µl diethylpyrocarbonate (DEPC, BDH) per one liter ddH_2O (0.01%). Shake and incubate for 2 hrs or overnight at 37°C. Autoclave to inactivate the residual DEPC.

A.11 Solution A

Mix 1 part water-saturated phenol, 1 part solution B and 0.1 part 2 M sodium acetate (pH 4). Supplement with 50 mM 2-mercapto-ethanol (Merck). Prepare a fresh solution just before use.

A.12 Agarose mini-gel

Melt in a microwave oven 0.25 g, 0.5 g or 0.75 g agarose (Hispanagar) in 45 ml ddH_2O to prepare a 0.5%, 1.0% or 1.5% solutions respectively. Cool the solution to 60°C before adding 5 ml 5x TBE-buffer and 2.5 µl of a 10 mg/ml ethidium bromide solution. Pour the solution into a min-gel apparatus and allow to solidify at 4°C.

A.13 5x Agarose gel-running buffer (TBE-buffer)

Dissolve 54.0 g Tris.base and 27.5 g boric acid in 900 ml ddH_2O. Add 20 ml 0.5 M EDTA solution (pH 8) and make up to 1000 ml with ddH_2O. Dilute the buffer 1 in 10 as a working solution.

A.14 6x Agarose gel-loading buffer (6x AGLB)

Dissolve 0.025 g bromophenol blue (0.25%) and 0.025 g xylene cyanol FF (0.25%) in 7 ml ddH_2O. Add 3 ml glycerol (30%) and sterilize the solution through a 0.45 µm syringe filter. Store in 1 ml aliquots at 4°C. Add 4 µl 6x AGLB to 20 µl PCR product and load on an agarose mini-gel. Electrophorese at 100 Volt/cm for 30 mins at RT.

A.15 Culturing and maintenance of Sf9 cells

Maintain *Spodoptera frugiperda* (Sf9) cells (Invitrogen) as a monolayer in TNMFH10 medium (see A.16 for details) under 100% air and 90% humidity at 27°C. Passage the cells twice per week. When confluent, dislodge the cells from the dish by pipetting the medium gently over the cells. Replate $1/5^{th}$ of the cell suspension with fresh TNMFH10 medium per 100 mm tissue culture dish (Corning). Subculture monolayer cultures for 2 weeks or more before seeding suspension cultures. Maintain suspension cultures under the same conditions as described above on a horizontal platform with orbital shaking at one rotation per second. Plate 5×10^5 cells/ml in 20 ml TNMFH10 medium in a 200 ml Ehrlenmeyer tissue culture flask (Corning). Subculture the cells when the cell density reach 3×10^6 cells/ml by replacing 80% of the cell suspension with fresh medium. Use only one-week-old suspension cultures for virus infections. Maintain frozen cell stocks in liquid nitrogen or at -80°C in 90% FCS plus 10% DMSO at 10^7 cells/ml. Use only cells in the logarithmic growth phase (2×10^6 cells/ml) and with a viability of 95-98% (use trypan blue exclusion determination) for cryopreservation. A doubling time (T½) of 18-22 hrs and a cell viability of 95-98% should be obtained during routine subculturing.

A.16 TNMFH medium

Dissolve 18.67 g Grace's powder medium (Highveld Biological) and 2.28 g $MgCl_2.6H_2O$ in 450 ml dd-H_2O. Dissolve (separately) 3.3 g yeastolate, 3.3 g lactalbumin and 26.68 g sucrose in 450 ml ddH_2O. Mix the two 450 ml solutions. Adjust the pH to 6.2 with 10 M NaOH and make up to 1000 ml with ddH_2O. Prefilter (Millipore type AP) and sterilize the medium through a 0.45 μm filter. Store at 4°C protected from light. Prepare TNMFH10 medium by adding 50 μg/ml gentamycin sulphate and 10% FCS to the TNMFH medium.

A.17 Determination of recombinant virus titer

Plate twelve 60 mm tissue culture dishes with $3x10^6$ Sf9 cells per dish and allow the cells to adhere overnight. Replace the medium with recombinant virus inoculum in TNMFH medium in 10-fold dilutions from 10^{-2} to 10^{-7}. Add 2 ml virus dilution per plate and incubate for 1 hr at RT on a rocking platform (30 secs per oscillation). Turn the dishes 90° every 15 mins. Aspirate the virus and overlay with 5 ml of a 1.5% agarose solution (Sea Plaque low melting point) containing 10% FCS, 1x TNMFH medium, 50 μg/ml gentamycin (Sigma) and 150 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, Sigma). Incubate at 27°C until blue plaques appear on the dishes. Count the number of plaques of a particular virus dilution and determine the virus titer [plaque forming units (PFU) per ml] as 1/virus dilution x number of plaques x 1/ml inoculum per plate.

A.18 Infecting suspension cultures for immunizations

Count a suspension culture with a haemocytometer and determine the cell viability with trypan blue exclusion. Use only cells with a viability of 97-98% or greater for virus infection. Calculate the amount of virus needed as follow:

$$\text{Milliliter inoculum needed} = \frac{\text{MOI (PFU/cell) x number of cells}}{\text{titer of virus (PFU/ml)}}$$

Resuspend the cell pellet in the calculated amount of virus inoculum. Add TNMFH10 medium to a cell density of 1×10^7 cell/ml and incubate for 1 hr at 27°C. Resuspend the cells by adding fresh medium to a cell density of $1\text{-}2 \times 10^6$ cells/ml. Transfer the cell suspension to an Ehrlenmeyer tissue culture flask and culture in suspension for 2-4 days as described under A.15 for suspension cultures. Monitor the expression of the recombinant protein after 3 days with immunofluoresence (see A.44 for details). Collect the culture medium for future inoculums and store at 4°C.

