PURINERGIC ANGIOGENESIS

An investigation of the role of extracellular nucleotide mediated signaling in angiogenesis.

by

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ABSTRACT

Angiogenesis refers to the growth and maturation of new vessels from pre-existing differentiated blood vessels. Co-ordination of angiogenic responses is crucial for a wide range of physiological and pathological processes. In particular, angiogenesis is hypothesized to facilitate tumour growth and promote metastasis, prompting research into anti-angiogenesis based cancer therapies.

CD39/NTPDase1 is an ectoenzyme expressed by vascular endothelium that hydrolyses extracellular nucleoside di- and triphosphates to the monophosphate derivatives. As such, this enzyme has a role in modulating endothelial responses to nucleotides that bind specific receptors termed Purinergic-type 2 (P2) receptors. Since a number of nucleotides, including ATP and UTP, have been shown to promote endothelial proliferation and migration in vitro, we propose that nucleotide signaling influences the process of angiogenesis in vivo.

A *Cd39*-null mouse has been developed in our group, and our studies show that deletion of *Cd39* results in disordered angiogenesis in several experimental models. The *Cd39*-null mouse exhibits limited angiogenesis following experimental myocardial infarction, in liver regeneration models and, most notably, during tumour angiogenesis. The mechanisms underlying these defects in angiogenesis have been a focus of our investigations.

We established primary pulmonary endothelial cell cultures from wild-type and *Cd39*-null mice. *Cd39*-null endothelial cells had defective attachment to vitronectin compared with wild-type, but exhibited normal attachment to collagen and fibronectin, suggesting differential integrin dysfunction, possibly implicating $\alpha_V\beta_3$. Treatment with $MnCl_2$, a non-specific integrin activator, corrected this defective adhesion. In addition, we were able to show decreased integrin-mediated intracellular signaling via focal adhesion kinase (FAK) together with extracellular signaling-regulated kinase-1 and -2 (ERK1/2) in *Cd39*-null endothelial cells. In keeping with our hypothesis that integrin dysfunction occurs secondary to P2-receptor desensitization, pre-treatment of the endothelial cells with soluble NTPDase resulted in the correction of both the adhesion and the intracellular signaling defects.

These findings demonstrate for the first time novel links between purinergic signaling, integrin function and angiogenesis. Our data raise the possibility of developing new therapies for a wide-range of human inflammatory vascular diseases.

CONFERENCE PROCEEDINGS

Aspects of the research included in this dissertation have been presented at international scientific conferences.

Decreased activity of endothelial $\alpha_V\beta_3$ integrin in the *Cd39*-null mouse is associated with defective tumor angiogenesis.

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TABLE OF CONTENTS

CHAPTER 1 – LITERATURE REVIEW
OVERVIEW OF ANGIOGENESIS

Page

1.1 Angiogenesis 12
1.2 A brief history of angiogenesis research 14
1.3 Tumour angiogenesis 17
1.3.1 The formation of tumour blood vessels 18
1.3.2 Characteristics of tumour vasculature 21
1.4 The role of angiogenesis in tumour metastasis 23
1.5 Angiogenic growth factors 26
1.5.1 The VEGF family 27
1.5.2 VEGF receptors 30
1.5.3 Fibroblast growth factor 31
1.5.4 The angiopoietins 32
1.6 Matrix biology 35
1.6.1 Structure and function of the extracellular matrix 35
1.7 Extracellular proteolysis 38
1.7.1 The matrix metalloproteinase family 39
1.7.2 The role of the matrix metalloproteinase family in angiogenesis
1.7.3 The plasminogen activator-plasmin system 43
1.7.4 The role of the plasminogen activator-plasmin system in angiogenesis 44
1.8 Integrins and angiogenesis 46
1.8.1 The integrin family of cell surface receptors 46
1.8.2 The role of integrins in angiogenesis 48
1.9 Anti-angiogenesis therapy 53
1.9.1 Angiogenesis inhibitors 54
1.9.2 Unique features of anti-angiogenesis therapy 60
1.9.3 Potential problems with anti-angiogenic cancer therapies 61
1.10 Experimental angiogenesis models 65
1.10.1 In vitro assays 66
1.10.2 Organ culture assays 70
1.10.3 In vivo assays 71

CHAPTER 2 – LITERATURE REVIEW
PURINERGIC SIGNALING

2.1 Purinergic signaling 77
- 2.1.1 Purinergic type-1 and type-2 receptors 78
- 2.1.2 ATP release mechanisms 81

2.2 Extracellular nucleotide metabolism 83
- 2.2.1 The E-NTPDase family 83
- 2.2.2 Ecto-5'-nucleotidase 85
- 2.2.3 The E-NPP family 86
- 2.2.4 Alkaline Phosphatases 86

CHAPTER 3 – HYPOTHESIS
CD39 IS AN IMPORTANT MODULATOR OF ANGIOGENESIS.

3.1 Potential roles for purinergic signaling in angiogenesis 89
- 3.1.1. Introduction 89
- 3.1.2. The role of adenosine in angiogenesis 89
- 3.1.3. Purinergic signaling in cultured endothelial cells 90
- 3.1.4. The importance of signaling complexes of P2-receptors, integrins and CD39. 92

3.2 Hypothesis – Deletion of *Cd39* inhibits angiogenesis 94

CHAPTER 4 – ESTABLISHED EXPERIMENTAL MODELS DELETION OF *Cd39* DISRUPTS ANGIOGENESIS IN VIVO

4.1 Development and characterization of the *Cd39*-null mouse 99
4.1.1 Generation of the *Cd39*-null mouse 99
4.1.2 Northern and Western blot confirmation of *Cd39* deletion 103
4.1.3 Ectonucleotidase activity in wild-type and *Cd39*-null tissues
4.2 Development of a seventh-generation back-crossed *Cd39*-null C57BL/6 mouse strain. 108
4.2.1 Introduction 108
4.2.2 Methods and results 110
4.3 Matrigel™ plug angiogenesis assays 116
4.3.1 Introduction 116
4.3.2 Results 117
4.3.3 Discussion 121
4.4 Liver Regeneration 122
4.4.1. Introduction 122
4.4.2. Results and discussion 127
4.5 Myocardial Infarction 132
4.5.1. Introduction 132
4.5.2. Methods and results 132
4.5.3 Discussion 135
4.6 Tumour Angiogenesis 138
4.6.1. Introduction 138
4.6.2. Methods and results 138
4.6.3. Discussion 152
4.7 Tumour Metastasis 153
4.7.1. Introduction 153
4.7.2. Methods and results 155
4.7.3. Discussion 159

CHAPTER 5 – NOVEL EXPERIMENTAL MODELS DELETION OF *Cd39* RESULTS IN VASCULAR INTEGRIN DYSFUNCTION IN VITRO

5.1 Isolation of murine pulmonary endothelial cells 163
 5.1.1 Methods 163
 5.1.2 Results 169
5.2 Characterization of pulmonary endothelial cells 170
 5.2.1 Methods 170
 5.2.2 Results 175
5.3 Endothelial proliferation assays 183
 5.3.1 Introduction 183
 5.3.2 Methods 185
 5.3.3 Results 187
5.4 Activation of endothelial ERK1/2 (p42/44-MAPK) by VEGF 196
 5.4.1 Methods 197
 5.4.2 Results 198
5.5 Endothelial apoptosis assays 200
 5.5.1 Introduction 200
 5.5.2 Methods 202
 5.5.3 Results 203
5.6 Matrix metalloproteinase expression 216
 5.6.1 Introduction 216
 5.6.2 Methods 218
 5.6.3 Results 220
5.7 Hypothesis: Deletion of *Cd39* and disordered P2-signaling causes vascular integrin dysfunction. 225
5.8 Adhesion assays 234
 5.8.1 Introduction 234
 5.8.2 Methods 238
 5.8.3 Results and discussion 241
5.9 Integrin-mediated intracellular signaling 253
 5.9.1 Introduction 253
 5.9.2 Methods 258
 5.9.3 Results 259
5.10 Differential expression of activated "ligand-receptive" $\alpha_V\beta_3$ by immunocytochemistry 269
 5.10.1 Introduction 269
 5.10.2 Methods 270

5.10.3 Results 271

CHAPTER 6 – DISCUSSION 276

CHAPTER 7 – APPENDICES

7.1 Abbreviations 284
7.2 Materials and suppliers 289

CHAPTER 8 – REFERENCES 297

CHAPTER 1: LITERATURE REVIEW

OVERVIEW OF ANGIOGENESIS

1.1 ANGIOGENESIS

As mammalian cells require nutrients and oxygen for their survival they are, therefore, are located within 100 to 200 µm of the nearest blood vessel, a distance defined by the diffusion limit of oxygen.[1] As cells divide, whether during embryogenesis or during physiological and pathological processes in the adult, new vasculature must be recruited and developed to supply the growing tissue. This occurs by the processes of vasculogenesis or angiogenesis.[2]

Vasculogenesis refers to the formation of immature blood vessels by the *in situ* differentiation of undifferentiated precursor angioblasts into endothelial cells. Although this process is traditionally associated with blood vessel formation in the developing embryo, bone marrow derived endothelial precursors have been also shown to contribute to new vessel formation in adult animals.[3]

Angiogenesis refers to the growth and maturation of new vessels from pre-existing differentiated blood vessels. This can occur by the process of endothelial sprouting or by intussusception,[4] the insertion of tissue columns into the lumen of pre-existing blood vessels.

The importance of angiogenesis arises because of its vital role in numerous different physiological and pathological processes, inclusive of inflammation. For example, angiogenesis is required for the physiological development of the neonatal retina[5] and for growth and maturation of the ovarian follicle during the female reproductive cycle.[6] In addition, angiogenesis has been implicated in a range of pathological states, including tumour growth, diabetic retinopathy and inflammatory states like arthritis and psoriasis.[7]

It is hoped that, by better understanding the process of angiogenesis, we might some day exert control over it for clinical benefit. For example, cardiac failure secondary to myocardial ischaemia may be prevented by drugs that promote angiogenesis,[8] while the growth of tumours may be limited by drugs that inhibit angiogenesis.[9] For this reason, understanding how new blood vessels form and develop has become the focus of much research interest during the last decade.

1.2 A BRIEF HISTORY OF ANGIOGENESIS RESEARCH

Research into angiogenesis has boomed in recent years. On average, more than 40 scientific papers are published in the field every week[10] and billions of dollars has already been spent on the research and development of drugs aimed at harnessing the potential of this process to treat human disease. However, the hypothesis that angiogenesis could be manipulated as therapy was initially greeted with much skepticism.

The highly vascular nature of most solid tumours was noted by Virchow, the father of anatomical pathology, in 1865, but the close relationship between tumour progression and its vascularization was only proposed by Judah Folkman in the 1970s. In a seminal paper in the New England Journal of Medicine in 1971, Dr. Folkman proposed that tumour growth and metastasis was intimately linked with the induction of new vasculature by the tumour and that, therefore, inhibiting blood vessels formation would limit cancer growth and metastasis.[11] Earlier that year, using a homogenate of Walker 256 carcinoma, Dr. Folkman's group had purified a fraction containing strong angiogenic activity. This active fraction was subsequently called "tumour angiogenesis factor" (TAF).[12]

Further angiogenic growth factors were isolated over the next two decades. In 1984, Shing et al. at Harvard Medical School purified fibroblast growth factor (FGF).[13] This was followed five years later by the purification of vascular endothelial growth factor (VEGF), by Dr. Napoleone Ferrara.[14]

Subsequent molecular cloning of VEGF proved that it was identical to a molecule called Vascular Permeability Factor (VPF) that had been discovered in 1983 by Dr. Harold Dvorak.[15]

In 1973, it was demonstrated that endothelial cells could be passaged in vitro.[16] This, and the development of numerous bioassays of angiogenesis, greatly advanced our ability to study angiogenesis *in vivo* and *in vitro*.

The hypothesis that tumours switch to an angiogenic state was developed in 1976 when it was shown that mammary tumours, implanted into rabbit eyes, induce new blood vessels.[17] In 1989, it was show that spontaneously arising tumours are not initially angiogenic, but that they undergo a switch to an angiogenic phenotype when a subset of tumour cells induces new capillaries to converge on the tumour.[18] It was later shown that this "switch" depends on the increased production of one or more of these angiogenesis stimulators.[19]

In his seminal paper in the NEJM in 1971, Dr. Folkman also hypothesized the existence of angiogenesis inhibitors.[11] By 1975, he had shown that cartilage inhibited tumour vascularization in a rabbit cornea assay.[20] This was followed by the discovery and development of numerous angiogenesis inhibitors, including: antibodies to natural stimulators of angiogenesis, like VEGF and FGF; agents aimed at preventing endothelial cell proliferation, like the fungal agent TNP-470; inhibitors of tissue metalloproteinases, like marimastat; inhibitors of the important angiogenic integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$; and, angiostatin, endostatin and tumstatin, extracellular matrix fragments which act as endogenous inhibitors of angiogenesis.[21]

In 1989, the first successful treatment of a human patient using an angiogenesis inhibitor was reported when Dr. Carl White, a pediatric radiologist, from Denver, used recombinant interferon alpha-2α to treat a patient with pulmonary hemangiomatosis.[22] This was followed three years later by the first clinical trial of an anti-angiogenic drug, TNP-470.[23]

By 1999, there were numerous clinical trials underway for both angiogenesis-stimulating and angiogenesis-inhibiting drugs. By that stage, anti-angiogenesis drugs were being studied in cancer patients, as well as other trials of angiogenesis-inhibition in patients with macular degeneration, diabetic retinopathy and psoriasis.[24] In contrast, clinical trials of "angiogenesis-stimulation" were being conducted for coronary artery disease, peripheral vascular disease, stroke and wound healing.[25]

In 2004, Avastin, a monoclonal antibody to VEGF, became the first U.S. Food and Drug Administration (FDA) approved therapy designed to inhibit angiogenesis. This occurred after the drug was shown to improve survival in patients with metastatic colon cancer by approximately five months.[26,27]

The huge clinical promise of angiogenesis-based therapies was finally starting to be realized.

1.3 TUMOUR ANGIOGENESIS

Although important roles have been suggested for angiogenesis in many conditions, it is its potential role in facilitating tumour growth, which sparked the initial interest in angiogenesis. In 1971, Judah Folkman proposed that tumour growth and metastasis are angiogenesis-dependent and that, therefore, blocking angiogenesis would prevent tumour growth.[11]

The process of tumour development has traditionally been described as the step-wise accumulation of genetic mutations that transform a normal cell into a cancer cell. Recently however, the induction of tumour vasculature has been included as a discrete step in tumour progression. The term "angiogenic switch" describes the acquisition of this angiogenic capacity by the developing tumour.[28] This concept divides tumour development into two distinct phases. The first avascular phase corresponds to small, clinically occult lesions with a maximum size of 2 mm. In these lesions, the lack of blood supply results in a balance between tumour cell proliferation and apoptosis. The second vascular phase is heralded by the increase in angiogenesis and exponential tumour growth.[19]

This angiogenic switch can occur at different stages of the tumour progression depending on the tumour type. The classical model hypothesizes that the switch to angiogenic phenotype occurs when pro-angiogenic factors outweigh anti-angiogenic ones. Pro-angiogenic factors may be upregulated by hypoxia, caused by tumour expansion beyond its blood supply, by the

activation of oncogenes or the inactivation of tumour suppressor genes, or by inflammatory cells infiltrating the tumour.[1]

The concept of an angiogenic switch provides a possible explanation for the puzzling existence of dormant occult tumours in a high percentage of autopsies performed on individuals dying of trauma. Up to a third of all women aged 40 to 50 have areas of occult breast cancer at autopsy despite only 1% incidence of clinical breast cancer in this age group.[29] Also, virtually all individuals aged 50-70 have *in situ* thyroid cancer but only 0.1% in this age group have clinical disease.[30] It is thought that endogenous angiogenesis inhibitors suppress the growth of these dormant tumours.[29]

1.3.1 The formation of tumour blood vessels

As mentioned previously, tumour vessels are formed by processes of vasculogenesis and angiogenesis. However, the percentage of tumour endothelial cells recruited from bone marrow precursors tends to be rather low in most tumours. Most tumour neovascularization occurs via a process termed sprouting angiogenesis.[19]

In the first stage of this process, the vessels adjacent to the tumour dilate and become leaky in response to the important angiogenic growth factor, vascular endothelial growth factor (VEGF). As tumours outgrow their blood supply and become hypoxic, hypoxia-inducible transcription factor (HIF-1) binds to hypoxia response elements (HRE) in the promoter region of the

VEGF gene, and upregulates the expression of VEGF.[31] Hypoxia is the most important stimulus for VEGF upregulation.[32]

An increase in vascular permeability depends on overcoming the actions of the soluble factor angiopoietin-1 (Ang-1) and the endothelial junctional molecules VE-cadherin and platelet/endothelial cell adhesion molecule (PECAM), as they stabilize resting blood vessels. The resulting increase in vascular permeability allows the extravasation of plasma proteins to form the provisional scaffold for endothelial cell migration.[2]

Before endothelial cells can migrate towards angiogenic stimuli, they first need to loosen their contact with adjacent endothelial and peri-endothelial cells. The angiogenic factor Ang2 has been implicated in this process as it blocks the vessel stabilizing effects of Ang1 at the endothelial receptor Tie-2.[33] Endothelial cells then digest the vascular basement membrane and extracellular matrix in a controlled fashion by secreting various extracellular proteases.[2] The most important proteases involved in this process are reviewed later in the literature review.

Once a path for migration has been cleared by proteolysis, endothelial cells proliferate and migrate towards the angiogenic stimulus. This process of "sprouting" is controlled by a balance of angiogenic activators and inhibitors. Molecules like VEGF, Ang1 and FGF promote endothelial cell proliferation and migration, while angiostatin, endostatin, antithrombin III, and numerous other factors inhibit it.[19]

Cell matrix receptors such as the integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ mediate cell-spreading and migration.[34] The endothelial cells often assemble as solid cords, which must, subsequently, acquire a lumen. Vessels may also increase their diameter and length by intercalation or thinning of endothelial cells or by fusion with pre-existing vessels.[2]

Maturation of these newly formed blood vessels requires the formation of a new basement membrane and the recruitment of pericytes and smooth muscle cells around the new endothelium.[7] Important mediators of this process include protease inhibitors, like PAI-1, which inhibit the degradation of newly formed extracellular matrix, platelet-derived growth factor-β (PDGF-β), which recruits smooth muscle cells, and transforming growth factor-1 (TGF-1) and Ang1/Tie2, which stabilize the interaction between endothelial cells and smooth muscle cells.[1] The maintenance of newly formed blood vessels depends on the survival of endothelial cells and hence on the balance between survival and pro-apoptotic signals. Molecules like VEGF and Ang1 promote endothelial survival, while many angiogenesis inhibitors, both endogenous and exogenous, promote endothelial apoptosis.[1] Close interaction between endothelial cells and pericytes also limits the requirement of endothelial cells for angiogenic factors that inhibit endothelial apoptosis.[35] The poor recruitment of pericytes in tumour vasculature may be the cause of the high incidence of vessel regression noted in tumours, to the fragility of these vessels as well as the sensitivity of tumour vasculature to the inhibition of VEGF.[36]

1.3.2 Characteristics of tumour vasculature

New blood vessels formed by physiological angiogenesis mature quickly after formation, resulting in a stable, remodeled vascular tree. In contrast, tumour vessels are characterized by continuous endothelial proliferation and vascular immaturity because of the loss in balance between pro- and anti-angiogenic factors.[37] Therefore, tumour vessels have certain unique features compared with normal vessels.

Firstly, tumour vessels have abnormal structural architecture. The vessels are dilated, tortuous and prone to develop shunts.[1] This results in areas of hypoxia within the tumour and may select for those cancer cells resistant to hypoxia.[38]

Secondly, tumour vessels are highly permeable compared with normal vessels. This occurs, inter alia, because of the increased expression of VEGF, a growth factor initially termed vascular permeability factor (VPF).[15]

Thirdly, tumour vessels are often characterized by abnormal and non-uniform expression of endothelial surface markers. This may explain the abnormal leukocyte attachment and migration noted to occur in tumour vasculature.[39] On the other hand, unique endothelial surface markers may allow the design of therapies targeted to tumour-associated, as opposed to normal, blood vessels.[40]

Finally, the morphological features of tumour vessels are very heterogeneous between different cancer types.[19] This has important implications for the development of anti-angiogenic therapies, as what is successful in one type of cancer, may not be successful in another.

1.4 ROLE OF ANGIOGENESIS IN TUMOUR METASTASIS

In addition to its role in allowing tumour growth, angiogenesis plays a significant role in the development of tumour metastases. Clearly angiogenesis is as important for the growth of any metastatic deposit, as it is for the primary tumour. However, angiogenesis also provides the route whereby cancerous cells can leave the primary tumour and enter the circulation. As discussed above, angiogenic vessels are typically immature, leaky and poorly enveloped in basement membrane and pericytes. This allows tumour cells to easily enter the blood stream and travel to distant sites.[41]

Although most tumour cells entering the circulation fail to produce clinically significant metastases, the number of metastasis is generally proportional to the number of tumour cells shed.[41] By allowing cancerous cells to enter the blood stream, increased angiogenesis should cause increased metastasis.

To summarize the role of angiogenesis in tumour metastasis: small primary tumours, initially devoid of blood supply, are vascularized by angiogenesis and start to grow. Cancerous cells then enter the immature new blood vessels by intravasation and migrate in the blood stream to distant sites. Tumour cells seed in distant vessels, migrate through the vessel walls and establish micrometastases. Induction of angiogenesis in these micrometastases heralds increased growth and the development of clinically significant metastatic tumours.

A review of a number of human cancers, including breast,[42] prostate,[43] lung[44] and cervix,[45] confirms that angiogenesis is crucial for tumour metastasis. In these tumours, increased tumour vascularity correlates with a higher incidence of metastases and a worsened prognosis.

The link between angiogenesis and metastasis has also been shown in experimental animal models involving established primary tumours. Treatment with angiogenesis inhibitors, in addition to causing regression of the primary tumour, decreases the development of tumour metastases. This has been shown for a wide range of angiogenesis inhibitors with a number of differing mechanisms of action. The angiogenesis inhibitors endostatin,[46] angiostatin,[47] thrombospondin,[48] TNP-40,[49,50] and thalidomide[51] have all been shown to decrease the incidence of tumour metastases in animal models.

In fact, endogenous angiogenesis inhibitors, secreted by the primary tumour, have been shown to inhibit the growth of secondary tumours.[52] In the primary tumour, local release of angiogenic factors overwhelms these endogenous inhibitors, promotes angiogenesis and allows tumour growth. But at distant sites, the long-acting endogenous inhibitors limit the growth of tumour metastases. In fact, surgical removal of primary tumours often leads to the development of clinically significant metastases.[9] This is now attributed to the loss of endogenous angiogenesis inhibitors from the primary.[29]

Clearly angiogenesis is an important factor in the development of metastatic tumours, and angiogenesis inhibition holds promise for both the prevention and treatment of tumour metastases.

1.5 ANGIOGENIC GROWTH FACTORS

A large number of growth factors have been implicated in promoting angiogenesis. Included in this group are, *inter alia*, the vascular endothelial growth factor (VEGF) family, fibroblast growth factor (FGF), the angiopoietins (Ang), platelet-derived growth factor-β (PDGF-β), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), and tumour necrosis factor-α (TNF-α).[2] Some of these, like VEGF, are relatively specific for endothelial cells, while others, like FGF, act on many different cell types.[53] Activation of the angiogenic switch requires an increase of these pro-angiogenic factors relative to angiogenesis inhibitors.[19]

However, any temptation to regard the pro-angiogenic effects of these growth factors as merely additive would be a gross simplification of this complex process. In reality, angiogenic growth factors need to act in a carefully coordinated manner on a number of different cell types, to promote effective angiogenesis.

The importance of understanding how these factors interact is that therapeutic manipulation of these factors holds considerable promise in the treatment of human disease. Studies are currently underway aiming at stimulating angiogenesis with VEGF or FGF in the treatment of ischaemic heart disease.[25] Similarly, angiogenic growth factors inhibitors are being tested as a treatment for, in particular, cancer.[9]

A comprehensive discussion of all the growth factors implicated in promoting angiogenesis is beyond the scope of this review. Instead, we have decided to mention three factors with particular relevance to our work, namely: the VEGF family, FGF, and the angiopoietins.

1.5.1 The VEGF Family

Physiological and pathological angiogenesis is dependant on the VEGF family of growth factors and their receptors. The members of this family, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PIGF, modulate angiogenesis by binding to tyrosine-kinase receptors on the cell surface. This induces receptor dimerization, and the activation of intracellular signaling pathways that influence angiogenesis responses.[54]

VEGF was originally isolated in 1983 as a tumour product which increased vessels permeability,[15] and was hence called vascular permeability factor (VPF). Six years later, VPF was found to be identical to a potent endothelial mitogen called vascular endothelial growth factor (VEGF).[14]

The six members of the VEGF family have distinct but overlapping biological functions. VEGF-A is the most potent angiogenic protein[54] known and is essential for both embryonic vasculogenesis and angiogenesis.[55] VEGF-A promotes angiogenesis in a number of ways. By increasing vascular permeability, it promotes fibrin deposition and provides a scaffold for endothelial cell migration.[56] VEGF-A also stimulates endothelial

proliferation and protects newly-formed vessels from regression by acting as an endothelial survival factor.[57] VEGF-A mediates these actions via the tyrosine-kinase receptors VEGF-receptor 1 (VEGFR-1) and VEGF-receptor 2 (VEGFR-2).[54]

The most important stimulus for VEGF-A upregulation is hypoxia.[32] As tumour outgrow their blood supply and become hypoxic, the hypoxia-inducible transcription factor (HIF-1) binds to hypoxia response elements (HRE) in the promoter region of the VEGF gene.[31] This upregulates VEGF expression and, by inducing angiogenesis, increases the blood supply to the hypoxic tumours. A number of growth factors, including transforming growth factors -α and -β (TGF-α, TGF-β), fibroblast growth factor (FGF) and epidermal growth factor (EGF), have also been shown to upregulate VEGF-A expression.[58] This implies cooperation between growth factors and hypoxia-responses in angiogenesis. Finally, a number of genetic transformations associated with oncogenesis, especially *ras* mutations, have also been shown to increase the expression of VEGF.[59]

Alternative splicing of VEGF-A results in several isoforms of different sizes, denoted 121-, 145-, 165-, 183-, 189-, and 206-isoforms. Each of these isoforms differs in its binding to extracellular matrix or the cell-surface receptor neuropilin-1 (NRP-1). For example, while 121 is soluble in bodily fluids, 165 and 189 bind heparin and are thus sequestered in the extracellular matrix.[54] Heparin-bound VEGF is thought to provide important directional cues to developing vessels.[60]

VEGF-B is structurally very similar to VEGF-A. It shares a common receptor with VEGF-A, VEGF receptor-1, and also has isoforms that differ in their heparin and neuropilin-1 binding.[61] However, the expression of VEGF-B is stable and it isn't upregulated by the various factors which upregulate VEGF-A expression.[62]

VEGF-C has only 30% structural similarity to VEGF-A, but induces similar effects on endothelial cell migration and permeability via VEGF-receptor 2.[63] Differential proteolytic processing of VEGF-C results in isoforms, which promote the development of lymphatics, or lymphangiogenesis.[64]

Like VEGF-C, VEGF-D binds VEGF-receptors 2 and 3, and both have important roles in both angiogenesis and lymphangiogenesis.[64]

The growth factor VEGF-E is only expressed following infection with certain strains of Orf virus, which causes pustular dermatitis in sheep, goats and, occasionally, humans. Interestingly, these lesions are highly vascularized and characterized by extensive endothelial cell proliferation.[65]

PlGF has been isolated from placental tissue and is structurally related to VEGF-A. Compared with VEGF-A, PlGF is a weak endothelial mitogen *in vitro*.[66] However, PlGF is considered an important angiogenic agent when acting synergistically with VEGF-A *in vivo*.[67] Genetic deletion of PlGF results in normal embryological development (VEGF-A deletion is lethal), but decreased pathological angiogenesis in adult animals making it an attractive target for anti-angiogenic therapy.[67]

be associated with the development of pre-eclampsia, implying that decreased activity of VEGF-A or PlGF may be responsible for endothelial dysfunction in pre-eclampsia.[70]

VEGFR-3 (Flt-4) is a high-affinity receptor for VEGF-C and VEGF-D and has a major role in lymphangiogenesis in adult animals.[71]

The neuropilins NRP-1 and NRP-2 are a class of non-tyrosine kinase receptors involved in VEGF signaling. They are expressed in neural cells, where they have a role in axonal guidance, and on endothelial cells, where they promote angiogenesis and lymphangiogenesis.[54] NRP-1 enhances the signaling of VEGF-A through VEGFR-2, while NRP-2 promotes VEGF-C signaling through VEGFR-3. In keeping with this finding, deletion of NRP-1 is embryonically lethal because of defective angiogenesis,[72] while NRP-2 deletion cause lymphatic defects.[73]

1.5.3 Fibroblast growth factor

Fibroblast growth factor 2 (FGF-2), also known as basic FGF, is one member of the large FGF family of growth factors. Like VEGF, it binds heparin and has strong pro-angiogenic actions.[74] In corneal and chick chorioallantoic membrane assays of angiogenesis, FGF-2 induces significant angiogenesis.[53]

However, in contrast to VEGF, FGF-2 exerts its actions on a wide range of cell types in several different physiological and pathological settings. It was

originally described as a mitogenic factor for fibroblasts involved in tissue regeneration, but is also neurotrophic and promotes neural cell survival. In angiogenesis, FGF-2 is responsible for endothelial cell proliferation and morphogenesis, and is thought to be an important inducer of the "angiogenic switch".[74]

Despite this wide range of actions, deletion of FGF-2 produces viable, fertile mice. The major abnormalities in FGF-2-null mice occur in cerebral development and in the neurological control of blood pressure, rather than in angiogenesis.[75]

It is possible that other angiogenic growth factors compensate for FGF-2 in this setting. Certainly, liver regeneration is normal in FGF-2-null mice and it has been suggested that VEGF compensates for the lack of FGF-2 in this model.[76]

These results have decreased the enthusiasm of those attempting to use FGF-2 antagonism as an anti-angiogenic therapy. However, angiogenesis stimulation using FGF-2 may yet prove useful clinically and trials are underway investigating FGF-2's role in treating ischaemic heart disease.[25]

1.5.4 The angiopoietins

The angiopoietins were discovered as ligands for the Tie-family of tyrosine kinase receptors, expressed by vascular endothelial cells. Although four members have been isolated (Ang1-4), only angiopoietin 1 (Ang-1) and

1.5.2 VEGF receptors

VEGF acts through the VEGF family of tyrosine kinase receptors, including VEGFR-1 (also known as Flt-1), VEGFR-2 (known as Flk-1 in the mouse and KDR in humans) and VEGFR-3 (Flt-4), and the non-tyrosine kinase neuropilin receptors (NRP-1 and NRP-2).[54]

VEGFR-2 (Flk-1/KDR) is activated by VEGF-A and plays a major role in vascular and lymphatic development in the embryo. VEGFR-2 deletion is embryonically lethal and characterized by significant defects in endothelial and haematopoietic cell function.[68] In the adult, VEGFR-2 is thought to be the major receptor mediating the physiological and pathological functions of VEGF-A.[54]

In contrast, VEGFR-1 (Flt-1) binds VEGF-A, VEGF-B and PIGF and is hypothesized to be an important negative regulator of VEGF function during embryogenesis. VEGFR-1 knock-out mice are characterized by an increase in endothelial progenitor cells which, despite normal migration and proliferation, have defective endothelial tube formation.[69] The pro-angiogenic effects of VEGF-B, a weak endothelial mitogen, have been hypothesized to include blocking VEGF-A binding to VEGFR-1 and, thus, increasing VEGF-A binding to the "angiogenic" VEGFR-2.[54]

A soluble form of VEGFR-1 exists and is thought to inhibit VEGF-A and PIGF function by binding these growth factors in the circulation. Recently, increased production of this soluble VEGFR-1 by the placenta was found to

angiopoietin 2 (Ang-2) have been studied so far. All of the angiopoietins bind and exert their vascular effects via Tie2. The ligands for Tie1 are less well known, but it is currently thought that, under certain circumstances, the angiopoietins might bind Tie1 as well as Tie2.[53]

Deletion of Ang-1[77] or Tie-2[78] produces similar effects *in vivo.* Unlike VEGF-null mice, these animals develop normal primary vasculature, but the vessels fail to remodel correctly into large and small vessels. In the heart, there is defective attachment of the endocardium to the underlying myocardium. At a microscopic level, there seemed to be abnormal association of endothelial cells with the underlying support cells and extracellular matrix in these mice.

In contrast, over expression of Ang-1 causes increases in vessel size (rather than vessel number, as would occur with VEGF over expression).[79] Also, whereas VEGF over expression led to leaky vessels, over expression of Ang-1 resulted in vessels with decreased permeability even after induction of inflammation or treatment with VEGF.[80]

These findings point to Ang-1 having important roles in promoting endothelial cell interaction with adjacent endothelial cells and with the extracellular matrix, pericytes and vascular smooth muscle cells. Vascular support cells produce Ang-1, which acts via endothelial Tie-2 receptors.[53]

In addition to increasing our understanding of angiogenesis, this knowledge may lead to important therapies for conditions associated with pathological,

leaky vessels. Ang-1 treatment might be of benefit in diabetic retinopathy, inflammation or brain oedema where leaky vessels cause pathology.[53]

In contrast to the stabilizing effects of Ang-1, Ang-2 is thought to promote vessel destabilization in the initial stages of angiogenesis by acting as a Tie-2 antagonist. In physiological angiogenesis in the ovary[81] and pathological angiogenesis in tumours,[82] Ang-2 expression is increased in actively remodeling vessels. The evidence for Ang-2 being a Tie-2 antagonist is increased by the finding that over expression of Ang-2 results in embryonic defects similar to the Ang-1 or Tie-2 knock-out mice.[81]

In liver regeneration, a process requiring controlled angiogenesis, Ang-2 levels rise early, whereas Ang-1 and Tie-2 only increase after several days.[83] This is consistent with Ang-2's role in angiogenesis initiation and the role of Ang-1 and Tie-2 in endothelial differentiation, maintenance of blood vessel integrity and the resolution phase of angiogenesis.[84]

1.6 MATRIX BIOLOGY

The extracellular matrix is no longer considered an inert structural scaffold for tissues, but rather a dynamic system that actively modulates blood vessel formation and function. This understanding has resulted in the development of the field of matrix biology as it pertains to angiogenesis.[85]

1.6.1 Structure and function of the extracellular matrix

On a structural level the extra-cellular matrix is divided into two distinct components: the basement membrane, a condensed matrix layer formed adjacent to numerous cell types, including endothelial cells, and epithelial cells; and the interstitial matrix. These two components both have as their basic structure a collagen scaffold, although these collagens and their three-dimensional architecture differ markedly in different types of extracellular matrix.[86]

The basement membrane consists of a highly cross-linked and insoluble composite of several glycoproteins.[87] In blood vessels, the basement membrane lies between the endothelial cell layer and the surrounding pericytes layer.[88] In all, over 50 proteins make up the structure of basement membranes, although collagens (especially Type IV collagen) make up over 50% of these.[85] The other major components include laminin, and heparin-sulphate proteoglycans (HSPGs) like perlecan and nidogen/entactin.[87] Different isoforms of type IV collagen, laminin and HSPGs can combine to

form organ-specific basement membranes with organ-specific matrix functions.

Endothelial cells and pericytes adhere to the extracellular matrix via integrins and other adhesion receptors. This adhesion and signals received from the vascular basement membrane influences cellular decisions towards growth, differentiation and apoptosis.[34]

Endothelial cells are normally quiescent when bound to the capillary basement membrane, suggesting that signals originating from intact basement membrane inhibit endothelial cell proliferation. The structure of intact basement membrane is highly complex and only certain domains of its various constituents are exposed to endothelial cells. In contrast, when the basement membrane is degraded, endothelial cells can interact with different domains of these molecules. This is an important concept, because it implies that the same set of components of the vascular basement membrane can impart distinct influences on endothelial cells at different stages of angiogenesis.[85]

During angiogenesis, the vascular basement membrane is first degraded by several extracellular proteases, including matrix metalloproteinases. This frees endothelial cells to proliferate and migrate, liberates growth factors (VEGF, FGF) sequestered in the extracellular matrix, and detaches pericytes that surround and support blood vessels.[85]

Endothelial cells now come into contact with interstitial provisional matrix components, like vitronectin, fibronectin, type-1 collagen and thrombin.

These molecules provide proliferative signals to endothelial cells, in contrast to intact basement membrane, which limits proliferation and migration.[85]

During the resolution phase of angiogenesis, growth factors promote the formation of intermediate and then mature basement membrane. Together with endothelial cells and pericytes, this vascular basement membrane promotes the formation of new, mature blood vessels.

Several components in the angiogenesis cascade interact closely with the vascular basement membrane and interstitial matrix. Extracellular proteases, for example the matrix metalloproteinase system, control angiogenesis by degrading matrix components. This facilitates, not only, the proliferation and migration of endothelial cells, but also, the generation of pro- and anti-angiogenic factors.[89]

Cell-surface integrins mediate interactions between endothelial cells and the extracellular matrix. Basement membrane components bind different integrins depending on whether they are intact or degraded, thus allowing differential signaling to endothelial cells.[34]

Finally, fragments of certain basement membrane components have been shown to have anti-angiogenic effects, which may act as physiological inhibitors of unwanted angiogenesis.[90]

Each of these topics is covered in detail in later in the literature review (Chapters 1.7.4, 1.8.1, 1.9.1).

1.7 EXTRACELLULAR PROTEOLYSIS

As previously discussed, the early steps in angiogenesis include: vessel wall disassembly; basement membrane and extracellular matrix digestion; and endothelial cell migration. It was shown over 20-years ago that these processes are mediated, at least in part, by the matrix metalloproteinase family and other extracellular proteases.[91]

However, excess matrix proteolysis is incompatible with angiogenesis as it results in destruction of the matrix scaffold required for cellular invasion as well as the development of aberrant vascular structures.[92] For this reason, fine control of extracellular proteases is required during angiogenesis.

This control is asserted on many levels. Firstly, just as the balance between positive and negative regulators of angiogenesis on endothelial cell activation status has led to the notion of an "angiogenic switch", so the concept of "proteolytic balance" has been developed to highlight the importance of protease-antiprotease equilibrium.[93]

Secondly, increasing evidence has emerged that proteases and anti-proteases are spatially arranged on the cell surface. The object is to concentrate proteolytic activity at the leading edge of cellular migration via the assembly of various components of the protease system into complexes on the cell surface.[94] For example, MMP-2, MT1-MMP, TIMP-2 and integrin $\alpha_V\beta_3$ have been co-localized in basolateral caveolae of endothelial cells.[95]

Thirdly, in addition to their role in matrix degradation and cellular invasion, extracellular proteases have been implicated in the regulation of cytokine activity. For example, matrix-bound VEGF and FGF may be released, and latent TGF-β activated by these proteases.[96]

Finally, extracellular proteases have been implicated in the generation of proteolytic fragments of the extracellular matrix and other molecules, a number of which have been shown to modulate angiogenesis. The angiogenesis inhibitors, angiostatin[52] and endostatin,[46] for example, are derived from the proteolysis of plasminogen and collagen XVIII, respectively.

1.7.1 The matrix metalloproteinase family

The matrix metalloproteinases (MMPs) are a multigene family of extra-cellular proteases involved in a range of physiological and pathological conditions. As a family, they share certain common features. Firstly, they all degrade at least one component of the basement membrane and all require two Zn^{2+} molecules for their activity. Secondly, they are inhibited by both metal chelators and tissue inhibitors of matrix metalloproteinases (TIMPs). Finally, they are all zymogens and require extra-cellular activation prior to being able to degrade extracellular matrix.[89]

All the MMPs share three common domains. In addition to the Zn^{2+}-containing catalytic domain, they have a prepeptide domain used for secretion and a propeptide domain that must be removed for activation. All

but MMP-7 also have a haemopexin domain adjacent to the catalytic domain which serves as the binding site for TIMPs, as well as, possibly, for cell-surface receptors. The gelatinases, MMP-2 and MMP-9. also contain a fibronectin-like region responsible for collagen binding. The membrane-type metalloproteinases (MT-MMPs) are unique in that they are anchored to the cell surface rather than secreted.[89]

The MMP family can be subdivided into five groups: the collagenases, the stromelysins, the gelatinases, the matrilysin, and membrane-type metalloproteinases. It is important to realize, however, that, although this classification system was developed on the basis of substrate specificity, there is some overlap between different members of the family.

Matrix metalloproteinase (MMP) function is regulated at three levels: firstly, at the level of gene expression; secondly, by control of MMP activation; and, thirdly, by inhibition by tissue inhibitors of matrix metalloproteinases (TIMPs).[89] This allows for tight cooperative control of MMP function. Disruption of this control has been associated with increased tumour cell invasion and progression.[97]

The expression of MMPs and TIMPs is regulated by a wide range of cytokines, steroid hormones and phorbol esters. In order to promote matrix digestion and cellular invasion, there must be a shift in the balance of protease to inhibitor. Cytokines and growth hormones, like VEGF[98] and FGF[99], appear to act in conjunction on both MMPs and TIMPs to create this state. In addition to cytokines and growth factors, MMP production can also be regulated by extracellular matrix components themselves.