A.19 Blood collection from the mouse eye

Anaesthetise the mouse with ketamine/xylazine at 0.00125 ml/g (half the recommended dose) or with CO_2. Collect blood from the inner corner of the eye with a heparinized capillary tube (60μl each) by twisting the tube to brake the surface capillary vessels. Plug the tube at the one side with

clay to prevent the blood from leaking out of the tube. Remove the cell fraction with centrifugation (1 min) in a standard microhematocrit. Brake the tube at the erythrocyte/plasma interphase by cutting a groove in the glass with a sharp blade. Blow the serum out of the cappillary tube via a rubber tube connection.

A.20 Culturing and maintenance of mouse myeloma cells

Maintain mouse myeloma cells in culture as described for human melanoma cell lines (see A.1 for details). When confluent, dislodge the cells from the dish with 1x versene-buffer at 37°C. Replate at 5 x 10^5 cells per 100 mm dish. Use only sub-confluent myeloma cells with a viability of 95% or more for fusions. Cryopreserve myeloma cells as described for human melanoma cells (see A.1 for details).

A.21 Monoclonal antibody production

On the day of fusion have ready two to three 100 mm tissue culture dishes with mouse myeloma cells of good viability. Dislodge the cells with 1x versene-buffer. Resuspend in RPMI medium and count the cells with a haemocytometer. Slaughter an immunized mouse with a high antibody titer using the cervical dislocation technique. Immobilize the animal on its dorsal side and sterilize the abdominal side with 70% ethanol. Remove the spleen with sterile instruments and transport to the laboratory in RPMI medium. Squeeze the spleen through a sterile stainless steel sieve with the plunger of a 2 ml syringe into a 100 mm tissue culture dish containing 5 ml RPMI medium. Wash the sieve with another 5 ml RPMI medium. Pipette the suspension up and down to break up the clumps and transfer to a 10 ml centrifuge tube. Allow the large pieces of tissue to settle by gravity for a few mins. Transfer the supernatant to a clean 10 ml tube.Wash the cells once with RPMI medium and resuspend in 2 ml RPMI medium. Count the spleen cells by making a 1 in 100 dilution in white cell diluting fluid (0.01% gentian violet and 3% acetic acid in ddH_2O) to lyse the erythrocytes.

Mix the spleen cells with 1/10^{th} the number of myeloma cells in RPMI medium in a 50 ml polypropylene tube. Centrifuge at 500 g for 5 mins. Aspirate the supernatant and gently loosen the pellet by tapping on the side of the tube. Add 1.5 ml polyethylene glycol (PEG) at 37°C dropwise over 2 mins to the cells with gentle mixing. Add 2 ml RPMI-medium (37°C) slowly over 2 min with gentle mixing. Add 8 ml RPMI-medium over the next 2 mins. Finally, add RPMI-medium to 45 ml. Centrifuge at 500 g for 5 mins. Aspirate the supernatant. Resuspend the cell pellet (with not to much pipetting) in HAT10-medium to a concentration of approximately 10^6 spleen cells/ml medium. Use HAT10-medium with FCS if the antigen is present in human serum. Dispense 1 ml aliquots per well of a 24-well tissue culture plate. Dispense 10^5 myeloma cells in one well of the plate for the control. These cells should all die within 3 days. Feed the cells with 0.5 ml HAT10-medium per well after an one week incubation period. Hybridoma clones should have grown within 10-14 days. Remove some of the supernatant when the medium starts to turn yellow and test for antibody.

Expand those hybridomas that tested positive for specific antibody into 60 mm tissue culture dishes in HT10-medium (HAT10-medium without aminopterin). Cryopreserve when enough cells have been obtained.

Clone selected hybridomas by limiting dilution to obtain single cell clones: Prepare a cell suspension at a concentration of approximately 10 cells/ml HT10-medium. Plate 200 µl aliquots into the wells of a 96-well flat-bottom tissue culture plate (Nunc) to obtain approximately 1-2 cells per well. If the culture supernatant was only weakly positive it may be necessary to prepare several 96-well plates or to clone initially at 10 cells per well. Feed the cells with 100 µl HT10-medium per well after 5-7 days of incubation. Prepare to screen for antibody when the clones become macroscopically visible and when the medium of some of the clones start to turn yellow. Sample the supernatants and test for antibody. Choose 2-3 positive clones for re-cloning. Expand the clones and cryopreserve. Repeat the cloning procedure to ensure that the final hybridoma clone will be from a single cell. Expand and cryopreserve several clones. Allow the hybridoma clone to adapt for growth in RP10-medium (Harlow & Lane, 1988; Pontecorvo, 1975).

A.22 Polyethelene glycol (PEG)

Weigh 2 g PEG (Merck, Mr = 4000 daltons) and autoclave. Add 2 ml RPMI at 37°C to the liquid PEG before it solidifies.

A.23 1000x Aminopterin

Dissolve 17.6 mg aminopterin (Sigma) in 25 ml ddH_2O by increasing the pH with 0.5 ml 5 N NaOH and neutralizing it again with 0.5 ml 5 N HCl. Make up to 100 ml with ddH_2O. Sterilize the solution through a 0.45 µm filter and store in 10 ml aliquots at -20°C protected from light. Dilute 1 in 1000 in RPMI medium to obtain a final concentration of 4×10^{-7} M as the working solution.

A.24 100x HT

Dissolve 0.34 g hypoxanthine (Sigma) in 125 ml ddH_2O by increasing the pH with 2.5 ml 10 N NaOH (add dropwise with stirring). Add 0.0968 g thymidine (Sigma), 0.0055 g glycine and 2.7515 g sodium pyruvate and make up to 250 ml with ddH_2O. Sterilize the solution through a 0.45 µm filter and store in 10 ml aliquots at -20°C. Dilute 1 in 100 in RPMI medium to give final concentrations of 1×10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine, 3×10^{-6} M glycine and 1×10^{-3} M sodium pyruvate.

A.25 HAT10-medium

Add 0.2 ml 1000x aminopterin, 2 ml 100x HT and 20 ml HUCS or FCS (Flow laboratories) to 200 ml RPMI medium.

A.26 Human umbilical cord serum (HUCS)

Collect and pool HUCS from different HIV-free patients. Heat-treat the serum, filter and store as described for FCS in A.3.

A.27 10x TST

Dissolve 60.6 g Tris.HCl (0.5 M), 58.4 g NaCl (1 M) and 5.0 ml Tween 20 (0.5%) in 900 ml ddH_2O. Adjust the pH to 7.6 with 1 M NaOH and make up to 1000 ml with ddH_2O. Store at RT. Dilute the buffer 1 in 10 as a working solution.

A.28 Sheep anti-mouse IgG (SAM) and mouse anti-beta galactosidase

A sheep anti-mouse IgG has been produced previously in the Department of Clinical Science and Immunology (Heussen-Schemmer *et al.*, 1993). The whole sheep serum was diluted 1 in 200 in PBS/0.1% BSA as a working solution. The mouse anti-beta galactosidase Mab was prepared previously in the department with standard monoclonal antibody technology.

A.29 Mouse anti-peroxidase Mab plus peroxidase (PAP)

An anti-peroxidase mouse Mab has been produced previously in the Department of Clinical Science and Immunology (Heussen-Schemmer *et al.*, 1993). The ascites fluid of mice injected with the anti-peroxidase hybridoma cells was diluted 1 in 200 in PBS containing 100 µg/ml horse radish peroxidase (Sigma or Seravec), 0.5% BSA and 0.01% thiomersal. The solution was stored at 4°C. Dilute 1 in 10 or 1 in 20 for ELISA's or Western blots.

A.30 ABTS substrate

Dissolve 2.1 g monohydrate citric acid (0.05 M) in 190 ml ddH_2O. Adjust the pH to 4 with 1 M NaOH and make up to 200 ml with ddH_2O. Store in 10 ml aliquots at -20°C. Thaw a frozen aliquot just before use. Add 100 µl 40 mM 2,2' azino-di (3-ethyl-benzthiazoline) sulfonic acid (ABTS, Sigma) and 10 µl 30% H_2O_2 to obtain a working solution.

A.31 Isotyping of Mabs

Coat a 96-well flat-buttom polystyrene ELISA plate (Nunc, MaxiSorp) with 10 µg/ml rabbit anti-mouse Ig (Serotec) in PBS for 2 hrs at RT. Add all the reagents at 50 µl per well for 30 mins at RT, except if mentioned differently. Wash five times with TST-buffer and block the plate with PBS/0.1% BSA. Add 24-48 hr serum-free CM from the hybridoma clones to be isotyped. Wash the plate as before. Add 20 µg/ml sheep isotyping sera (Serotec) specific for mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA. Wash the plate as before. Add 20 µg/ml peroxidase-conjugated rabbit anti-goat Ig (Dako, cross-react with sheep antibodies). Wash the plate 10 times with TST-buffer. Add 100 µl per well ABTS in citrate buffer as substrate (see A.29 for details). Measure the absorbance at 405 nm after 30 mins (Coligan *et al.*, 1994a).

A.32 Ascites production

Prime Balb/c mice with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane, Aldrich) intraperitoneally (i.p.). Inject 1-5 x 10^6 hybridoma cells in 0.5 ml PBS per mouse i.p. 1-2 weeks after priming. Collect the ascites from the extended abdomen after approximately 2 weeks. Centrifuge (800 g, 5 mins) the ascites and use the antibody-containing supernatant to purify the antibody with caprylic acid and ammoniumsulphate precipitations.