Thrombospondin-1 (TSP-1), for example, is an extracellular matrix protein, which is also a stimulator of MMP-9 production by endothelial cells.[100]

With the exception of the MT-MMPs, all MMPs need to be activated before they can degrade matrix components. This is performed in two general ways. Firstly, extracellular proteases such as trypsin 2, cathepsins, elastins and plasmin can directly activate MMPs. The plasminogen-activator/plasmin system, in particular, has been implicated in MMP activation and in the control of tumour cell invasion and angiogenesis.[94,101] Secondly, MMPs, once activated, are capable of activating themselves and each other. This mode of activation is associated, in particular, with the gelatinases, MMP-2 and MMP-9.[89]

Tissue inhibitors of matrix metalloproteinases (TIMPs) are small molecules expressed in various tissues and body fluids. There are four members of the TIMP family: TIMP-1, TIMP-2, TIMP-3, and TIMP-4.[102] Increased levels of TIMPs have, in general, been associated with decreased tumour cell invasion and metastatic potential, although, it is important to remember that it is the ratio of protease to inhibitor which influences matrix proteolysis.

1.7.2 The role of matrix metalloproteinases in angiogenesis

The fact that MMPs are required for angiogenesis has been firmly established.[103] The use of natural or synthetic MMP inhibitors has demonstrated that matrix metalloproteinases are required during the initial sprouting phase of angiogenesis. For example, increased expression of

TIMP-1[104] and TIMP-2[105] inhibited tumour-associated angiogenesis, while a range of synthetic MMP inhibitors have demonstrated anti-angiogenic efficacy.[89] Unfortunately, most synthetic inhibitors lack specificity for single MMPs, making elucidation of the relative importance of each MMP in angiogenesis difficult.

Interestingly, not only are MMPs and TIMPs important during the initial sprouting phase of angiogenesis, but inhibition of MMP activity has been shown to be a requirement for vessel stabilization during the resolution phase. Failure to switch-off MMP activity in this phase is associated with regression of newly-formed blood vessels.[106]

The gelatinases, MMP-2 and MMP-9, have been shown to be especially important in both *in vitro* and *in vivo* models of angiogenesis. A MMP-9 knock-out model demonstrated that MMP-9 is vital for adequate angiogenic revascularization of ischaemic tissues following hindlimb ischaemia.[107] Similarly, the MMP-2-null mouse displayed reduced tumour angiogenesis, resulting in smaller tumour volumes following intra-dermal injection of B16-BL6 melanoma cells and Lewis lung carcinoma cells (approximate decreases of 39% and 24%, respectively).[108]

The importance of MMP-2's co-localization with integrin $\alpha_V\beta_3$ in caveolae on the cell surface was demonstrated by the use of an organic inhibitor, which blocked the binding of MMP-2 to $\alpha_V\beta_3$, without affecting either the catalytic activity of MMP-2, or the vitronectin binding of $\alpha_V\beta_3$. This inhibitor successfully limited tumour growth in a murine model by blocking

tumour angiogenesis.[109] This underscores the importance of the correct spatial organization of MMPs in protein complexes on the cell surface.

1.7.3 The plasminogen activator–plasmin system

Plasmin is a broad-spectrum protease responsible for, directly, the hydrolysis of fibrin and, indirectly, the degradation of many extracellular proteins following its activation of pro-MMPs. Plasmin is activated from plasminogen by two separate serine proteases, the urinary-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA).[93] Despite catalyzing the same reaction, uPA is thought to be more important in tissue remodeling and tPA in vascular fibrinolysis.[110]

Many different cell types secrete pro-uPA and elevated levels of production are associated, in particular, with malignant cells. Pro-uPA binds with high affinity to the uPA receptor (uPAR) on the surface of numerous cells, including endothelial cells. Plasmin and plasminogen also associate with uPA on cell membranes and result in increased efficiency of subsequent plasmin-mediated proteolysis.[110]

An additional level of control of this protease system is achieved by specific, physiological inhibitors of plasmin (for example, α2-antiplasmin) and of the PAs (i.e. PAI-1 and PAI-2).[93] Plasminogen activator inhibitor-1 (PAI-1) is considered to be the principal regulator of fibrinolysis in the vasculature and recent findings suggest that it may also have a role in regulating cell migration, adhesion and tumour angiogenesis.[111] PAI-1 is secreted by

vascular endothelial cells, platelets, hepatocytes, fibroblasts, and many tumour cells. It is stabilized in its active conformation by binding to vitronectin, both in the circulation and in the extracellular matrix.[110]

1.7.4 The role of the plasminogen activator-plasmin system in angiogenesis

The generation of knock-out models of the PA-plasmin system allowed the study of this system's role in angiogenesis *in vivo*. Previous *in vivo* and *in vitro* studies had suggested that the PA-plasmin system had a crucial role during angiogenesis. However, unexpectedly, developmental and wound healing-associated angiogenesis was unaffected in the uPA-/-, uPAR-/-, tPA-/-, and plasminogen-null mice.[93] Interestingly, in one tumour angiogenesis model, although uPA was localized to newly-formed blood vessels in wild-type mice, uPA was replaced by tPA in uPA-/- mice. This implies that plasminogen activators are able to compensate for each other.[112]

In other experimental models, however, angiogenesis is affected by deletion of components of the plasminogen activator-plasmin system. In a murine model of myocardial infarction, postinfarction myocardial revascularization and remodeling was severely impaired in uPA-/- mice. This occurred because of impaired endothelial and immune cell migration into the infarcted area.[101]

Perhaps more important than the individual members of the PA-plasmin family, are the dynamic interactions of these molecules at the cell surface.

Numerous *in vivo* studies have demonstrated that the interaction between uPA and uPAR is crucial for angiogenesis.[93] For example, a fusion protein which functions as a high-affinity uPAR antagonist inhibited FGF-induced angiogenesis into a Matrigel™ plug.[113]

The presence of the PA inhibitor PAI-1 is an absolute requirement for tumour angiogenesis. In a murine model of transplanted malignant keratinocytes, the absence of PAI-1 in the host resulted in marked impairment of both tumour cell invasion and host-derived neovascularization.[114]

Two potential explanations exist for why PAI-1 is essential for angiogenesis. Firstly, by preventing excessive ECM degradation, PAI-1 may help to maintain the matrix scaffold endothelial cells use during migration. Secondly, a complex set of interactions between PAI-1, uPAR, integrins and vitronectin has been described which may affect the adhesive and migratory properties of endothelial cells.[93]

It should be clear from this brief review that the involvement of extracellular proteases in angiogenesis is extremely complex. However, the evolution of numerous levels of proteolysis control perhaps reflects the physiological and pathological importance of this system. Furthermore, increased understanding will provide the rational basis for the development of effective anti-angiogenesis therapies aimed at manipulating matrix proteolysis.

1.8 INTEGRINS AND ANGIOGENESIS

1.8.1 The integrin family of cell surface receptors

Integrins are a family of heterodimeric, transmembrane glycoproteins that mediate cell-extracellular matrix and, occasionally, cell-cell adhesion. Each integrin is composed of a single α- and β-subunit, with the specific combination conferring ligand specificity to the integrin. In humans, there are at least 18 different α-subunits and 8 different β-subunits, which can assemble into the 24 known integrins. Endothelial cells express several of these integrins, including: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_5\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$.[34]

Of the endothelial integrins, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are predominantly collagen receptors although they can bind laminin, while $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ are primarily laminin receptors. $\alpha_5\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ all bind to extra-cellular matrix proteins via an arginine-glycine-asparagine (RGD)-binding site. The main ligand for $\alpha_5\beta_1$ is fibronectin and for $\alpha_V\beta_5$ is vitronectin. The promiscuous integrin $\alpha_V\beta_3$ can bind vitronectin, fibronectin, von Willebrand factor, thrombospondin, osteopontin, laminin and denatured collagen.[34]

The cytoplasmic tails of integrins are generally short and devoid of enzymatic activity. Hence, integrins transduce signals from the extracellular matrix by interacting with cytoplasmic kinases and with the cytoskeleton. Upon binding to the extracellular matrix, integrins cluster together in the cell membrane and form a cytoskeletal and signaling complex which results in

the assembly of actin filaments into larger stress fibres. This results in further integrin clustering and the formation of, what is known as, a focal adhesion complex.

The assembly of focal adhesions results in the recruitment and activation of various protein tyrosine kinases, including focal adhesion kinase (FAK), Src-family kinases, Abl, and integrin-linked kinase (ILK). This large number of intracellular signaling targets allows the induction of a diverse range of cellular behaviours.[115]

In addition, many integrins are not constitutively active, but rather, are expressed on the cell surface in an inactive state. The most obvious example of this is the platelet integrin $\alpha_{IIb}\beta_3$, which only binds its ligands and causes thrombosis after being activated in response to platelet agonists, including ADP. If the platelet integrin were constitutively active this would result in disseminated intravascular thrombosis.

Recent studies of the crystal structure of integrins have suggested a mechanism for this activation. The extracellular domain of integrin $\alpha_V\beta_3$ in the inactive, unbound state is folded over hiding the RGD-binding site. Upon activation, this straightens and allowing interaction with the extracellular matix.[116]

1.8.2 The role of integrins in angiogenesis

The first indication that integrins may have a role in angiogenesis is that certain integrins, in particular $\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_2\beta_1$, are barely detectable in quiescent vessels but are greatly upregulated in angiogenic endothelium.[117] Studies using genetic manipulation have confirmed that certain integrins are crucial for effective angiogenesis. Furthermore, antagonists to a number of different integrins have been shown to be effective in limiting angiogenesis in both *in vitro* and *in vivo* experimental models.[34] A number of these angiogenesis inhibitors are currently being tested in human clinical trials.

The most important of these angiogenic integrins are detailed and discussed below.

i) $\alpha_5\beta_1$-integrin

Genetic ablation of the α_5-integrin is embryonically lethal and results in the formation of a severely abnormal embryonic vascular network.[118] Also, $\alpha_5\beta_1$ antagonists have been shown to inhibit non-VEGF-induced angiogenesis in both chick chorioallantoic membrane (CAM) assays and in murine tumours.[119] Finally endostatin, an endogenous angiogenesis inhibitor, is known to inhibit endothelial cell migration by binding to $\alpha_5\beta_1$.[120]

ii) $\alpha_1\beta_1$ and $\alpha_2\beta_1$-integrins

The importance of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ in angiogenesis is indicated by the finding that VEGF upregulates these collagen receptors and by the use of an $\alpha_2\beta_1$ inhibitor to inhibit experimental tumour angiogenesis.[121] Surprisingly, however, genetic ablation of α_1 and α_2 results in viable and fertile mice.

The α_2-null mice also has normal wound healing[122] and, although α_1-null mice do have defective tumour angiogenesis, the mechanism for this is thought to be the upregulation of MMP7 and MMP9 and the subsequent increased formation of the angiogenesis inhibitor, angiostatin.[123]

iii) $\alpha_V\beta_3$ and $\alpha_V\beta_5$-integrins

Although several members of the integrin family have been implicated in angiogenesis, the largest body of data has linked $\alpha_V\beta_3$ and $\alpha_V\beta_5$ with promoting neovascularization. $\alpha_V\beta_3$, in particular, is greatly upregulated by FGF and is prominent on proliferating endothelial cells.[124]

Crosstalk between $\alpha_V\beta_3$ and growth factor receptors is crucial to coordinate, fine-tune and amplify angiogenesis. For example, VEGF, acting via its receptor Flk-1, up regulates $\alpha_V\beta_3$. In turn, $\alpha_V\beta_3$ associates with Flk-1 and amplifies the activity of VEGF on vascular endothelial cells.[125]

$\alpha_V\beta_3$ also associates with a number of other molecules with important roles in angiogenesis. For example, MMP-2 is known to co-localize with $\alpha_V\beta_3$ in

caveolae on the cell surface[95] and merely preventing this interaction is sufficient to inhibit murine tumour growth.[109]

Further evidence of the role of α_V-integrins in angiogenesis came from the discovery that antagonists of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ are able to inhibit neo-vascularisation. For example, a function-blocking antibody directed at the extracellular domain of $\alpha_V\beta_3$ (LM609) has been shown to block angiogenesis in arthritis, retinopathy of prematurity, and tumour angiogenesis models.[34] Vitaxin, a humanized version of LM609, is currently undergoing human clinical trials.[126]

Also, a single molecule inhibitor of both $\alpha_V\beta_3$ and $\alpha_V\beta_5$ inhibits murine tumour angiogenesis.[127] Inhibiting both integrins may have therapeutic benefit because it has been shown that these two integrins mediate preferential responses to different angiogenic factors: $\alpha_V\beta_3$ for FGF, and $\alpha_V\beta_5$ for VEGF.[128]

These results suggested that genetic ablation of α_V, β_3 or β_5 would result in extensive vascular defects. However, each of these single-gene knock-out animals had relatively normal vascular development during embryogenesis. Furthermore, it was recently published in the journal Nature that β_3/β_5 double knock-out mice have increased, rather than decreased, tumour angiogenesis.[129]

A comment by Peter Carmeliet in that issue of Nature suggested possible explanations for this discrepancy between the pharmacological and genetic

ablation data.[130] Firstly, other integrins may have substituted for the genetically ablated integrins in the β_3/β_5-null mice. Although individual extracellular matrix components can bind multiple integrins, no compensatory integrin over-expression was noted in the β_3/β_5-null mice, making this explanation less likely.

Secondly, β_3/β_5-independent angiogenesis pathways may have been induced to compensate for the deletion of these integrins. It is known that β_3 and β_5 are not required for embryonic vasculogenesis, so, perhaps, a more "embryonic" mode of angiogenesis was utilized to supply the growing adult tumours. Or, circulating endothelial precursors from the bone marrow may have substituted for the impaired sprouting of local vessels.

Thirdly, the $\alpha_V\beta_3/\beta_5$ antagonists mentioned previously may have blocked several other integrins, as well. If this is true, it may mean that synergistic inhibition of several integrins is required to successfully inhibit angiogenesis.

Finally, it may be that our basic understanding of the function of integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ is incomplete. The authors of the β_3/β_5-null angiogenesis study suggest that $\alpha_V\beta_3$ may not only stimulate angiogenesis, it may inhibit it. They suggest that ligated and unligated integrins could provide positive and negative signals to the cell, only allowing new vessel formation in the presence of appropriate extracellular ligands. Integrin antagonists may mimic the unligated state, inhibiting angiogenesis, while absence of these

integrins would result in the loss of this negative signal and enhanced angiogenesis.

Increased expression of Flk-1 has been noted on β_3-null endothelial cells, suggesting that β_3 could, indeed, negatively regulate aspects of angiogenesis, including VEGF receptor expression.

It is worth remembering that the efficacy of $\alpha_V\beta_3/\beta_5$ antagonists is not questioned by this study and such inhibitors may yet be shown to have important therapeutic benefits. However, it is clear that we require a more thorough understanding of both the precise mechanisms of these inhibitors as well as the exact roles of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins in angiogenesis.

1.9 ANTI-ANGIOGENESIS THERAPY

Angiogenesis inhibitors are a relatively new class of cancer drugs, whose biological and biochemical characteristics differ markedly from conventional cytotoxic chemotherapy. Angiogenesis inhibitors, for the most part, target vascular endothelial cells in an effort to block tumour angiogenesis. This approach of targeting cells, which support the cancer, rather than the cancerous cells themselves, is an attractive approach because these cells are genetically stable, and thus less likely to accumulate mutations, which would render them resistant to therapy.[9]

Folkman et al. have divided angiogenesis inhibitors into two main groups, direct and indirect angiogenesis inhibitors.[9] Direct angiogenesis inhibitors target endothelial cells and stop them proliferating, migrating and avoiding apoptosis in response to a pro-angiogenic growth factors, like VEGF, PDGF or FGF. Because they target genetically stable endothelial cells, they are less likely to induce acquired drug resistance.

Indirect angiogenesis inhibitors block the expression or activity of a tumour-expressed protein that promotes angiogenesis. Activating mutations in oncogenes have been shown to cause tumour cells to upregulate angiogenic proteins and downregulate inhibitors of angiogenesis.[131] For example, human osteosarcoma cells implanted into mice form only microscopic avascular tumours. However, after transfection with the Ras oncogene, these cells doubled their VEGF expression and downregulated thrombospondin-1. This

resulted in the development of large, neovascularized tumours within two weeks.[132]

Thus, several drugs targeting tumour oncogenes and tumour suppressor genes have been shown to elicit anti-angiogenic activity. Trastuzumab, an antibody that blocks ERBB2 receptor tyrosine kinase signaling, suppresses cancer cell production of TGFβ, angiopoeitin-1, PAI-1 and possibly VEGF. It also upregulates the expression of the angiogenesis inhibitor thrombospondin-1.[133]

Therefore, although trastuzumab was designed to target a cell-surface receptor overexpressed by certain cancers, it also acts as an indirect inhibitor of angiogenesis by blocking the angiogenic output of the tumour.

1.9.1 Angiogenesis inhibitors

A wide range of angiogenesis inhibitors have been developed and tested in animal experiments and in human clinical trials, but an exhaustive discussion of all of these is beyond the scope of this review. Therefore, we have decided to focus on some of the anti-angiogenesis therapies, which are in the later stages of clinical trials or have already been successfully used in humans.

i) Interferon-α

IFN-α was demonstrated, over twenty years ago, to reversibly inhibit the migration of cultured endothelial cells. This was followed by the discovery that it downregulates the expression of FGF in tumour cells[134] and that it has in vivo anti-angiogenic activity at low doses in murine studies.[135]

The first demonstration of the efficacy of anti-angiogenic therapy in human patients was the use of IFN-α in the successful treatment of a 12-year-old patient with pulmonary haemangiomatosis.[22] Since then, low-dose IFN-α has been used to treat life- or sight-threatening haemangiomas, high-grade giant cell tumours refractory to conventional therapy, and angioblastomas. These tumours are all characterized by the expression of FGF as their major angiogenic factor.[9]

ii) Thalidomide

Another previously existing drug, which was subsequently shown to have anti-angiogenic activity is thalidomide. Thalidomide successfully inhibited the angiogenic response to VEGF and FGF in rabbit corneal pocket assays as well as limiting the growth of carcinomas in both rabbits and mice. In 1999, thalidomide was shown to be clinically active in human patients with advanced multiple myeloma. In the initial study, 32% of patients demonstrated a clinical benefit, as evidenced by a decrease in the serum levels of myeloma protein.[136] Within three years, 160 clinical trials of thalidomide were being undertaken worldwide in various human cancers. In

fact, today thalidomide is considered one of the most effective agents for the treatment of multiple myeloma.[9]

Despite these clinical benefits, the mode of action of thalidomide remains controversial. The anti-angiogenic effects of the drug are thought to be important as plasma levels of pro-angiogenic molecules FGF and VEGF dropped following thalidomide treatment, especially in patients with a positive clinical response. Also, treatment with thalidomide has been shown to decrease the production of TNF-α, a known angiogenic factor. However, specific inhibitors of TNF-α have little activity or no activity in either murine models or human trials of multiple myeloma. Thalidomide itself has direct action on myeloma cells, but only at doses above therapeutically utilized levels.[137] One theory postulates that thalidomide acts on both cancer cells and endothelial cells in exerting its clinical benefit.

iii) Avastin

In February 2004, Bevacizumab (Avastin), a humanized monoclonal antibody to VEGF, was approved by the Food and Drug Administration (FDA) in the United States for the treatment of metastatic colon cancer.[138] This occurred after the results of a successful phase III trial of this drug were published in the New England Journal of Medicine.[57]

This trial compared treatment with irintecan, fluorouracil and leucovorin (IFL) plus avastin with IFL treatment alone in patients with previously untreated metastatic colorectal cancer. The addition of avastin increased the

median duration of survival from 15.6 months to 20.3 months, and the duration of progression free survival from 6.2 months to 10.6 months.

Phase 1 and 2 trials had previously identified hemorrhage, thromboembolism, proteinuria, and hypertension as possible side-effects of avastin treatment. However, in this study only hypertension was significantly increased in the avastin-treated group, and this was easily treated with standard oral anti-hypertensive therapy. Apart from the cardiovascular side-effects, gastro-intestinal perforation was identified as a small but significant risk.

However, within six months of receiving approving avastin, the FDA issued a warning to healthcare professionals regarding the increased risk of adverse cardiovascular events in avastin-treated patients. The risk of arterial thromboembolic events (4.4% vs. 1.9%), cerebrovascular arterial events (1.9% vs. 0.5%) and cardiovascular arterial events (2.1% vs. 1.0%) were all increased in patients receiving Avastin in combination with chemotherapy. This was particularly common in patients over the age of 65.[139]

A possible mechanism for these findings, albeit concerning a different disease process, was published in the New England Journal of Medicine at the same time that avastin was approved for use. In this study it was shown that serum and placental concentrations of the soluble VEGF receptor (sFlt-1) were increased in women with preeclampsia.[140] This protein acts by binding to PIGF and VEGF, thus preventing their interaction with endothelial receptors for these molecules and causing endothelial dysfunction. In keeping with this hypothesis, exogenous administration of

sFlt-1 to pregnant and non-pregnant rats induces hypertension, proteinuria, and glomerular endotheliosis.[70]

It is tempting to hypothesize that, by binding circulating VEGF, avastin prevents its physiological interaction with non-tumour vascular endothelium and results in hypertension and an increased risk of cardiovascular events. Of course, the risk of these events should be viewed in the context of avastin's proven success in improving the overall survival of patients with metastatic colorectal cancer.

iv) Extracellular matrix fragments

Proteolytic digestion of the extracellular matrix and coagulation factors during the sprouting phase of angiogenesis develops endogenous protein fragments, which possess anti-angiogenic activity.[85]

The first of these to be discovered was angiostatin, a proteolytic fragment of plasminogen,[52] which was isolated by O'Reilly et al. in 1994. This was followed three years later by the discovery of endostatin, an internal fragment of collagen XVIII, in the same laboratory.[46]

Endostatin had been shown to inhibit the proliferation of endothelial cells in vitro by binding integrin $\alpha_5\beta_1$, prompting study into its potential inhibitory effect on tumour growth. In a seminal Nature paper in 1997, it was shown that, not only did endostatin prevent tumour growth in mice, but also that there was no development of resistance to this therapy.[141] This initial

observation has been followed by a large number of animal tumor studies with results ranging from complete block of tumor growth, to the absence of any significant effect.[142]

As a further guide to translation of this therapy to human disease, it has been shown that continuous infusions of endostatin are more effective than intermittent therapy,[143] and that combination therapy with either radiotherapy or cytotoxic chemotherapy is effective. Phase I and II trials are currently underway.[142]

Tumstatin is one of several endogenous angiogenesis inhibitors derived from the proteolytic digestion of type-IV collagen. Tumstatin binds the $\alpha_V\beta_3$ integrin on endothelial cells and inhibits endothelial-cell proliferation and neovascularization. It has been shown that tumstatin has no effect on $\alpha_V\beta_3$-/- endothelial cells, whereas endostatin, which binds $\alpha_5\beta_1$, has preserved activity.[144]

Studies on mice in which the $\alpha 3$ chain of type-IV collagen has been deleted led to important discoveries about the physiological control of angiogenesis. $\alpha 3$-null mice develop no endogenous tumstatin and tumours implanted into $\alpha 3$-null mice were associated with excessive neovascularization and enhanced tumour growth compared with wild-type animals. However, if tumstatin was replaced, tumour growth was reduced below wild-type levels.[145] This implies that tumour growth in normal tissues does not occur at ceiling rates, but is inhibited, at least partially, by the endogenous development of tumstatin and, presumably other, endogenous inhibitors.

In fact, it has long been known that individuals with Down syndrome have a very low incidence of solid tumours even though their life expectancy has increased markedly in the last century. Individuals with Down syndrome have subsequently been shown to have higher levels of circulating endostatin, possibly because they possess three copies of collagen XVIII on chromosome 21. It is an intriguing possibility this excessive endostatin acts as a form of cancer prophylaxis in these individuals.[90]

1.9.2 Unique features of anti-angiogenesis therapy

Treating a tumour's blood supply, rather than the actual cancerous cells involved a major paradigm shift in the field of oncology. It is, therefore, not surprising that a number of the traditional principles of cancer chemotherapy do not apply to anti-angiogenesis therapies.

Firstly, tumour regression following anti-angiogenic therapy is often slow and can take up to a year.[146] On the other hand, rapid tumour regression usually accompanies successful cytotoxic chemotherapy. A patient with "stable disease" but no tumour regression on cytotoxic therapy might be considered a treatment failure because of the almost inevitable development of tumour drug resistance. But, anti-angiogenic treatment may not be as prone to the development of resistance allowing prolonged periods of tumour growth arrest.[141]

Secondly, the traditional methods of administering cytotoxic chemotherapeutic agents in cancer patients may not apply to angiogenesis inhibitors. It has been shown in animal studies that many angiogenesis inhibitors are most effective when given at a dose and schedule, which maintains a constant concentration of the drug in the circulation.[143] This is in contrast to conventional cytotoxic chemotherapy, which is usually given for short periods at the maximum tolerated dose, followed by intervals without therapy in order to allow bone marrow and gastrointestinal recovery.

1.9.3 Potential problems with anti-angiogenic cancer therapies

The obvious initial question posed about any new therapy concerns its efficacy. Despite great successes in the treatment of cancers in experimental animal models, we must await the result of human clinical trials before we can say whether inhibition of angiogenesis will be successful in treating human cancer. In the meantime, it has been proposed that anti-angiogenic therapy will never be able to treat all types of cancer. In particular, it has been suggested that angiogenesis inhibition will fail to treat slow-growing or poorly vascularized tumours.

Perhaps because rapidly-growing tumours are generally more sensitive to cytotoxic chemotherapy, it has been assumed by some oncologists that slow-growing tumours would be resistant to anti-angiogenic therapy. However, in numerous animal models, angiogenesis inhibitors have been shown to be effective in the treatment of slow-growing tumours.[9] In fact, in a murine model of tumour angiogenesis involving two types of human bladder cancer

– one rapidly growing and highly vascularized, the other slowly growing and poorly vascularized – the slow-growing tumour exhibited the greater response to angiogenesis inhibition.[147]

Similarly, poorly vascularized tumours have been successfully treated by angiogenesis inhibitors in animal models. The significant point is that any tumour large enough to become clinically significant has already undergone neovascularization. In fact, highly vascular tumours often require a higher dose of angiogenesis inhibitor or a combination of inhibitors in order to secure a tumour response.[9]

Although these findings don't substitute for human clinical trials, they are encouraging in that they suggest that anti-angiogenesis therapy may prove beneficial in a wide range of different cancers.

Perhaps the greatest benefit to anti-angiogenic therapy for the treatment of human cancer would be that patients could avoid the wide range of side-effects associated with traditional cytotoxic chemotherapy. However, some side-effects to angiogenesis inhibition would be expected.

Angiogenesis is required in certain physiological settings in the adult, including wound-healing and follicular development in the ovary. In addition, the requirement of angiogenesis for normal embryogenesis may contraindicate anti-angiogenesis therapy during pregnancy.[9] The solution to this problem would be to weigh the risks and benefits involved when treating a potentially fatal malignancy, and to develop angiogenesis inhibitors that target pathological, and not physiological, angiogenesis.

Individual anti-angiogenesis therapies may also have unpredicted side-effects. The development of strokes by patients on Avastin is a case in point. Although we now have a possible mechanism why this occurs (vide supra), the development of hypertension and strokes was certainly not predicted on the basis of angiogenesis inhibition.

Another concern raised is that anti-angiogenic therapy may result in hypoxia, which is known to select for tumour cells resistant to apoptosis, and may limit the efficacy of the treatment.[148] Potential responses to this problem include the fact that even hypoxia-resistant cells cannot withstand total anoxia following regression of tumour microvessels. Also, additional therapeutic strategies could be devised to target HIF-1α and other transcription factors involved in the hypoxia response.[9]

Clinicians have raised the concern that combination therapy with conventional cytotoxic and anti-angiogenic drugs would result in decreased delivery of the cytotoxic agents. However, recent work suggests that anti-angiogenic therapy may, in fact, normalize tumour vasculature.[149] Tumour vessels are typically very leaky resulting in raised tissue pressure within the tumour and poor blood flow. By preventing this leakage, angiogenesis inhibitors have been shown, at least initially, to increase the delivery of cytotoxic agents.[150] Chronic anti-angiogenic therapy, on the other hand, results in either a stabilization or gradual reduction in total tumour blood flow. Furthermore, the combination of angiostatin or endostatin with conventional chemotherapy in a murine model of liver metastasis resulted in enhanced anti-tumour activity.[151]

In conclusion, although many questions still remain regarding the use of angiogenesis inhibitors in humans, animal experiments and early clinical trials provide hope that the promise of effective anti-angiogenic cancer therapies may yet be realized.

1.10 EXPERIMENTAL ANGIOGENESIS MODELS

One of the major problems in angiogenesis research has been modeling this complex process experimentally.[152,153] In vivo angiogenesis is a carefully controlled process, which integrates many different cell types, making it difficult to model in vivo and in vitro. However, discovering the cellular mechanisms underlying angiogenesis and testing novel angiogenesis inducers and inhibitors requires the development of reliable, reproducible and efficient experimental angiogenesis models.

As covered in the previous chapter the process of angiogenesis requires endothelial cells to, firstly, break through their surrounding basement membrane and, secondly, to migrate towards the source of the angiogenic stimulus. Behind this migrating front, endothelial cells must proliferate to produce the cells required to form the new vessels. Finally, this new outgrowth of endothelial cells needs to reorganize into patent three-dimensional tube structures. Each of these specific elements: basement membrane disruption, cell migration, cell proliferation, and tube formation, can be a target for intervention, and each can be tested in vitro.[153] However, because angiogenesis requires the incorporation of several different cell types, several in vivo assays have also been developed.

A brief discussion of the major in vitro and in vivo angiogenesis assays is discussed below, including an evaluation of the advantages and limitations of each method.

1.10.1 In vitro assays

The use of culture systems of endothelial cells to study angiogenesis creates several problems. Firstly, it is now accepted that all endothelial cells are not alike, but rather differ greatly in their response to growth factors and inhibitors depending on the source of the endothelial cells.[154] In the past, the major source of endothelial cells used to study angiogenesis in vitro were bovine aortic endothelial cells and human umbilical vein endothelial cells. These macrovascular endothelial cells have differing in vitro phenotypes to microvascular endothelial cells, which are the main responding cells during angiogenesis. In addition, microvascular endothelial cells differ depending on which organ or, even, which blood vessels within that organ they were isolated from.[154,155] Also, endothelial cells in tumour-induced blood vessels, as apposed to those in quiescent microvasculature, show marked upregulation of specific integrins and cell adhesion molecules.[156,157] In vitro experiments that fail to take these factors into account may miss subtle features in the angiogenesis process.

The second major problem is obtaining sufficient cells for experimentation from primary tissues. Inevitably, this requires expanding cell cultures in vitro, but significant changes have been observed in endothelial cells following prolonged culture. These include alteration in surface receptors, proliferation rates and activation status.[158]

Finally, the major responding cells during angiogenesis are quiescent microvascular endothelial cells. Endothelial cells have one of the lowest

proliferation rates in vivo, but can divide rapidly upon stimulation.[159] Since cultured endothelial cells are, by definition, activated, proliferating cells, they are poor model for many in vivo endothelial behaviours.

Despite these limitations, three main in vitro endothelial cell assays are widely used in angiogenesis research. These are: cell proliferation assays, cell migration assays, and tube formation assays.

i) Cell proliferation assays

Measuring endothelial cell proliferation following growth factor stimulation or specific inhibition is often used as a preliminary test of angiogenesis inducers or inhibitors. There are several well-established assays for assessing cell proliferation, including thymidine incorporation, BrdU uptake and the MTT assay.

In the thymidine incorporation assay, [^{3}H] Thymidine is incorporated into the newly synthesized DNA of proliferating cells. Subsequent quantification of [^{3}H] thymidine is performed by scintillation counting of autoradiography. In the BrdU assay, radioactive thymidine is replaced by the thymidine analogue 5-bromo-2-deoxyuridine (BrdU), which partially replaces thymidine during the process of DNA synthesis. Following partial denaturation of double stranded DNA, this BrdU is detected immunochemically allowing the assessment of the population of cells, which are synthesizing DNA.[160]

The MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to form dark blue formazan crystals. These crystals accumulate in healthy cells and can be liberated and solubilised using detergent. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay and results read on a multiwell scanning spectrophotometer (ELISA reader).[161]

In addition to the general problems common to all in vitro assays, certain additional caveats must be observed when using endothelial cell proliferation assay. Firstly, a potent stimulus of endothelial cell proliferation may have actions on a number of different cell types in several in vivo settings making it a poor target for anti-angiogenesis therapy. For example, although FGF-2 is a potent endothelial mitogen, it also acts on a wide range of other cell types, and deletion of this gene has minimal effects on angiogenesis in vivo.[75,162] This may be because other growth factors compensate for FGF-2 in this setting, and FGF-2 inhibition may still be an important anti-angiogenic therapy in combination with other agents. However, this emphasizes the importance of testing angiogenic growth factors in multiple in vitro and in vivo settings.

Secondly, an agent that decreases endothelial cell proliferation may not be specific for endothelial cells, but rather a general inhibitor of cellular proliferation. The effect noted would, therefore, not be a valid representation of anti-angiogenic activity.

ii) Cell migration assays

Several tests can be used to assess the migratory response of endothelial cells to angiogenesis promoting or inhibiting factors. The most frequently used is the modified Boyden chamber in which endothelial cells are placed on the upper layer of a cell-permeable filter and permitted to migrate towards a test factor placed in the media below the filter. Cells that have migrated through the filter are then fixed, stained and counted manually.[153]

In addition to the general problems of endothelial cell heterogeneity and lack of cellular interactions common to all in vitro assays, several specific difficulties occur with migration assays. Firstly, microvascular endothelial cells are more delicate than standard large-vessel endothelial cells (BAEC or HUVEC), often resulting in poor cellular migration. Secondly, enumeration of traversed cells is both tedious and prone to significant ranges of error.

iii) Tube formation assays

One of the most specific in vitro tests for angiogenesis is measuring the formation of three-dimensional tubes by endothelial cells in culture.[163]

Initially tube formation, while spontaneous in culture, occurred slowly because of the time taken for endothelial cells in culture to lay down appropriate extracellular matrix. However, with the discovery of Matrigel™,

endothelial cells could be induced to form tubes within 24 hours and the test gained prominence as an in vitro assay of angiogenesis.

A caveat is that non-endothelial cells, for example fibroblasts, may also respond to Matrigel™ by forming tubes.[164] Secondly, different populations of endothelial cells differ in their ability to form tubes. Mouse lung endothelial cells, such as those used in this project, fail to form tubes on Matrigel™ (S.W. Jackson, unpublished observation) rendering this a poor test of angiogenesis in this setting.

1.10.2 Organ culture assays

i) Aortic Ring assays

Organ culture has become increasingly used as an assay of angiogenesis in vitro, because of the recognition that angiogenesis in vivo involves not only endothelial cells but also their surrounding cells. The most common organ culture assay currently used is the rat aortic ring assay, in which isolated rat aorta is cut into segments and placed in culture, generally on a Matrigel™ matrix. Over the next 7-14 days, the explants are monitored for outgrowths of endothelial cells and for the effect of addition of various test substances.[153]

The advantages of this assay include: firstly, that it models the in vivo situation by including both endothelial and non-endothelial surrounding cells; and, secondly, that the endothelial cells are not pre-selected for a

proliferative phenotype by passaging in culture. On the other hand, the use of aortic tissue is not ideal, as angiogenesis is primarily a microvascular, not macrovascular phenomenon.[153]

1.10.3 In vivo assays

i) Chick chorioallantoic membrane (CAM) assays

In the chick chorioallantoic membrane assay the entire egg contents of a chick embryo are transferred to a plastic culture dish after 72 h of incubation. After 3–6 days in culture, the vascular chorioallantoic membrane (CAM) develops. Angiogenic stimulation can then be assessed in response to tumour grafts, immunocompetent allografts, or test substances placed onto the membrane. Inhibitors can be assessed for their effect on the normal development of the CAM vasculature or on induced angiogenesis.[153]

The CAM assay has numerous advantages including the ease of performing the assay, the ready availability of experimental material, the ability to test multiple agents on a single CAM, and the ability to monitor the reaction throughout the course of the test. Disadvantages include the use of chicken cells, which may not correlate well with other animal and human models, and the fact that the CAM, itself, is undergoing rapid morphological changes during the course of the assay.[153]

ii) Corneal angiogenesis assays

The corneal angiogenesis assay is considered a very useful in vivo angiogenesis test because the cornea itself is avascular and, therefore, any vessels seen after the administration of angiogenesis-inducing tissues or factors must be new vessels. This is in contrast to the CAM assay where test substances modulate pre-existing vasculature.

The original method was developed for rabbit eyes in Dr. Folkman's laboratory.[165] Briefly, a pocket is made in the cornea into which tumour cells or test substances (incorporated into slow-release materials such as ELVAX (ethylene vinyl copolymer) or Hydron) are introduced. Angiogenesis is evidenced by the ingrowth of new vessels from the peripheral limbic vasculature. To test anti-angiogenic factors, one can monitor the effect of orally or systemically administered inhibitors on this process.

As with the CAM assay, angiogenesis can be visualized throughout the course of the experiment, in this case, using a slit lamp. Previously, visualization of the corneal neovascularization was achieved by injecting India ink, but today, fluorochrome-labeled high-molecular weight dextran has become the method of choice. This latter method has the added advantage that the angiogenic effect can be quantified accurately using histographic analysis of fluorescent intensity.[166]

Aside from its historical significance in the development of the theory of angiogenesis, the corneal assay has several advantages over other tests. For

example, one can monitor the progress of angiogenesis with time; there is no background vasculature in the cornea; and, the assay has been modified for use in mice. On the other hand, the surgical technique is demanding and very little space is available for introducing test materials. Also, inflammatory reactions are common and, as a model of angiogenesis, the assay is atypical precisely because the cornea is avascular.

iii) Matrigel™ plug assays

Matrigel™ is a solubulized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in ECM proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin.[167]

Following subcutaneous injection, the Matrigel™ matrix polymerizes to produce biologically active matrix material resembling the mammalian cellular basement membrane. Test cells or substances can be included in this matrix to assess the angiogenic response. After 7-21 days, the plug of Matrigel™ is harvested and examined histologically for invasion by new vasculature.[168]

Although the quantification of these new vessels in histological sequences is tedious, it is accurate.[168] In addition, plasma volume in the Matrigel™ may be measured by fluorescence using fluorescein isothiocyanate (FITC)-labeled dextran.[169]

Compared with the CAM assay and the corneal angiogenesis assay, the Matrigel™ plug assay has significant advantages. It is a better model of physiological and pathological angiogenesis because it can be used in adult mice (or in other animals) and because it can be performed in normal vascularized tissue. Unfortunately, one cannot visualize the development of new blood vessels with time.

iv) Implanted tumour angiogenesis assays

The traditional method for testing cytotoxic oncology therapies has been adapted to study tumour angiogenesis, namely, models of murine tumour inoculation. This can be achieved by either inoculating murine tumours into syngeneic mice, or human tumours into nude mice.[170] Angiogenesis inhibitors can then be tested by monitoring their effects on the growth of implanted tumours. Similarly, the importance of various angiogenic growth factors or natural angiogenesis inhibitors on murine tumour angiogenesis can be tested in knock-out or transgenic mice.

Naturally, this method provides a more accurate model of tumour angiogenesis than the previous in vivo and in vitro models. However, it has been repeatedly shown that certain cytotoxic drugs have marked effects on implanted murine tumours, but minimal effect on human cancers.[170] Differences between implanted murine tumours and spontaneously arising human cancers may also impact on the response to anti-angiogenic therapy.

Secondly, most tumours used in murine implantation models are very fast growing in contrast to most human cancers.[170] This is particularly pertinent when testing cytotoxic cancer therapies, which target rapidly dividing cells, but may also impact on anti-angiogenesis therapies.

Thirdly, anti-tumour therapies in murine models are tested for action against the primary inoculated tumour, while metastatic disease is the most common cause of death in human cancer. Also, tumours are usually implanted in ectopic sites (often subcutaneous) in murine models, rather than at the original site of tumour growth. Given the importance of the local microenvironment on tumour growth[171], the response of anti-tumour agents in this setting may be artificial.

Fourthly, the use of nude mice in human tumour models results in abnormal immune anti-tumour effects to spontaneously arising tumours in immuno-competent hosts.[172] Also, several murine tumour lines used for implantation may no longer be syngeneic with the host.[173]

Finally, differences in drug metabolism between mice and humans may make it difficult to repeat successful drug protocols in human patients. For example, the maximum tolerated dose of cytotoxic agents is often significantly greater in mice than humans.[174] Although this is especially pertinent to cytotoxic therapies, which rely on intermittent doses of maximal therapy, it may also impact on anti-angiogenic therapies.

Because of these problems with tumour implantation models, mice with a predisposition to develop spontaneous tumours have been developed. It is

hoped that these will provide greater similarities to human cancers. For example, *ras* oncogene transgenic mice have been shown to develop mammary carcinomas with similar features to human breast cancer with regard to chemotherapy sensitivity, namely sensitivity to doxorubicin and resistance to cisplatin.[175]

In general, such tumours also share greater histological similarities with human tumours and behave similar to human tumours, especially with regard to the development of metastases. Also, because the mice are engineered via over-expression of an oncogene or deletion of a tumour suppressor gene, they develop a wide range of different tumours in various organs. The problems with tumour immunogenicity or the need for immunodeficient mice are also avoided. Finally, these models can be used to test chemoprevention, a particularly attractive options considering the recent findings that natural angiogenesis inhibitors prevent cancer development.