A.33 Caprylic acid and ammoniumsulphate purification of antibodies

Add 200 ml 0.06 M acetate buffer (pH 4) to 100 ml ascites fluid or 10x concentrated serum-free hybridoma conditioned medium. Adjust the pH to 4.8 with 1 M HCl if necessary. Add 3.3 ml n-octanoic acid (caprylic acid, Sigma) dropwise with stirring and continue to mix for 30 mins at RT. Centrifuge at 10 000 g for 30 mins at 4°C. Keep the supernatant which contains the antibody and filter through a 0.45 µm syringe filter. Dialyze twice against 50 volumes of PBS containing 2 mM EDTA. Precipitate the immunoglobulin by slowly adding ammonium sulphate crystals to 50% saturation (100% saturation = 70.5 g/100 ml). Centrifuge as described before. Dissolve the precipitate in PBS and dialyze twice against 50 volumes of PBS. Measure the absorbance at 280 nm (OD_{280} of 1.38 = 1 mg/ml IgG). Store in suitable aliquots at -20°C.
Polyclonal antibodies can also be purified with this method. Add 200 ml 0.06 M acetate buffer (pH 4) to 100 ml serum. Adjust the pH to 4.8 with 1 M HCl if necessary. Adjust the amount of caprylic acid added according to the species, ie. 8.2 ml for rabbit, 7.6 ml for horse and human, 6.8 ml for bovine, 8.0 ml for goat and 7.6 ml for sheep (Reik *et al.*, 1987).

A.34 5x Tris-glycine reservoir buffer

Dissolve 15.1 g Tris.base and 94.0 g glycine in 900 ml ddH_2O. Add 50 ml 10% SDS and make up to 1000 ml with ddH_2O. Dilute the buffer 1/5 as a working solution for mini-gels and 1/10 for large gels.

A.35 30% Acrylamide mix

Dissolve 29.2 g acrylamide (Sigma) and 0.8 g N,N'-methylene-bis-acrylamide (BDH) in 90 ml ddH_2O and make up to 100 ml. The pH should be approximately 7. Sterilize the solution through a 0.45 µm filter and store at 4°C protected from light for up to 6 months.

A.36 SDS sample buffer

Reagent	2x	6x
4x Tris/SDS, pH 6.8	**2.5 ml**	**7.0 ml**
SDS	**0.4 g**	**1.0 g**
Bromophenol blue	**2 mg**	**6 mg**
Glycerol	**2.0 ml**	**3.6 ml**

Dissolve the above mentioned reagents in 4x Tris/SDS, pH 6.8 buffer and make up to 10 ml with ddH_2O. Sterilize through a 0.45 µm syringe filter and store in 0.5 ml aliquots at -20°C.

A.37 4x Tris/SDS, pH 6.8

Dissolve 6.05 g Tris.base in 90 ml ddH_2O. Adjust the pH to 6.8 with 1M HCl. Correct the pH to 6.8 after 30 mins and make up to 100 ml with ddH_2O. Sterilize through a 0.45 µm filter. Add 0.4 g SDS and store at 4°C.

A.38 4x Tris/SDS, pH 8.8

Dissolve 36.4 g Tris.base in 150 ml ddH_2O. Adjust the pH to 8.8 with 1 M HCl. Correct the pH to 8.8 after 30 mins and make up to 200 ml with ddH_2O. Sterilize through a 0.45 µm filter. Add 0.8 g SDS and store at 4°C.

A.39 Separating (running) gel

Mix in the following order: Final acrylamide conc. in the running gel (%)

Stock solutions	5	7.5	10	12	15
ddH_2O	**8.75**	**7.50**	**6.25**	**5.25**	**3.75**
30% Acrylamide mix	**2.5**	**3.75**	**5.00**	**6.00**	**7.50**
4x Tris/SDS, pH 8.8	**3.75**	**3.75**	**3.75**	**3.75**	**3.75**
10% Ammonium persulphate	**0.05**	**0.05**	**0.05**	**0.05**	**0.05**
TEMED*	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**

* N,N,N',N'-tetramethylethylenediamine (Sigma)

Pipette the solution between two pre-sealed (use 1% agarose) glass plates of the gel apparatus. Overlay the solution with water-saturated iso-butanol to prevent the inhibition of polymerization by oxygen. Allow the polymerization reaction to complete for 1 hr at RT. Note: Prepare fresh ammonium persulphate daily.

A.40 4% Stacking gel

Mix in the following order: 3.05 ml ddH_2O, 0.65 ml 30% acrylamide mix, 1.25 ml 4x Tris/SDS (pH 6.8), 0.025 ml 10% ammonium persulphate and 0.005 ml TEMED. Pour the stacking gel immediately and place the teflon comb into position. Allow the polymerization reaction to complete for 15 mins at RT.

A.41 Gel electrophoresis

Remove the comb from the gel and rinse the wells with 1x Tris-glycine reservoir buffer to remove any unpolymerized acrylamide. Run the protein samples in 1x SDS sample buffer under reduced (200 mM dithiothreitol, DTT, Sigma) or non-reduced conditions. Boil samples for 3-5 mins prior to electrophoresis unless proteolytic activity or protein complexes are of importance. Electrophorese mini-gels at 100 V/cm or at constant current of 10 mAmps for 2 hrs at RT or at 4°C. Stain gels with 0.1% Coomassie brilliant blue (Sigma) solution in 30% methanol and 10% glacial acetic acid for 1 hr at RT. Destain with 30% methanol and 10% glacial acetic acid solution for several hours.

A.42 Western blot

Cut a piece of Immobilon-P PVDF membrane (Millipore) to the size of a mini-gel. Pre-wet for a few seconds in 100% methanol and then for 1 min in transfer buffer. Cut two pieces of Whatmann filter paper (3 mm chr) to the same size as the PVDF membrane and pre-wet in transfer buffer. Use two carbon blocks (20 x 15 x 2 cm) as electrodes and two pieces of absorbant material (20 x 15 cm) as reservoirs for the transfer buffer. Prepare the western blot sandwich as follows (from bottom to top) and place in a plastic container:

- Carbon block (negative electrode, **top**)
- Absorbant pad
- Filter paper
- Polyacrylamide gel
- PVDF membrane
- Filter paper
- Absorbant pad
- Carbon block (positive electrode, **bottom**)

Blot a 12 x 6 x 1 mm mini-gel for 1 hr at 220 mAmps.

A.43 Transfer buffer

Dissolve 6.05 g Tris.base (0.05 M) and 14.4 g glycine (0.2 M) in 200 ml methanol and make up to 1000 ml with ddH_2O. Store at 4°C. The pH should be approximately 8.3.

A.44 Enhanced chemiluminescence (ECL)

Use the chemiluminescent western blot detection system from Boerringer and Mannheim to enhance the peroxidase signal on a blot. Dilute solution B (H_2O_2) 1 in 100 in solution A (luminol and 4-iodophenol) and incubate for 30 mins at RT. Add 125 µl of the detection reagent per cm^2 membrane for 1 min. Drain excess detection reagent from the blot. Seal the blot with "Cling Wrap" and expose immediately to Cronex 4 X-ray film for 10 secs to 5 mins. Determine the exposure time experimentally. Develop the film with Kodak X-ray developer for 5 mins. Stop development with 2% acetic acid solution for 1 min and fix with Kodak fixative for 5 mins. Rinse the X-ray film thoroughly with running tap water and air dry.

A.45 Multi-well cell monolayers for immunocytochemistry

Prepare teflon-coated, 8-well (6 mm diameter) slides with double-sided sellotape (2.5 cm wide) and Whatman no.50 filter paper according to a standard method (Veenstra & Dowdle, 1992). Pipette 20 µl of a cell suspension ($1x10^6$ cells/ml in RP10 medium) unto each well of a prepared slide. Allow the slide to dry overnight at RT before removing the tape and filter paper. Store the slides for up to one week in a dessicator or wrap the slides in alminium foil and store for an indefinite period at -20°C.

Fix the cells with 50% methanol/50% acetone for 90 secs at RT and transfer immediately to 1x TBS for 3-5 mins (use Coplin-jars). Block with 0.1% BSA in PBS for 15 mins. Add 20 µl of the primary antibody (10 µg/ml in PBS/0.1% BSA) to the appropriate wells and incubate for 30 mins in a moist chamber. Wash the slide twice with PBS. Flood the slide (500 µl) with a fluorescein-labelled secondary antibody and incubate for 30 mins. Wash the slide twice with PBS. Counterstain the cell nuclei with ethidium bromide (1 µg/ml in PBS) for 1 min and wash twice with PBS. Mount the cells temporally in PBS/30% glycerol or permanently in Mowiol mounting medium. View under the fluorescent micropscope using a blue excitation filter.

A.46 Mowiol mounting medium

Add 2.4 g Mowiol 4-88 (polyvinylalcohol, Hoechst) to 6 ml glycerol. While stirring, add 6 ml ddH_2O and leave for several hours at RT. Add 12 ml 0.2 M Tris (pH 8.5) and incubate for 1 hr at 50°C with occasional stirring. Add 1,4-diazobicyclo-[2.2.2]-octane (Dabco) to 2.5% to reduce immunofluorescence fading. Store in 2 ml aliquots at -20°C. Centrifuge at 12 000 g for 5 mins to remove insolubles before using the mounting medium.

A.47 DAB substrate

Prepare 25 mg/ml diaminobenzidine (Sigma) as a stock solution in ddH_2O and store in 1 ml aliquots at -20°C. Dilute 1 in 50 in PBS containing 0.03% H_2O_2 and 0.03% $CoCl_2$ as a working solution.

A.48 Purification of human plasminogen (Plg)

Collect 1 liter fresh citrated blood. Centrifuge the blood at 700 g for 10 mins at RT. Sterilize the plasma through a 0.45 μm filter and dilute with 2 parts PBS. Apply the diluted plasma at a flow-rate of 1.5 ml/min to a lysine-Sepharose column (Pharmacia) equilibrated with PBS at 4°C. Wash the column once with PBS and then with 1 M KCl in PBS (pH 7.4) containing 40% ethylene glycol. Wash the column with PBS containg 3 mM EDTA until the baseline OD_{280} is stable. Elute the Plg as a single peak (OD_{280}) with 0.2 M ε-aminocaproic acid (ε-ACA) in PBS (pH 7.4). Collect 2 ml aliquots and pool the Plg-containing fractions. Mix an equal volume of chloroform with the eluant. Remove the aqueous phase and dialyse 3x against PBS. Determine the Plg concentration by measuring the absorbance at 280 nm (OD_{280} of 1.7 = 1 mg/ml). The method yields more than 99% pure Plg (Castellino & Powell, 1981; O' Reilly *et al.*, 1996). Use the same protocol to prepare Plg-free FCS which is collected as the flow-through.