However, there are still significant disadvantages to these models. The major problem is that, because these tumours develop spontaneously, they also develop unpredictably and sporadically. This requires the breeding, typing and monitoring of a large number of mice, which is both expensive and labour intensive. In addition, these tumours may still not mimic human tumours exactly. This is especially important when testing newer designer drugs, which target specific intracellular signaling mediators. For example, a Ras farnesyltransferase inhibitor is able to dramatically shrink spontaneous breast carcinomas in *ras* oncogene transgenic mice, but is only cytostatic in murine models of human mammary carcinoma.[176]

CHAPTER 2: LITERATURE REVIEW

PURINERGIC SIGNALING

2.1 PURINERGIC SIGNALING

Although the potent extracellular actions of purine nucleotides and nucleosides on the mammalian cardiovascular system was first recognized over seventy years ago, research in the field of purinergic signaling was initially limited.[177] But, in the 1960s, a non-adrenergic, non-cholinergic component of the autonomic nervous system was identified in several tissues. Burnstock proposed that this novel neurotransmitter was ATP and coined the term "purinergic" signaling.[178]

The field was then bolstered by the discovery of specific extracellular receptors for nucleotides and nucleosides as well as by the discovery of a family of enzymes, which modulate this signaling by hydrolyzing the released nucleotides.

Today we known that ATP, UTP, ADP and UDP signaling plays a key role in a diversity of tissue functions that include fast excitatory neurotransmission, developmental processing, pulmonary function, nociception, auditory and ocular function, the apoptotic cascade, astroglial cell function, metastasis formation, bone and cartilage disease, and platelet aggregation/hemostasis.[179]

2.1.1 Purine Receptors

Extracellular purines (ATP, ADP and adenosine) and pyrimidines (UTP, UDP) are important signaling molecules, which mediate a wide range of biological effects via cell-surface receptors called purine receptors. Purine receptors are subdivided into P1-receptors, which recognize adenosine, and P2-receptors, which recognize ATP, UTP, ADP and UDP.[180]

Adenosine/P1-receptors have been further subdivided into A_1, A_{2A}, A_{2B}, and A_3 receptor subtypes, according to their molecular, biochemical and pharmacological features. Each of these receptors is a G-protein coupled receptor.

P2-receptors are divided into two major families: P2X, which are ligand-gated ion channels; and P2Y, which are seven-transmembrane spanning G-protein coupled receptors.

i) P2X-Receptors

P2X-Receptors are ATP-gated ion channels, which mediate rapid (within 10ms) and selective permeability to cations (Na^+, K^+, and Ca^{2+}). Seven subtypes, each with distinct pharmacological profiles, have been cloned and denoted $P2X_{1-7}$.[180] Based on their agonist efficacy and desensitization characteristics, P2X receptors have been divided into three groups.[181] Group 1 includes $P2X_1$ and $P2X_3$, which have high affinity for ATP (EC_{50}=1μM) and are rapidly desensitized. Group 2 includes $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_6$,

which have lower affinity for ATP (EC_{50}=10μM) and show slow desensitization and sustained depolarization currents. Group 3 is represented by $P2X_7$, which has very low affinity for ATP (EC_{50}=300-400μM), shows little or no desensitization, and, in addition to functioning as an ATP-gated ion channel, can also function as a non-selective ion pore.[182]

ii) P2Y-Receptors

P2Y receptors are G-protein coupled receptors that bind purine and/or pyrimidine nucleotides and their derivatives. At least seven receptors of this class have been cloned and designated $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$.[180]

As with P2X receptors, different P2Y receptors are divided according to agonist specificity. $P2Y_1$ is stimulated by ATP, whereas $P2Y_2$, $P2Y_4$, and $P2Y_6$ respond to uracil (pyrimidinergic) nucleotides. $P2Y_2$ is activated by UTP and ATP, while $P2Y_4$ is activated by UTP and, less efficiently, by ATP. $P2Y_6$ is activated by UDP. ATP is the major agonist of $P2Y_{11}$.[179] The platelet ADP receptor, $P2T_{AC}$, has subsequently been shown to be $P2Y_{12}$.[183]

P2Y receptors are seven-transmembrane-spanning proteins, which couple to different G proteins. Most P2Y receptors activate phospholipase C (PLC), resulting in the formation of inositol 1,4,5-triphosphate (IP_3) and the mobilization of cytosolic Ca^{2+}. However, the activation of $P2Y_1$ and $P2Y_2$ results in the inhibition of adenylate cyclase through G_i and $P2Y_{11}$ stimulates both PLC and adenylate cylase pathways. In addition, the activation of

$P2Y_{12}$ receptors on platelets results predominantly in the inhibition of adenylate cyclase.[177]

P2Y receptors as a class are considered less likely to desensitize than other G protein-coupled receptor subfamilies. However, desensitization of P2Y-receptors does occur, especially in the context of the *Cd39*-null mouse. Like P2X receptors, different P2Y receptors desensitize at different rates.[180]

$P2Y_1$ and $P2Y_2$ are relatively resistant to desensitization, although they can be induced to do so in experimental conditions. On the other hand, IP_3 responses following stimulation of $P2Y_4$ decline within minutes of stimulation and are not reproducible, indicating desensitization.[180] Also, desensitization of the $P2Y_1$ ADP-receptor on platelets has been demonstrated in several experimental models, including the *Cd39*-null mouse.[184]

The mechanism of desensitization in these cases may involve, as with other G protein coupled receptors, phosphorylation of the receptor by a protein kinase, or uncoupling of the receptor from its associated G protein.[177]

2.1.2 ATP release mechanisms

The cytosolic concentration of ATP is in the range of 3-5 mM, while its extracellular concentration is very low. Despite this huge concentration gradient, ATP and other nucleotides are prevented from diffusing out of cells by the lipid bilayer. In pathological conditions, loss of cell viability or cytolysis results in indiscriminate ATP release, while in physiological conditions the release of intracellular nucleotides is highly controlled and regulated. This physiological release of ATP never exceeds 10μmol/L - equivalent to less than 0.1% of the total intracellular ATP pool.[177]

There are at least three mechanisms for the regulated release of nucleotides: i) non-conductive, facilitated diffusion through an ATP transporter, permease, or flippase, ii) conductive ATP transport through an ATP release channel, and iii) exocytosis of ATP-filled vesicles or granules.[185]

Non-conductive transporters for ATP release have been extensively studied in the mitochondrial membrane but their nature in the plasma membrane is more controversial. It has been suggested that the cystic fibrosis transmembrane conductance regulator (CFTR) may transport ATP as well as other large anions.[177]

The nature of conductive ATP release channels is equally controversial. A recent theory is that, rather than transporting ATP itself, CFTR may regulate an ATP-transporting maxi anion channel similar to the mitochondrial porin or outward rectifier Cl^- channel.[183]

Exocytosis of ATP-filled vesicles is known to be a major mechanism of ATP release, especially in platelets, neurons and neuroendocrine cells. The dense granules of mast cells, and adrenal medullary chromaffin cells contain, in addition to their other agonists, ATP and ADP at millimolar concentrations. In this case, ATP and its metabolites act as neurotransmitters or cotransmitters with classical neurotransmitters or histamine. In addition, the dense granules of platelets store a combined ATP plus ADP concentration approaching one molar.[177]

2.2 EXTRACELLULAR NUCLEOTIDE METABOLISM

Intercellular signaling generally requires mechanisms for signal inactivation. For example, during neurotransmission, catecholamine signals are inactivated by cellular reuptake of catecholamines, while acetylcholine is subject to extracellular hydrolysis. Nucleotides are hydrolyzed by an extracellular hydrolysis pathway, which results in the formation of the respective unphosphorylated nucleoside and free phosphate. The nucleoside can then be taken up into the cell and reused.[186] In the case of ATP/ADP, the hydrolysis product, adenosine, also has signaling properties via P1-receptors (vide supra).

The currently known ectonucleotidases include the E-NTPDase family (ecto-nucleoside triphosphate diphosphohydrolase family), the E-NPP family (ectonucleotide pyrophosphatase/phosphodiesterase family), alkaline phosphatases, and ecto-5'-nucleotidase.[187]

2.2.1 The E-NTPDase family

Members of the E-NTPDase family can hydrolyze nucleoside-5'-triphosphates and nucleoside-5'-diphosphates with varying efficiency (figure 2.2.1). They are membrane bound and face the extracellular medium, although cleaved and soluble forms have been described. In most cases, K_m-values are in the low micromolar range and catalytic activity in physiological to alkaline pH range (pH 7.0 – 8.5)[188] and the presence of

divalent cations, such as Ca^{2+} and Mg^{2+}. All members of this family are characterized by the presence of five highly conserved sequence domains called apyrase conserved regions, which presumably have an important role in catalytic activity.[186]

NTPDase1 (CD39) hydrolyzes ATP and ADP equally well, resulting in the generation of AMP[189]. In contrast, NTPDase2 (CD39L1) has a strong preference of nucleoside triphosphates and hydrolyzes ATP and ADP at a molecular ratio of 1:0.03[190]. Both these enzymes have broad tissue distribution and numerous functional roles, but their differential expression in the vasculature suggests an important role in the control of platelet aggregation. NTPDase1 (CD39) is the major ectonucleotidase expressed on the endothelium, while NTPDase2 (CD39L1) is expressed on pericytes surrounding blood vessels. It has been hypothesized that on intact endothelium CD39 may limit platelet aggregation by hydrolyzing ADP, a potent stimulus for platelet aggregation. Endothelial damage would result in the exposure of platelets to NTPDase2 (CD39L1), which preferentially produces ADP from ATP, thus promoting platelet aggregation and haemostasis.[191] The role of CD39 in modulating the agonist activity of nucleotides on platelets in vivo is demonstrated in figure 2.2.2.

NTPDase3 has an intermediate enzymatic action and a molecular ratio of ATP:ADP of 1:0.3.[192]

NTPDase4 shares the general structure of NTPDase1-3, but has a different cellular localization. Two closely related forms have been described, locating to the Golgi apparatus and lysosomal/autophagic vacuoles,

respectively. The Golgi enzyme has highest activity on UDP and a role has been suggested in glycosylation reactions of proteins and lipids.[187]

NTPDase5 (CD39L4) has a high preference for nucleoside-5'-diphosphates, especially UDP, GDP. The murine orthologue of this enzyme was allocated to the endoplasmic reticulum, but expression of human NTPDase5 in COS-7 cells resulted in a secreted, soluble form. Similarly, NTPDase6 is a diphosphohydrolase associated with the Golgi apparatus, but it also has a secreted form.[187]

The functional role of these enzymes is controversial. Rat NTPDase5 has been suggested to promote reglycosylation reactions important for glycoprotein folding, while NTPDase6 may have a similar role to NTPDase4 in the Golgi apparatus.[187]

2.2.2 Ecto-5'-nucleotidase

Ecto-5'-nucleotidase (also known as the lymphocyte surface protein CD73) is a GPI-anchored enzyme that is a maturation marker on both B and T lymphocytes. The enzyme catalyzes the final step of extracellular nucleotide degradation, the hydrolysis of nucleoside 5'-monophosphates to the respective nucleoside and free phosphate. In this manner, ecto-5'-nucleotidase is the major enzyme responsible for the formation of extracellular adenosine. By activating P1-receptors, ecto-5'-nucleotidase may have a variety of different functions depending on its cell and tissue expression.[186,187]

2.2.3 The E-NPP family

Members of the E-NPP family of ectonucleotidases possess a broad range of substrate specificity. The have both alkaline phosphodiesterase and nucleotide pyrophosphatase activity, and can thus hydrolyze cAMP, ATP, and ADP, as well as NAD^+ to AMP and nicotinamide mononucleotide.[186,187] A mouse deficient in NPP1, a member of this family, develops excessive bone formation around growth plates, joints and tendons, suggesting a role in the balance of calcification and inhibition of calcification in tissues.[193]

2.2.4 Alkaline Phosphatases

Alkaline phosphatases degrade not only nucleoside 5'-tri-, di-, and mono-phosphates, but also release inorganic phosphate from a variety of organic compounds, like proteins. Unlike the E-NTPDase and E-NPP families, the K_m of this group of enzymes is in the millimolar range, and, as such, the alkaline phosphatases have received little attention in the field of purinergic signaling.[186,187]

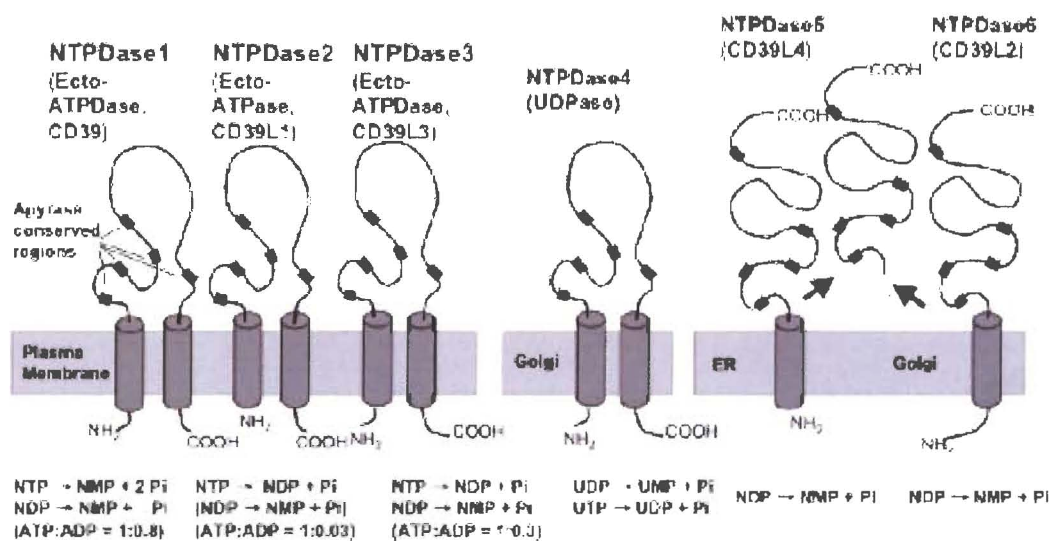


Figure 2.2.1 - The E-NTPDase family of ecto-nucleotidases.

A diagrammatic representation of the E-NTPDase family showing NTPDases1-6. Their alternate names are shown in parentheses below. Apyrase conserved regions are demonstrated as black boxes in the extracellular domain. The enzymatic actions and ATPase:ADPase ratios of each ectonucleotidase are shown below the diagrams. Note that NTPDases1-3 are expressed in the plasma membrane with their enzymatic domains facing the extracellular space. In contrast, NTPDases 4-6 reside in either the Golgi or endoplasmic reticulum. (Image: Figure 1 in Zimmermann, H. Extracellular metabolism of ATP and other nucleotides. *Naunyn-Schmiedeberg's Arch Pharmacol* (2000) 362:299–309. Figure reprinted with permission).

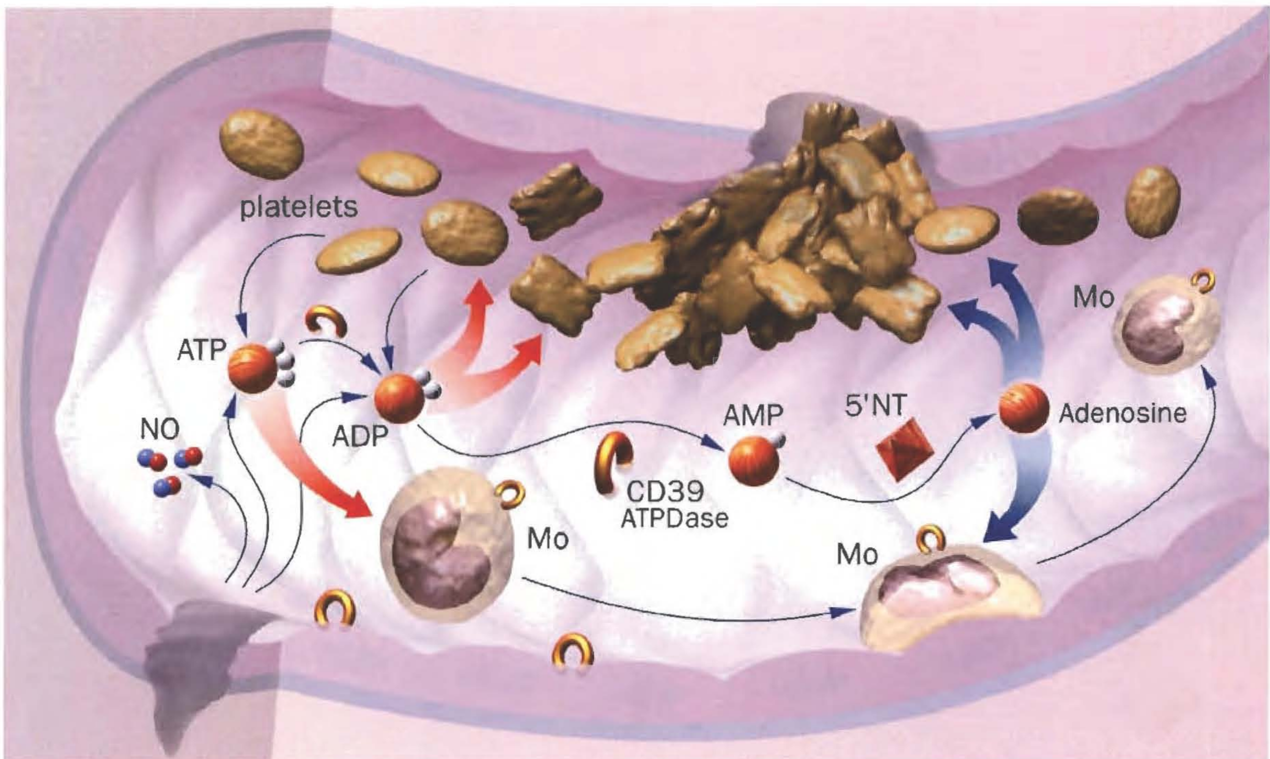


Figure 2.2.2 - Graphical representation of the action of CD39 on extracellular nucleotides, ATP and ADP.

ATP and ADP are released at the site of tissue damage, where they induce platelet activation. CD39 (represented by the gold rings on the endothelial surface and on the surface of monocytes (Mo)) hydrolyzes ATP and ADP to AMP, thus limiting platelet aggregation. AMP is further hydrolysed by 5'-nucleotidase (5'NT) to adenosine, which has anti-inflammatory and anti-aggregatory effects. (Image courtesy of Dr. Simon Robson).

3.1 POTENTIAL ROLES FOR PURINERGIC SIGNALING IN ANGIOGENESIS

3.1.1. Introduction

Although it was hypothesized over twenty years ago that nucleotides may be capable of acting as angiogenic agents,[194] the importance of purinergic signaling in angiogenesis has only recently been realized. Evidence for the role of purines in angiogenesis has come, in particular, from studies on the effects of adenosine, ATP, and UTP on endothelial cells in vitro and on angiogenesis in vivo.

In addition, recent studies have suggested that macromolecular complexes of purinergic receptors, integrins, and, possibly, CD39, are important for the normal function of each of these components.[195-198] This spatial orientation at the cell surface of P2-receptors and integrins implies important links between purinergic signaling and integrin function.

These findings suggest that purinergic signals may influence angiogenesis.

3.1.2. The role of adenosine in angiogenesis

Adenosine has long had a putative role in the promotion of angiogenesis. For example, adenosine promotes the growth of several macrovascular endothelial cell lines, but has no effect on dermal fibroblasts or cardiac

myoblasts.[199] In addition, adenosine promotes angiogenesis in chick chorioallantoic membrane assays.[199]

In vivo, adenosine promotes wound healing by activating A_{2A} receptors and stimulating microvessel formation.[200] This increase in neovascularization was shown to occur by endothelial progenitor recruitment (vasculogenesis), as well as by local vessel sprouting (angiogenesis).[201] In keeping with this finding, A_{2A} deficient mice have disordered wound healing characterized by abnormal granulation tissue development.[200]

The mechanisms for adenosine's angiogenic properties are largely unexplained, but adenosine has been shown to upregulate VEGF expression in several cell types in vitro and in angiogenic tissue in vivo.[199] This may be one of many mechanisms whereby adenosine modulates the complex process of angiogenesis.

3.1.3. Purinergic signaling in cultured endothelial cells

Extracellular nucleotides have been shown to induce a number of different cellular behaviours in endothelial cells, including proliferation, migration and apoptosis.[202] Each of these may influence angiogenesis in vivo, implying important roles for purinergic signaling in angiogenesis.

In this context, both ATP and ADP have been shown to induce proliferation of cultured endothelial cells.[202] However, there is contradictory information on their roles in endothelial migration. Satterwhite et al. have reported that

UTP, but not ATP, promotes cardiac endothelial migration,[203] while Kaczmarek has reported similar chemotactic effects for ATP and UTP on human umbilical endothelial cells.[204] This is an example of endothelial cell heterogeneity limiting interpretation of in vitro results as a correlate for in vivo angiogenesis. UTP also has a number of vasoactive effects on endothelial cells, and modulates vascular tone and blood flow by stimulating the release of vasodilatory compounds, such as NO and PGI_2.[205] UTP has also been shown to be a potent in vivo angiogenic stimulus in chick-chorioallantoic membrane assays.[203]

The mitogenic and chemotactic effects of UTP might be mediated by either $P2Y_2$ or $P2Y_4$. In settings where both UTP and ATP induced cellular migration, $P2Y_2$ is implicated, while when UTP alone is chemotactic, $P2Y_4$ is the most likely P2-receptor involved. $P2Y_6$ responds primarily to UDP, which does not induce endothelial migration.[203]

In addition, to their roles in promoting endothelial cell proliferation and migration, extracellular nucleotides have also been implicated in the induction of endothelial apoptosis. ATP has been shown to cause apoptosis in cultured endothelial cells via differing mechanisms. In some studies, ATP and ADP caused apoptosis directly, probably via activation of $P2X_7$,[206] whereas, in other studies, ATP only caused apoptosis following degradation to adenosine (ADP not studied in this setting).[207]

3.1.4. The importance of signaling complexes of P2-receptors, integrins and CD39.

Cellular signaling by G protein–coupled receptors (GPCRs), such as P2Y-receptors, involves not only the coupling of the receptors to G proteins, but also the formation of large protein complexes that assist in transmitting an extracellular signal to an intracellular response.[208,209] In this context, the purinergic receptor $P2Y_2$ forms a critical macromolecular complex with the angiogenic integrin $\alpha_V\beta_3$, implying important functions for this P2-receptor in angiogenesis.[195]

$P2Y_2$ contains an integrin-binding, arginine-glycine-aspartic acid (RGD) domain in its first extracellular loop, suggesting that it may bind the integrins $\alpha_V\beta_3/\beta_5$. Co-localization of α_V-integrins with $P2Y_2$ was decreased ten-fold following mutation of this RGD sequence to RGE. This mutation also greatly impaired phosphorylation of FAK following UTP treatment, as did an antibody to α_V.[195]

Direct association between $P2Y_2$ and α_v-integrins was also necessary for UTP-induced cell migration. Cells transfected with the RGE mutant $P2Y_2$ demonstrated poor migration and stress fibre formation following UTP treatment compared with wild-type $P2Y_2$.[210]

In addition, $P2Y_2$ possesses two SH3 binding sites on its intracellular carboxy terminus, which have been shown to be important in the activation of the non-receptor tyrosine kinases Src and Pyk2. Activation of $P2Y_2$ results in the phosphorylation of the PDGF receptor and EGF receptor, but

this feature is lost following deletion of the SH3 domains. Also, wild-type $P2Y_2$, but not SH3-deleted $P2Y_2$, co-localizes with the EGF receptor following treatment with UTP.[196] This data suggests that agonist induced binding of Src to SH3 domains on $P2Y_2$ facilitates Src activation and the recruitment of growth factor receptors into a protein complex with $P2Y_2$.

In addition, enrichment of various components of the signaling cascade in plasmalemmal microdomains, called caveolae, has been shown to be crucial for normal signal transduction.[211] Various growth factor receptors, G-protein coupled receptors, integrins and intracellular signal transducers are targeted to caveolae. In the context of purinergic signaling, we have shown, in collaboration with Agnes Kittel in Hungary, that CD39 and $P2Y_1$, co-localize within caveolae in human placenta.[197] CD39 undergoes a crucial post-translational palmitoylation, which results in this association in lipid rafts.[198]

These results suggest that purinergic receptors, in particular $P2Y_2$, may target to large macromolecular complexes on the cell surface. This interaction may provide crucial links between P2-receptors and integrins or growth factor receptors during angiogenesis. In addition, the finding that CD39 colocalizes with P2-receptors and, possibly, endothelial integrins and growth factor receptors in caveolae suggests that CD39 may also have important angiogenic effects.

3.2 HYPOTHESIS: DELETION OF *Cd39* INHIBITS ANGIOGENESIS.

As discussed above, purinergic signals may modulate angiogenesis in a number of ways. Aberrant regulation of purinergic signals via P2-receptors has been observed in *Cd39*-null mice, suggesting that deletion of *Cd39* may influence angiogenesis. However, the numerous effects of CD39 on purinergic signaling could result in many different pro- and anti-angiogenic effects.

Firstly, the pro-angiogenic actions of adenosine predict important consequences of *Cd39* deletion on angiogenesis. CD39 hydrolyses extracellular ATP and ADP to AMP, which is subsequently degraded to adenosine by ecto-5'-nucleotidase. By removing the majority of endothelial ectonucleotidase activity, deletion of *Cd39* would result in decreased adenosine generation. This may limit angiogenesis in a manner similar to A_{2A} receptor deletion.[212,213]

Secondly, extracellular nucleotides, in particular ATP and UTP, have been shown to have potent mitogenic and chemotactic effects on endothelial cells.[203,204] By hydrolyzing these extracellular nucleotides, CD39 may have anti-angiogenic actions. On the other hand, deletion of *Cd39* has been associated with differential P2-receptor desensitization secondary to excess nucleotide concentrations. For example, it was initially predicted that deletion of *Cd39* would result in enhanced thrombogenesis secondary to

excess ADP. Unexpectedly, platelet ADP-receptor ($P2Y_1$) desensitization caused a mild bleeding diathesis in *Cd39*-null mice.[184]

In a similar manner, desensitization of endothelial P2-receptors in *Cd39*-null mice may result in decreased mitogenic and chemotactic responses to extracellular nucleotides and, hence, defective angiogenesis. This dual role of CD39 in both limiting P2-mediated signaling and protecting P2-receptors from desensitization makes it difficult to predict the in vivo phenotype of the *Cd39*-null mouse.

Thirdly, extracellular nucleotides have been implicated in inducing endothelial cell apoptosis, albeit via differing mechanisms.[206,207] ATP has been shown to cause endothelial apoptosis, directly, by activation of $P2X_7$ and, indirectly, by the generation of adenosine. These differing mechanisms raise interesting questions about the role of CD39 in this setting. CD39 is responsible for both the hydrolysis of ATP and, in turn, for the generation of adenosine via ecto-5'-nucleotidase. CD39 may thus protect angiogenic endothelial cells from apoptosis by hydrolyzing extracellular ATP, or promote apoptosis by generating adenosine.

Finally, deletion of *Cd39* may affect the formation of macromolecular complexes of P2-receptors and integrins on the surface of endothelial cells. It has been shown that $P2Y_2$ colocalizes with $\alpha_V\beta_3$ and that this interaction is important for the mitogenic effects of UTP.[210] $P2Y_2$ may not be unique in this regard. Numerous other endothelial P2-receptors may also colocalize with integrins or growth factor receptors in the cell membrane.

Deletion of *Cd39* may disrupt these important macromolecular complexes in a number of ways. Firstly, deletion of *Cd39* has been associated with P2-receptor desensitization on the basis of increased extracellular nucleotide concentration. This desensitization is mediated, as with other G protein coupled receptors, by phosphorylation of the receptor by a protein kinase, or uncoupling of the receptor from its associated G protein. Each of these actions could disrupt the association of the purinergic receptor with other members of the putative macromolecular complex.

The second manner in which deletion of *Cd39* may disrupt these signaling complexes is independent of the ectonucleotidase activity of CD39. Since CD39 targets to caveolae in the cell membrane,[198] it may associate with a wide range of integrins, growth factor receptors or P2-receptors that also concentrate in caveolae. In this context, CD39 and $P2Y_1$ both target to caveolae in endothelial cells in the placenta.[197] It is possible that the CD39 protein may be important for the structural integrity of cell signaling complexes within endothelial caveolae. This would occur independently of the nucleotide hydrolyzing actions of CD39.

In conclusion, a number of different lines of evidence suggest that endothelial behaviours, and, hence, angiogenesis, may be crucially affected by purinergic signals. Since CD39 is the major modulator of nucleotide signaling in the vasculature, we hypothesize that CD39 has crucial roles in both physiological and pathological angiogenesis in vivo.

It should be stressed that, although the bulk of this work deals with the effects of purinergic signals on endothelial cells during angiogenesis,

nucleotides certainly modulate the cellular behaviors of other cell types. For example, transmigration of *Cd39*-null monocyte/macrophages through Matrigel is decreased *in vitro*.[214] This suggests that *Cd39* deletion may be associated with pluripotent defects in cellular activation and migration.

CHAPTER 4: ESTABLISHED EXPERIMENTAL MODELS

DELETION OF *Cd39* DISRUPTS ANGIOGENESIS IN VIVO

This chapter represents a synopsis of prior work involving several experimental models of angiogenesis where I was involved peripherally. I would like to acknowledge and thank each of the researchers responsible for this important work, which demonstrates the presence of defective angiogenesis in *Cd39*-null mice.

Firstly, I am indebted to Keiichi Enjoyji for allowing me to include a discussion of the initial generation of the *Cd39*-null mouse in this dissertation. In addition, Dr Enjoyji developed the technique I used to breed and type *Cd39*-null mice.

Christian Goepfert performed the Matrigel™ plug assay, which was the initial experiment demonstrating an angiogenesis defect in *Cd39*-null animals. Sun Xiaofeng performed the liver regeneration experiments, while Mika Ogawa demonstrated defective angiogenesis in ischaemic muscle in a murine myocardial infarction model. Finally, Tomokazu Hoshi performed the *in vivo* tumour angiogenesis assays, including the tumour metastasis models.

I am grateful to Dr. Simon Robson, for allowing me to include a discussion of this work as a prelude to the in vitro experiments presented in Chapter 5.

4.1 DEVELOPMENT AND CHARACTERIZATION OF THE *Cd39*-NULL MOUSE

4.1.1 Generation of the *Cd39*-null mouse

The *Cd39*-null mouse was developed by homologous recombination using a replacement construct targeted to exon 1 of the murine *Cd39* gene. The *Cd39* targeting vector was constructed containing two regions of homology to the murine *Cd39* gene: a 7.5-kb fragment upstream of exon 1 flanked by EcoR1 restriction sites; and a 1.5-kb fragment downstream of exon 1 flanked by the restriction enzymes BAMH1 and Pst1.[184] (figure 4.1.1, a)

A neomycin resistance gene was included in the targeting vector between the two homologous sequences, allowing positive selection of ES cells that have stably incorporated the construct. A thymidine kinase gene sequence was added outside the two flanking regions to allow for negative selection of those colonies, which have undergone random incorporation of the targeting vector (figure 4.1.1, b).[184]

The linearized targeting vector was transfected into D3 ES cells by electroporation. G418 sulphate and gancyclovir were added to the culture

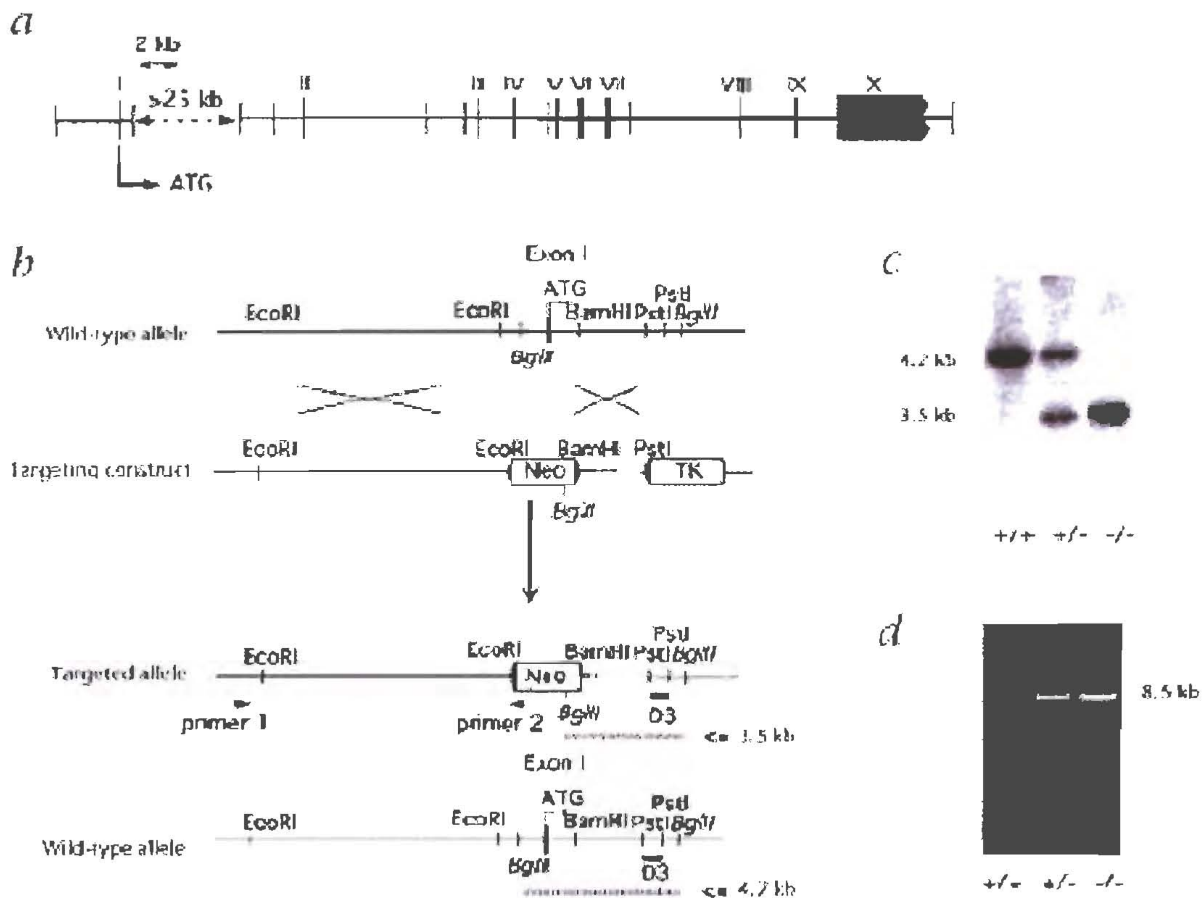


Figure 4.1.1 - Generation of the *Cd39*-null mouse

a) Restriction enzyme site map of mouse *Cd39* gene. *Bam*HI restrictions sites are denoted by thin vertical lines; exons shown by thick vertical lines. >25kb section represents a large intron between exons I and II.

b) Targeting of the *Cd39* gene. D3 (Thick horizontal bar), probe used for Southern blot analysis; Hatched horizontal bars, fragments generated with the D3 probe (sizes shown on the right); PCR primers (small arrowheads) used for the confirmation of homologous recombination; TK, thymidine kinase; Neo, neomycin resistance gene.

c) Southern blot analysis with the D3 probe showing homologous recombination at 3' end. 4.2-kb or 3.5-kb *Bgl*II fragments generated by D3 hybridization to wild-type or targeted alleles. Genotype denoted blow the blot.

d) PCR analysis of clones at the 5' end (primers 1 and 2 used to generate an 8.5-kb product). Genotype denoted blow the blot.

Image: Figure 1 in Enjyoji, K. et al. Targeted disruption of cd39/ATP diphospho-hydrolase results in disordered hemostasis and thromboregulation. *Nature medicine* 5, 1010-7 (1999). Reprinted with permission.

media to select for colonies in which homologous recombination of the targeting vector had occurred at exon 1 of the *Cd39* gene (figure 4.1.1, b).[184]

G418 is an aminoglycoside related to gentamicin, which inhibits both prokaryotic and eukaryotic protein synthesis and is, thus, toxic to murine ES cells. Successful homologous recombination of the targeting vector into the ES cell genome results in expression of the neomycin resistance gene. The product of this gene, aminoglycoside 3'phosphotransferase, inactivates G418 by phosphorylation and allows the survival and positive selection of successfully recombined ES cells.[215]

However, random insertion of the targeting vector into the ES cell genome would also result in the expression of the neomycin resistance gene. Therefore, a thymidine kinase gene was added outside of the two homologous sequences in the targeting vector. During homologous recombination, sequences outside the regions of homology to the target gene are lost. On the other hand, random integration results in the insertion of the entire targeting vector into the genome because recombination usually occurs at the ends of the construct. Expression of the thymidine kinase gene activates the anti-viral drugs acyclovir and gancyclovir, rendering them toxic to eukaryotic cells.[216] Therefore, addition of gancyclovir negatively selects those ES cells in which random integration of the targeting vector has occurred.

Resistance to both G418 and gancyclovir suggests a high likelihood of successful homologous recombination at the correct site in the ES cell genome.

Resistant colonies were selected and analyzed for successful homologous recombination at the 3'- and 5'-ends. Successful recombination at the 3'-end was confirmed by *Bgl*II digestion, and Southern blot analysis of the generated fragments using the probe D3 (figure 4.1.1, c). The *Bgl*II restriction site within the neomycin resistance gene resulted in the generation of a 3.5kb fragment in the targeted allele, as opposed to the 4.2kb fragment in the wild-type allele.[184]

Homologous recombination at the 5'-end was confirmed by PCR generation of an 8.5kb fragment. Primer 1 (5'–AACACTGCACTGGATATC-CTGTCTGTTGTTGTGC–3') was designed from the EcoR1 fragment, outside of the 5'-end of the targeting vector.
Primer 2 (5'–CTGCGTGCAATCCATCTTGTTCAATGGCCGATCCC–3') localized to the neomycin resistance gene in the targeting vector.[184] (figure 4.1.1, d)

Successfully targeted clones were then injected in blastocysts from C57BL/6 mice. The success of incorporation of ES cells into the final animal was assessed by the ES cell contribution to coat colour and the development of a male phenotype. Since the ES cell injection protocol involves the injection of male agouti ES cells into a female black host embryo (C57BL/6) the degree of chimerism is assessed by the percentage of coat colour that is agouti and by the birth of a phenotypically male mouse.[216]

Germline transmission was then assessed by breeding chimeric founder mice with C57BL/6 mice and observing for the birth of Agouti pups. Successful

deletion of *Cd39* was assessed by PCR analysis using primers named D3M, NEOP2 and MEC8P (sequences shown below). Primers D3M and MEC8P were used to probe the wild-type allele, while D3M and NEOP2 target the *Cd39*-null allele.

D3M 5'-ACTGTTTATATCCCAAGGAGCTGGCATAGG-3'
NEOP2 5'-TACCCGTGATATTGCTGAAGAGCTTGGCGG-3'
MEC8P 5'-GACAGACGAGGGAAGAGGAAGG-3'

4.1.2 Northern and western blot confirmation of *Cd39* deletion

To confirm successful deletion of *Cd39*, mRNA was isolated from various tissues, separated on 1% agarose gels and transferred to GeneScreenPlus membranes. ^{32}P-labeled *Cd39* cDNA probes were then developed by PCR amplification, using the primers, 5'–GACAGACGAGGGAAGAGGAAGG –3 and 5'–TCTTCCAGCCAGCTCAGCTTGGGC–3'. The mRNA membranes were then hybridized overnight with the ^{32}P-labeled probes, washed and exposed to Biomax films with the Biomax intensifying screen.[184]

Figure 4.1.2 (a) confirms the absence of *Cd39* mRNA from lung, spleen, placenta, liver, kidney, heart and brain tissue isolated from *Cd39*-null animals. Mice heterozygous for the *Cd39* gene deletion have decreased *Cd39* mRNA expression.[184]

Antibodies to murine Cd39 were developed by direct inoculation of *Cd39* cDNA in pcDNA3 into rabbits. Figure 4.1.2 (b) demonstrates the use of this antibody in a western blot analysis of lung homogenates from wild-type and *Cd39*-null mice, as well as of COS-7 cells transfected with a murine *Cd39* expression vector or an empty vector. This confirms the absence of *Cd39* protein expression in *Cd39*-null mouse lung homogenates. The specificity of the antibody is confirmed by the expression of Cd39 in expression vector transfected COS-7 cells. Cd39 exists as a native 78kDa protein and a 56kDa proteolytic form.[184]

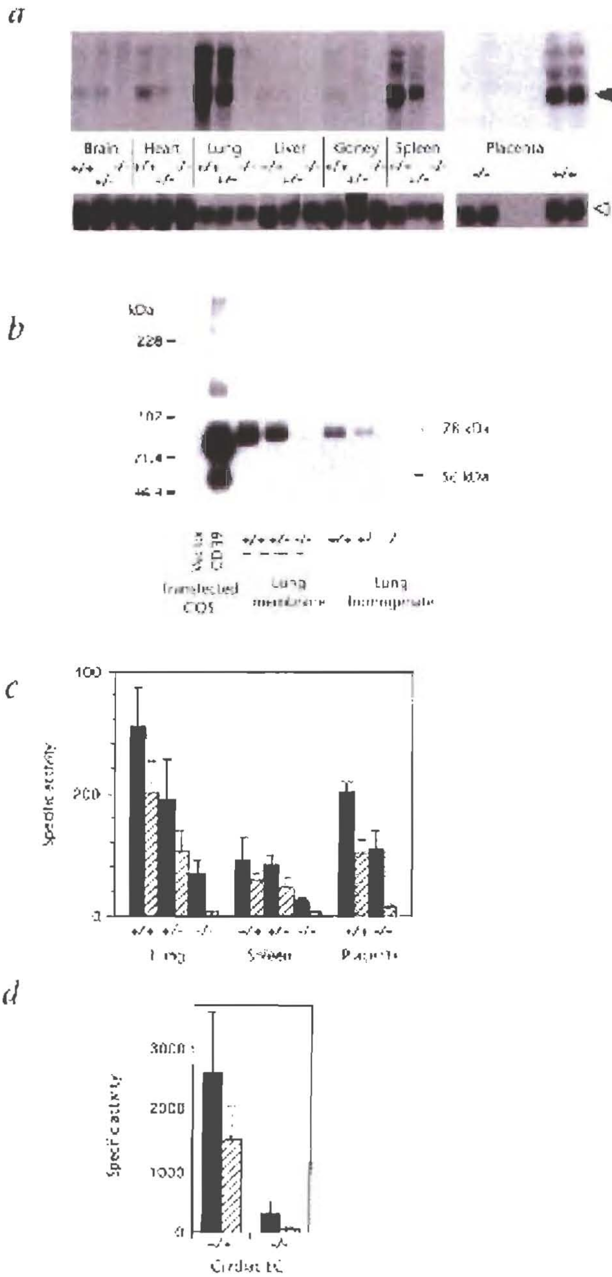


Image: Figure 2 in Enjyoji, K. et al. Targeted disruption of cd39/ATP diphospho-hydrolase results in disordered hemostasis and thromboregulation. *Nature medicine* 5, 1010-7 (1999). Reprinted with permission.