A.49 Preparation of Plg-free human fibrinogen (Fg)

Dissolve 1 g of human Fg (Kabi) in 50 ml 0.1 M KPO_4-buffer (pH 6.4, mix 72.2 ml 1 M KH_2PO_4 and 27.8 ml 1 M K_2HPO_4 and make up to 1000 ml with ddH_2O). Make up to 100 ml with ddH_2O and tumble overnight at 4°C. Centrifuge at 1000 g for 10 mins at RT and discard the pellet. Precipitate the supernatant by adding slowly 1/3 the volume saturated ammonium sulphate at RT. Allow to stand for 1 hr (stir occasionally with a glass rod) and centrifuge as before. Dissolve the "gummy" pellet in 20 ml 0.6 M NaCl (with gentle stirring at 37°C) and adjust the pH to 7.4 with ammonium hydroxide. Dialyse the solution overnight at RT against 0.6 M NaCl containing 10^6 U/ml penicillin and 0.2 g/l streptomycin sulphate. Determine the Fg concentration by measuring the absorbance at 280 nm (OD_{280} of 1.3 = 1 mg/ml). Adjust the concentration to 10 mg/ml with 0.6 M NaCl. Dilute one part Fib with 5 parts 0.12 M lysine-HCl in 0.005 M $NaPO_4$-buffer (pH 7). Bring the solution to 0°C in a salt-ice bath. Discard any precipitate that may form. Add 8 ml 95% ethanol to every 100 ml solution (at 0°C) and stir constantly with a glass rod to prevent the Fg from solidifying. Centrifuge the solution as before and redissolve the precipitate in 10 ml 0.6 M NaCl at 37°C. Dialyse overnight at RT against PBS (containing antibiotics). Pre-filter (Millipore type AP) and sterilize through a 5.0 and 0.45 μm filters. Measure the absorbance at 280 nm and adjust the concentration to 5 mg/ml. Store at –20°C (Mosesson, 1962).

0.12 M Lycine-HCl in 0.005 M $NaPO_4$-buffer

Dissolve 10.96 g lysine-HCl and 0.45 g $Na_2HPO_4.2H_2O$ in 500 ml ddH_2O. Dissolve 10.96 g lysine-HCl and 0.34 g $NaH_2PO_4.H_2O$ in 500 ml ddH_2O. Combine the two solutions to obtain a pH of 7.

A.50 Labelling of Fg with ^{125}Iodine (Salacinski *et al.*, 1981)

Dissolve 1 mg Iodogen (1,3,4,6-tetrachloro-3',6',-diphenylglycoluril, Pierce) in 50 ml chloroform. Prepare 1 ml aliquots and allow the chloroform to evaporate overnight at 4°C. Store at -80°C and thaw just before use.

Prepare a Sephadex-G10 column in PBS. Wash the column once with FCS and 10x with PBS. Block the column with 2 ml cold Fg (5 mg/ml) and wash finally once with PBS. Incubate 5 mg cold Fg in 400 µl PBS with 2.5 mCi ^{125}Iodine (Amersham) in a pre-prepared Iodogen tube for 4 mins at RT. Separate the hot Fg from the free ^{125}Iodine on the Sephadex-G10 column. Collect fractions of 7 drops per tube and count 5 µl of each fraction on a Gamma-counter. Pool the hot Fg from the first peak which will be followed by a higher peek of free ^{125}Iodine. Filter the hot Fg through a PBS-saturated 0.45 µm syringe filter. Dilute the hot Fg 1 in 100 in 500 µl PBS and count three 5 µl aliquots on the Gamma-counter. Calculate the mean cpm/ml as the (mean cpm of the three aliquots) x 100 x 200.

Incubate 900 µl RP10 with 100 µl trichloro-acetic acid and 5 µl hot Fg for 30 mins on ice. Centrifuge at 12 000 g for 1 min. Count the protein pellet and supernatant separately and estimate the efficiency of ^{125}Iodine-incorporation as follows: (cpm of pellet) / (cpm of pellet + cpm of supernatant) x 100. Determine the hot Fg concentration by measuring the absorbance at 280 nm.

A.51 Preparation of ^{125}Iodine fibrin plates (Strickland & Beers, 1976)

Require 6.5 x 10^5 cpm/ml of hot Fg to prepare plates. Calculate the volume (µl) of hot Fg needed as (6.5 x 10^5 cpm/ml) / (cpm/ml of hot Fib). Calculate the amount (µg) of hot Fg needed as (mg/ml hot Fg) x (µl hot Fg). Need 150 µg/ml Fg in total. Calculate the amount of cold Fg needed as (total amount) – (hot amount). Calculate the volume of cold Fg needed as (cold amount) / (mg/ml cold Fg). Need 5 ml Fg per 24-well plate (Linbro or Cell-Cult). Make up the calculated amounts of hot and cold Fg with ddH_2O (37°C) and add 200 µl per well. Incubate the plate for 4 days at 37°C to dry. Store the plates for up to 3 months at RT in a fume hood behind lead bricks.

A.52 Fibrin plate assay (Wilson & Dowdle, 1978)

The principle of the assay is the conversion of insoluble Fg to soluble fibrin peptides by plasmin, which in turn is generated by the activation of Plg by PAs. PA activity can thus be measured indirectly by counting the amount of ^{125}I-labelled fibrin peptides that are released from the plate.

Incubate a ^{125}I-fibrin plate for 1-2 hrs at 37°C with 1 ml RPMI per well. Wash the plate twice with 1 ml PBS per well and then with 1 ml 0.1 M Tris-buffer (pH 8.1) per well. Dry the plate with suction and keep at 4°C.

Prepare a Plg-cocktail by mixing 250 µl 0.1 M Tris-buffer (pH 8.1) with 20 µl 0.1 mg/ml PIF Plg and 10 µl 8 mg/ml PIF BSA. Prepare a PA standard (tPA or uPA) starting at 2.5 IU/ml PA with 12 doubling dilutions. Add 20 µl of each dilution to 280 µl Plg-cocktail per well to generate a PA

standard starting at 0.05 IU per well. Add 20 µl of each sample to be tested (neat or diluted) to 280 µl Plg-cocktail per well. Dilute all samples in RPMI medium containing 3% Plg-free FCS (RP3, see A.47 for details). Control wells include (a) 300 µl 0.25% trypsin solution in PBS to give the total radioactivity present in each well, (b) 280 µl Plg-cocktail plus 20 µl RP3 to detect background lysis due to plasmin contamination of Plg, (c) as control b, but without Plg to detect protease contamination of the other reagents, (d) the sample, but without Plg to detect Plg independent protease activity. Include two test-wells containing 0.05 IU per well PA and two extra wells with trypsin on the plate.

Incubate the plate for 1 hr in a 37°C incubator with 90% humidity. Remove 50 µl time-points from the test-wells and from the extra trypsin-wells and count for 1 min in the gamma-counter. Take only the full time-point when the test-wells give cpm of between 25-30% of the trypsin-wells. Continue incubation if less than 25%. Take three time-points (30-60 mins each). Plot the standard curve for PA with the log(%trypsin) on the y-axis and mUnits PA on the X-axis. Convert each sample to mUnits PA/ml and express as mUnits PA/10^6 cells for tissue culture supernatants.

A.53 Protease and inhibitor free (PIF) BSA

Subject a 20 mg/ml BSA solution (Boehringer Mannheim) to acid-treatment to destroy protease inhibitors by adjusting the pH to 3 with 2 M HCl for 2 hrs at RT and then restore the pH to 7.4 with 2 M NaOH. Treat the BSA solution with 0.01 M di-isopropyl fluorophosphate (DFP, Sigma) for 2 hr at 40°C to inactivate proteases. Dialyse the solution overnight against 0.1 M Tris (pH 8.1) at 4°C. Pre-filter with a Millipore type AP and sterilize through a 0.45 µm syringe filter. Measure the absorbance at 280 nm (OD_{280} of 6.15 = 10 mg/ml) and adjust the concentration to 8 mg/ml. Store the PIF BSA in aliquots at –20°C. Obtain PIF Plg using the same protocol.

A.54 Activation of Sepharose CL-4B with Cyanogen bromide (CNBr).

Dissolve 0.5 g CNBr (Sigma) in 1 ml acetonitrile (62.5% solution, wt/vol). Use a fume hood to perform all the steps. Wash 25 ml Sepharose CL-4B (Pharmacia) in a sintered-glass funnel with 10 volumes ddH_2O. Transfer the beads to a 50 ml polypropylene tube and resuspend in 25 ml 0.2 M Na_2CO_3-buffer. Activate the sepharose by adding the 1 ml CNBr-solution dropwise over 1 min with slow stirring. Stir the slurry for another 7 mins. Transfer immediately to a sintered-glass funnel and wash with 10 volumes ice-cold 1 mM HCl. Wash with 2 volumes ice-cold 0.1 mM HCl and finally with 2 volumes coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Transfer the activated sepharose immediately to an equal volume of coupling buffer containing the antibody or protein (5-10 mg/ml) to be coupled (Coligan *et al.*, 1994b).

A.55 Immobilization of antigens or antibodies to sepharose

Dialyze the antibody or antigen that must be coupled to the activated sepharose against coupling buffer. Adjust the concentration to 5-10 mg/ml. Incubate (with tumbling) equal volumes of activated

sepharose with the antibody or antigen for 2 hrs at RT or overnight at 4°C. Determine the coupling efficiency by measuring the absorbance of the supernatant at 280 nm. The OD_{280} reading should be significantly less compared to what was obtained before the coupling procedure. Block the remaining active groups with 0.2 M glycine-buffer (pH 8) for 2 hrs at RT. Wash excess adsorbed antibody or antigen away with three consecutive washes coupling buffer (pH 8.3) alternating with acetate buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 4). Equilibrate the antibody or antigen coupled beads in PBS and store at 4°C with 0.05% sodium azide as an anti-bacteriostatic agent. Use a suitable syringe fitted with a porous polyethylene disk to prepare a column. Prepare a precolumn by blocking activated sepharose with 0.2 M glycine-buffer (pH 8) for 2 hrs at RT and then equilibrate the beads in PBS. Use the precolumn to remove non-specific binding to sepharose beads (Coligan *et al.*, 1994b).

A.56 Preparation of 2c-tPA

Prepare Plg-sepharose as described in A.48 and A.53 – A.54 and equilibrate the beads in 0.1 M Tris (pH 8) containing 0.02% Triton X100. Mix 1 mg/ml recombinant tPA in 0.1 M Tris (pH 8) containing 0.02% Triton X100 with an equal volume of Plg-sepharose and tumble at RT. Remove 50 µl aliquots every 60 mins and assay the supernatant (1 in 100 dilution) with a chromogenic assay (American Diagnostica) for plasmin activity. The reaction is completed when a plateau is reached (approximately after 3 hrs). Record the protein concentration with the BCA protein assay (Pierce) using BSA as the standard. Assay the activity of 2c-tPA by recording the rate of hydrolysis of the chromogenic substrate CH_3SO_2-D-cyclohexyltyrosyl-Gly-Arg-p-nitroanilide (American Diagnostica). Measure the absorbance of released nitroanilide at 405/680 nm. Use 2c-tPA with a known activity as the standard and express the activity as IU/ml and as IU/mg.