Figure 4.1.2

Confirmation of Cd39 deletion in the Cd39-null mouse by analysis of mRNA and protein expression as well as functional ectonucleotidase activity.

a) Northern blot analysis Cd39 mRNA expression from adult mouse organs. Genotypes shown below the blot. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) controls are shown below.

b) Western blot analysis of transfected COS-7 cells using a Cd39-specific polyclonal antibody. Lane 1: empty vector; lane 2: Cd39 expression vector; lanes 3-8: lung homogenates and membrane fractions from wild-type and Cd39-null mice. Genotypes shown below the blot. 78kDa: native protein; 56kDa: proteolytic form

c) ATPase ■, ADPase □, activity of various murine organs (pmol/min) by phosphate release following addition of ATP and ADP. Genotypes shown below graph.

d) ATPase ■, ADPase □, activity of wild-type and *Cd39*-null murine endothelial cell cultures. Genotypes shown below the graph.

4.1.3 Ectonucleotidase activity in wild-type and *Cd39*-null tissues

The effect of *Cd39* deletion on specific ATPase and ADPase activity was determined using tissues from wild-type and *Cd39*-null mice. Preparations of lung, spleen and placenta were analyzed for phosphate release following addition of ATP or ADP. Figure 4.1.2 (c) shows that ADPase activity was substantially decreased in both *Cd39*-null and *Cd39*-heterozygote mice. ATPase activity was also decreased, although the decrease was less substantial than the effect on ADPase activity.[184]

Microvascular endothelial cell cultures from wild-type and *Cd39*-null mice were also analyzed for ATPase and ADPase activity. Figure 4.1.2 (d) shows the almost complete absence of ATPase and ADPase activity on *Cd39*-null endothelial cells. From this data, the contribution of other ectonucleotidases to nucleotide hydrolysis in cultured endothelial cells was estimated to be 12% for ATP hydrolysis and 3% for ADP hydrolysis. The functional significance of this in vitro is the finding that wild-type endothelial cells are able to prevent platelet aggregation responses to ADP, while *Cd39*-null cells were not as efficient.[184] (Fig 4.1.3)

In conclusion, prior work in the Robson laboratory has confirmed that *Cd39* was successfully deleted by targeted homologous recombination. This *Cd39*-null mouse has no *Cd39* mRNA or protein expression and has a major defect in extracellular nucleotide hydrolysis.

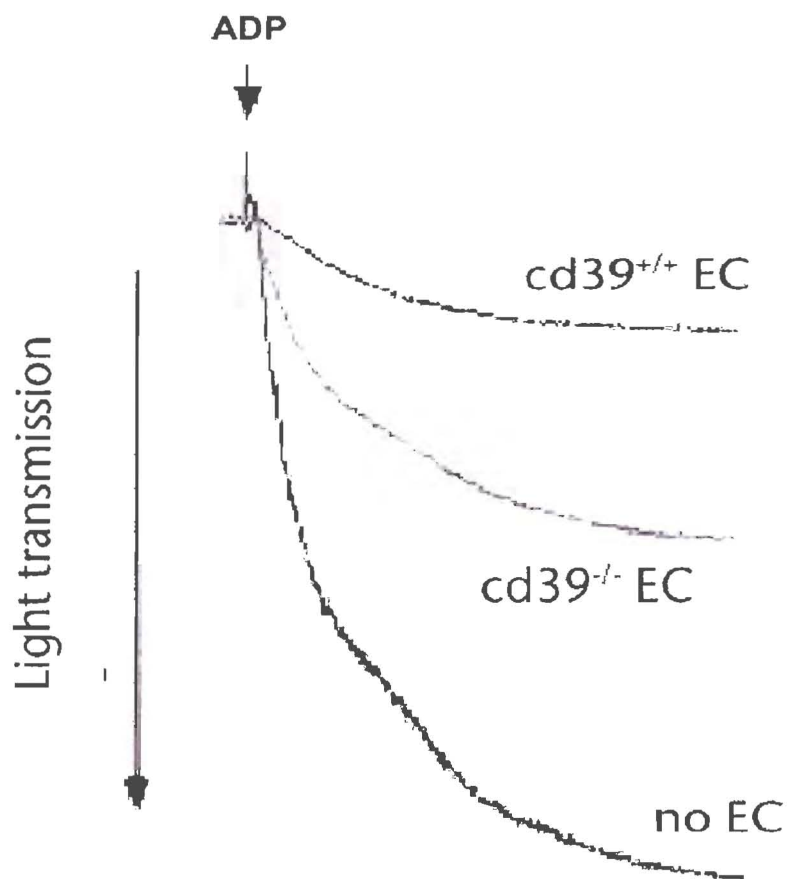


Figure 4.1.3 - Decreased inhibition of platelet aggregation by Cd39-null endothelial cells. 10μM ADP was added to human platelets in the presence of wild-type, Cd39-null, or no endothelial cells and platelet activation measured by changes in light transmission. Platelets in the presence of wild-type (cd39+/+) endothelial cells experience almost no aggregation in response to ADP.

Image: Figure 3 in Enjyoji, K. et al. Targeted disruption of cd39/ATP diphospho-hydrolase results in disordered hemostasis and thromboregulation. *Nature medicine* 5, 1010-7 (1999). Reprinted with permission.

4.2 DEVELOPMENT OF A SEVENTH-GENERATION BACK-CROSSED *Cd39*-NULL MOUSE STRAIN

4.2.1 Introduction

The injection of *Cd39* gene-deleted embryonic stem (ES) cells into C57BL/6 host embryos resulted in the development of chimeric founder mice. The success of incorporation of ES cells into the final animal was assessed by the ES cell contribution to coat colour and the development of a male phenotype. Since the ES cell injection protocol involves the injection of male agouti ES cells into a female black host embryo (C57BL/6) the degree of chimerism is assessed by the percentage of coat colour that is agouti and by the birth of a phenotypically male mouse. Germline transmission was then assessed by breeding the male chimeric founder mouse with a female C57BL/6 mouse and observing for the birth of Agouti pups.[217] Successful deletion of *Cd39* was then confirmed by DNA extraction, polymerase chain reaction (PCR) amplification of the targeting sequence and Southern blot analysis.[184]

Inbred mice and ES cells are genetically homogeneous and homozygous at all loci because they have been derived by at least 20 generations of sequential brother-to-sister crosses. The mouse which develops following successful incorporation of ES cells into host embryos is, by definition, a mixed chimera because D3 ES cells were originally derived from 129/SvPas mice and the host embryo was derived from a C57BL/6 mouse. Mice with

mixed genetic backgrounds are not ideal for experimental analysis because it is not possible to produce an identical mixed background for control.

A congenic mouse strain is defined as a mouse strain which is isogenic to the inbred wild-type strain, but which contains the gene sequence responsible for target gene deletion.[217] Thus, a congenic C57BL/6 *Cd39*-null mice is isogenic to a C57BL/6 wild-type mouse, except that it does not express the *Cd39* gene. The development of congenic mouse strains by the sequential back-crossing of chimeric mice with isogenic mice works on the principle that each back-cross reduces the chimeric gene content by 50%.[217] Typically, congenic murine strains are defined as those that have been backcrossed over twenty generations.

For our experiments, we developed a seventh-generation back-crossed C57BL/6 *Cd39*-null mouse by sequential back-crossing of chimeric *Cd39*-null mice with C57BL/6 wild-type mice. The chimeric *Cd39*-null male was initially bred with a wild-type C57BL/6 female to generate the generation denoted F1. This generation is considered heterozygous at all loci because half of the loci are derived from the chimera and half from the C57BL/6 mouse. Those progeny that contain the deleted gene were then identified by DNA extraction, polymerase chain reaction (PCR) amplification of the targeting sequence and Southern blot analysis. These identified mice are heterozygous for *Cd39* deletion because they derive from founder *Cd39*-null males and wild-type females.

The second generation backcrossed mice were developed by breeding F1-generation *Cd39*-null heterozygotes with C57BL/6 wild-type mice. These

second generation mice contain 25% of the original chimera's genome. As with the F1-generation, *Cd39*-null heterozygote second-generation mice were identified by PCR and Southern blotting of DNA extracted from mouse tails. Successive generations were developed by repeatedly backcrossing chimeric mice with C57BL/6 wild-type mice, each time reducing the original chimeric genome content by 50%.

In vivo experiments were performed on C57BL/6X129svj mixed-chimera wild-type/*Cd39*-null mice or on C57BL/6 wild-type mice and seventh-generation back-crossed *Cd39*-null C57BL/6 mice as described in methodology. All in vitro experiments reported in this dissertation were performed on seventh-generation back-crossed *Cd39*-null C57BL/6 mice with C57BL/6 wild-type controls. As these mice contain only 0.78125% of the original chimeric genome, C57BL/6 wild-type mice can be considered appropriate murine controls. Naturally, further back-crossing of mice is important for further purification in the chromosomal region around *Cd39*. Efforts to generate a truly congenic (twenty generations backcrossed) C57BL/6 *Cd39*-null mouse are ongoing in the laboratory.

4.2.2 Methods and results

a) Murine husbandry

- All breeding involved placing one male and one female, both at least 6-weeks-old, together in a cage. The females were then observed for pregnancy and the males removed prior to the birth of pups. Leaving

the male in the cage with new pups or disturbing a mother with young pups increases the likelihood that pups will be cannibalized, a risk with C57BL/6 mothers.

- After a gestation of approximately 3 weeks pups are born. The pups were then weaned at 4-weeks of age, typed by tail biopsy and DNA extracted for PCR and Southern blot analysis (vide infra).

b) Development of a seventh-generation back-crossed *Cd39*-null C57BL/6 mouse strain

- The chimeric *Cd39*-null male was bred with a wild-type C57BL/6 female to generate the generation denoted F1. F1-generation mice heterozygous for *Cd39*-deletion were identified by PCR and Southern blot analysis and these mice used in subsequent backcrosses.
- The second generation backcrossed mice were developed by breeding F1-generation *Cd39-null* heterozygotes with C57BL/6 wild-type mice. As with the F1-generation, *Cd39-null* heterozygote second-generation mice were identified by PCR and Southern blotting of DNA extracted from mouse tails.
- Successive generations were developed by repeatedly backcrossing chimeric *Cd39*-heterozygote mice with C57BL/6 wild-type mice and identifying heterozygote progeny by PCR amplification of the targeted gene sequence.
- The seventh generation *Cd39*-heterozygote mice were used to generate homozygote *Cd39*-null mice. Seventh-generation *Cd39*-heterozygote males and seventh-generation *Cd39*-heterozygote

females were bred together. Their progeny were then analyzed by PCR and Southern blotting to identify homozygous *Cd39*-null animals.

- Seventh-generation homozygous *Cd39*-null males and females were bred to produce animals for experimentation.
- All in vivo and in vitro experiments in this dissertation were performed using seventh-generation homozygous *Cd39*-null C57BL/6 male mice between 8 and 16 weeks of age.

c) Extraction of DNA from mouse tails

- At the time of weaning (4-weeks old) the mice were tagged for identification using an ear punch and a tail biopsy collected for DNA extraction.
- DNA was extracted from tail samples using the DNeasy® Tissue kit (Qiagen, CA, USA).
- The tail sample was initially digested overnight at 55°C in 180μl lysis buffer (buffer ATL) containing 20μl proteinase K.
- After digestion, digested samples were washed in buffers AL, AW1 and AW2. Excess buffer was removed by centrifugation in a DNeasy® spin column.
- The DNeasy® mini spin column was then transferred to a clean 1.5ml microcentrifuge tube and 200μl of the elution buffer (AE) added.
- The samples were then centrifuged at 8000 rpm for 1 minute, and the eluted DNA stored at 4°C until use.

d) Murine genotyping by amplification of specific wild-type and *Cd39*-null DNA sequences

- Samples for polymerase chain reaction (PCR) amplification were prepared, each containing the following:
  - 2.5μl 10X *Ex Taq* buffer (Takara Bio Inc. Japan)
  - 2.0μl dNTP (Takara Bio Inc. Japan)
  - 0.125μl *Ex Taq* polymerase Hot Start Version (Takara Bio Inc. Japan)
  - 1.0μl DNA template (from rodent tail DNA extraction)
  - 2.0μl primer mix (Wild-type or *Cd39*-null primers)
- Primers used:
- D3M 5'-ACTGTTTATATCCCAAGGAGCTGGCATAGG-3'
- NEOP2 5'-TACCCGTGATATTGCTGAAGAGCTTGGCGG-3'
- MEC8P 5'-GACAGACGAGGGAAGAGGAAGG-3'
- Primers D3M and MEC8P were used to probe the wild-type allele, while D3M and NEOP2 target the *Cd39*-null allele.
- DNA fragments were amplified by 30 cycles polymerase chain reaction protocol, compromising 1:15min at 94°C, 3:30min at 66°C, and 10min at 72°C.
- Amplified samples were analyzed by Southern blotting (1% agarose gel containing 1μl ethidium bromide per 100ml agarose).
- Figure 4.2.1 shows an example of Southern blot analysis of extracted DNA from wild-type, heterozygous and homozygous *Cd39*-null mice. Wild-type mice produce only a wild-type fragment, *Cd39*-null only

the *Cd39*-null fragment and heterozygous mice, both fragments. The wild-type and *Cd39*-null fragments are both approximately 3 kB long.

- The successful generation of homozygous *Cd39*-null C57BL/6 mice allowed the study of the role of Cd39 in angiogenesis *in vivo*.

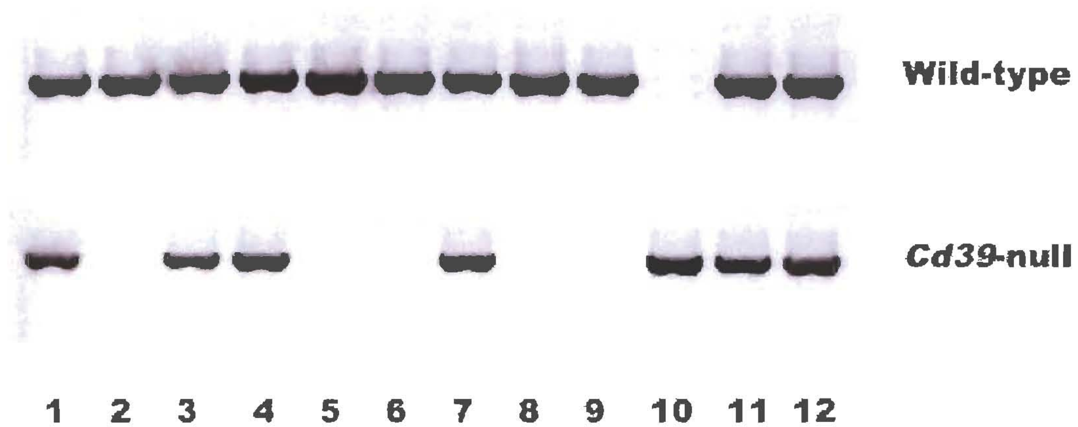


Figure 4.2.1 - Mouse genotyping

Representative example of Southern blot analysis of mouse genotype. DNA was extracted from the tails of 12 different mice. Samples represent PCR amplification of extracted DNA using primers recognizing wild-type and *Cd39*-null DNA sequences. Murine genotype is represented by the presence of wild-type, Cd39-null or both DNA sequences.

Mice numbered 2, 5, 6, 8 and 9 are wild-type. Mice numbered 1, 3, 4, 7, 11 and 12 are *Cd39* heterozygotes. Mouse number 10 is *Cd39*-null.

4.3 MATRIGEL™ PLUG ANGIOGENESIS ASSAYS

4.3.1 Introduction

The Matrigel™ plug assay has been used as a successful in vivo model of angiogenesis.[168] Matrigel™ is a solubulized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular membrane proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin.[167] Following subcutaneous injection into a research animal, the Matrigel™ polymerizes to produce a matrix resembling normal mammalian cellular basement membrane. After 1-3 weeks, the plug of Matrigel™ is removed and examined histologically for invasion by new blood vessels.

Incorporation of growth factors and other substances into this matrix has facilitated the study of their *in vivo* requirements for angiogenesis. In addition to VEGF and βFGF, sphingosine-1-phosphate appears to be particularly important in the regulation of angiogenesis.[218]

Sphingosine-1-phosphate is a bioactive sphingolipid, which is released by activated platelets. It has been shown to promote both VEGF- and βFGF-induced angiogenesis in Matrigel™ and appears to be important for the development of adherens junctions between endothelial cells and for the formation of mature basement membrane. In this way, sphingosine-1-phosphate promotes new vessel morphogenesis and maturation.[219]

In order to evaluate the role of CD39 and purinergic signaling in angiogenesis, Matrigel™ plugs were implanted into wild-type and *Cd39*-null mice and the invasion of new vasculature was evaluated. Sphingosine-1-phosphate, VEGF and βFGF were added to the Matrigel™ to promote vascular proliferation, Mice were euthanased at 14 days, the Matrigel™ plugs removed and analyzed by immunohistochemistry.

4.3.2 Results

The isolated Matrigel™ plugs from wild-type and *Cd39*-null mice were analyzed at day 14 for the development of angiogenesis, as evidenced by increased vascular density at the interface between the Matrigel™ and the underlying tissue and by in-growth of vessels into the matrix.

Wild-type mice demonstrated a marked increase in vessel density at this interface as well as significant ingrowth of vessels into the Matrigel™ (Figure 4.3.1) Vessels in the adjacent skin demonstrated vascular activation as evidenced by development of thin-walled, pericyte-deficient vessels, termed "mother vessels". Of the new vasculature invading the Matrigel™, most were surrounded by pericytes, which stained positively with PDGFβ, and possessed a formed basement membrane of laminin and perlecan. Monocyte-macrophages invaded the Matrigel™ in a wave before the advancing endothelial cells.[214]

In contrast, *Cd39*-null mice had abnormal angiogenic responses. Vessels in the tissue adjacent to the Matrigel™ showed, like the wild-type, some initial

angiogenic activation, as evidenced by the development of mother vessels, but this occurred to a lesser degree than the wild-type. Migration of endothelial cells into the Matrigel™ was absent in the *Cd39*-null mice (Figure 4.3.1). There was also a defect in the migration of monocyte-macrophages and pericytes into the Matrigel™ (Figure 4.3.2).[214]

In the wild-type, the interface between Matrigel™ and adjacent skin was characterized by dense connective tissue septa invaded the Matrigel™ and a significant number of mixed cells within the matrix. However, in the *Cd39*-null animals, an inflammatory zone developed between the Matrigel™ and normal tissue, consisting of a compact cellular rim of cells with failure of significant cellular migration into the Matrigel™. This inflammatory zone contained distinct layers of monocytes, endothelial cells and pericytes at the border of the Matrigel™.[214]

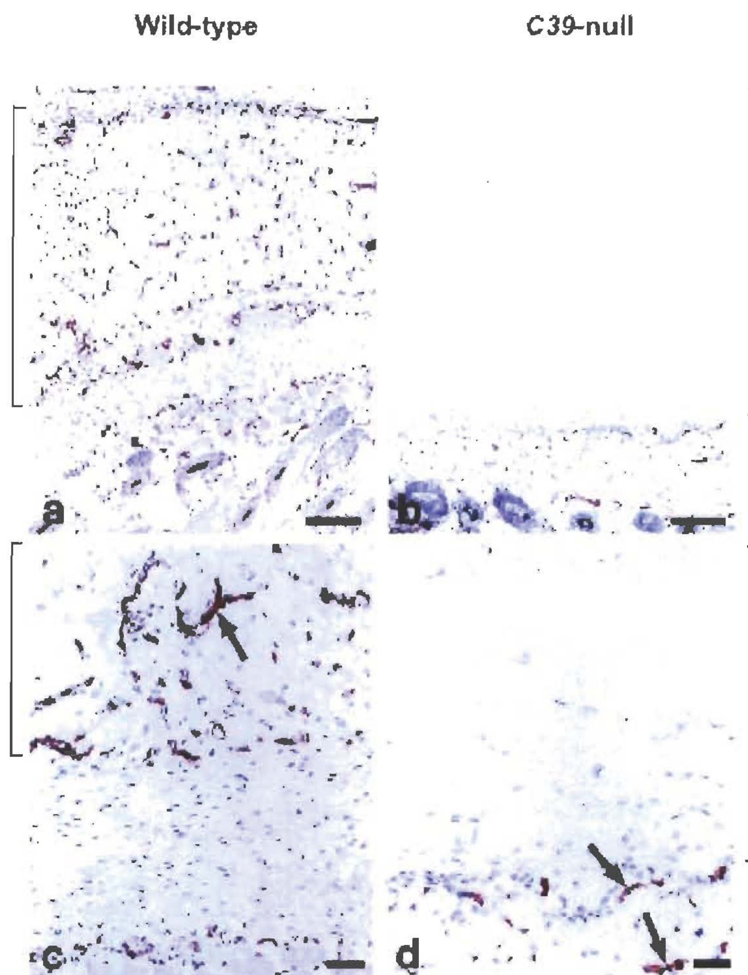


Figure 4.3.1 - Matrigel plug immunohistochemistry. Wild-type (*a*, *c*) and *Cd39*-null (*b*, *d*) matrigel plugs are stained with antibodies recognizing CD31. Bracketed areas represent Matrigel. *a* and *c*, CD31+ endothelial cells are seen invading Matrigel plugs and forming new vessels in wild-type mice. *b* and *d*, CD31+ endothelial cells are unable to penetrate Matrigel in *Cd39*-null animals. Bars=25 μm (*c*, *d*) and 200 μm (a and b).

Image: Figure 1 in Goepfert C et al. Disordered cellular migration and angiogenesis in cd39-null mice. *Circulation* 104, 3109-15 (2001). Reprinted with permission.

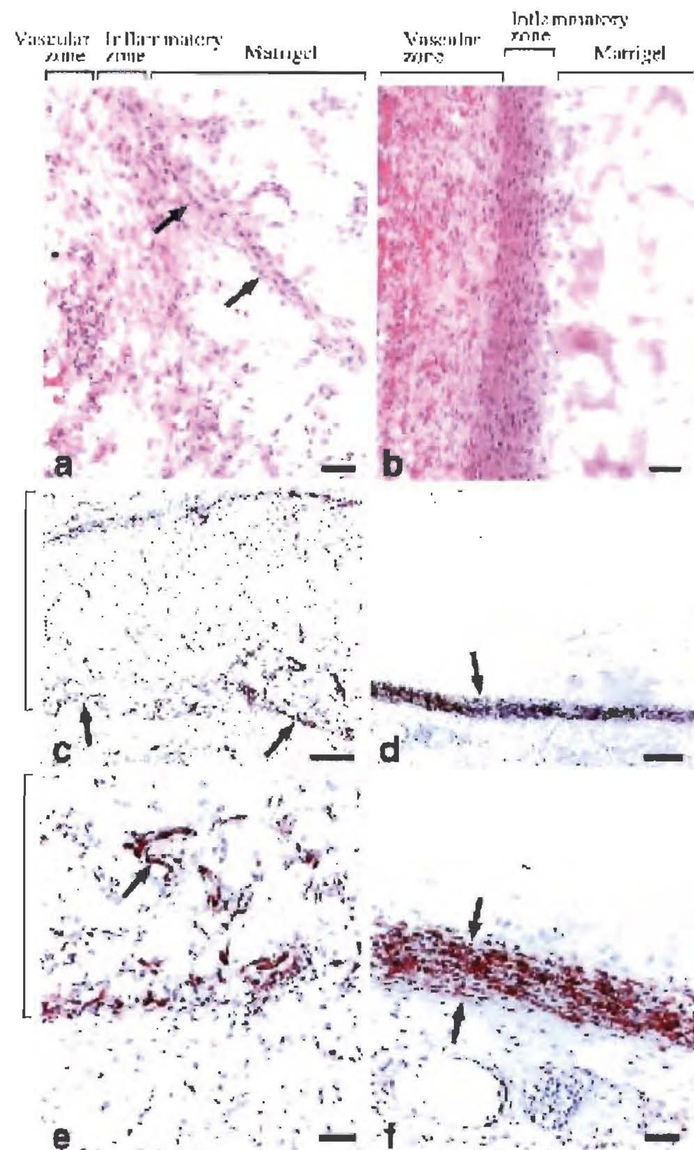


Figure 4.3.2 - Matrigel plug immunohistochemistry.

***a-b*: Hematoxylin-eosin stain of the interface between dermis and Matrigel in wild-type (*a*) and *Cd39*-null (*b*) mice. Note the lack of cellular infiltration of Matrigel in *Cd39*-null animals.**

***c-f*: F4/80-macrophage stain of Matrigel plugs in wild-type (*c, e*) and *Cd39*-null (*d, f*) mice. While extensive invasion of macrophages occurs in the wild-type, a compact rim of macrophages develops at the dermis/Matrigel interface in Cd39-null mice.**

Bars=25 μm (*a, b, e, f*) and 200 μm (*c, d*).

Image: Figure 2 in Goepfert C et al. Disordered cellular migration and angiogenesis in cd39-null mice. *Circulation* 104, 3109-15 (2001). Reprinted with permission.

4.3.3 Discussion

Matrigel™ plug angiogenesis assays have provided a useful methodology for studying angiogenesis in vivo. Despite the addition of proangiogenic factors VEGF, FGF and sphingosine-1-phosphate into the Matrigel™, there was complete failure of angiogenesis in the *Cd39*-null mice. Several of the initial phases of angiogenesis, including vascular activation and loosening of peri-endothelial pericyte support, occurred in *Cd39*-null animals. However, there were significant defects in migration of all cellular lines into the Matrigel™ and a complete failure to form new vasculature within the Matrigel™.[214] These results suggest that physiological and pathological angiogenesis may be affected by the deletion of *Cd39* and prompted further study of angiogenesis during liver regeneration, myocardial infarction and tumour growth and metastasis in *Cd39*-null mice.

4.4 LIVER REGENERATION

4.4.1 Introduction

Liver regeneration is a vitally important physiological process, which occurs after liver resection, liver transplantation and after toxic or viral hepatic injury.[220] In addition to hepatocyte proliferation, the formation of new hepatic sinusoids by angiogenesis is considered crucial for effective liver regeneration.[83] This has been demonstrated, at least in an animal model, by showing that the angiogenesis inhibitor angiostatin can inhibit liver regeneration in vivo, despite having no direct actions on hepatocytes.[221]

Initially, after significant liver resection, hepatocytes are induced to proliferate with peak DNA synthesis occurring at 24 hours (figure 4.4.1). This creates avascular parenchymal islands of hepatocytes without normal sinusoidal architecture (figure 4.4.2). In response, sinusoidal endothelial cells begin to proliferate, reaching a peak at day 3, and infiltrate these parenchymal islands in order to re-establish vascular supply (figure 4.4.1).[83] Normal hepatic weight and architecture is established by about day 8 in murine models of 70% hepatectomy.[221]

The molecular mechanism for this induction of angiogenesis includes the increased expression of VEGF, the angiopoietins (ang-1 and -2), and their receptors (flt-1, flk-1, Tie-1 and Tie-2) in the regenerating liver (figure 4.1.3). The expression of VEGF and its receptors (flt-1 and flk-1) start increasing soon after liver resection, and peak at day 3, in parallel with the

peak in endothelial cell proliferation. Ang-2 expression precedes VEGF expression and remains elevated for 6 days.[83] This corresponds with previous studies suggesting that Ang-2, by inhibiting Ang-1 and disrupting blood vessels, has an important role in initiating angiogenesis.[81]

In contrast, Ang-1 and the receptors Tie-1 and Tie-2 have slow induction responses following liver regeneration and reach peaks at days 4 – 7. This is consistent with the role of Tie-1 in endothelial differentiation, maintenance of blood vessel integrity and the resolution phase of angiogenesis.[84]

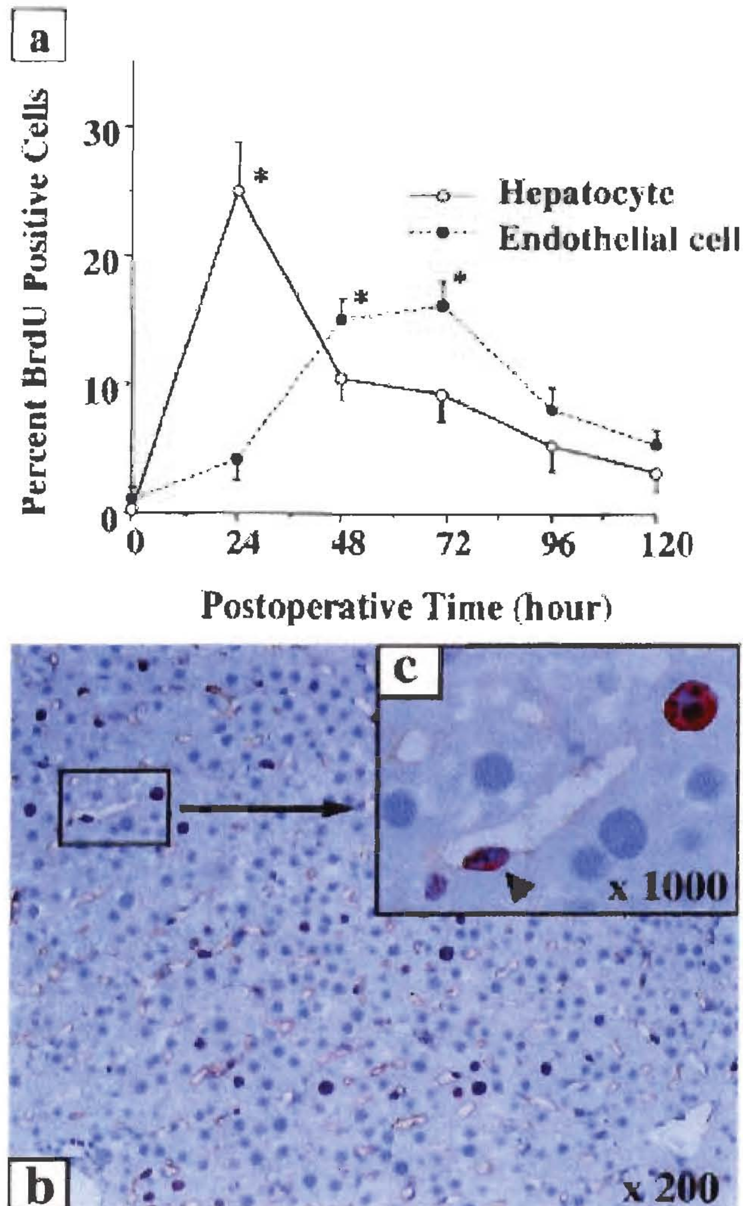


Figure 4.4.1 - Proliferative responses of hepatocytes and endothelial cells following 70% hepatectomy.

a) Percentage of hepatocytes and endothelial cells in the proliferative state by BrdU uptake.

b) The identification of proliferating endothelial cells by double immunohistochemical staining. Red nuclear staining denotes BrdU uptake, while the endothelial membrane is stained brown using an antibody to RECA-1.

c) Arrowhead denotes a proliferating endothelial cell nucleus.

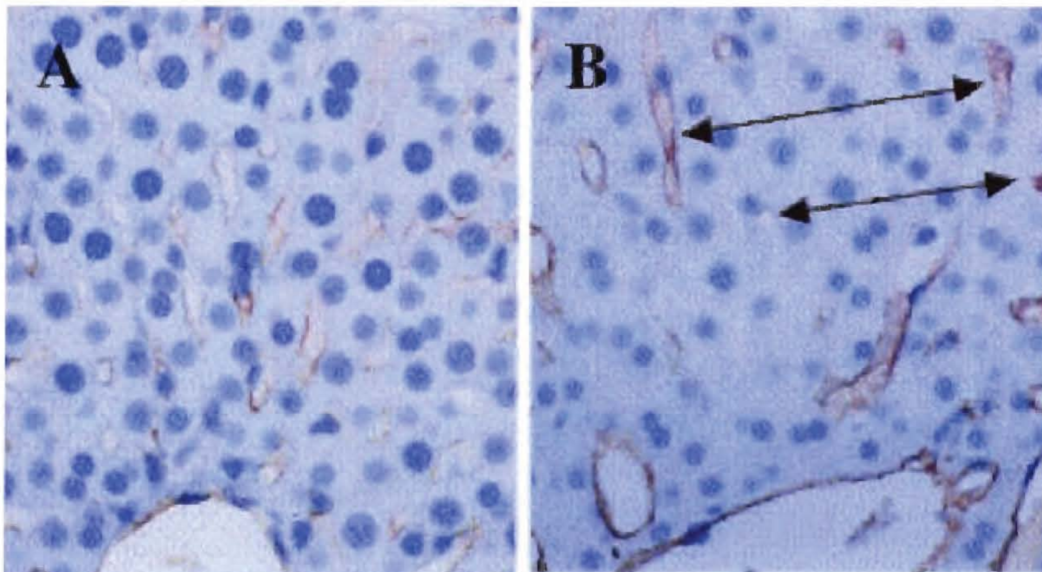


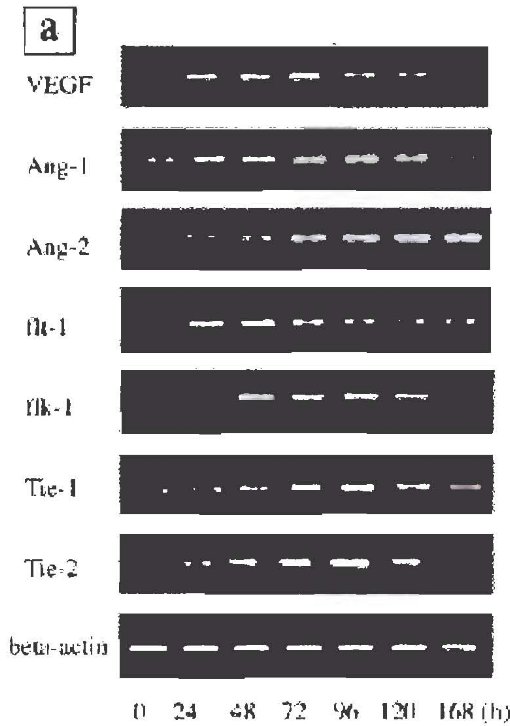
Figure 4.4.2 - Changes in hepatic architecture during liver regeneration.

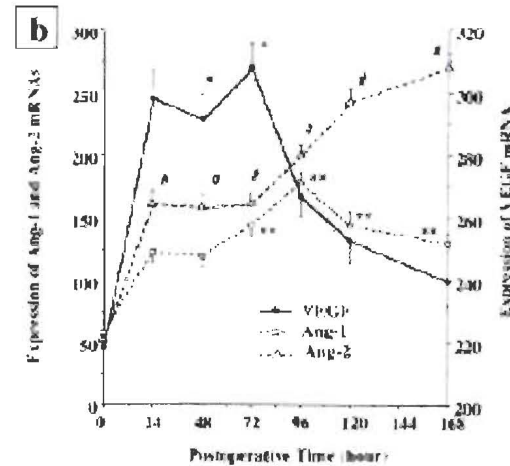
a) **Pre-hepatoctomy state showing that most hepatocytes have at least one facet of contact with an endothelial sinusoid.**

b) **At 3 days post hepatectomy, wide gaps are noted between intervening sinusoids as hepatocyte proliferation outstrips endothelial proliferation (arrows). Normal hepatic architecture is re-established by wild-type in animals by 8 days after 70% hepatectomy. Endothelial staining denoted by antibody raised against rat endothelial cell antigen-1 (RECA-1).**

Figures 4.4.1-4.4.2

Sato, T. et al. Sinusoidal endothelial cell proliferation and expression of angiopoietin/Tie family in regenerating rat liver. *J Hepatol.* 34, 690-8 (2001). Reproduced with permission.





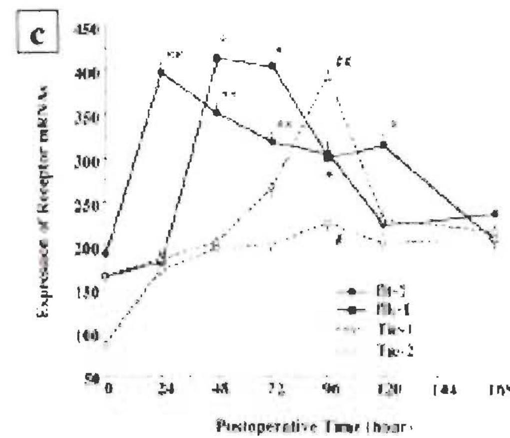


Figure 4.4.3

Hepatic expression of angiogenic growth factors following 70%-hepatectomy.

a) Northern blot analysis of angiogenic growth factors and growth factor receptor mRNA expression after 70%-hepatectomy. VEGF mRNA expression peaked at 72 hours, while Ang-1 peaked at 96 hours. Significant increases in Ang-2 mRNA were also noted, with peak levels significantly delayed compared with VEGF and Ang-1. All four receptors were elevated after hepatectomy, with flt-1, and then flk-1, the first to increase. Both Tie-1 and Tie-2 were elevated, but their peak expressions coincided with the resolution phase of angiogenesis.

b) Graphic representation of angiogenic growth factor mRNA expression following 70%-hepatectomy.

c) Graphic representation of angiogenic growth factor receptor mRNA expression following 70%-hepatectomy.

Sato, T. et al. Sinusoidal endothelial cell proliferation and expression of angiopoietin/Tie family in regenerating rat liver. *J Hepatol.* 34, 690-8 (2001). Reproduced with permission.

4.4.2 Results and discussion

Standard 70% hepatectomy was performed on age-, sex- and type-matched wild-type and *Cd39*-null mice.[222] Mice were observed for mortality, and surviving mice euthanased and livers analyzed by immunohistochemistry at 14 days.

In keeping with previous studies using the 70%-hepatectomy model of liver regeneration, wild-type mice had no mortality and normal hepatic size and architecture was restored by day 8 (Sun Xiaofeng, unpublished data).

In contrast, 30% of the *Cd39*-null mice died within the first few days after liver resection secondary to massive vascular thrombosis, haemorrhage and shock. It is our view that this occurred because of vascular perturbations and, in particular, platelet dysfunction in the *Cd39*-null mouse, and not because of defective angiogenesis. Previous work in the laboratory has shown that the *Cd39*-null mouse has a major defect in platelet function and vascular haemostasis, probably secondary to platelet $P2Y_1$ desensitization and decreased aggregatory responses to ADP.[184]

However, of the mice that survived the initial vascular complications associated with hepatectomy, up to 50% had persisting lethargy, anorexia and jaundice secondary to impaired liver regeneration (Sun Xiaofeng, unpublished data).

At 5 days after 70%-hepatectomy, wild-type livers demonstrated both hepatocyte and endothelial cell proliferation, as evidenced by BrdU uptake. In contrast, the *Cd39*-null animals had normal hepatocyte proliferation, but limited endothelial cell proliferation (figure 4.4.4, a-b). Also, Tunel staining for apoptosis showed that there was marked endothelial cell apoptosis in the *Cd39*-null, but not in the wild-type mice (figure 4.4.4, c-d) (Sun Xiaofeng, unpublished data).

This combination of poor endothelial cell proliferation and enhanced apoptosis in *Cd39*-null mice following liver resection, resulted in the failure to re-establish normal hepatic sinusoidal architecture and the persistence of avascular islands of hepatocytes up to 7 days (Fig 4.4.5). In other words, despite normal hepatocyte proliferation, liver regeneration in *Cd39*-null mice was impaired because of defective angiogenesis. Whereas wild-type mice re-established normal hepatic architecture by 8 days, in *Cd39*-null mice, late liver injury, as manifested by islands of ischaemic hepatic necrosis, were seen up to 14 days after resection (Fig 4.4.6) (Sun Xiaofeng, unpublished data).

These results demonstrate that normal Cd39 function is required for physiological angiogenesis during liver regeneration. This prompted further research into the role of *Cd39* deletion in patho-physiological angiogenesis following experimental myocardial infarction.

Figure 4.4.4 (a)

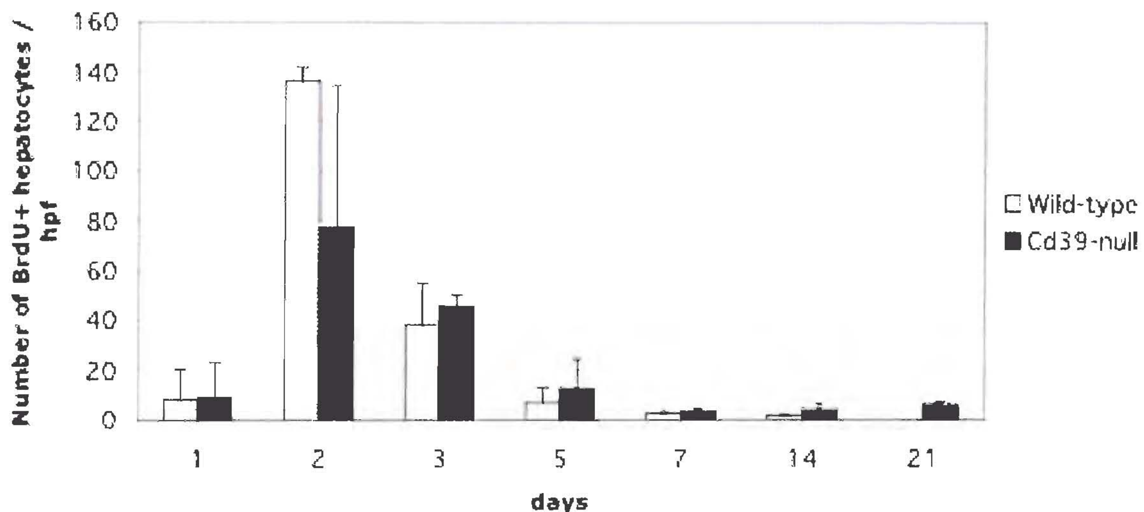


Figure 4.4.4 (b)

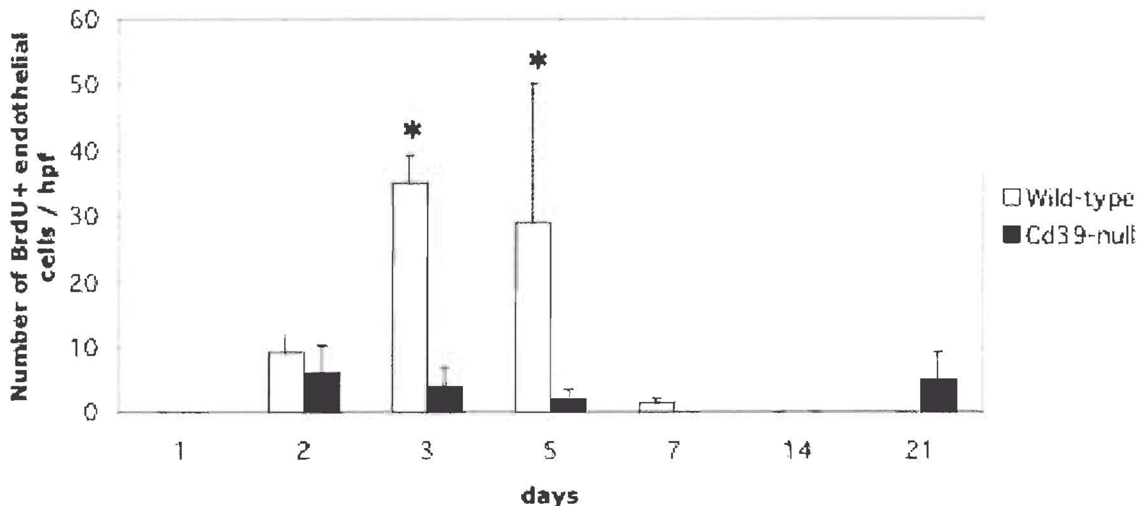


Figure 4.4.4 - Graphs representing the proliferation of hepatocytes (a) and endothelial cells (b) in wild-type and *Cd39*-null endothelial cells after 70%-hepatectomy. There is similar proliferation of hepatocytes (a) in *Cd39*-null mice, but markedly decreased endothelial cell (b) proliferation, compared with wild-type mice. Bar charts represent the average number of BrdU-positive hepatocytes (a) or endothelial cells (b) per high-powered field (hpf). *, $P<0.05$. (Sun Xiaofeng, unpublished data).

Figure 4.4.4 (c)

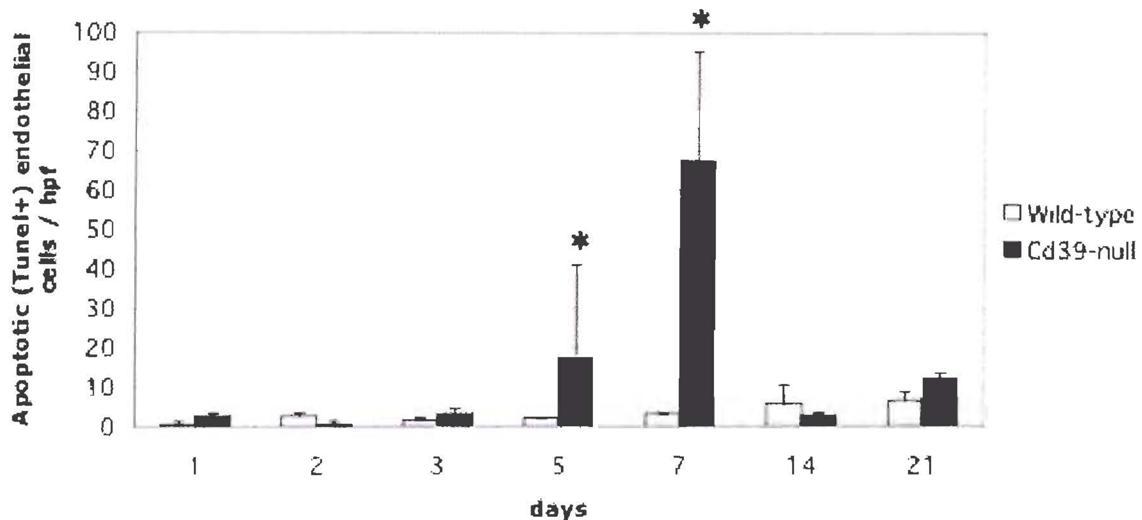


Figure 4.4.4 (c) - Graph representing endothelial cell apoptosis in wild-type and *Cd39*-null endothelial cells after 70%-hepatectomy. Between days 5 and 7, there is marked apoptosis of *Cd39*-null, but not wild-type, endothelial cells. Bar charts represent the average number of apoptotic endothelial cells per high-powered field (hpf) by Tunel staining. *, $P<0.05$. (Sun Xiaofeng, unpublished data).

Figure 4.4.4 (d)

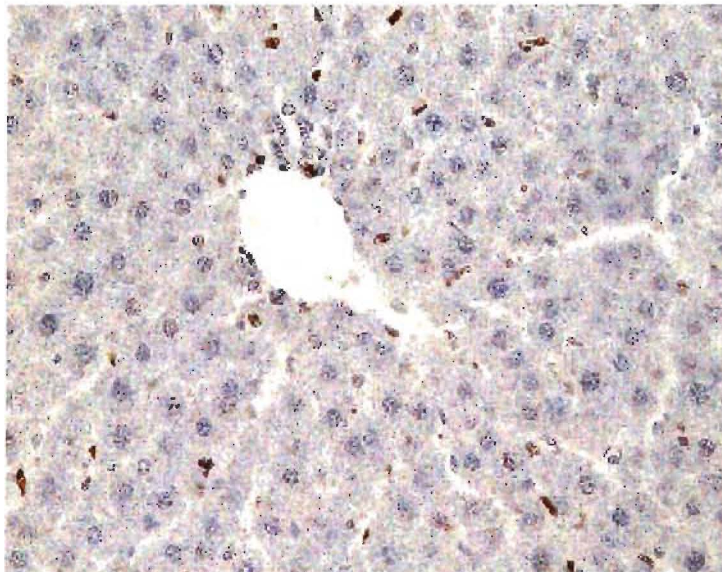


Figure 4.4.4 (d) - Tunel Stain showing endothelial cell apoptosis in regenerating *Cd39*-null mouse liver 5 days after 70%-hepatectomy. Minimal apoptosis was noted in wild-type livers. (Sun Xiaofeng, unpublished data).

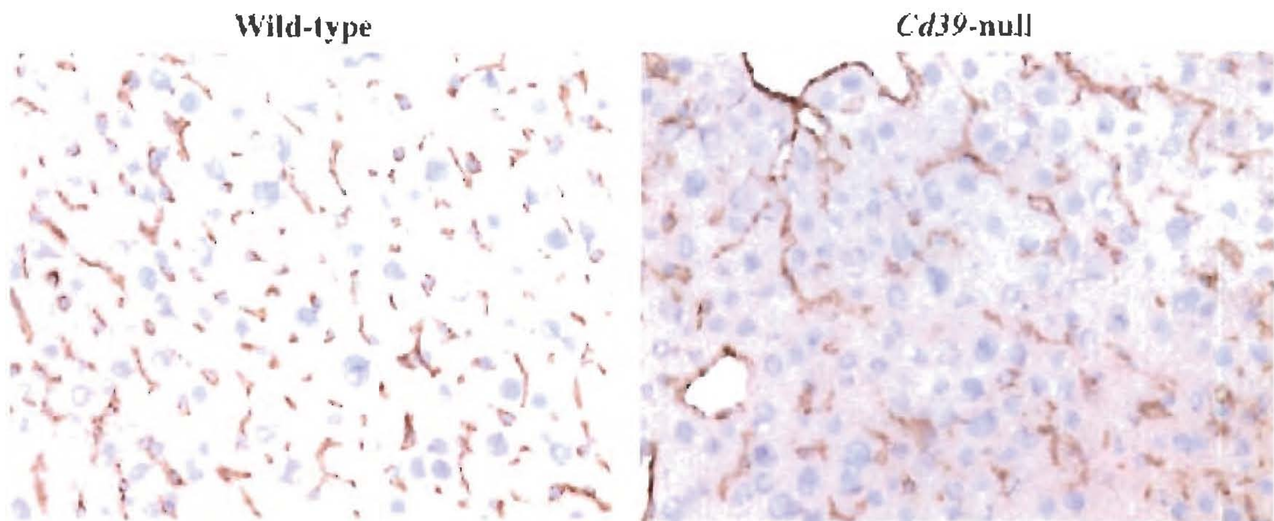


Figure 4.4.5 - CD31-staining of endothelial cells at day 7 post 70%-hepatectomy in wild-type and *Cd39*-null mice. Wild-type mice shows normal hepatic architecture with each hepatocyte having at least one facet of contact with the sinusoidal endothelium (left). *Cd39*-null mouse has wide, irregular gaps between adjacent sinusoidal and several hepatocytes lack contact with the vasculature (right). (Sun Xiaofeng, unpublished data).

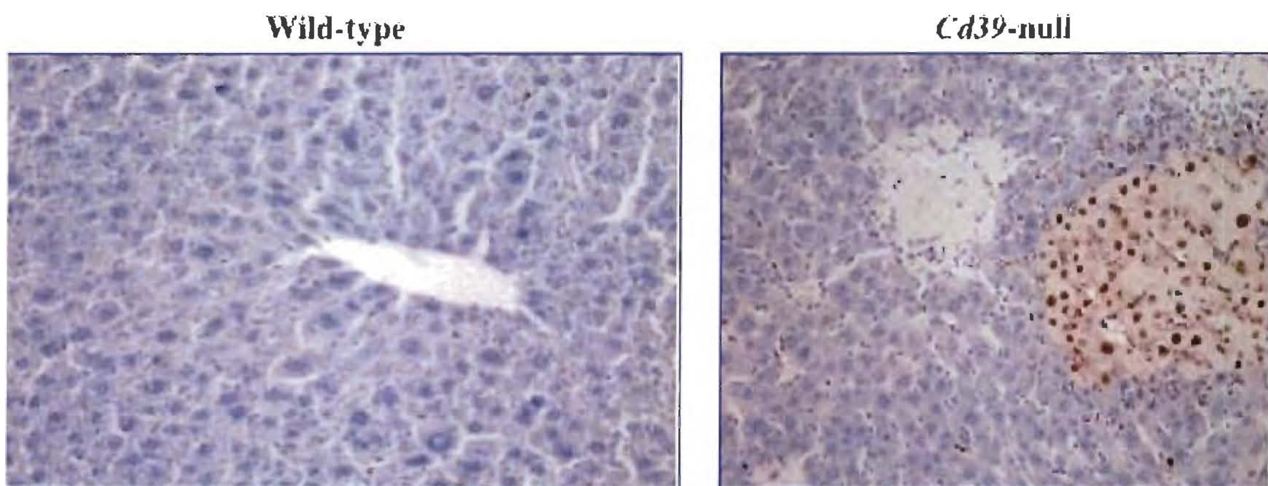


Figure 4.4.6 - Wild-type and *Cd39*-null liver sections at day 14 after 70%-hepatectomy. Wild-type shows normal architecture (left), while cd39-null have large areas of hepatic necrosis secondary to defective revascularization (right). Apoptotic areas are denoted by brown Tunel staining. (Sun Xiaofeng, unpublished data).

4.5 MYOCARDIAL INFARCTION

4.5.1 Introduction

Heart disease, in particular ischaemic heart disease, remains the major cause of death in western societies.[223] As a novel approach to the treatment of this disease, several researches have attempted to use pro-angiogenic therapy to induce myocardial collateral formation and increase myocardial blood flow. At least 17 clinical trials of myocardial angiogenesis have been performed to date, involving over 900 patients. Despite initial promising results in non-randomized trials, none of the double-blind randomized trials showed clinical benefits. However, newer approaches like gene therapy may yet prove useful as treatments for ischaemic heart disease.[224]

It is with this in mind, that the role of *Cd39* deletion was studied in a murine model of myocardial infarction.

4.5.2 Methods and results

The left anterior descending coronary artery was ligated in wild-type and *Cd39*-null mice (n=5,5), inducing massive infarction of the anterior left ventricle. After recovery, the mice were monitored daily and the surviving mice euthanased at 28 days. Myocardial tissue was examined by immunohistochemistry following spontaneous death or euthanasia.

Figure 4.5.1 shows the survival curve of wild-type and *Cd39*-null mice after myocardial infarction. Within 4 days, two-thirds of the wild-type mice had died because of cardiac rupture (Mika Ogawa, unpublished data). This correlates with previous work showing that myocardial rupture, occurring in the first 4 days, is the commonest cause of death following experimental myocardial infarction.[101] Almost uniformly fatal, cardiac rupture causes 5 to 31% of all in-hospital mortality following acute myocardial infarction in humans.[225]

However, no *Cd39*-null mice died of cardiac rupture and *Cd39*-null mice initially had a survival benefit over their wild-type counterparts. But, from one week after myocardial infarction, *Cd39*-null mice started to die of progressive cardiac failure and at 4 weeks had similar mortality rates to wild-type mice. The *Cd39*-null mice that died exhibited congested, oedematous lungs and livers. No wild-type mice died of cardiac failure (Mika Ogawa, unpublished data).

Histological examination of hearts from wild-type and *Cd39*-null mice demonstrated that CD45-positive inflammatory cells failed to migrate into *Cd39*-null infarcts (figure 4.5.2). The CD45 antigen, also known as leukocyte common antigen (LCA), is a tyrosine phosphatase expressed by all cells of haematopoietic origin, with the exception of red blood cells and platelets.[226] In addition, the migration of CD31-positive endothelial cells into *Cd39*-null infarcts was significantly decreased. Post-infarct myocardial healing in *Cd39*-null mice was thus characterized by poor inflammatory cell infiltration of the necrotic myocardium and defective angiogenesis during myocardial remodeling (Mika Ogawa, unpublished data).

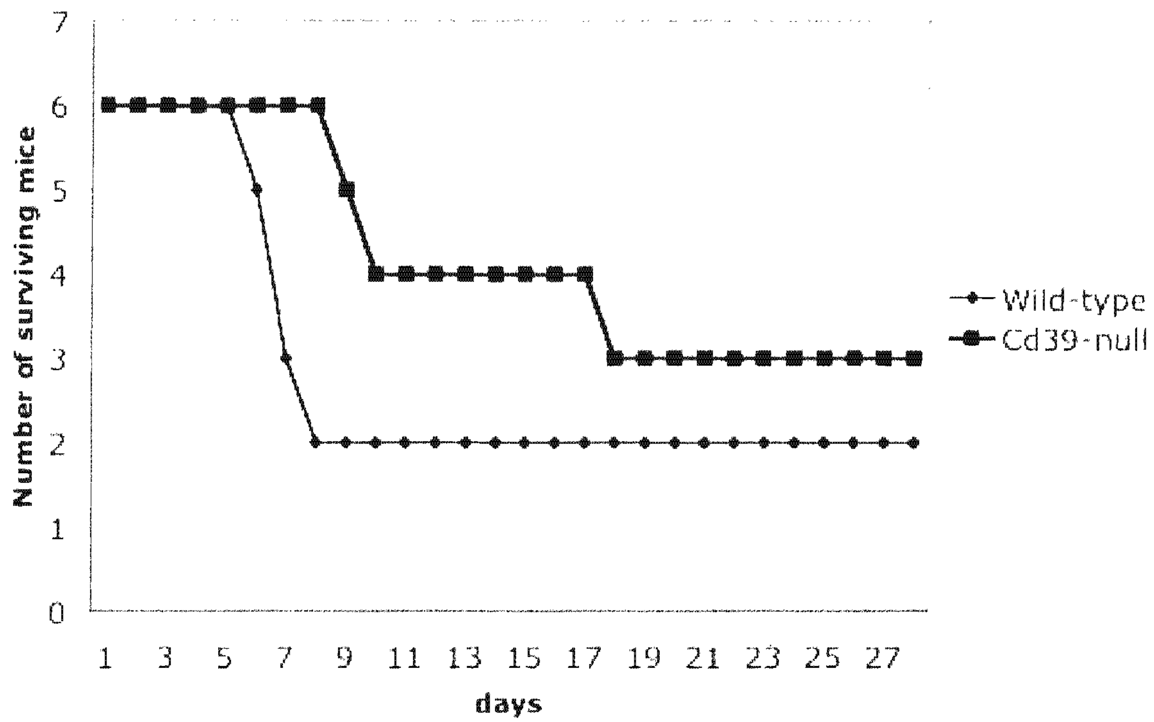


Figure 4.5.1 - Survival curve of wild-type and *Cd39*-null mice following myocardial infarction (n=6,6). Two-thirds of wild-type mice died within the first week after myocardial infarction because of cardiac rupture. In contrast, no *Cd39*-null mice developed cardiac rupture, but half the *Cd39*-null mice had delayed mortality because of progressive cardiac failure. (Mika Ogawa, unpublished data).

4.5.3 Discussion

The explanation for the unusual finding that *Cd39*-null mice have decreased cardiac rupture but increased cardiac failure following experimental infarction is that this inflammatory cell infiltration and revascularization is both beneficial and detrimental following myocardial infarction.

On the one hand, revascularization is required for myocardial remodeling after myocardial infarction. Without remodeling, the mice develop to progressive cardiac failure and die. On the other hand, during revascularization and remodelling, the extracellular matrix within the infarct must first be digested. Inflammatory cells and endothelial cells migrate into the infarct and secrete a range of extracellular proteases including the matrix metalloproteinases and the plasminogen activator-plasmin family of proteases. These proteases weaken the infarcted myocardial wall and predispose to cardiac rupture.

In keeping with this hypothesis, infarcted myocardial tissue from *Cd39*-null mice had significantly lower levels of gelatinase metalloproteinase (MMP-2 and MMP-9) activity, than wild-type infarcts (data not shown). We believe that this decrease in protease activity occurred because of the failure of inflammatory and endothelial cells to migrate into the infarct rather than because of an actual decrease in matrix metalloproteinase production. We have shown, for example, that there is no difference in the production of MMP-2 and MMP-9 by wild-type and *Cd39*-null endothelial cells in vitro (chapter 5.6).

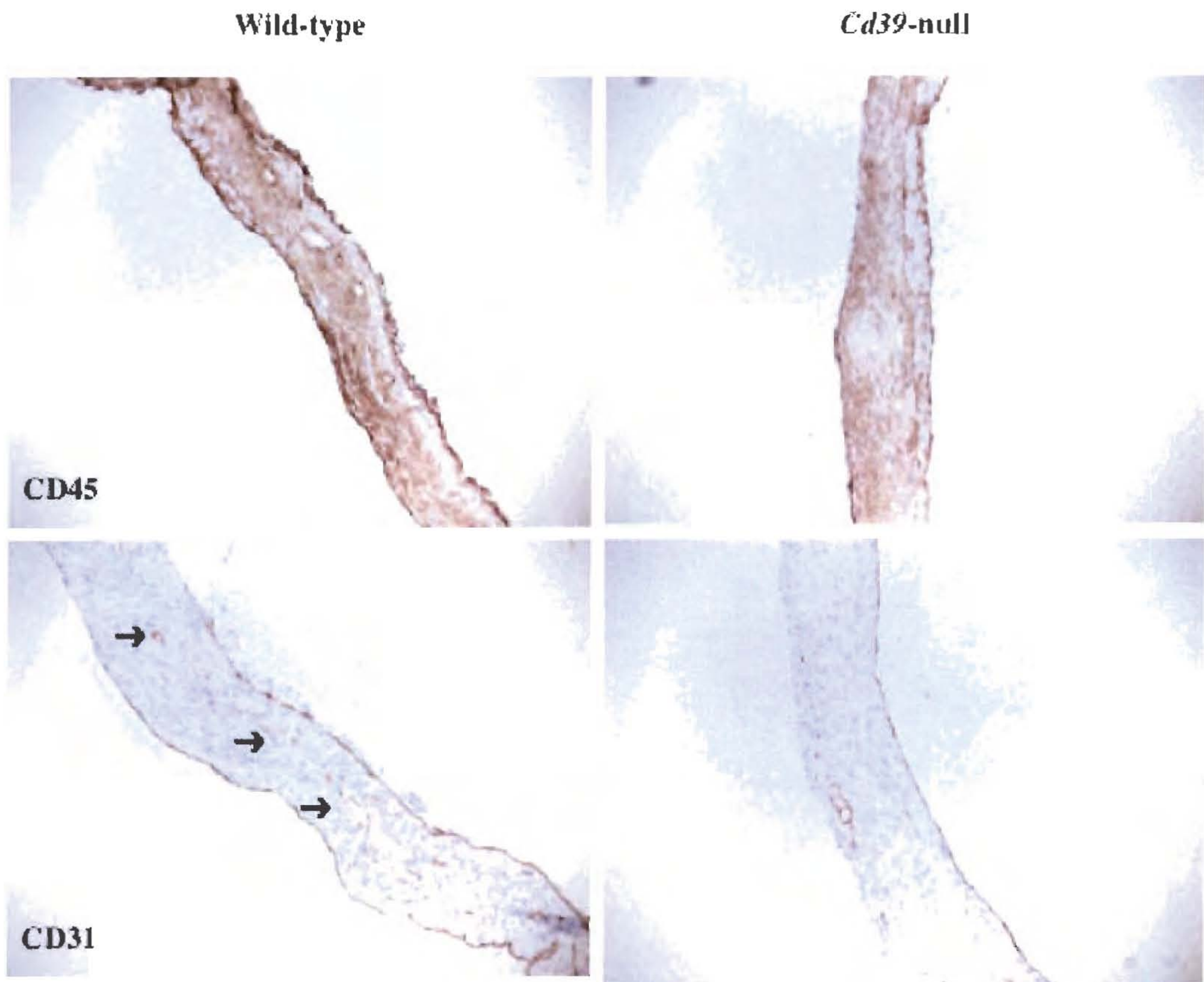


Figure 4.5.2 - Immunohistochemical staining of infarcted myocardial tissue from wild-type (left) and *Cd39*-null (right) mice. The upper panels show increased migration of CD45-positive inflammatory cells into wild-type (left), compared with Cd39-null (right), infarcts. Similarly, new blood vessels formation (arrows, CD31-positive endothelial cells) is noted within infarcted tissue in wild-type hearts (left, lower panel). Few new blood vessels developed in Cd39-null infarcts (right, lower panel). (Mika Ogawa, unpublished data).

The beneficial and detrimental effects of myocardial remodeling after myocardial infarction was confirmed in another study using genetically engineered mice. The urokinase-type plasminogen activator knock-out mouse (u-PA –/–) was completely protected, and gelatinase-B (MMP-9)-null mouse partially protected, from cardiac rupture following acute myocardial infarction. But, like the *Cd39*-null mice, the u-PA-/- mouse showed impaired infarct revascularization and developed fatal cardiac ischaemia, arrhythmias and cardiac failure.[101]

The question remaining is, could we limit matrix digestion sufficiently to prevent cardiac rupture, but still allow myocardial remodeling after acute myocardial infarction? The researchers involved in the above study were able to show that temporary administration of plasminogen-activator inhibitor-1 (PAI-1) or the matrix metalloproteinase inhibitor TIMP-1 protected against cardiac rupture without compromising remodeling.[101] This shows, at least in an animal model, that temporary angiogenesis inhibition could have therapeutic benefit in preventing cardiac rupture after acute myocardial infarction. This would certainly prove a novel and unexpected use of anti-angiogenesis therapy.

4.6 TUMOUR ANGIOGENESIS

4.6.1. Introduction

The importance of angiogenesis in facilitating the growth of tumours has been stressed earlier in this dissertation. Understanding the precise molecular mechanisms involved in the complex process of tumour angiogenesis is required to realize the ultimate aim of developing effective anti-angiogenic cancer therapies.

Deletion of *Cd39* is associated with defective angiogenesis in experimental models of liver regeneration and myocardial infarction, implying that tumour angiogenesis may also be impaired. For this reason, the effects of *Cd39* deletion on in vivo experimental models of angiogenesis were examined.

4.6.2. Methods and results

C57BL/6X129svj strain wild-type and type-matched *Cd39*-null mice were inoculated with 3.0 X 10^6 cells B16-F10 melanoma (B16-F10, ATCC: CRL-6457, n=8,6), B16-CG (stable β-HCG expression vector transfected) melanoma[227] (n=5,4) or LLC cells (LLC, ATCC: CRL-1642, n=4) into the dorsal subcutaneous space. Tumour size was measured daily and the tumour volume calculated using the formula $V=\pi L.W^2/6$, where L is the longer diameter, and W the short diameter.

The urine of mice inoculated with B16-CG melanoma cells was collected as described,[227] and urine β-HCG and creatinine measured using a free β-HCG ELISA Kit (Alpha Diagnostic, Texas) and a Creatinine Kit (Sigma diagnostics, Inc. St. Louis MO), respectively.

Wild-type and *Cd39*-null mice were also injected with 5.0 X 10^5 B16-F10 melanoma into the subperitoneal plane of an abdominal wall flap. The tumours were examined at 2-day intervals, the tumour size measured and new blood vessels growing into the tumour were counted by microscopy. After 15 days, both groups of mice were sacrificed and the tumours analyzed by immunohistochemistry.

i) Subcutaneous tumour growth

Since angiogenesis has been shown to be impaired in *Cd39*-null mice during liver regeneration and post myocardial infarction, the effect of deletion of the bulk of vascular endothelial cell NTPDase activity on transplanted tumour growth was studied *in vivo*. Matched wild-type and *Cd39*-null mice were injected subcutaneously with murine melanoma or lung carcinoma cells. Lewis lung carcinoma is a cell line established from the lung of a C57BL mouse, which was implanted with primary Lewis lung carcinoma.[228] The cell line is highly tumourigenic in C57BL mice, but seldom spontaneously metastatic. However, it is often used in experimental models of metastasis by intravenous injection of tumour cells.[229] B16-F10 is a murine melanoma cell line, which is tumourigenic in C57BL/6J mice.[209]

As predicted, the growth of both B16-F10 melanoma and Lewis lung carcinoma was significantly attenuated in *Cd39*-null mice (figure 4.6.1). Wild-type mice had significantly larger B16-F10 (figure 4.6.2, a) and LLC (figure 4.6.2, b) tumour volumes at one week after inoculation. Tumour growth then entered the exponential phase in wild-type mice, in keeping with the induction of an angiogenic phenotype. In contrast, tumour growth in *Cd39*-null mice was severely retarded to 15 days (Tomokazu Hoshi, unpublished data).

The release of β-HCG following implantation of B16-F10 melanoma cells stably transfected with β-HCG vector (B16-CG) was also used to estimate tumour volume.[227] β-HCG excretion correlates well with tumour volume in this model (R^2=0.96, data not shown). Daily urine β-HCG excretion was significantly decreased in *Cd39*-null mice, in keeping with the observed poor tumour growth in these animals (figure 4.6.3) (Tomokazu Hoshi, unpublished data).

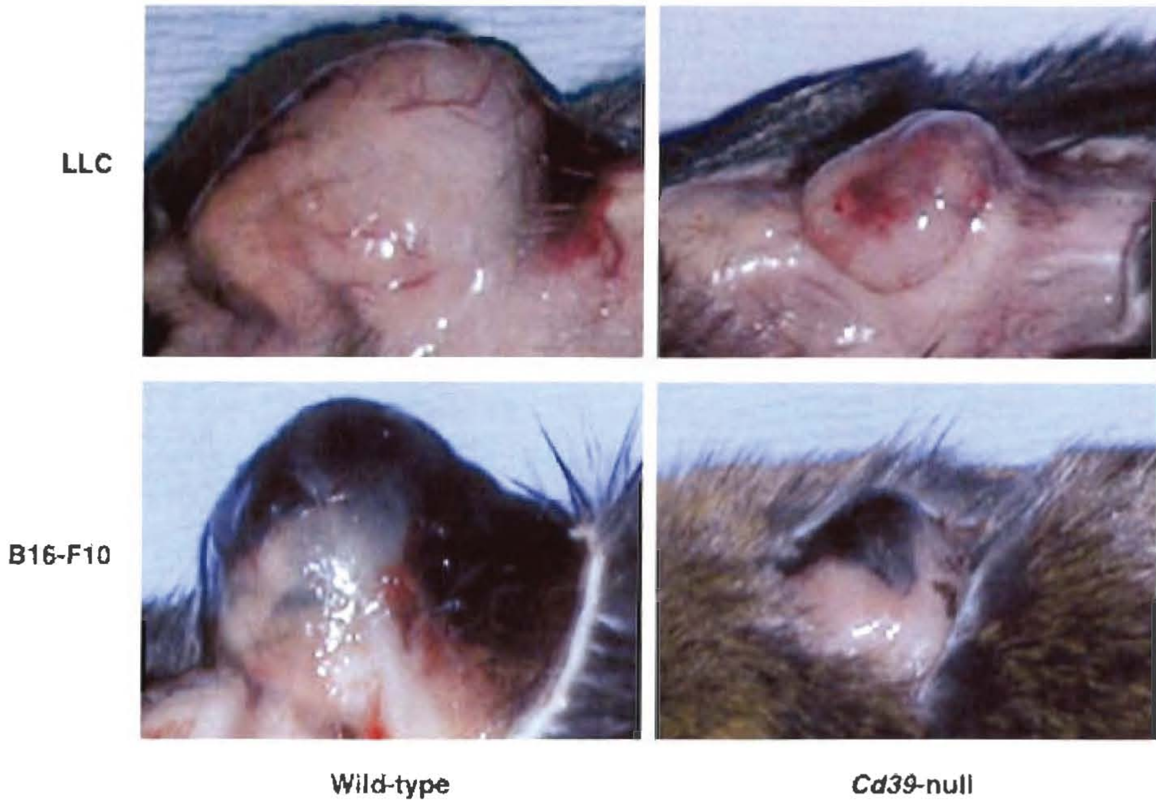


Figure 4.6.1 - Representative examples of tumour growth in wild type (left) and *Cd39*-null (right) mice at 7 days after implantation. The upper tumour is Lewis lung carcinoma and the lower is B16-F10 melanoma. (Tomokazu Hoshi, unpublished data).

Figure 4.6.2 (a)

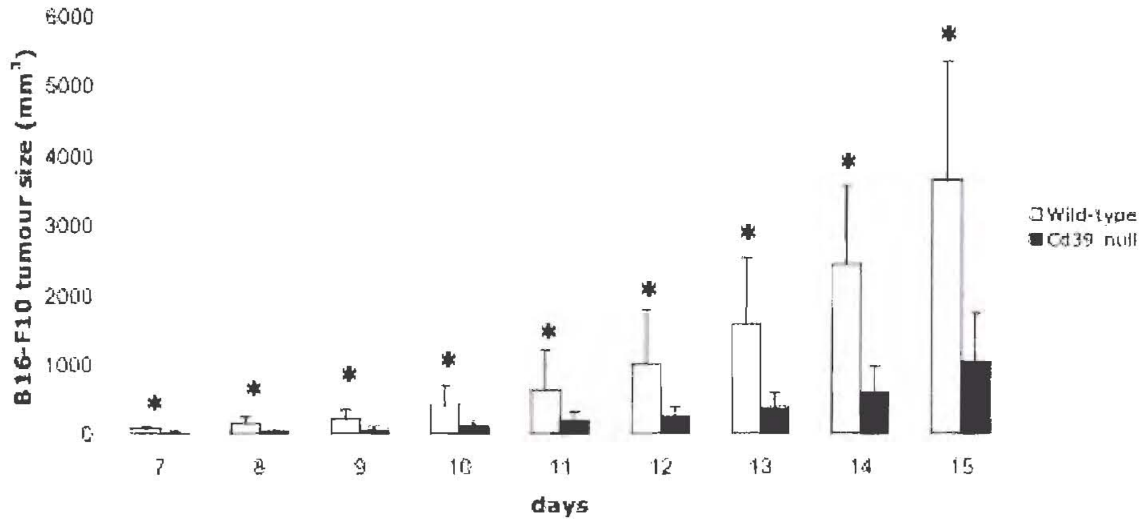


Figure 4.6.2 (b)

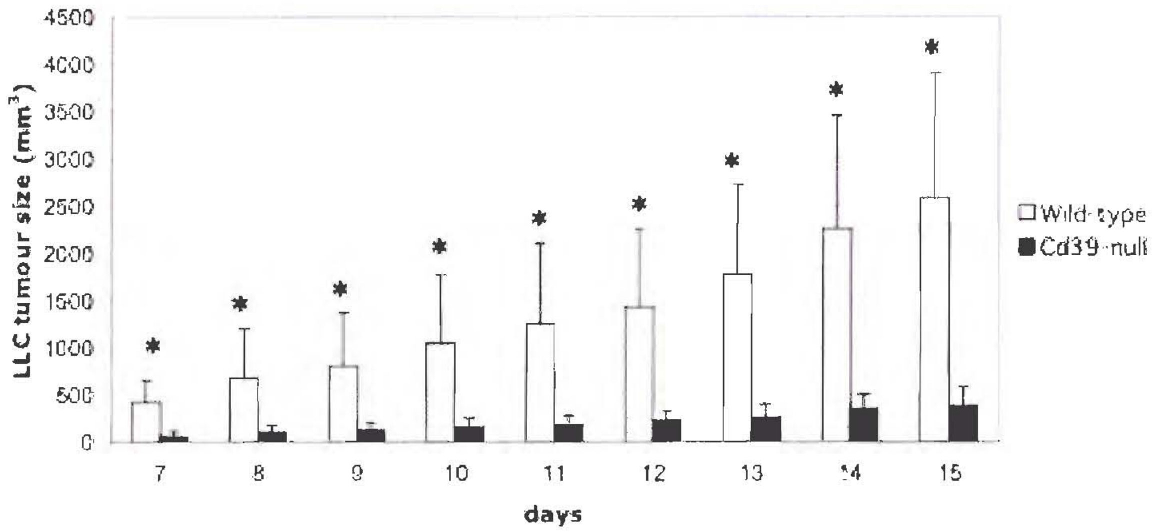


Figure 4.6.2 - Measurement of murine B16-F10 (a) and LLC (b) subcutaneous tumor growth in wild type (□) and *Cd39*-null (■) mice. Bars show mean tumor volume in mm^3 ± s.t.d. *, $P<0.05$. (Tomokazu Hoshi, unpublished data).

Figure 4.6.3

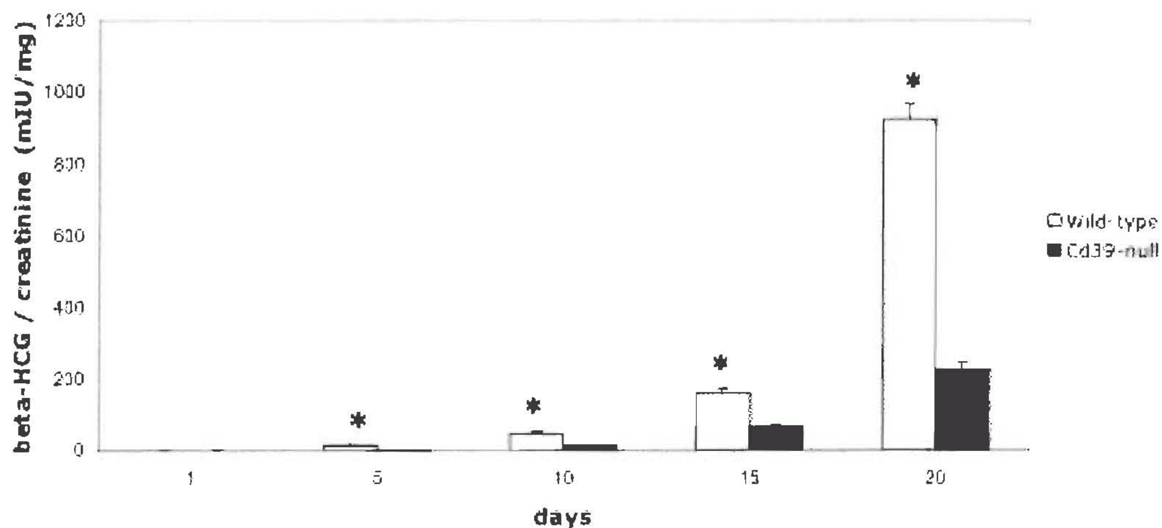


Figure 4.6.3 - β-HCG excretion following subcutaneous inoculation with B16-CG tumors. Wild type (□), *Cd39*-null (■). Bars show urinary β-HCG/creatinine (mIU/mg) ± s.t.d. *, $P<0.05$. (Tomokazu Hoshi, unpublished data).

ii) Subperitoneal tumour growth and direct visualization of angiogenic vessels.

Inoculation of B16-F10 tumours into the subperitoneal abdominal wall allowed direct observation of tumour growth. Direct visualization of B16-F10 tumours in wild-type and *Cd39*-null animals 7 days after implantation confirmed poor tumour growth in *Cd39*-null mice (figure 4.3.4). In addition, this model allowed direct visualization of new vascularization of the tumour by dissection microscopy. By 2 days after tumour injection, wild-type mice had evidence of new vessels infiltrating the tumour and the number of vessels increased steadily over the next week (Fig. 4.3.5). This did not occur in *Cd39*-null mice, in keeping with our hypothesis that defective angiogenesis is responsible for the observed decreases in tumour growth (Tomokazu Hoshi, unpublished data).

Figure 4.6.4

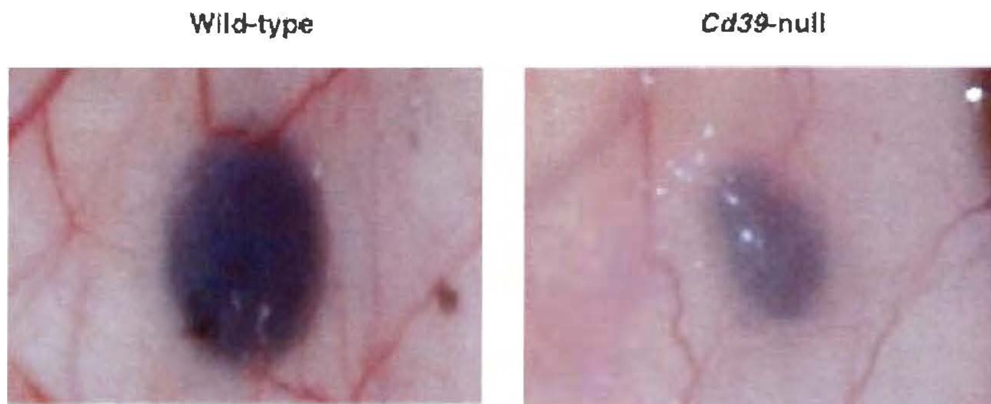


Figure 4.6.4 - Macroscopic appearance of subperitoneal B16-F10 tumor at 7 days after implantation in wild type (left) and *Cd39*-null (right) mice. Visualization under light microscopy allowed the direct counting of angiogenic blood vessels invading the tumour. ((Tomokazu Hoshi, unpublished data).

Figure 4.6.5

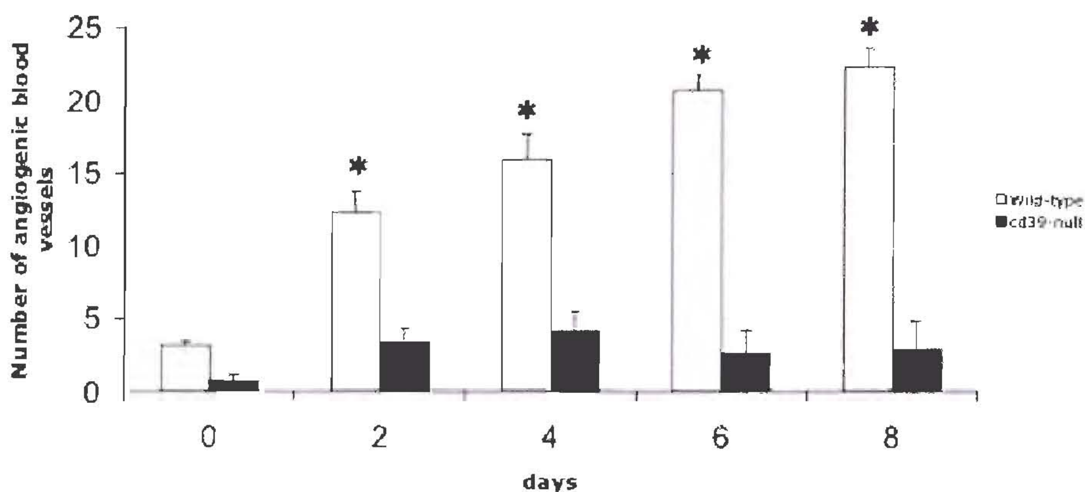


Figure 4.6.5 - Vessel counts in subperitoneal B16-F10 tumors in wild type (□) and *Cd39*-null (■) mice. Bars show mean number of vessels ± s.e.m. *, $P<0.05$. (Tomokazu Hoshi, unpublished data).

iii) Immunocytochemistry

Failure of angiogenesis in *Cd39*-null mice was confirmed by immunohistochemical staining of tumour sections. Staining with the endothelial marker CD31-1 showed deep ingrowth of blood vessels into the tumour mass in wild-type mice (arrow, figure 4.6.6, *a*). In contrast, tumours in *Cd39*-null mice were devoid of vessels, which were confined to the interface between the tumour and adjacent normal tissue (arrow, figure 4.6.6, *b*).