A.57 p-Nitrophenyl phosphate (PNPP) soluble substrate for ELISA

Mix 97 ml diethanolamine (liquefy at 50°C, Merck) with 700 ml ddH_2O and 100 mg $MgCl_2.6H_2O$. Adjust the pH to 9.8 with 1 M HCl and make up to 1000 ml with ddH_2O. Store protected from light at 4°C. Dissolve 10 mg/ml PNPP (Boehringer Mannheim) in the above buffer and store in 1 ml aliquots at -20°C. Dilute an aliquot 1 in 10 in the above buffer just before use.

A.58 PAI1 data for controls

n = 38 Controls	ng/ml Total PAI1	ng/ml Latent PAI1	ng/ml Active PAI1	% active PAI1	Gender	Age
1	38	13	25	66	F	48
2	17	9	8	47	F	50
3	11	5	6	55	F	38
4	87	27	60	69	F	51
5	43	17	26	60	F	34
6	18	10	8	44	F	40
7	17	8	9	53	F	52
8	29	14	15	52	F	53
9	64	21	43	67	F	78
10	20	7	13	65	F	29
11	18	8	10	56	M	31
12	16	10	6	**38**	F	49
13	14	6	8	57	F	26
14	14	7	7	50	F	27
15	**271**	54	217	80	F	48
16	66	22	44	67	M	28
17	10	3	7	70	F	45
18	64	10	54	84	M	25
19	43	15	28	65	M	39
20	25	8	17	68	M	51
21	82	18	64	78	M	53
22	32	13	19	59	M	60
23	41	14	27	66	F	39
24	80	22	58	73	F	37
25	33	14	19	58	F	43
26	15	7	8	53	F	54
27	70	24	46	66	M	32
28	**326**	69	257	79	M	34
29	29	11	18	62	F	42
30	67	18	49	73	F	56
31	81	26	55	68	F	48
32	23	9	14	61	F	35
33	86	45	41	48	M	23
34	60	26	34	57	M	29
35	35	12	23	66	F	31
36	26	6	20	77	F	50
37	32	13	19	59	F	27
38	162	75	87	54	F	36
Min	10	3	6	38	**M = 11**	23
Max	326	75	257	84	**F = 27**	78
Mean	57	18	39	62		41.3
SD	66	17	52	10.5		11.9
Median	34	13	22	64		39.5

A.59 PAI1 data for patients with PM

n = 21 PM	Stage	ng/ml Total PAI1	ng/ml Latent PAI1	ng/ml Active PAI1	% active PAI1	Gender	Age
1	1	40	14	26	65	M	64
2	1	39	28	11	**28**	F	50
3	2	45	21	24	53	M	59
4	2	43	17	26	60	M	58
5	2	22	13	9	**41**	F	60
6	2	53	25	28	53	M	62
7	1	32	15	17	53	M	48
8	2	60	23	37	62	M	62
9	1	69	24	45	65	M	56
10	2	31	14	17	55	M	54
11	2	196	49	147	75	M	56
12	1	16	7	9	56	F	45
13	2	**694**	279	415	60	F	63
14	1	46	24	22	48	M	64
15	2	**1257**	797	460	**37**	M	69
16	2	45	29	16	**36**	F	71
17	1	120	29	91	76	M	51
18	2	29	13	16	55	F	63
19	2	15	5	10	67	F	57
20	2	41	23	18	44	M	73
21	2	22	9	13	59	M	80
	Min	15	5	9	28	**M = 14**	45
	Max	1257	797	460	76	**F = 8**	80
	Mean	139	69	69	55		60.2
	SD	295	176	127	12.4		8.5
	Median	43	23	22	55		60

A.60 PAI1 data for patients with AM

n = 18 AM	Stage		ng/ml Total PAI1	ng/ml Latent PAI1	ng/ml Active PAI1	% Active PAI1	Gender	Age
1	3		**528**	227	301	57	M	43
2	4		78	21	57	73	M	70
3	4		135	62	73	54	F	63
4	4		83	60	23	**28**	M	59
5	4		37	21	16	**43**	F	64
6	3		181	140	41	**23**	F	?
7	3		49	31	18	**37**	F	58
8	3		12	6	6	50	F	50
9	3		24	11	13	54	M	?
10	4		36	14	22	61	F	83
11	4		25	17	8	**32**	M	85
12	3		15	11	4	**27**	F	36
13	3		189	112	77	**41**	F	59
14	4		110	70	40	**36**	F	79
15	3		40	28	12	**30**	M	58
16	3		9	5	4	44	F	38
17	3		11	4	7	64	M	21
18	4		27	9	18	67	M	88
		Min	9	4	4	23	**M = 8**	21
		Max	528	227	301	73	**F = 10**	88
		Mean	88	47	41	46		59.6
		SD	124	59	69	15.1		19
		Median	39	21	18	44		59

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