Significant numbers of macrophages migrated to the interface between tumour tissue and adjacent normal tissue as well as into the viable and necrotic areas (denoted, *) within the tumours in wild-type animals (arrow, figure 4.6.6, *c*). In contrast, few macrophages were noted in *Cd39*-null sections (arrow, figure 4.6.6, *d*), in keeping with our previous finding that *Cd39*-null macrophages manifest defective migration through endothelial monolayers *in vitro*. Tumours and surrounding tissues in wild-type and *Cd39*-null animals contained similar amounts of B and T-lymphocytes as well as granulocytes (Table 4.6.1). Platelet deposition (integrin α_{IIb}–positive) was noted in vessels within the tumours in wild-type mice (arrow, figure 4.6.6, *e*), but was only seen surrounding tumours in *Cd39*-null animals (arrow, figure 4.6.6, *f*).

Basal lamina deposition, based on anti-perlecan staining, was noted around tumour vessels and muscle bundles in wild-type mice (arrow, figure 4.6.6, *g*). In contrast, in tumours grown in *Cd39*-null mice (figure 4.6.6, *h*), which

were largely devoid of vasculature, basal lamina divided individual tumour cell nests into gland-like structures (*). Similar encasement of tumour cells by basal lamina was absent in tumours in wild-type mice (figure 4.6.6, *g*) indicating a higher level of tumour cell differentiation in tumours in *Cd39*-null mice. In addition to enhanced growth, tumours in wild-type mice were capable of invading the underlying dermal muscle fascia (*, figure 4.6.6, *i*). In contrast, in tumours grown in *Cd39*-null mice (*j*), tumour cell invasion was absent and the tumour mass and muscle layer (*) was clearly separated (figure 4.6.6, *j*) (Christian Sundberg, unpublished data).

Maturation of angiogenic tumour vasculature requires the recruitment of pericytes and smooth muscle cells around the infiltrating endothelium. In tumours implanted into wild-type mice, endothelial cells (blue) were partially enveloped by pericytes (green) and surrounded by interstitial fibroblasts (red) (arrow, figure 4.6.7, *d*). In contrast, tumours in *Cd39*-null mice were devoid of endothelial cells, pericytes and fibroblasts, indicating pluripotent cellular migration defects in *Cd39*-null mice. Pericyte-enveloped vessels were confined to the adjacent normal tissue (arrow, figure 4.6.7, *h*). Markers of blood vessel activation such as dissolution of the enveloping basal lamina, increased pericyte expression of NG2 and PDGFβ-receptors, decreased pericyte expression of smooth muscle α-actin, and increased endothelial expression of VEGFR2-receptors was apparent in vessels at the interface between tumour and normal adjacent tissue in wild-type but not in *Cd39*-null mice (Table 4.6.1). Platelet (green) and fibrin (red) deposition, central components of the provisional pro-angiogenic matrix was noted in and around infiltrating vessels in wild-type tumours (arrow, figure 4.6.7, *l*),

but in *Cd39*-null mice was confined to the rim of tissue surrounding the tumour (arrow, figure 4.6.7, *p*). Thus, despite deposition of a provisional matrix in normal tissue adjacent to the tumour, blood vessels failed to appropriately activate and migrate into the tumour mass in *Cd39*-null mice (Table 4.6.1) (Christian Sundberg, unpublished data).

These results confirm that tumour angiogenesis is defective in the *Cd39*-null mouse. The failure to adequately vascularize implanted tumours resulted in significantly decreased tumour growth in *Cd39*-null animals.

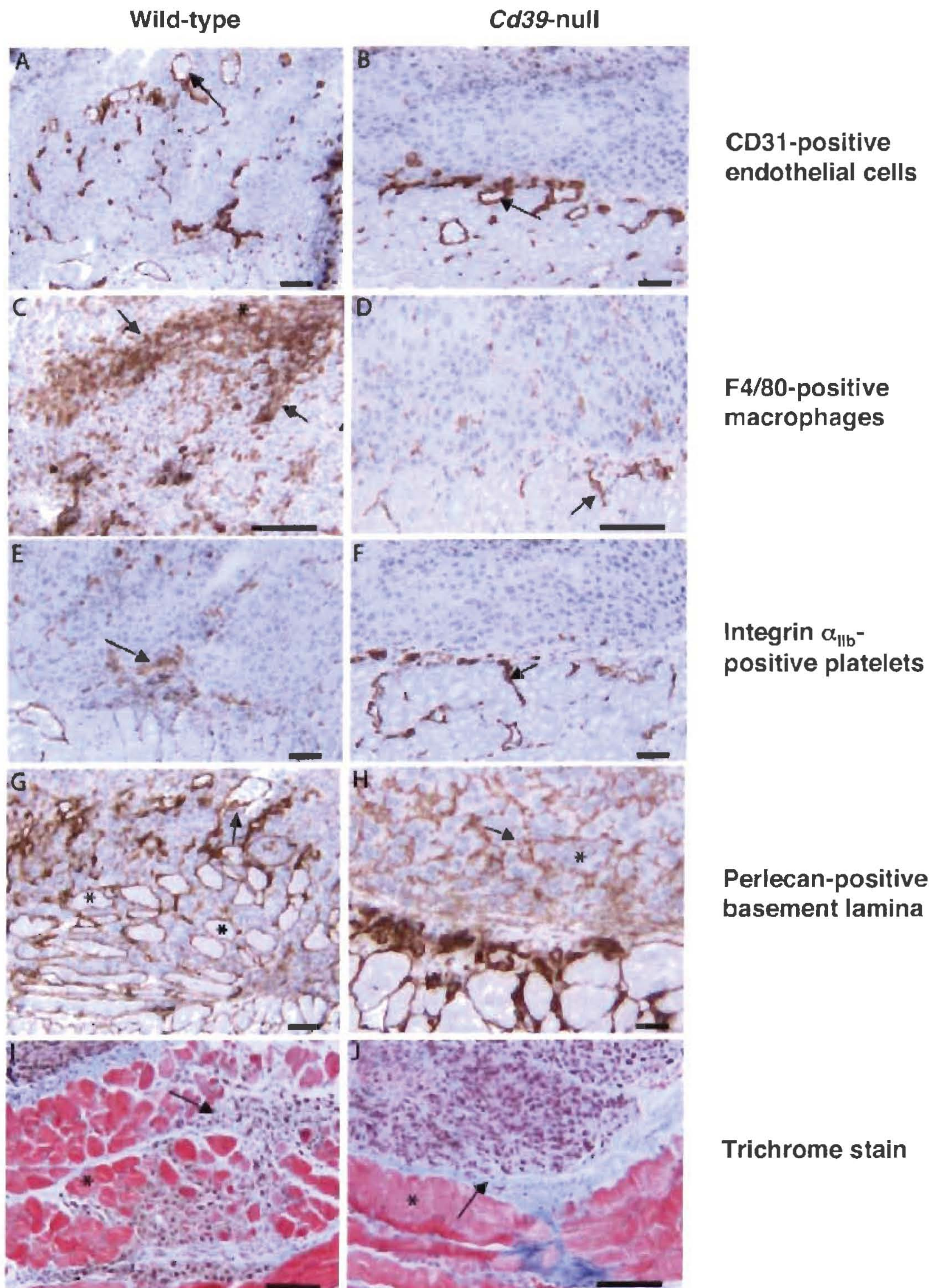


Figure 4.6.6 - Immunohistochemistry staining of implanted tumours in wild-type and Cd39-null mice. Bars=20μm. (Christian Sundberg, unpublished data).

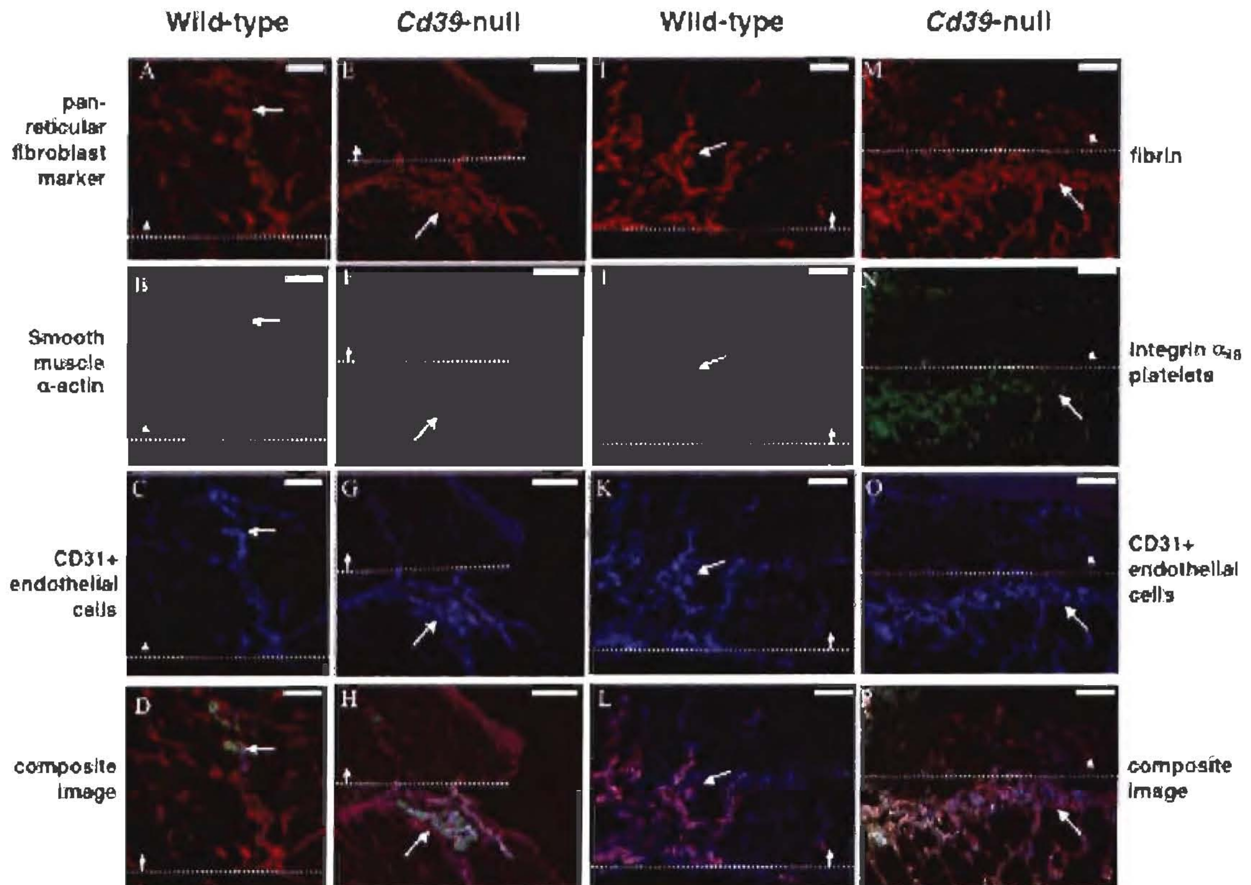


Figure 4.6.7 - Triple immunofluorescent staining of implanted tumors. Tumors grown in the subcutaneous space in wild type (*a-d, i-l*) and *Cd39*-null (*e-h, m-p*) mice with antibodies recognizing; pericytes and fibroblasts (pan-reticular fibroblast marker) (*a,e*); pericytes and smooth muscle cells (smooth muscle α actin) (*b,f*); fibrin (*i,m*); platelets (integrin α_{IIb}) (*j,n*); endothelial cells (CD31) (*c, g, k and o*) and their composites (*d, h, l and p*). The dotted white line delineates the interface between the tumor mass and adjacent normal tissue. The small arrow indicates the direction of the tumor. Solid bars=20μm. (Christian Sunberg, unpublished data).

Table 4.6.1.
Distribution of cell-type specific markers, cellular activation markers and extracellular matrix components in wild-type and *Cd39*-null mice following tumour implantation (Christian Sundberg, unpublished data).

	Normal Dermis	**Tumour Interface**	**Tumour Tissue**
<u>**Endothelium**</u>			
PECAM-1			
Wild-type	++	++	++
Cd39-Null	++	++	-
Panendothelial Marker			
Wild type	++	++	++
Cd39-null	++	++	-
VEGFR2-receptor			
Wild-type	+	++	++
Cd39-null	+	++	-
<u>Pericytes/Myofibroblasts</u>			
α-SMA			
Wild-type	++	+	++
Cd39-null	++	++	-
PDGFβ-receptors			
Wild-type	+	++	++
Cd39-null	+	+	-
NG2			
Wild-type	+	++	++
Cd39-null	+	+	-
Reticular Fibroblasts			
Wild-type	+	++	++
Cd39-null	+	++	-
<u>**Basal Lamina**</u>			
Perlecan			
Wild-type	++	+	++*1
Cd39-null	++	++	++*2
Fibrin			
Wild-type	-	++	++
Cd39-null	-	++	+
<u>**Inflammatory cells**</u>			
F4/80			
Wild-type	+	++	++
Cd39-null	+	+	+
Platelets			
Wild-type	+	++	++
Cd39-null	+	++	-
B-cells			
Wild-type	+	-	-
Cd39-null	+	-	-
T-cells			
Wild-type	+	-	-
Cd39-null	+	-	-
Granulocytes			
Wild-type	+	-	-
Cd39-null	+	-	-

Key: - = no expression. + = sparse expression. ++ = abundant expression. NA= not applicable.
*1= associated with vasculature and muscle bundles. *2 associated with tumour nests.

4.6.3 Discussion

These results suggest that abnormal purinergic signaling in the *Cd39*-null vasculature results in profound defects in tumour growth and angiogenesis. This is consistent with the finding of defective angiogenesis during liver regeneration and after myocardial infarction in *Cd39*-null mice.

Cd39-null mice exhibit the normal initial phases of angiogenesis, including mother vessel formation, increased vascular permeability and fibrin deposition. However, there is a profound defect in the migration of endothelial and inflammatory cells into tumours in *Cd39*-null animals. There is also abnormal resolution of angiogenesis in *Cd39*-null mice characterized by poor recruitment of surrounding pericytes and smooth muscle cells to new blood vessels. As embryonic vascular development is normal in *Cd39*-null mice[184], this data suggests that purinergic signaling has differential roles in physiological blood vessel development as opposed to pathological angiogenesis.

Perhaps most importantly, these results suggest that pharmacological ectonucleotidase blockade may have a novel therapeutic role in clinical oncology practice.

4.7. TUMOUR METASTASIS

4.7.1. Introduction

Angiogenesis is crucially important in the process of tumour metastasis. In addition to allowing tumours to grow, cancerous cells can enter angiogenic blood vessels and travel in the circulation to distant organs. The development of tumour metastases correlates with increased vascularity of the primary tumour, and, at least in animal models, anti-angiogenic therapy prevents both tumour growth and tumour metastasis. This suggests that the angiogenesis defect of the *Cd39*-null mouse could also result in decreased tumour metastasis in these animals.

Although an increased number of tumour cells entering the circulation generally correlates with increased metastasis, the vast majority of circulating cancer cells are eliminated before seeding by immune and non-immune defense mechanisms.[230] A 1 cm primary breast cancer can release up to $2x10^6$ cancerous cells into the circulation per day,[231] but very few of these ever become clinically significant metastases.

The first possible explanation for this is that endogenous angiogenesis inhibitors prevent the growth of those cancerous cells that do establish distant micrometastases. Anti-angiogenic factors released by the primary tumour include angiostatin and endostatin and these may prevent the growth of micrometastases.

There is also much evidence linking haemostatic factors, in particular platelets and fibrinogen, to increased tumour cell survival in the circulation. Platelet activation is thought to facilitate tumour metastasis by, inter alia, protecting circulating tumour cells from natural killer (NK) cell-mediated elimination.[232]

It was initially expected that the *Cd39*-null mouse would have greatly increased platelet activation and that spontaneous thromboses would develop because of decreased hydrolysis of pro-aggregatory ADP. However, deletion of *Cd39* was associated with a mild bleeding diathesis and poor platelet activation *in vivo* and *in vitro*. The demonstrated explanation is that decreased ectonucleotidase activity causes excess intravascular ADP concentrations and desensitization of the major platelet ADP receptor, $P2Y_1$.[184]

Increased fibrin deposition has also been noted in the pulmonary, renal, cardiac, cerebral and splenic vasculature of *Cd39*-null mice.[184] This probably represents activation of secondary hemostatic responses by activated endothelial cells in *Cd39*-null animals.

The *Cd39*-null mouse thus provides an interesting model for studying tumour metastasis because it has defects in both angiogenesis and in platelet activation and fibrin deposition. This decreased platelet function could cause decreased survival of circulating tumour cells in *Cd39*-null mice, while defective angiogenesis could prevent the growth of those tumour cells that did form micrometastases. Both factors would tend to limit the formation of

metastatic tumours. On the other hand, increased fibrin deposition would tend to increase the formation of micrometastases.

4.7.2. Methods and results

Because B16-F10 and LLC tumours seldom form tumour metastasis spontaneously after subcutaneous implantation, tumour cells were injected directly into the inferior vena cava and the mice observed for the development of pulmonary metastases. 1×10^5 cells LLC or 1.5×10^5 B16-F10 cells were injected into the inferior vena cava of C57BL/6X129svj strain wild-type and type-matched *Cd39*-null mice. After 17 days the mice were euthanased and the pulmonary metastases analyzed for size and number.[233]

Wild-type lungs became massively infiltrated with metastatic melanoma and lung carcinoma and, at 15 days after intravascular injection, tumour metastases had replaced the entire lung (figure 4.7.1). In contrast, *Cd39*-null lungs had limited tumour loads. Wild-type lung weights were also significantly increased over *Cd39*-null levels. (1738 ± 276 mg wild-type vs. 604 ± 42 mg *Cd39*-null. $P<0.01$ (Fig. 4.7.2)) (Tomokazu Hoshi, unpublished data).

To study the effect of apyrase on tumour metastasis, 0.3×10^5 B16-F10 cells plus or minus 0.2U/g soluble apyrase (grade VII apyrase, Sigma-Aldrich, Saint Louis, MO) were injected in the inferior vena cava of wild-type and *Cd39*-null mice. After 13 days, mice were euthanased and surface metastatic foci counted. In keeping with the increased in lung weights in wild-type

animals, 15 days after intravascular inoculation of 0.3×10^5 B16-F10 cells, the number of visible surface metastases was significantly greater in wild-type versus *Cd39*-null mice (205 ± 12 wild-type vs. 74 ± 62 *Cd39*-null. $P<0.05$).

The addition of apyrase, a soluble NTPDase, with the tumour cells at the time of inoculation, increased the number of visible metastases in both wild-type and *Cd39*-null mice (figure 4.7.3). The number of metastases increased by 60% in wild-type (205 ± 12 wild-type vs. 331 ± 33 wild-type plus apyrase, $P<0.02$) and by almost 300% in *Cd39*-null animals (74 ± 62 *Cd39*-null vs. 282 ± 37 *Cd39*-null plus apyrase. $P<0.005$). Importantly, there was no significant difference between the number of metastases in wild-type and *Cd39*-null mice after addition of apyrase (331 ± 33 wild-type plus apyrase vs. 282 ± 37 *Cd39*-null plus apyrase. $P>0.1$) (Tomokazu Hoshi, unpublished data).

Figure 4.7.1

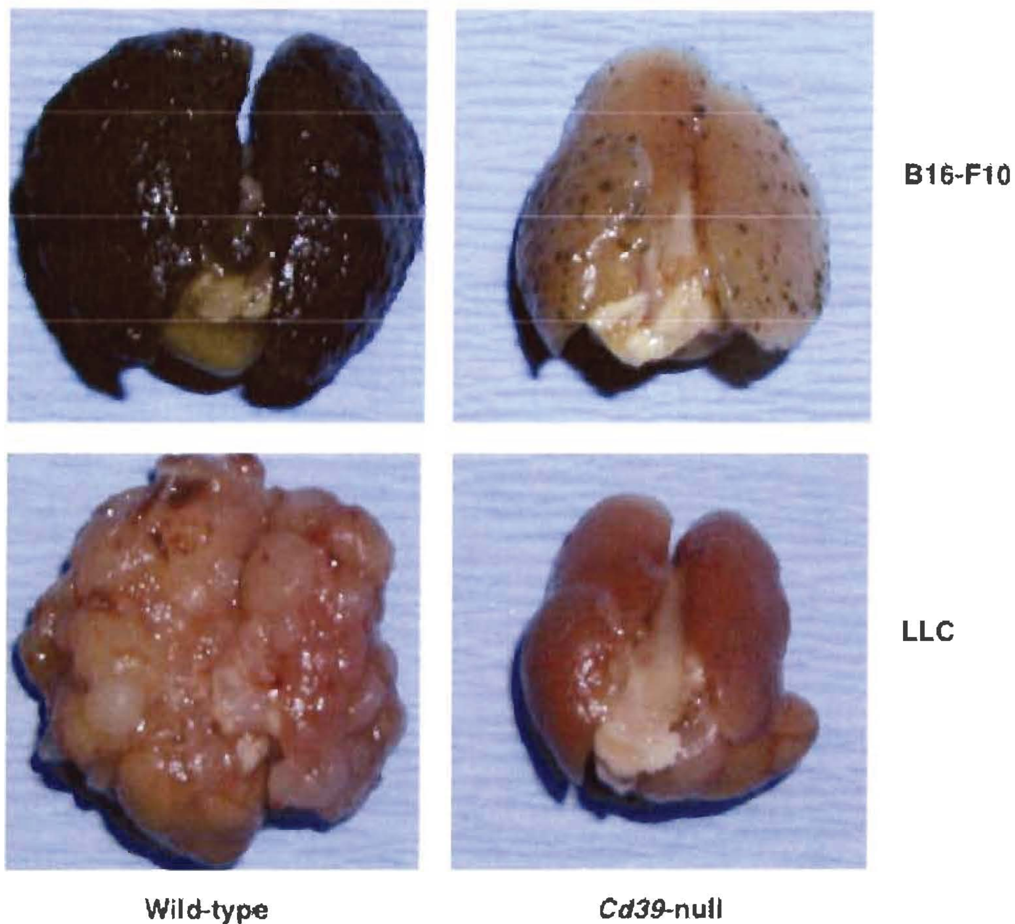


Figure 4.7.2

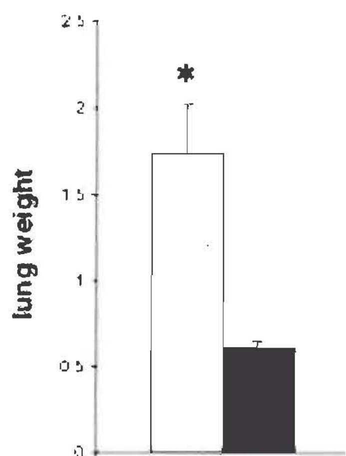


Figure 4.7.1

Representative examples of wild type (left) and *Cd39*-null (right) mouse lungs at 15 days after tumour injection. Upper panels are of B16-F10 melanoma metastases, and lower of Lewis lung carcinoma metastases. (Tomokazu Hoshi, unpublished data).

Figure 4.7.2

Wild type (□) and *Cd39*-null (■) lung weights 15 days after intravenous tumour injection. Mice were injected with 1.5×10^5 B16-F10 cells per 25g body-weight into the inferior vena cava. Bars represent mean weight ± s.e.m. *, $P<0.006$. (Tomokazu Hoshi, unpublished data).

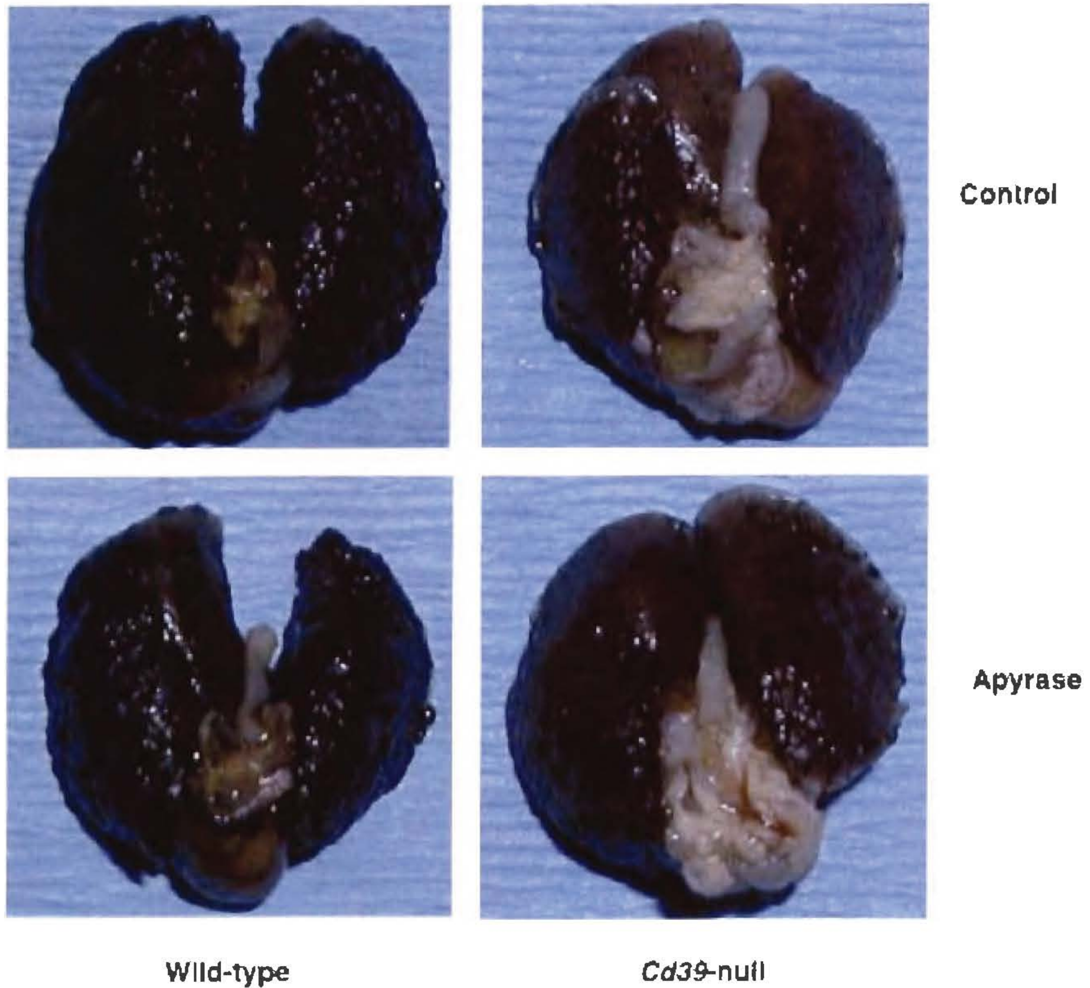


Figure 4.7.3 - Representative examples of wild type (left) and *Cd39*-null (right) mouse lungs at 15 days after B16-F10 tumour injection. Lower panels show the effect of apyrase while the upper panels represent control experiments. (Tomokazu Hoshi, unpublished data).

4.7.3. Discussion

Angiogenesis is required for the growth of both primary and metastatic tumours, and the lack of tumour metastases may reflect the angiogenesis defect of the *Cd39*-null mouse. In addition, platelet activation has been shown to facilitate tumour metastasis by protecting circulating tumour cells from NK cell-mediated elimination.[232] It has previously been shown that deletion of *Cd39* results in platelet dysfunction, because of $P2Y_1$ receptor desensitization secondary to increased pericellular nucleotide concentrations.[184] Platelet dysfunction in *Cd39*-null mice would render intravascular tumour cells more prone to immunological elimination and may further explain the decreased development of pulmonary metastases in *Cd39*-null mice.

This does not explain how the addition of soluble apyrase at the time of tumour inoculation increases the number of tumour metastases in *Cd39*-null mice to wild-type levels. A single dose of apyrase should not be sufficient to correct platelet function in *Cd39*-null mice in vivo.

Perhaps the most likely explanation is that apyrase modulates integrin activation states on the tumour cells themselves prior to injection and that these integrins facilitate binding to the pulmonary vasculature and the establishment of micrometastases.

If this is the case, it has important implications for the development of ectonucleotidase-based therapies. Soluble derivatives of CD39 have been

proposed as effective anti-thrombotic or anti-inflammatory therapies,[234] but our findings suggest that these may also increase the growth and/or metastasis of tumours.

CHAPTER 5:
NOVEL EXPERIMENTAL MODELS

DELETION OF *Cd39* RESULTS IN VASCULAR INTEGRIN DYSFUNCTION IN VITRO

Deletion of *Cd39* is associated with *in vivo* defects in both physiological and pathological angiogenesis. This results in abnormal angiogenesis in Matrigel™ plug, liver regeneration and myocardial infarction models of angiogenesis. With important therapeutic implications, deletion of *Cd39* also results in the decreased growth of both implanted and metastatic tumours.

Angiogenesis is known to involve several stages. Firstly, vessels dilate and become leaky in response to VEGF. This allows extravasation of plasma proteins, which form the provisional scaffold for endothelial migration.[2] This initial stage was preserved in *Cd39*-null animals undergoing angiogenesis in Matrigel™ plug and implanted tumour experimental models. In fact, increased fibrin deposition has been noted in the tissues of *Cd39*-null animals.[184]

Secondly, endothelial cells must proliferate and migrate towards the source of the angiogenic stimulus.[2] This migration is dependant on the action of extra-cellular proteases, including matrix metalloproteinases.[93] Cell matrix receptors like integrin $\alpha_V\beta_3$ and $\alpha_V\beta_5$ are also crucial mediators of endothelial cell migration.[34]

Finally, endothelial cells must form mature blood vessels in a process that requires endothelial tube formation, production of a new basement membrane and the recruitment of pericytes and smooth muscle cells around the new endothelium.[2] Excessive vessel formation is controlled by the induction of endothelial apoptosis. Several growth factors, in particular VEGF, promote endothelial survival, while many angiogenesis inhibitors, both endogenous and exogenous, promote endothelial apoptosis.[1] Close interaction between endothelial cells and pericytes protects endothelial cells from apoptosis following growth factor withdrawal.[35,36]

In order to further characterize the angiogenesis defect of the *Cd39*-null mice *in vitro*, we established primary endothelial cell cultures from wild-type and *Cd39*-null mice. We used these endothelial cells to model each of the above angiogenic steps *in vitro*. In particular, we studied the proliferative responses, resistance to apoptosis and production of matrix metalloproteinase of wild-type and *Cd39*-null endothelial cells. We also studied integrin activity in wild-type and *Cd39*-null endothelial cells, with a particular focus on integrin $\alpha_V\beta_3$.

5.1 ISOLATION OF MURINE PULMONARY ENDOTHELIAL CELLS

5.1.1 Methods

a) Solutions needed

- PBS/Heparin: 1 unit heparin/ml

- Endothelial Cell Media:

  200 ml Ham's F-12
  200 ml DMEM (low glucose)
  100 ml Fetal Bovine Serum
  Penicillin: Final concentration 100U/ml
  Streptomycin: Final concentration 100μg/ml
  L-Glutamine: Final concentration - 2mM
  Endothelial Mitogen: 25mg
  Heparin: 50mg
  Filter Sterilize and store at 4°C

- 0.1% Collagenase:

  Prepare 0.2% collagenase (Worthington type I) in 50ml PBS with Ca^{2+}/Mg^{2+}
  Autodigest at 37°C for 1 hour.
  Dilute to 0.1% in PBS with Ca^{2+}/Mg^{2+}

Filter Sterilize and store at 4°C

b) Preparation of antibody-conjugated beads

- Negative Selection Beads:

  10μl rat anti-mouse anti-FcγIII/II antibody
  50μl sheep anti-rat IgG dynabeads
  500μl PBS (with 2% FBS added)

- Positive Selection Beads:

  10μl rat anti-mouse ICAM-2 antibody
  50μl sheep anti-rat IgG dynabeads
  500μl PBS (with 2% FBS added)

- Incubate antibody-bead conjugates overnight at 4°C.
- Before use, wash three times in PBS (plus 2% FBS) by repeatedly placing the beads in the magnet, aspirating the supernatant and resuspending the beads in 8ml PBS (plus 2% FBS).

c) Tools required

- Surgical / Dissecting tools
- 14-gauge blunt cannula
- 140μm sieve
- 10 ml syringe
- 25-gauge needle

d) Isolation of murine pulmonary endothelial cells

Three age-, sex- and genotype-matched mice were used per preparation. The ideal age is 8-13 weeks old.

i) DAY 0

- The mice were anaesthetized by sub-peritoneal injection of avertin (20mg/ml) and euthanased by cervical dislocation.
- Avertin stock solution was prepared by dissolving 2.5g 2,2,2-Tribromoethanol in 5ml 2-methyl-2-butanol, and diluting the solution to a final volume of 200ml.
- Prior to dissection, the mouse was cleaned with 70% ethanol and the chest cavity opened.
- The lungs were perfused with approximately 8 ml of cold PBS/Heparin solution. This is done by injecting the solution into the right ventricle and cutting the left atrium to allow drainage of perfused liquid.
- The lungs and heart were collected into cold Ham's F-12.
- This procedure was repeated for each of the three mice.

The following steps were performed under the tissue culture hood

- Two 10cm tissue culture dishes were opened and 10 ml 70% ethanol aliquoted into the first dish and 10 ml endothelial media into the other.

- The lungs and heart in Ham's F-12 were poured into a 10cm tissue culture dish.
- The individual lung lobes were dissected away from the heart, dipped briefly in 70% ethanol and then placed into the media.
- Once all the lung pieces had been collected in media, all the tissue pieces were moved to a dry tissue culture dish.
- The tissue pieces were minced into very small pieces using sharp dissection scissors, aiming for a pâté/mousse consistency.
- The minced tissue was transferred to a 50ml tube and digested for 1 hour in 15ml 0.1% collagenase at 37°C. The tube was shaken occasionally to aid the digestion process.

Following digestion, the samples were returned to the tissue culture hood

- The digested tissue was homogenized by repeatedly drawing the collagenase/tissue mixture through a blunt 14-gauge cannula. Aim for about 5-10 slow passages to avoid over-homogenization.
- The mixture was filtered through the 140μm sieve into a 10cm tissue culture dish.
- The filtered cells were collected in 20ml endothelial cell media and spun down at 1200 rpm at 4°C for 5 minutes.
- The supernatant was carefully aspirated and the pellet resuspended in 12ml endothelial media. The resulting mixture was plated into two gelatin-coated T75 flasks.
- After 3-16 hours, the plates were washed well with PBS to remove any red blood cells or non-adherent dead cells.

ii) DAY 1 Negative selection

- Prior to the negative selection, the cells were cooled at 4°C for up to one hour to slow the cellular processes and avoid non-specific uptake of beads.
- Anti-FcγIII/II receptor antibody-coated beads in 3ml endothelial media were added to the adherent cells and incubated at 4°C for one hour. The plates were occasionally shaken to spread the beads across the plate.
- After visualizing the attachment of the magnetic beads to FcγIII/II-expressing cells, the cells were trypsinized and resuspended in 15ml endothelial media.
- The tube containing the media was placed into a magnet and the supernatant collected.
- These cells from the supernatant were plated into two gelatin-coated T75 plates.

iii) DAY 3 First positive selection

- As with the negative sort, the plates were first washed with PBS and cooled at 4°C for up to one hour prior to positive selection.
- Anti-ICAM-2 antibody-coated beads in 3ml endothelial media were added to the adherent cells and incubated at 4°C for one hour. The plates were occasionally shaken to spread the beads across the plate.
- After visualizing the attachment of the magnetic beads to ICAM-2-expressing endothelial cells, the cells were trypsinized and resuspended in 15ml endothelial media.
- The tube containing the media was placed into a magnet and the supernatant discarded.
- The cells from the pellet were plated into one gelatin-coated T75 plate. Halving the number of tissue-culture plates compensates for the fact that only a small percentage of the cells present prior to positive selection are endothelial.

iv) DAY 5-8 Second positive selection

- Depending on the yield and rate of growth of endothelial cells after the first positive selection, a second sort was performed on day 5-8. Selections were optimally performed when the cell culture was 70% confluent.
- After the second positive sort, the culture was split to two or three gelatin-coated T75 plates, depending on the yield of the endothelial preparation.

5.1.2 Results

Depending on the success of the preparation, once these two or three T75 plates are confluent, one can split the plates 1:3. Once these plates are confluent, the cells are cryopreserved in fetal bovine serum and 10% DMSO and stored at -180°C. Storing the cells at 500,000 cells per vial allows enough cells for one 10 cm plate upon replating.

In all, one can expect 15-20 vials of 500,000 cells following a successful mouse pulmonary endothelial cell preparation using three mice.

5.2 CHARACTERIZATION OF PULMONARY ENDOTHELIAL CELLS

5.2.1 Methods

a) Immunocytochemistry.

- The cells were grown to confluence in 6-well tissue culture plates, then the plate placed on ice.
- After washing with PBS, the cells were fixed for 3 minutes with a solution of 75% Acetone, 20% cold distilled water, 5% of buffered formalin (10% solution).
- The sample was then blocked with 7% rabbit serum (diluted in PBS) for 30 minutes at room temperature.
- The cells were incubated overnight at 4°C with the following primary antibodies:
  - Control: Rat IgG2A control (1μl/ml)
  - Specific: Rat anti-mouse CD31 (PECAM) (1μg/ml)
- The next morning, the wells were blocked with H_2O_2 (1:300 solution) for 10 min at room temperature. The plates were washed twice with PBS both before and after blocking.
- The cells were then incubated with rabbit anti-rat biotinylated secondary antibody (2μg/ml) for one hour at room temperature.
- Avidin-biotin complex, a mixture of streptavidin and biotinylated horse-radish peroxidase, was prepared and placed on the cells.

- Colour was developed by the addition of 3,3'-diaminobenzidine (DAB), which produces a brown end product by the action of horse-radish peroxidase.
- Haematoxylin and Eosin Staining was then performed as follows.
    - Haematoxylin for 15 seconds.
    - HCl (1%), Ethanol (70%) solution for 15 seconds.
    - NH_4OH for 15 seconds.
- Excess liquid was removed and the cells covered with crystal mount and placed at 37°C for 1 hour.

b) Immunofluorescence

- Wild-type and *Cd39*-null mouse lung endothelial cells were grown to 70% confluence in normal endothelial media, then detached with trypsin (0.05%) and allowed to attach to the gelatin-coated slides for 1 hour.
- Plated cells were then fixed with 4% paraformaldehyde for 20 minutes at room temperature.
- Fixed cells were treated with 0.5% Triton-X for 3 minutes, then blocked with 5% goat serum for 20 minutes at room temperature.
- Slides were incubated with primary anti-α_V antibody (1:200 dilution) for 1 hour at room temperature.
- After washing, the secondary antibody (anti-mouse fluorescent antibody) was applied at 1:200 concentration for 1 hour at room temperature.

- Nuclear staining was obtained using a 1:5000 solution of Hoescht for 5 minutes at room temperature.
- The slides were then covered and examined under a fluorescent microscope.

c) CD39 protein expression in wild-type and *Cd39*-null pulmonary endothelial cells.

Antibodies to murine Cd39 had previously been developed by direct inoculation of *Cd39* cDNA in pcDNA3 into rabbits.[184] We confirmed the expression of Cd39 protein by cultured mouse lung endothelial cells by Western blot analysis using this antibody.

i) Solutions used

- **Lysis Buffer**

RIPA Buffer - 10ml
Aproprotinin - 50μl
Leupeptin - 20μl
PMSF - 100μl of 100mM stock solution
NaFl - 50μl of 1M stock solution
DTT - 10μl of 1M stock solution

- **SDS-PAGE running buffer (10X)**

Glycine - 143 g

SDS - 10 g

Trizma - 30 g

Dilute in 1000ml water to produce a 10X solution

- **Transfer buffer (10X)**

  Tris - 58.4 g

  Glycine - 29.6 g

  Dilute in 1000ml water to produce a 10X solution

  Prior to use, dilute the 10X solution in 20% methanol, and 70% distilled water

- **PBS/tween solution**

  Phosphate buffered saline (PBS) - 1 litre

  Tween - 500μl (final concentration 0.5%)

- **5% Milk in PBS/tween**

  Dry non-fat milk powder - 5g

  PBS/tween - 10ml

ii) Experimental protocol

- Wild-type and *Cd39*-null mouse lung endothelial cells were cultured to 70% confluence in normal endothelial media. Prior to experimentation, the cells were starved overnight in a basal media containing 0.5% fetal bovine serum (FBS) and no endothelial mitogen.

- The adherent cells were collected in a 300μl solution of lysis buffer, centrifuged at 14000 rpm for 10 minutes, the supernatant conserved and the nuclear pellet discarded.
- Sample protein concentrations were determined using the Biorad DC Protein Assay.
- 30μg aliquots were then boiled in SDS-PAGE Sample Buffer containing 2% β-mercaptoethanol.
- The samples were then separated by SDS-PAGE electrophoresis at a constant voltage of 80mV in a 4-15% acrylamide gel. Constituents of the running buffer constituents detailed above.
- Protein was then transferred to PVDF membrane by Western blot at constant current of 75mA for 2 hours.
- The membranes were then blocked in 5% milk PBS/tween solution for 1 hour, prior to probing with primary antibody (1:1000 concentration rabbit anti-Cd39 antibody).
- The following day, the membranes were washed, then incubated with 1:10 000 concentration secondary antibody for one hour.
- Signal was then developed using the Supersignal chemiluminescent substrate (Pierce Biotechnology, Inc. Rockford, IL.).

5.2.2 Results

Prior to using the mouse lung endothelial cells in experiments, we had to analyze the endothelial purity generated by this preparation technique. All experiments in this project, including the endothelial characterizations of this chapter, were performed using endothelial cells after 5-8 passages in culture.

a) Cellular morphology

Initial confirmation of a high degree of endothelial purity using this technique came from visualization of the morphology of these cells in culture. Endothelial cells are described as having a typical "cobble-stone" morphology and evidence of contact inhibition under phase-contrast microscopy. Figure 5.2.1 shows a representative example of pulmonary endothelial cells in culture demonstrating this classical picture. Prior to positive selection, the cultures contain many elongated and spindle shaped cells, which probably are contaminating fibroblasts. These cells also grow in clumps because of a lack of contact inhibition. During purification these cells are removed and the classical endothelial morphology is seen.

b) Immunocytochemistry

In order to more accurately assess the endothelial purity of the mouse lung endothelial cell preparation, plated cells at passage 8 were stained with the

endothelial marker, CD31. CD31, also known as PECAM-1 (Platelet Endothelial Cell Adhesion Molecule-1), is a member of the immunoglobulin superfamily that mediates cell-to-cell adhesion. It is constitutively expressed on the surface of adult and embryonic endothelial cells and weakly expressed on peripheral leukocytes and platelets.[235]

Even at this late passage stage, the endothelial purity of the mouse lung endothelial cell preparation, as assessed by CD31 expression, was >98% (Figure 5.2.2 and 5.2.3). Only occasional cells fail to stain with CD31, indicating that use of cells after numerous passages is not associated with the overgrowth of contaminating cells.

In order to further characterize the endothelial nature of these cells we stained passaged cells for von Willebrand Factor, α_V-integrin, and β_3-integrin expression. von Willebrand Factor (vWF) is a multimeric plasma glycoprotein synthesized by endothelial cells and megakaryocytes. In plasma, vWF functions as a carrier for Factor VIII and is a mediator of initial platelet adhesion to subendothelium and of platelet-platelet interaction. vWF is concentrated in Weibel-Palade bodies in the cytosol of endothelial cells.[236]

The $\alpha_V\beta_3$ integrin is thought to have a crucial role in the control of angiogenesis and is upregulated in vivo in proliferating angiogenic endothelium.[117] Since cultured cells also exist in an activated, proliferating state we decided to test the expression of $\alpha_V\beta_3$ by immunocytochemistry.

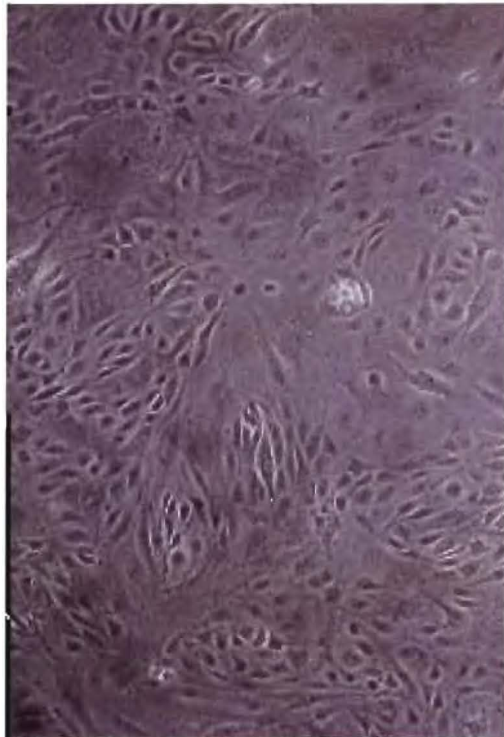


Figure 5.2.1 - Phase contrast microscopy of mouse lung endothelial cells in culture, showing typical cobblestone appearance of cultured endothelial cells

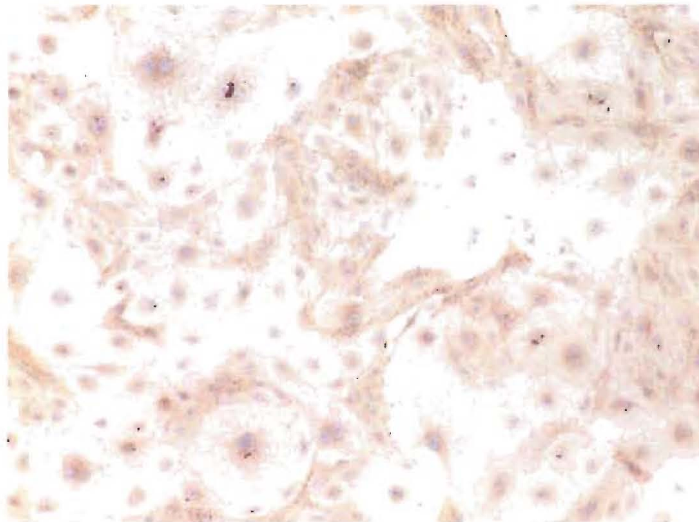


Figure 5.2.2 - Anti-CD31 immunocytochemistry stain of cultured murine pulmonary endothelial cells

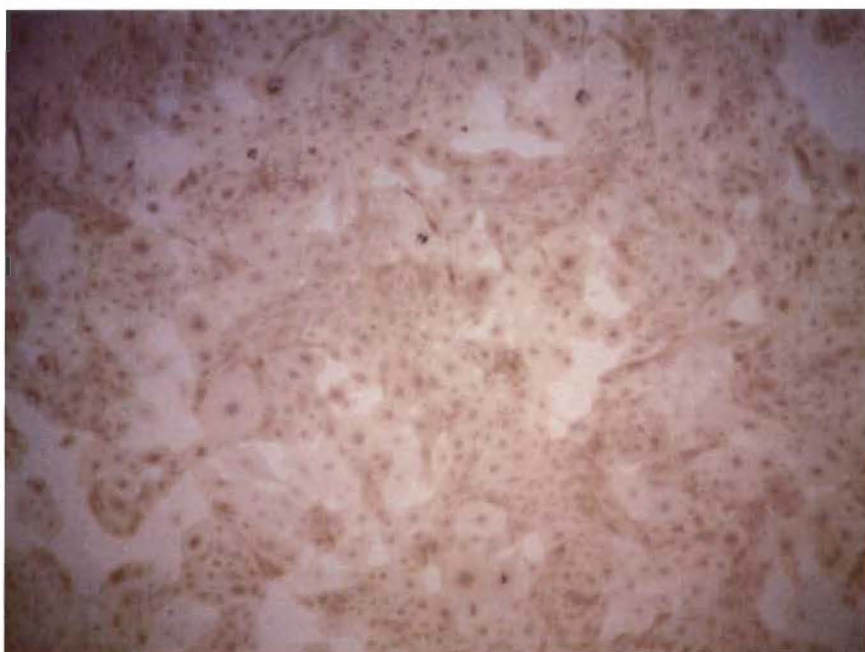


Figure 5.2.3 - Anti-CD31 staining of cultured pulmonary endothelial cells.

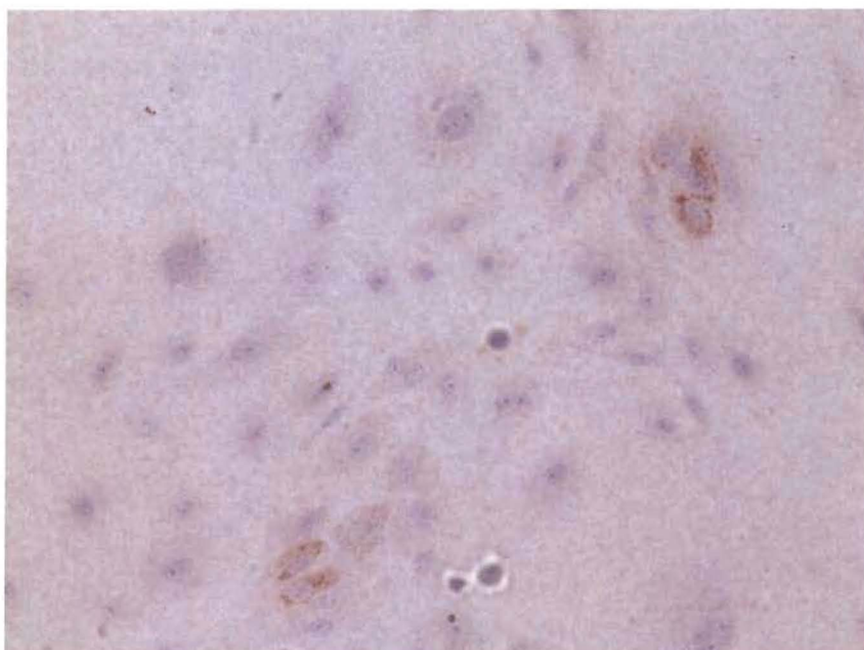


Figure 5.2.4 - von Willebrand factor expression on cultured pulmonary endothelial cells.

Unlike the uniform CD31 staining that was noted on the mouse lung endothelial cells, only occasional cells stained for von Willebrand factor (Fig 5.2.4). Although von Willebrand factor is normally expressed by endothelial cells, the loss of this factor in cultured endothelial cells following multiple passages is well described.[237] Also, the cultured cells failed to stain for α_V- or β_3-integrin (data not shown) but this is probably just indicative of low integrin expression levels and poor sensitivity of immunohistochemical integrin staining.

c) Immunofluorescence

As discussed in the literature review, integrin $\alpha_V\beta_3$ is thought to be crucial for normal endothelial function during angiogenesis. Therefore, because standard immunohistochemistry failed to show integrin $\alpha_V\beta_3$ expression on our endothelial cell culture, we used immunofluorescence to stain pulmonary endothelial cells for α_V expression.

Figure 5.2.5 shows representative examples of α_V-integrin expression on the surface of wild-type and *Cd39*-null endothelial cells. It can be seen that the α_V-subunit of integrin $\alpha_V\beta_3$ has preserved expression on the surface of cultured murine pulmonary endothelial cells. This is a further indicator of the endothelial nature of these cells.

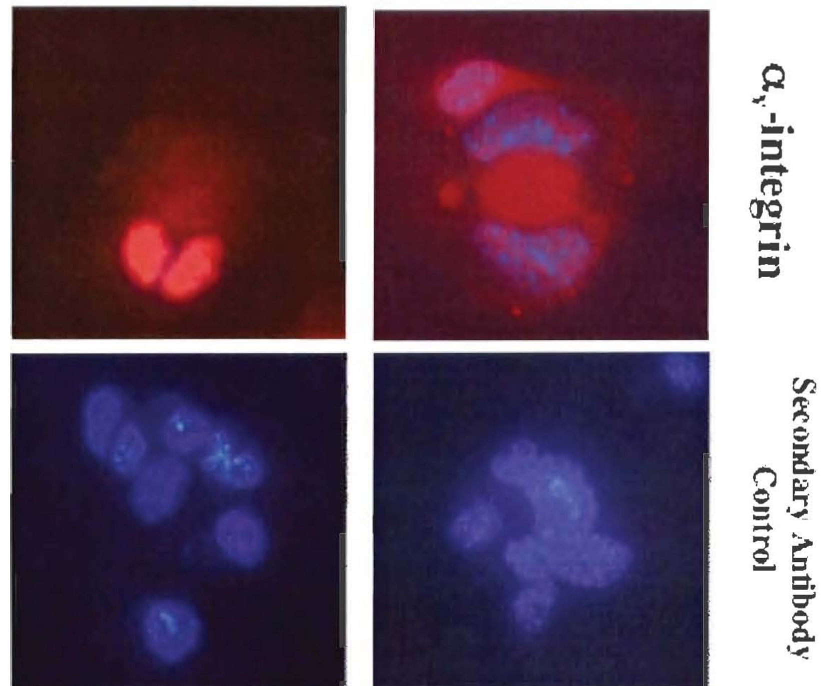


Figure 5.2.5 - Immunofluorescence staining of α_v-integrin expression (red) on wild-type (left) and *Cd39*-null (right) endothelial cells (upper panel). Negative control with only secondary antibody (Fluorescein-conugated rabbit anti-rat IgG) shown below. Cell nuclei are stained blue.

d) Cd39 protein expression

Although the expression of Cd39 on murine endothelium in vivo and its successful deletion in the *Cd39*-null mouse has been confirmed previously in our laboratory,[184] we wished to confirm that Cd39 is still expressed by passaged wild-type mouse lung endothelial cells. If Cd39 expression is down-regulated in culture in a manner analogous to the loss of vWF, it may compromise our future *in vitro* experiments.

Antibodies to murine Cd39 had previously been developed by direct inoculation of *Cd39* cDNA in pcDNA3 into rabbits.[184] We confirmed the expression of Cd39 in cultured mouse lung endothelial cells by Western blot analysis using this antibody. Whole lung lysate from wild-type C57/B6 mice was used as a positive control. As can be seen in Fig 5.2.7, wild-type mouse lung endothelial cells continue to express Cd39, while Cd39 expression is predictably absent in *Cd39*-null endothelial cells. Experiments documenting the presence of appropriate ATPase and ADPase activity on the surface of cultured microvascular endothelial cells were not repeated as this had previously been shown by others in the laboratory.[184]

In conclusion, the use of this protocol for isolation of pulmonary endothelial cells from wild-type and *Cd39*-null mice resulted in high-purity, expandable endothelial cultures, which continue to express a number of important endothelial surface proteins, including CD31, integrin $\alpha_V\beta_3$, and Cd39.

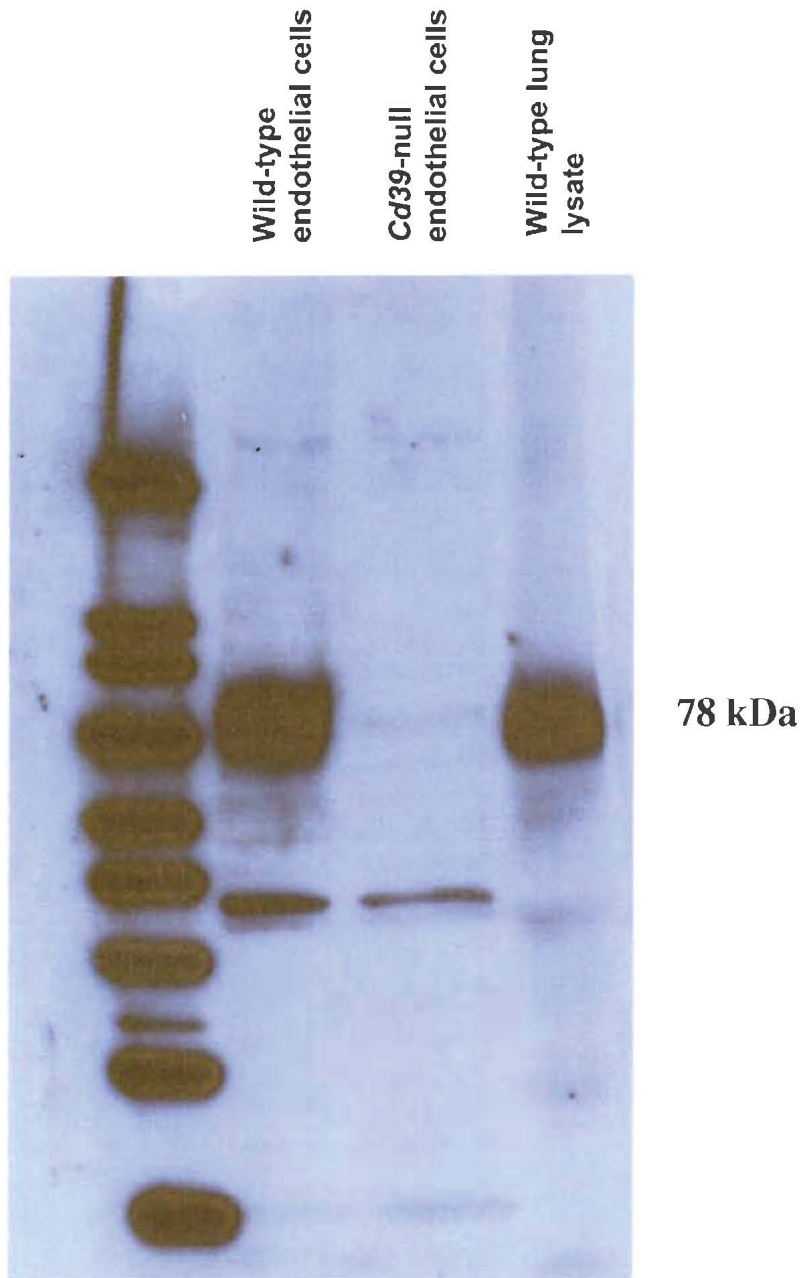


Figure 5.2.7 - Western-blot analysis of Cd39 expression on wild-type, and *Cd39*-null endothelial cells as well as wild-type whole lung lysates. Cd39 protein band is at 78 kDa.

5.3 ENDOTHELIAL PROLIFERATION ASSAYS

5.3.1 Introduction

Resting endothelial cells have extremely low cellular turnover rates. However, when activated during physiological and pathological angiogenesis, endothelial cells proliferate rapidly to provide cells for the developing vasculature. 10% of endothelial cells in tumours take up BrdU or [^{3}H] thymidine, indicating cellular proliferation, as opposed to 0.2% of resting endothelial cells.[159]

We have shown that *Cd39*-null mice have poor endothelial cell proliferation during liver regeneration, as opposed to wild-type animals, which show brisk endothelial proliferation on days 3-5 following hepatic resection. This, along with increased endothelial cell apoptosis in *Cd39*-null mice, results in defective angiogenesis and disordered liver regeneration following 70%-hepatectomy in *Cd39*-null mice. (Sun Xiaofeng, unpublished data).

Increasing evidence has emerged over the last several years, that signaling complexes of growth factor receptors, purinergic receptors, integrins and possibly *Cd39*, form in caveolae on the surface of endothelial cells.[195,198] It is hypothesized that these signaling complexes serve to integrate signals from growth factors, nucleotides and the extracellular matrix to promote endothelial proliferation, differentiation or migration. Deletion of *Cd39* could disturb these signaling complexes in several ways. Firstly, absence of the Cd39 protein in caveolae on the cell surface could prevent normal

structural assembly of this putative signaling complex. Secondly, absent ectonucleotidase activity could result in increased local nucleotide concentrations at the cell surface and result in P2-receptor over-activation or desensitization. This might result not only in defective nucleotide signaling, but also could cause defects in growth factor signaling because of alterations in signaling complex assembly and function. P2-receptor desensitization involves, as with other G-protein coupled receptors, phosphorylation of the receptor by a protein kinase, or uncoupling of the receptor from its associated G-protein.[177] This phosphorylation or uncoupling may affect targeting of P2-receptors to signaling complexes and, hence, signaling complex function.

During angiogenesis, endothelial cells come into contact with different extracellular matrix proteins, which provide important signals to these proliferating, migrating endothelial cells.[85] It is possible that different integrins, binding different matrix substrates, could co-localize in these putative signaling complexes and result in differing effects in vivo and in vitro depending on which matrix component the cell is bound.

For example, the integrin $\alpha_V\beta_3$, which binds RGD-sequences on both vitronectin and fibronectin, has been shown to associate with the purinergic receptor $P2Y_2$, providing a possible link between nucleotide signaling and integrin function.[195] Deletion of *Cd39* may thus inhibit endothelial cell proliferation on some, but not all, basement membrane substrates.

Because of these putative links between growth factor receptors, integrins and nucleotide-mediated signaling, we measured the in vitro proliferation of

wild-type and *Cd39*-null endothelial cells, firstly, in response to various growth factors, and, secondly, on a number of different extracellular matrix substrates.

5.3.2 Methods

a) BrdU uptake proliferation assay

- 96-well plates were coated overnight at 37°C with gelatin (1μg/ml), vitronectin (3μg/ml) or fibronection (40μg/ml). Prior to use, the wells were washed once with PBS.
- Wild-type and *Cd39*-null endothelial cells were cultured to 70%-confluence, then trypsinized, seeded at 10 000 cells/well in the matrix-coated 96-well plates and starved overnight in basal endothelial media containing 0.5% FBS.
- The following morning, the cells were stimulated with control media containing 20% FBS, or control media (20% FBS) plus insulin (10nM), vascular endothelial growth factor (VEGF, 10ng/ml), hepatocyte growth factor (HGF, 50ng/ml), fibroblast growth factor (FGF, 10ng/ml), or insulin-like growth factor 1 (IGF-1, 10ng/ml).
- After 24 hours, the proliferating cells were pulsed with BrdU (10μM) for 8 hours.
- BrdU uptake was measured using the BrdU Labeling and Detection Kit III (Roche Diagnostics Corp. Indianapolis, IN)

- Briefly, this involves first fixing the cells with 70% ethanol in HCl (0.5M), then incubating the cells with nuclease buffer to expose the incorporated BrdU.
- BrdU was then probed with a peroxidase-conjugated anti-BrdU antibody.
- Peroxidase substrate solution was added to the plates and the resulting green colour measured in a microtitre plate reader at 405nm with a reference wavelength of 490nm.
- The experiments were performed in triplicate and the results expressed as the average absorbance ± standard deviation.

b) [^{3}H] thymidine uptake proliferation assay

- The experiment was performed in the same manner as the BrdU uptake proliferation assay, except that, after 24 hour incubation with growth factors, the cells were pulsed for 8 hours with 1μCi/well methyl-^{3}H-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ).
- After 8 hours, the plates were washed in cold PBS and incubated for 15 minutes in 10% trichloroacetic acid (TCA).
- The cells were then washed with water, and 200μl of 0.2N NaOH was added to solubilize the cells.
- This volume was added to scintillation fluid and analyzed using the Beckman Liquid Scintillation System.

5.3.3 Results

a) Assessment of endothelial cell proliferation by incorporation of BrdU

The BrdU assay works on the principle that 5-bromo-2-deoxyuridine (BrdU) is a thymidine analogue, which partially replaces thymidine during the process of DNA synthesis. Following partial denaturation of double stranded DNA, this BrdU is detected using a peroxidase-conjugated anti-BrdU antibody. The development of a green colour following addition of peroxidase substrate is then measured on a microplate reader.[160] Incorporation of BrdU occurs during the S-phase of the cellular growth cycle and is, thus, a measure of in vitro cellular proliferation.

When plated on gelatin, wild-type and *Cd39*-null endothelial cells had equal BrdU incorporation in the 20% serum control group and in response to 20% serum plus 10ng/ml VEGF, 10nM insulin, 10ng/ml IGF-1, 50ng/ml HGF, or 10ng/ml FGF. Each of these growth factors failed to demonstrate in vitro the defective proliferation of *Cd39*-null endothelial cells, which had been noted in vivo. (Figure 5.3.1, Table 5.3.1).

Unfortunately, the growth factors failed to induce increased proliferation over the baseline control in either wild-type or *Cd39*-null endothelial cells. It has previously been shown that VEGF, in particular, can induce endothelial proliferation in vitro. In a study using primary hepatic sinusoidal endothelial cells, VEGF was shown to promote a 75% increase in proliferation over

baseline.[238] Unfortunately, in this study, the serum concentration used at baseline was not published making comparisons with our results difficult.

In various other studies using different endothelial cell populations, FGF, insulin, IGF-1 and HGF were each shown to promote endothelial proliferation to varying degrees,[239,240]

The lack of growth factor effects in this experiment can be explained by the high serum concentration used. Our earlier experiments using either no serum or low serum concentrations (0.5% FBS) resulted in no proliferation in either the wild-type or *Cd39*-null endothelial cells in both the control and growth factor-stimulated groups. (Shaun Jackson, unpublished results) The mouse lung endothelial cells used in our experiments are exquisitely sensitive to removal of serum and become apoptotic within hours. (Shaun Jackson, unpublished results). For this reason, we decided to use 20% FBS for the remainder of our proliferation assays.

Although, this results in the absence of significant increases in endothelial cell proliferation following growth factor stimulation, we are still able to compare the proliferation rates of wild-type and *Cd39*-null endothelial cells. The consistent feature in this experiment is that there is no decrease in *Cd39*-null endothelial cell proliferation in vitro compared with wild-type cells.

b) Assessment of endothelial cell proliferation by incorporation of ^{3}H-thymidine

In the thymidine incorporation assay, [^{3}H] Thymidine is incorporated into the newly synthesized DNA of proliferating cells. Subsequent quantification of [^{3}H] thymidine is then performed by scintillation counting of autoradiography. The measurement of BrdU incorporation by proliferating cells is often used in proliferation assays instead of ^{3}H-thymidine, because this avoids the use of potentially dangerous radioactive isotopes. However, the assessment of cellular proliferation has been shown to be equal using either method.[241]

We decided to confirm our BrdU uptake results by measuring the proliferation of wild-type and *Cd39*-null endothelial cells by ^{3}H-thymidine incorporation. (Figure 5.3.2, Table 5.3.2) As expected, the proliferation of wild-type and *Cd39*-null endothelial cells was equal in the 20% FBS control group (wild-type 100±39.2% vs. *Cd39*-null 97±44.1%. P=0.47). The proliferation of wild-type and *Cd39*-null endothelial cells was also equal in the VEGF-stimulated group (wild-type 135±19.4% vs. *Cd39*-null 133.4±36.6%. P=0.47).

Interestingly, in this experiment, there was a trend towards increased proliferation in the VEGF-treated groups. This is consistent with robust VEGF-induced activation of ERK1/2 in wild-type and *Cd39*-null endothelial cells (vide infra, Chapter 5.4). However, the increase in proliferation was not significant in either the wild-type (P= 0.12) or *Cd39*-null (P=0.17) groups.

The most likely explanation for the disparity between growth factor activation and cellular proliferation, is that phosphorylation of ERK1/2 is a more sensitive index of VEGF activity than proliferation, which is dependant on a number of other factors, including serum concentration.

The consistent feature, however, is that there is no decrease in the in vitro proliferation of *Cd39*-null endothelial cells compared with wild-type cells when plated onto gelatin.

c) Endothelial cell proliferation on different extracellular matrix components

Although we showed no difference in the proliferation of wild-type and *Cd39*-null endothelial cells on gelatin, we have hypothesized that *Cd39*-null cells might have poor proliferation on fibronectin or vitronectin because the integrin $\alpha_V\beta_3$ associates with purinergic receptors in cell-membrane signaling complexes. $\alpha_V\beta_3$ binds RGD-sequences on a number of extracellular matrix components, including fibronectin and vitronectin.

However, we showed no difference between the proliferation of wild-type and *Cd39*-null endothelial cells on either fibronectin and vitronectin (Figure 5.3.3, Table 5.3.3). There was equal BrdU uptake in wild-type and *Cd39*-null endothelial cells plated on fibronectin (wild-type 116.2%±11.6% vs. *Cd39*-null 123.5±14.3%. P=0.26) and on vitronectin (wild-type 33.4±17,9% vs. *Cd39*-null 37.7±8.5%. P=0.36). In keeping with previous results, the BrdU uptake of wild-type and cells *Cd39*-null plated on gelatin was also

equal (wild-type 100%±18.9% vs. *Cd39*-null 114.9±18.6%. P=0.19). These experiments were performed with 20% FBS and no added growth factors. Similar results were obtained for the growth factor treated groups (data not shown).

Although $\alpha_V\beta_3$ can bind the RGD-sequences of both fibronectin and vitronectin, there is a marked difference between the proliferation of endothelial cells on these two matrix substrates. The proliferation of wild-type and *Cd39*-null endothelial cells on fibronectin was equal to the proliferation noted for these cells on gelatin. However, there was markedly decreased proliferation of both wild-type and *Cd39*-null cells on vitronectin. BrdU uptake in wild-type and *Cd39*-null endothelial cells plated on vitronectin was approximately 1/3 of cells plated on gelatin.

Because vitronectin inhibits the proliferation of endothelial cells in our experiments, the in vitro proliferation assay is a poor model to study the interaction between vitronectin and $\alpha_V\beta_3$ in wild-type and *Cd39*-null endothelial cells. Therefore, we used adhesion assays to further study this interaction. These results are presented in chapter 5.8.

Table 5.3.1: Endothelial cell BrdU uptake in wild-type and *Cd39*-null endothelial cells stimulated with various endothelial growth factors. Experiment performed on 96-well plates pre-coated with gelatin (1μg/ml). Results expressed as a percentage of wild-type control BrdU uptake ± standard deviation.

	Control	**VEGF (10ng/ml)**	**Insulin (10nM)**	**IGF-1 (10ng/ml)**	**HGF (50ng/ml)**	**FGF (10ng/ml)**
Wild-type	100±20.9%	98.4±20.8%	96±24.4%	105.5±21.6%	96.4±11.5%	118.5±25%
***Cd39*-null**	93.3±20.6%	113.2±30.7%	100.9±13.3%	89.3±15.6%	87.8±15.4%	110.2±35.8%

Table 5.3.2: Endothelial cell ^{3}H-thymidine uptake in wild-type and *Cd39*-null endothelial cells stimulated with control (20% FBS) or VEGF (10ng/ml plus 20% FBS). Experiment performed on gelatin-coated 96-well plates. Results expressed as a percentage of the wild-type control group ± standard deviation.

	Control	**VEGF (10ng/ml)**
Wild-type	100±39.2%	135±19.4%
***Cd39*-null**	97±44.1%	133.4±36.6%

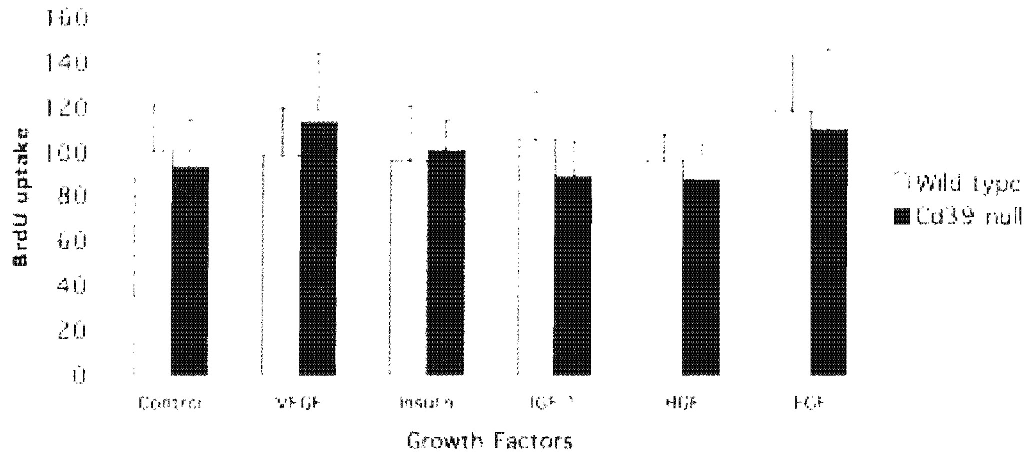


Figure 5.3.1 - Proliferation of wild-type and *Cd39*-null endothelial cells following growth factor stimulation. Proliferation is measured by the cellular uptake of BrdU, with results expressed as a percentage of wild-type control BrdU uptake. Bars show mean cellular BrdU uptake ± s.t.d.

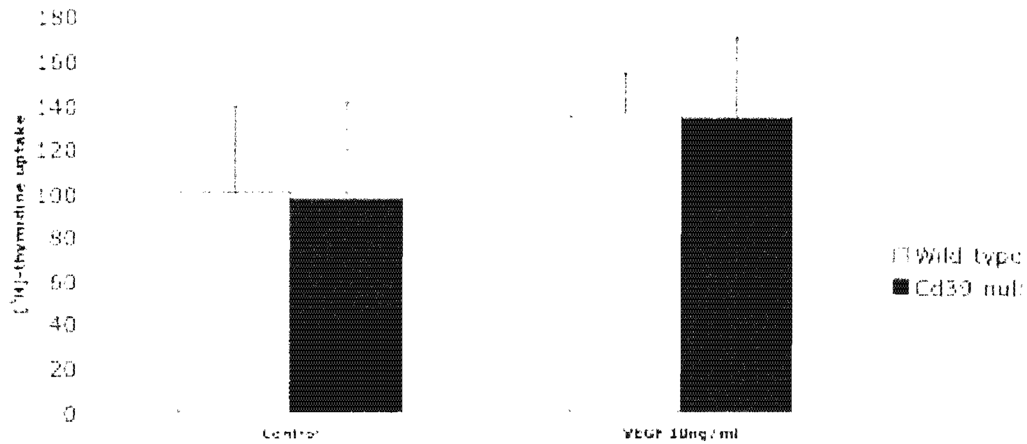


Figure 5.3.2 - Endothelial cell proliferation measured by the cellular uptake of [^{3}H]-thymidine. Results are expressed as a percentage of wild-type control [^{3}H]-thymidine uptake. Bars show mean cellular [^{3}H]-thymidine uptake ± s.t.d.

Table 5.3.3: Endothelial cell BrdU uptake in wild-type and *Cd39*-null endothelial cells stimulated with 20% FBS on 96-well plates pre-coated with gelatin (1μg/ml), fibronection (40μg/ml), or vitronectin (3μg/ml). Results expressed as a percentage of wild-type control BrdU uptake (gelatin sample) ± standard deviation.

	Gelatin	**Fibronectin**	**Vitronectin**
Wild-type	100±18.9%	116.2±11.6%	33.4±17,9%
***Cd39*-null**	114.9±18.6%	123.5±14.3%	37.7±8.5%

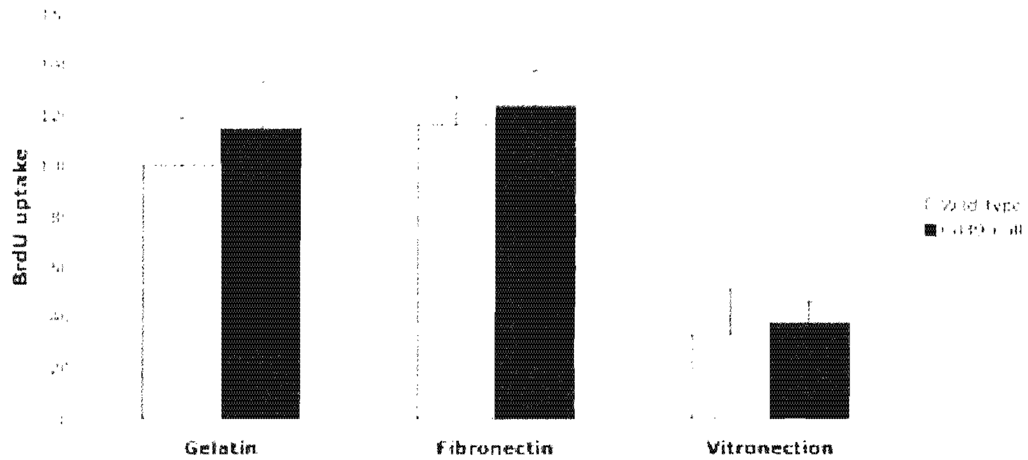


Figure 5.3.3 - Proliferation of wild-type and *Cd39*-null endothelial cells on various extracellular matrix substrates. Proliferation is measured by the cellular uptake of BrdU, with results expressed as a percentage of wild-type control BrdU uptake. Bars show mean cellular BrdU uptake ± s.t.d.

5.4 ACTIVATION OF ENDOTHELIAL ERK1/2 (p42/44-MAPK) BY VEGF

5.4.1 Introduction

We have hypothesized that there is a defect in growth factor signaling in *Cd39*-null endothelial cells in that deletion of *Cd39* results in disruption of growth factor signaling complexes. Although we failed to show a difference in the proliferation of wild-type and *Cd39*-null endothelial cells in response to VEGF, the high serum concentrations required in this experimental model prevented us from concluding that no defect exists in the intracellular signaling of individual growth factors.

We chose to study the intracellular signaling cascades of VEGF, in particular, because VEGF is the most potent angiogenic protein known.[54] VEGF promotes angiogenesis by increasing vascular permeability,[56] by stimulating endothelia proliferation and by protecting newly-formed vessels from regression by acting as an endothelial survival factor.[57] VEGF mediates several of these actions by the activation of mitogen activated protein kinases (MAPKs), in particular p42/44-MAPK (also known as extracellular signal-regulated kinase 1/2 (ERK1/2)).[54] Activation of ERK1/2 protects endothelial cells from apoptosis and promotes proliferation by promoting entry into the cell cycle.[242]

Because of the importance of VEGF-induced activation of endothelial ERK1/2 in angiogenesis, we measure this activation in wild-type and *Cd39*-

null endothelial cells. ERK1/2 activation was assessed by Western blot analysis using a phosphospecific antibody recognizing Threonine 202/Tyrosine 204 phosphorylation of ERK1/2 (Cell Signaling Technology, Inc. Danvers, MA).

5.4.1 Methods

- Wild-type and *Cd39*-null mouse lung endothelial cells were cultured to 70% confluence in normal endothelial media. Prior to experimentation, the cells were starved overnight in a basal media containing 0.5% fetal bovine serum (FBS) and no endothelial mitogen.
- The cells were then stimulated for 5 minutes with media containing VEGF (10ng/ml) or control media without growth factors or serum.
- Western blot analysis was performed as described in 5.2.1(c), using anti-phospho-ERK1/2 (Thr202, Tyr204) as the primary antibody.
- After development, the membranes were stripped using Restore Stripping Buffer (Pierce Biotechnology, Inc. Rockford, IL.) and reprobed with antibodies to unphosphorylated ERK1/2 to correct for differences in protein loading for each sample.
- Unphosphorylated ERK1/2 levels were then measured as above.
- Results are expressed as the ratio of phosphorylated ERK1/2 to total ERK1/2. Results are the average of three independent experiments ± standard deviation.

5.4.2 Results

VEGF induced rapid and equal phosphorylation of ERK1/2 in wild-type and *Cd39*-null endothelial cells at 5 minutes (wild-type 100±6.8% vs *Cd39*-null 99.7%±6.6%. P=0.48. Figure 5.4.1. Table 5.4.1.). This implies that deletion of *Cd39* does not result in defective VEGF-signaling. One could argue that without prior stimulation with nucleotides, the P2-receptor desensitization that we have hypothesized would disrupt signaling complexes on the surface of endothelial cells would not have occurred. However, we have shown defective *Cd39*-null endothelial cell adhesion and integrin signaling in the absence of nucleotide pre-treatment (results shown in chapters 5.8 and 5.9). Because pre-treatment with soluble NTPDase corrected these defects, we have concluded that certain P2-receptors on *Cd39*-null endothelial cells are desensitized at baseline in culture – probably because of local paracrine nucleotide release by endothelial cells.

Interestingly, the unstimulated ERK1/2 activation state of wild-type endothelial cells was significantly greater than that of *Cd39*-null endothelial cells (wild-type 18.2±2.3% vs *Cd39*-null 6.2%±2.0%. P=0.001). Thus, even at the baseline state in culture, there is a difference in the activation of state of the important signaling molecule ERK1/2. However, this difference was not seen following VEGF stimulation leading us to feel confident in concluding that no defect in VEGF signaling exists in *Cd39*-null endothelial cells in vitro.

Table 5.4.1: Relative phosphorylation of ERK1/2 following stimulation of wild-type and *Cd39*-null endothelial cells for 5 minutes. Relative ERK1/2 phosphorylation is expressed as the intensity of the phosphorylated ERK1/2 divided by the total ERK1/2. Results are expressed as a percentage of maximal phosphorylation ± standard deviation.

	Control	**VEGF (10ng/ml)**
Wild-type	18.2±2.3%	100±6.8%
***Cd39*-null**	6.2±2%	99.7±6.6%

Figure 5.4.1

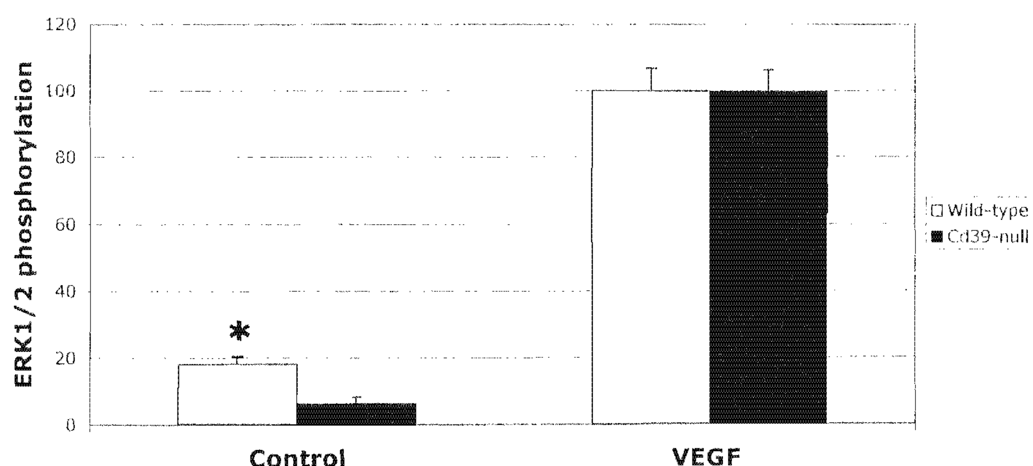


Figure 5.4.1 - Phosphorylation of ERK1/2 in wild-type and *Cd39*-null endothelial cells following stimulation with VEGF for 5 minutes. Results shown are a ratio between phosphorylated and unphosphorylated (total) ERK1/2 for each experimental condition. Results are expressed as a percentage of wild-type VEGF-stimulated ERK1/2 phosphorylation. Bars show mean ERK1/2 phosphorylation ± s.t.d.

5.5 ENDOTHELIAL APOPTOSIS ASSAYS

5.5.1 Introduction

The balance between endothelial cell survival and apoptosis has been shown to play a major role in the development of tumour vasculature. In order to promote angiogenesis, tumour cells frequently secrete endothelial cell survival factors such as VEGF. Immature blood vessels seem particularly prone to endothelial cell apoptosis and vessel regression following the inhibition of this tumour-derived VEGF.[36]

In addition, tumours transplanted into mice deficient in the pro-apoptotic molecules Bax or acid sphingo-myelinase were more resistant to radiotherapy and contained a lower incidence of endothelial cell apoptosis than the same tumours transplanted into wild-type animals.[243]

Therefore, the inhibition of endothelial survival factors or the activation of endothelial cell apoptosis is an attractive anti-tumour strategy.[244] In fact, several anti-angiogenic therapies currently undergoing clinical trials seem to exert at least part of their anti-tumour action by promoting endothelial cell apoptosis, including neovastat,[245] angiostatin[47] and endostatin.[246]

Purine signaling has an important role to play in altering this balance between cellular survival and apoptosis in a number of important physiological and pathological settings. For example, the turnover of keratinocytes in the stratified squamous epithelium of the skin is controlled

by differential expression of P2-receptors in each distinct epithelial layer. Proliferating cells in the basal stratum spinosum express $P2Y_1$, while differentiated keratinocytes express $P2X_5$ and apoptotic surface cells in the stratum corneum express $P2X_7$. A similar functional relationship has been demonstrated in intestinal epithelial cells, astrocytes, thymocytes and immune cells.[247]

Extracellular ATP and adenosine have both been shown to cause apoptosis in cultured endothelial cells, although the mechanism differs in different endothelial cell populations. In a study of porcine endothelial cells, extracellular ATP and ADP, probably acting through $P2X_7$, were shown to activate NF-κB and induce apoptosis.[206] On the other hand, in cultured human and bovine pulmonary artery arterial cells, extracellular ATP and adenosine both caused apoptosis, but the effect of ATP was dependent on the generation of adenosine by ectonucleotidases and on the uptake of adenosine into cells.[207]

These differing mechanisms for the induction of endothelial cell apoptosis by extracellular nucleotides raise interesting questions about the role of CD39 in this setting. CD39 is responsible for both the hydrolysis of ATP and, in turn, for the generation of adenosine via CD73. Immature angiogenic endothelial cells in *Cd39*-null mice might, therefore, be prone to apoptosis because of high levels of extracellular ATP, or protected from apoptosis because of the failure to generate adenosine. For this reason, we chose to investigate the role of extracellular nucleotides in the development of apoptosis in cultured wild-type and *Cd39*-null mouse lung endothelial cells.

5.5.2 Methods

a) Staining apoptotic cells using ApopTag® Peroxidase Kit

- Wild-type and *Cd39*-null endothelial cells were grown to 70% confluence in 6-well tissue culture plates, then treated with 200μM or 2mM ATP for 4 hours.
- Apoptosis induction was assessed by staining the plated cells with the ApopTag® Peroxidase Kit (CHEMICON International, Temecula, CA)
- The plated cells were fixed in 1% paraformaldehyde in PBS, pH 7.4 for 10 minutes at room temperature.
- After three washes with cold PBS, the endogenous peroxidase was quenched in 3% H_2O_2 in PBS for 5 minutes.
- After three more washes with cold PBS, equilibration buffer (ApopTag® Peroxidase Kit, CHEMICON) was placed on the cells for at least 10 seconds, and then working strength TdT enzyme (ApopTag® Peroxidase Kit, CHEMICON) was incubated on the cells for 1 hour at 37°C.
- After washing, anti-digoxigenin peroxidase conjugate (ApopTag® Peroxidase Kit, CHEMICON) was applied for 30 minutes at room temperature and the colour was developed by staining with peroxidase substrate (ApopTag® Peroxidase Kit, CHEMICON).
- Plates were then counterstained in 0.5% methyl green and covered.

- Positively stained apoptotic cells were then counted under the microscope and results expressed as the average number of cells stained per high-power field in three separate tissue culture wells.

b) Measurement of endothelial apoptosis by flow cytometry

- Wild-type and *Cd39*-null endothelial cells were treated with staurosporine 200nM or a range of concentrations of ATP, and Adenosine for 16 hours.
- Both adherent and floating cells were then harvested, washed twice with cold PBS and fixed overnight in 100% ethanol at -20°C. After fixation, the cells were again washed twice in cold PBS before being stained in propidium iodide buffer for one hour at room temperature.
- The propidium iodide buffer consisted of 0.1% TritonX-100, 0.1 mM EDTA (pH 7.4), 0.05 mg/ml RNAse A (50 U/mg) and 50 μg/ml propidium iodide in phosphate buffered saline (pH 7.4).
- DNA staining of permeabilized cell stained with propidium iodide was then performed by fluorescence-activated cell sorter (FACS) analysis with excitation at 488nm (blue) and detection at 620nm (red).

5.5.3 Results

a) Staining apoptotic cells using ApopTag® Peroxidase Kit

Induction of apoptosis results in the activation of a nuclease, which cuts genomic DNA between nucleosomes and generates DNA fragments with

lengths corresponding to multiples of 180 base pairs. This results in the generation of multiple free 3'-OH DNA termini, which are absent in normal and proliferative nuclei.[248] The reagents provided in the ApopTag® Peroxidase Kit (CHEMICON International, Temecula, CA) are designed to label these free 3'-OH DNA termini in situ with digoxigenin-labeled nucleotides using the enzyme terminal deoxynucleotidyl transferase (TdT). An anti-digoxigenin antibody is then allowed to bind to the digoxigenin-conjugated nucleotide oligomer. The peroxidase reporter molecule conjugated to the antibody generates an intense localized stain from chromogenic substrates, allowing the detection of apoptotic cells.[249]

We compared the induction of apoptosis in plated wild-type and *Cd39*-null endothelial cells following 4 hours of treatment with ATP at concentrations of 200μM and 2mM. Apoptotic cells where then stained with the ApopTag® Peroxidase Kit and apoptotic cells counted manually. Apoptosis is reported as the average number of apoptotic cells per high-power field with three wells of a 6-well plate per condition.

Figure 5.5.1 and table 5.5.1 show apoptosis induction rates for wild-type and *Cd39*-null endothelial cells following treatment with 200μM and 2mM ATP. As expected from previous studies, higher concentrations of ATP induced increased apoptosis in both wild-type and *Cd39*-null endothelial cells. However, there was no significant difference in apoptosis induction between wild-type and *Cd39*-null cells at either 200μM ATP (P=0.15) or at 2mM ATP (P=0.13).

Although a trend towards increased apoptosis in *Cd39*-null endothelial cells is observed at the higher ATP concentration, counting of apoptotic cells is both tedious and prone to wide ranges of error. For this reason, we cannot conclude that *Cd39*-null endothelial cells are more prone to apoptosis following treatment with ATP. In order to increase the accuracy of our assessment of apoptosis induction, we decided to measure DNA fragmentation by flow cytometry.

b) Measurement of endothelial apoptosis by flow cytometry

An alternate method for assessing DNA fragmentation in apoptotic nuclei is the measurement by flow cytometry of the percentage of nuclei with hypodiploid content.[250] This is performed by staining nuclear DNA with propidium iodide, a dye that binds the DNA helix and fluoresces strongly red. Because propidium iodide also stains double-stranded RNA, this is removed by the prior addition of ribonuclease A (RNAse A).

Following staining of permeabilized cells with propidium iodide and FACS analysis, the normal stages of the cell cycle are denoted by the major diploid peak (G_0/G_1 phase), a hyperdiploid region (S phase) and a tetraploid peak (G_2/M phase). The region below the G_0/G_1 peak is designated A_0 and is indicative of cells undergoing apoptosis-associated DNA degradation. (Figure 5.5.2).

Table 5.5.1 Number of apoptotic cells per high-power field following staining of apoptotic nuclei (± standard deviation)

ATP Concentration	Control	200μM	2mM
Wild-Type	0.3 ± 0.6	1.5 ± 1.1	5.6 ± 3.1
***Cd39*-null**	0.7 ± 0.0	2.9 ± 0.8	13.6 ± 7.2
P-values	0.21	0.15	0.13

Figure 5.5.1:

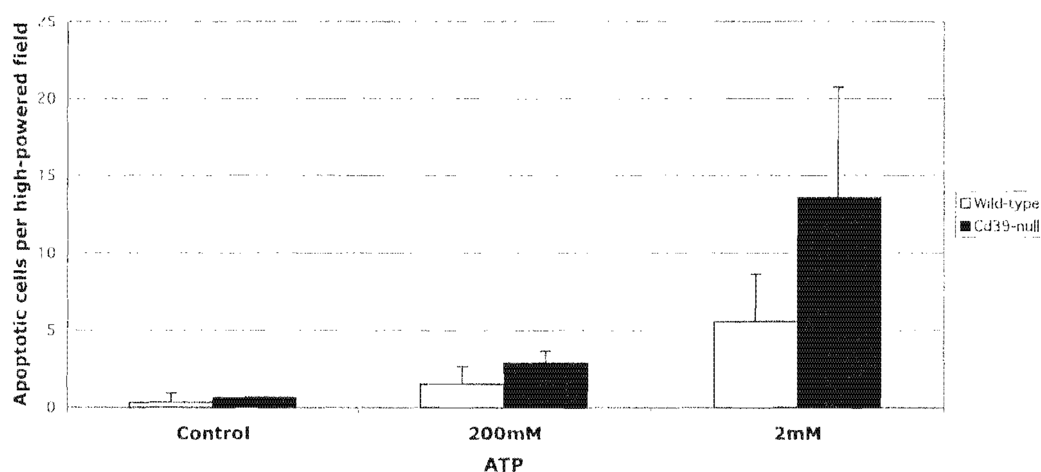


Figure 5.5.1 - ATP induction of apoptosis in wild-type and *Cd39*-null endothelial cells by immunohistochemical staining of apoptotic cells using the ApopTag® peroxidase kit. Results are expressed as the number of apoptotic cells per high-powered field ± s.t.d.

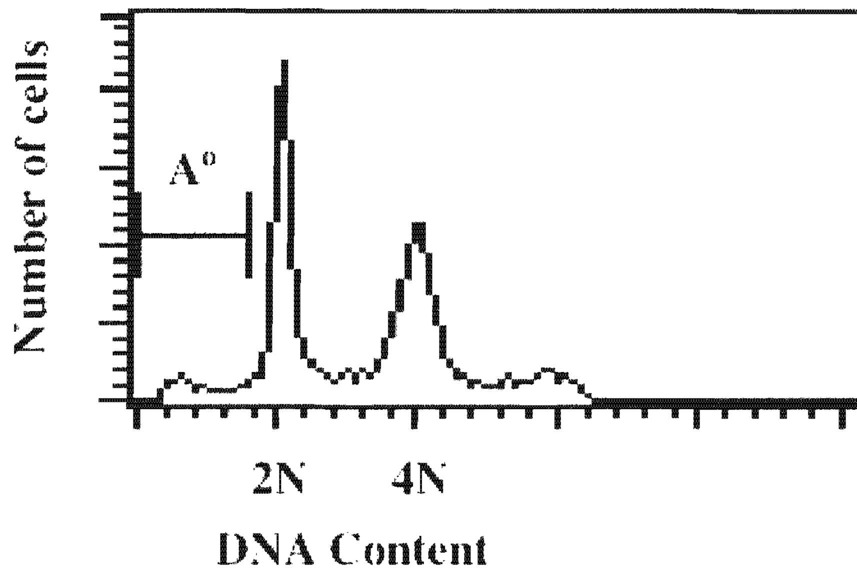


Figure 5.5.2 - Cell cycle analysis by FACS

Normal stages of the cell cycle following propidium iodide staining of nuclear DNA and FACS analysis. The major diploid peak (2N) represents G_0/G_1, the hyperdiploid region between 2N and 4N the S phase, and the tetraploid peak (4N) the G_2/M phase. The region A° represents apoptotic cells undergoing DNA degradation.

For flow cytometry analysis, wild-type and *Cd39*-null endothelial cells were grown to confluence and then treated with varying concentrations of ATP and adenosine for 16 hours. Staurosporine (200nmol/l) was used as a positive apoptotic control. Staurosporine, derived from the bacterium Streptomyces staurosporeus, is a relatively non-selective protein kinase inhibitor, which is often used experimentally to induce apoptosis.[251] After induction of apoptosis, both adherent and floating cells were harvested, permeabilized and stained with propidium iodide. The cells were analyzed by flow cytometry and the region below the G_0/G_1 peak gated. Apoptosis induction results are expressed as the percentage of the total cell population that is gated and are an average of three independent experiments.

This protocol provides a far more accurate method of determining apoptosis induction than can be achieved by manually counting cells stained using the ApopTag® kit. Annexin V staining is a very sensitive index of cellular plasma membrane pertubation and was not used. We did, however, determine the induction of apoptosis in wild-type and *Cd39*-null endothelial cells following treatment with ATP or adenosine by cytofluorometric analysis using propidium iodine. We added adenosine because we wished to determine whether differences in apoptosis induction between wild-type and *Cd39*-null endothelial cells occurred because of differences in ATP concentrations at the cell surface or because of altered adenosine generation.

In addition, we decided to use lower ATP concentrations for the FACS experiments than was used in the ApopTag® experiments. Instead of using 200μM and 2mM ATP, we used 100μM and 1mM ATP. The reasons for this decision were: firstly, that these concentrations of ATP would better reflect the nucleotide concentrations to which endothelial cells are exposed physiologically;[180] and, secondly, that using high concentrations ATP solutions in vitro could expose the endothelial cells to additional stresses unrelated to nucleotide signaling. For example, preparation of a 100mM ATP stock solution requires the addition of 170mM NaOH solution to correct to pH to 7.4. Therefore, the addition of high concentrations of ATP would alter the Na^+ concentration of the culture media and may also alter the pH because of limitations in the accuracy of our pH correction.

Figure 5.5.3 and Table 5.5.2 show apoptosis induction of wild-type and *Cd39*-null endothelial cells following treatment with 100μM and 1mM ATP. Figure 5.5.4 and Table 5.5.3 show corresponding results for 100μM and 1mM adenosine treatment.

As can be seen from these results, both wild-type and *Cd39*-null cells showed minimal induction of apoptosis by either ATP or adenosine treatment. The reasons for this are not readily apparent because previous studies in our laboratory have shown apoptosis induction of 9.6% and 16.1% following treatment of human umbilical vein endothelial cells (HUVECs) with 100μM and 1mM ATP, respectively.[252] Adenosine was not used to induce endothelial apoptosis in this project.

It is possible that the different source of endothelial cells used in these previous experiments account for the observed differences in results obtained. Primary pulmonary endothelial cells may be more resistant to apoptosis than commercial HUVEC cell lines. Indeed, 200nM staurosporine induced 69.24% apoptosis in HUVECs but induced only half the amount of apoptosis in our pulmonary endothelial cells.

In our experiments, 1mM ATP induced significantly greater apoptosis in *Cd39*-null endothelial cells than wild-type cells (wild-type 1.2 ± 0.06% vs. *Cd39*-null 1.7 ± 0.1%. P=0.001). Although statistically significant, these results are not clinically significant as induction of less than 2% apoptosis could not explain the angiogenesis defect observed in *Cd39*-null animals. Similarly, although there was also statistically significant increases in apoptosis induction in *Cd39*-null cells following 100μM and 1mM adenosine treatment (P=0.005 and 0.004, respectively), the level of apoptosis induction would almost certainly not have a significant effect in vivo.

An interesting explanation for this finding could be that C57BL/6 mice are relatively more resistant to the cytotoxic effects of high-dose ATP because of a naturally occurring mutation in the $P2X_7$ receptor.[253] Adriouch et al. have reported an allelic mutation (P451L) in the cytoplasmic tail of $P2X_7$ in a number of commonly used mice, including C57BL/6 (but not BALB/c). This mutation results in significantly decreased ATP-induced $P2X_7$ pore formation.[253] This is consistent with previous work showing that transfected murine $P2X_7$ is less sensitive to ATP than the rat or human counterpart.[254,255]

As all of our *in vitro* experiments use endothelial cells derived from C57BL/6 mice, it is possible that the lack of demonstrated apoptosis occurs independent of Cd39 ectonucleotidase function -- an attractive hypothesis given the significant effect of ATP on human umbilical vein endothelial cells using the same experimental methodology. It would be interesting to repeat these experiments on a BALB/c background, but unfortunately, we have been unable to successfully breed BALB/c *Cd39*-null mice to date, perhaps because of this backgrounds increased sensitivitiy to ATP.

However, given these in vitro findings, we can be relatively confident in concluding that the observed increase in endothelial cell apoptosis in *Cd39*-null animals during liver regeneration is not explained by altered induction of endothelial apoptosis by extracellular ATP or adenosine. Alternate mechanisms, for example changes in vascular integrin function, may be responsible for this finding.

Finally, it is interesting to note that our positive control, staurosporine, induced significantly greater apoptosis in wild-type endothelial cells than *Cd39*-null cells (wild-type 36.4 ± 4.4% vs. *Cd39*-null 25.5 ± 5.2. P=0.024). *Cd39*-null mice have an increased incidence of spontaneous malignancy, in particular liver tumours and lymphoproliferative malignancies, later in life (Sun Xiaofeng, unpublished observation). Of course, we have not formally studied the induction of apoptosis in hepatocytes or lymphoid cells, but it is intruiging to postulate that this increased tumour formation is due, in part, to decreased cellular apoptosis in *Cd39*-null tissues.

Table 5.5.2: Percentage apoptotic cells following ATP treatment by FACS analysis (± standard deviation)

ATP	**0**	**100μM**	**1mM**	**Staurosporine 200nM**
Wild-type	1.8 ± 0.2	1.7 ± 0.5	1.2 ± 0.1	36 ± 4.4
***Cd39*-null**	2.1 ± 0.4	2.1 ± 0.3	1.7 ± 0.1	26 ± 5.2
P Values	0.16	0.17	0.001	0.02

Table 5.5.3: Percentage apoptotic cells following adenosine treatment by FACS analysis (± standard deviation)

Adenosine	**0**	**100μM**	**1mM**	**Staurosporine 200nM**
Wild-type	1.8 ± 0.2	1.6 ± 0.1	2.2 ± 0.2	36.4 ± 4.4
***Cd39*-null**	2.1 ± 0.4	2.2 ± 0.2	3.6 ± 0.4	25.5 ± 5.2
P values	0.16	0.005	0.004	0.02

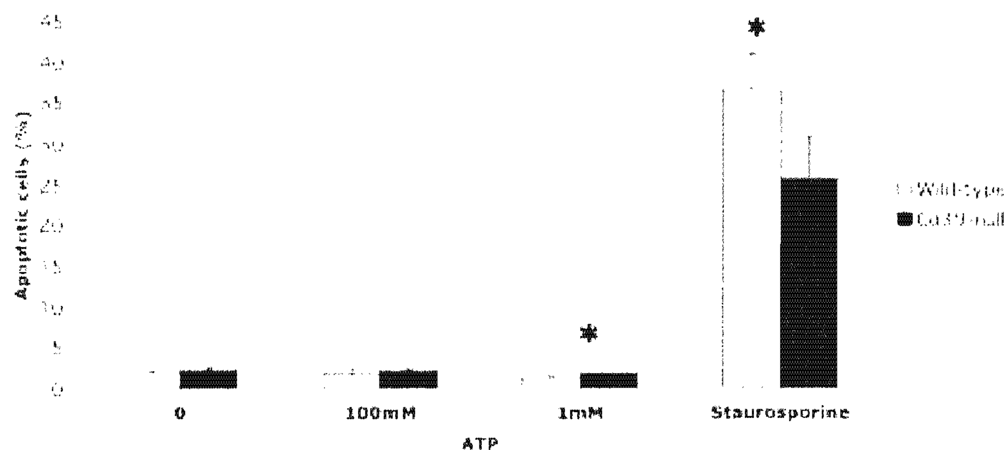


Figure 5.5.3 (a) - ATP induction of apoptosis in wild-type and *Cd39*-null endothelial cells by FACS analysis. Results are expressed as the number of gated apoptotic cells ± s.t.d. *, $P<0.05$. Figure 5.5.3 (b) (shown overleaf) shows FACS analysis of nuclear DNA content. Gated area A° represents hypodiploid apoptotic DNA.

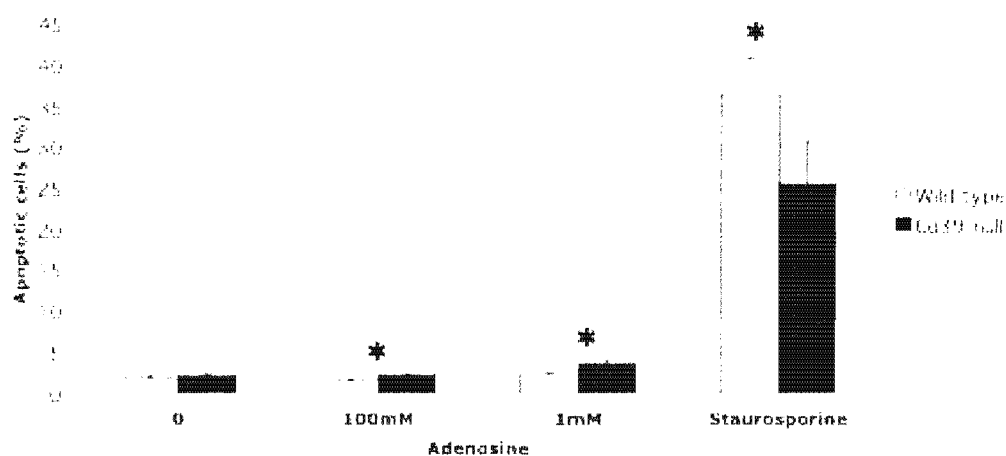


Figure 5.5.4 (a) - Adenosine induction of apoptosis in wild-type and *Cd39*-null endothelial cells by FACS analysis. Results are expressed as the number of gated apoptotic cells ± s.t.d. *, $P<0.05$. Figure 5.5.4 (b) (shown overleaf) shows FACS analysis of nuclear DNA content. Gated area A° represents hypodiploid apoptotic DNA.

Figure 5.5.3 (b)

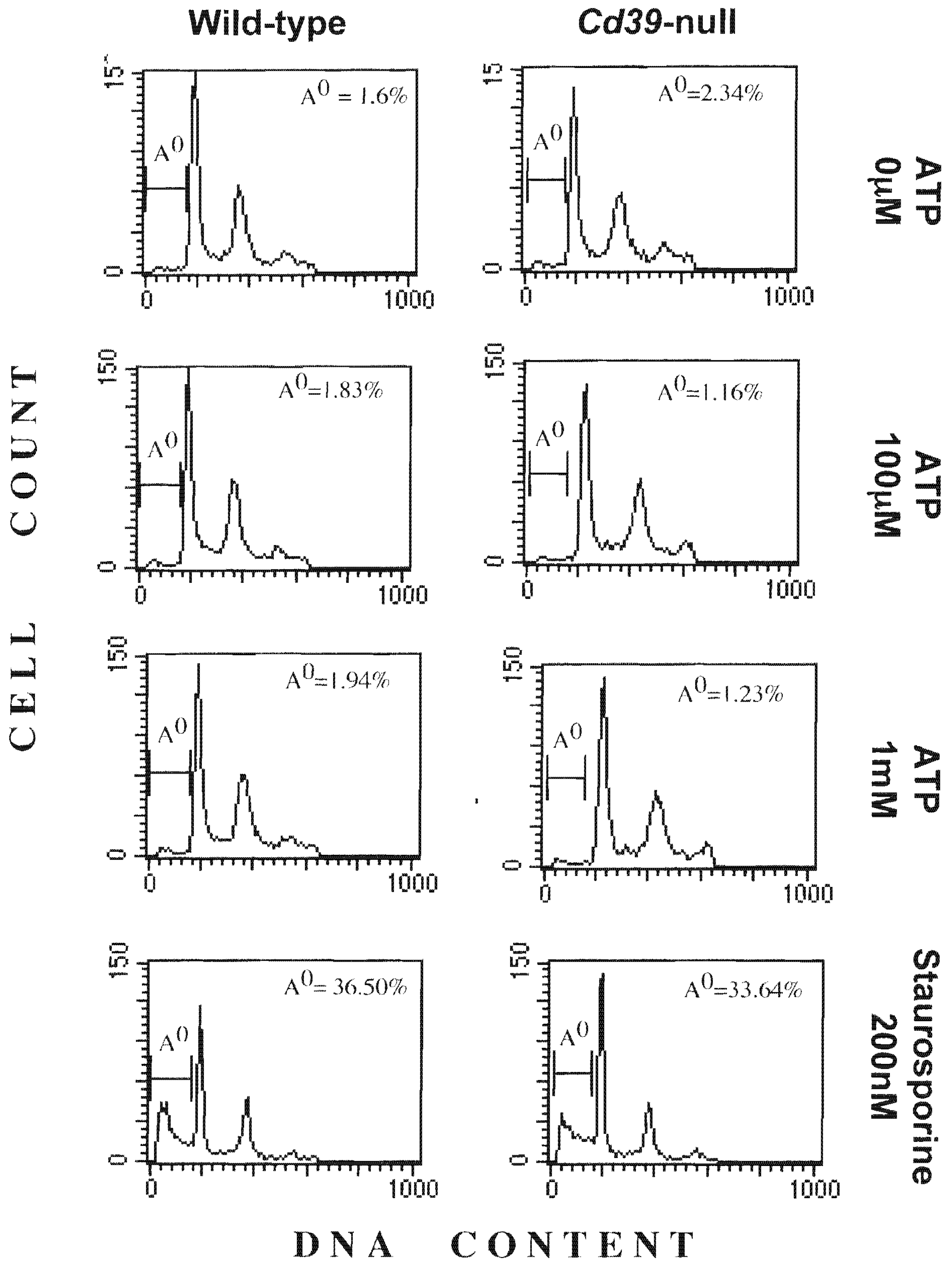
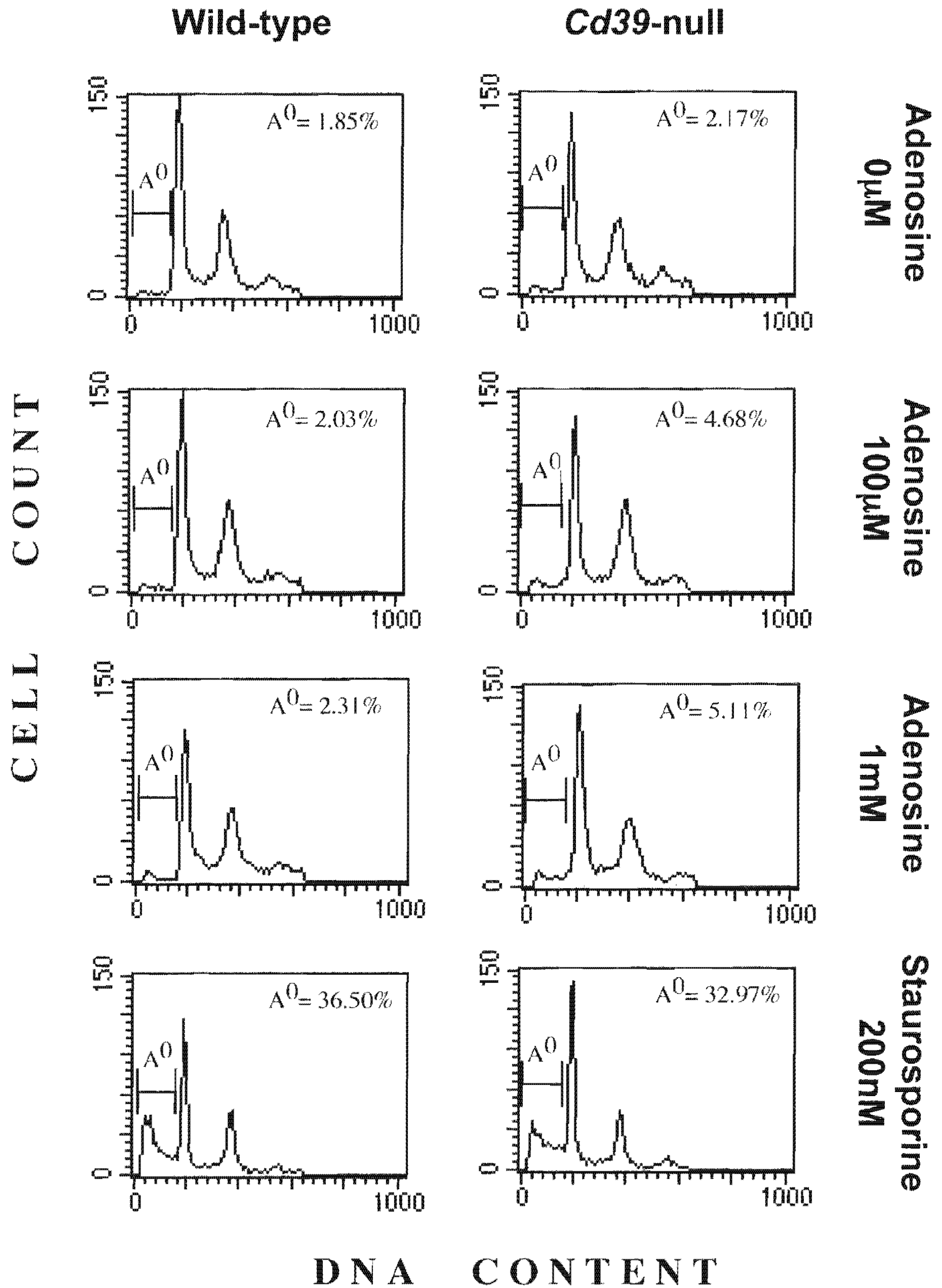


Figure 5.5.4 (b)



5.6 MATRIX METALLOPROTEINASE EXPRESSION

5.6.1 Introduction

The matrix metalloproteinase system has been shown to be crucially important in the control of angiogenesis.[89,93] Matrix metalloproteinases are responsible for the digestion of extracellular matrix by migrating endothelial cells during the sprouting phase of angiogenesis. This is demonstrated by the use of natural and synthetic MMP inhibitors to inhibit angiogenesis, and by the finding that deletion of MMP-2 and MMP-9 in mouse models results in poor physiological and pathological angiogenesis.[89,93,107]

However, excessive digestion of connective tissue can have detrimental consequences. For example, cardiac rupture following acute myocardial infarction has been cited as a complication of inflammatory cells-mediated digestion of the infarcted myocardium. In a murine model of myocardial infarction, deletion of urokinase-type plasminogen activator (u-PA) or gelatinase B (MMP-9) protected mice against myocardial rupture, a complication that affected 30% of the wild-type mice. However, the lack of matrix digestion in theses mice also limited angiogenesis and myocardial remodeling, and predisposed these mice to cardiac arrhythmias and to cardiac failure. Interestingly, administration of plasminogen activator inhibitor-1 (PAI-1) or a MMP inhibitor (TIMP-1) protected wild-type mice against rupture without affecting infarct remodeling, and, as such, may prove an effective therapy to prevent rupture following myocardial infarction.[101]

Of relevance to the field of purinergic angiogenesis, is the observation that *Cd39*-null mice are also protected from cardiac rupture following the induction of myocardial infarction. However, like the u-PA-/- and MMP-9-/- mice, these mice demonstrate poor infiltration of inflammatory cells and defective angiogenesis in the infarcted tissue. This prevents effective remodeling of the myocardium and causes the *Cd39*-null mice to die of cardiac failure in the weeks following the infarction.

The expression of matrix metalloproteinases in the infarcted tissue of *Cd39*-null mice is significantly decreased compared with infarcts in wild-type mice (Mika Ogawa, unpublished results). While this offers a probable explanation for the absence of cardiac rupture in *Cd39*-null mice, it does not answer whether this occurs because of decreased production of MMPs by *Cd39*-null inflammatory and endothelial cells, or whether the lack of MMPs is merely the result of defective invasion of MMP-producing cells. It is quite possible that any angiogenesis inhibitor could prevent cardiac rupture following acute myocardial infarction by blocking the migration and action of those cells which digest and weaken the infarct.

For this reason, we decided to measure the production of MMPs by wild-type and *Cd39*-null endothelial cells in vitro. It has previously been shown that endothelial cells release vesicles that contain MMP-2 and MMP-9. By a process of ultracentrifugation we were able to purify these endothelial vesicles and measure their metalloproteinase expression by enzyme zymography.[256]

5.6.2 Methods

- Wild-type and *Cd39*-null endothelial cells were grown to 70% confluence in 6-well plates. Prior to plating, cells were counted to ensure an equal number of cells in each well.
- The experimental groups were:

- Control - normal endothelial media plus 0.5% serum
- VEGF - 10ng/ml VEGF plus endothelial media with 0.5% serum

- For the culture media MMP expression experiments, 500μl of media was incubated with each well for 48 hours.
- For the vesicle purification experiments, 500μl of media was incubated in each well for 9 hours.
- In the vesicle purification experiments, media (500μl) was collected and centrifuged at 600g for 15 minutes, and then the supernatant centrifuged at 1500g for 15 minutes, in order to remove any floating cells or debris.
- The supernatant was then centrifuged at 100,000g for 1 hour at 4°C in order to pellet the secreted endothelial vesicles.
- The pellet was then dissolved in 80μl PBS and stored at -80°C until analysis by zymography.
- For zymography, 7.5% SDS-PAGE gels containing 1mg/ml gelatin type B were prepared. The samples (either endothelial media, or purified vesicles) were then diluted in SDS-PAGE sample buffer (20μl 4x buffer) in non-reducing conditions without heating, and 45μl of sample/SDS-PAGE sample buffer separated by electrophoresis.

- The gels were then washed in 2.5% Triton X-100 for 60 minutes at room temperature.
- The gels were incubated overnight at 37°C in collagenase buffer containing 50mmol/l Tris-HCl, 10mmol/l $CaCl_2$, and 150mmol/l NaCl.
- The gels were stained for 1 hour in Coomassie Blue in a 4:1:5 mixture of methanol, acetic acid and water.
- The gels were then destained in the same 4:1:5 mixture of methanol, acetic acid and water.
- Matrix metalloproteinase activity was visualized as distinct clear bands, indicating hydrolysis of the gelatin substrate in the gel.
- Prior to performing the experiment, zymography was performed on samples of normal endothelial media plus varying concentrations of serum, to control for the presence of matrix metalloproteinases in fetal bovine serum (FBS).
- The amount of gelatin digestion was quantified by inverting the image and then measuring the band intensity by densitometry.
- Experiments were repeated in triplicate.

5.6.3 RESULTS

Serum is known to contain matrix metalloproteinases, but is also needed for endothelial cell survival. Primary mouse lung endothelial cells are particularly sensitize to total absence of serum and become apoptotic within a few hours. Therefore, we had to determine what concentration of serum would allow endothelial survival without confounding our experiment by introducing exogenous proteases.

We separated fresh endothelial media containing varying concentrations of fetal bovine serum (FBS) in a SDS-PAGE gel containing 1mg/ml gelatin. After incubation in collagenase buffer, we stained the gel in coomassie blue and measure the clear bands of gelatin digestion. Figure 5.6.1 shows a representative example of a gel digested by the varying concentrations of fetal bovine serum. Figure 5.6.2 is a graphical representation of serum metalloproteinase activity in this experiment.

From this we can confirm that there is negligible MMP-2 or MMP-9 in the endothelial media if 1% FBS or less is used. Therefore, for the remainder of the experiments we used 0.5% FBS, as experience had shown that this concentration of serum was sufficient for mouse lung endothelial cell survival.

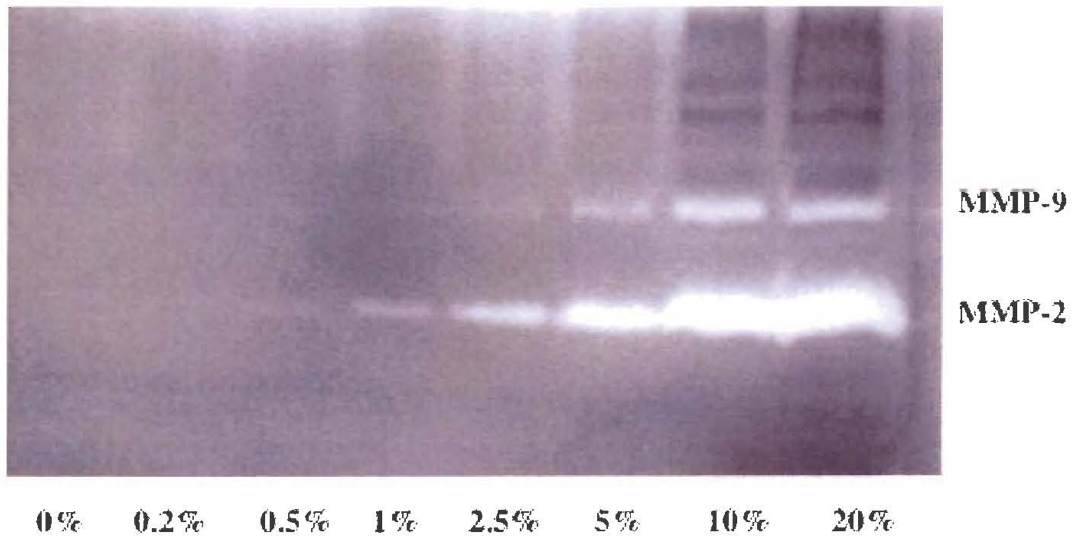


Figure 5.6.1 - Measurement of the endogenous matrix-metalloproteinase activity of fetal bovine serum (FBS) by zymography. Clear bands represent areas of gelatin digestion and correspond to matrix-metalloproteinase 2 (MMP-2) and matrix-metalloproteinase-9 (MMP-9).

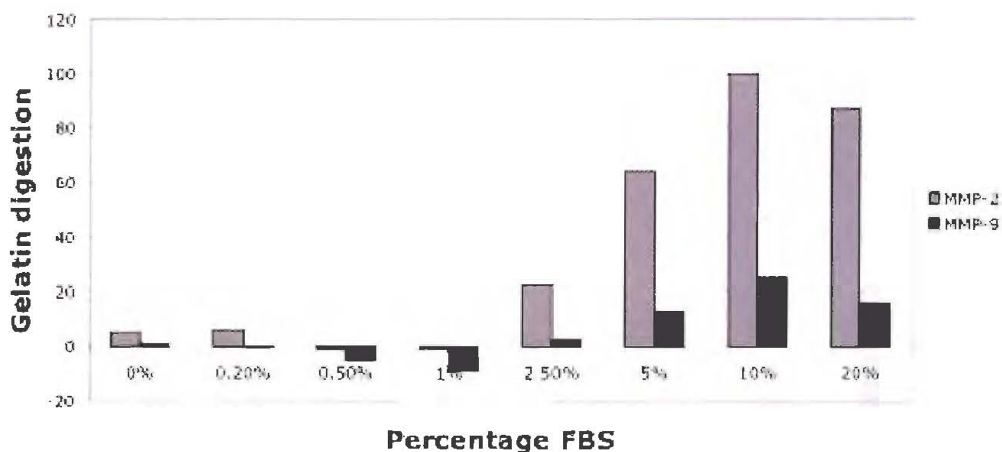


Figure 5.6.2 - Graphical representation of the endogenous matrix-metalloproteinase activity of fetal bovine serum (FBS). The gel shown in Fig. 5.6.1 was inverted digitally and the resulting bands measured by densiometry. Results are expressed as a percentage of maximal digestion.

To measure the production of matrix metalloproteinases by wild-type and *Cd39*-null endothelial cells, we measured MMP activity in the culture media after incubation on endothelial cells as well as the MMP activity of purified endothelial vesicles. Our 2 experimental conditions were a control group of 0.5% FBS and a treatment group of 10ng/ml VEGF in 0.5% FBS.

The culture media had no detectable MMP activity even after prolonged (48 hour) incubation on endothelial cells (results not shown). This is not unsurprising as MMPs usually act locally around endothelial cells and significant dilution would occur after release into the culture media.

However, after purification of endothelial vesicles from the culture media by ultracentrifugation, we were able to detect significant MMP-2 activity. Figure 5.6.3 shows the activity of MMP-2 in wild-type and *Cd39*-null endothelial cells in the control samples and following VEGF treatment. Surprisingly, VEGF failed to induce increased MMP-2 expression in either wild-type or *Cd39*-null endothelial cells. Control media without endothelial cells showed minimal MMP activity (not shown). This contrasts with published data that VEGF induces MMP expression in cultured endothelial cells.[256] However, in that paper, the researchers used 2.5% serum with VEGF, indicating that perhaps the combination of serum and growth factor is required for upregulation of MMP expression.

Importantly, there was no significant difference in the expression of MMP-2 by wild-type or *Cd39*-null endothelial cells in either the control or VEGF-treated groups. In the control group, the wild-type cells had higher expression levels (100% in the wild-type vs 70% in the *Cd39*-null) but this

was not statistically significant (P=0.13). This trend was reversed in the VEGF-treated group with *Cd39*-null cells higher (70% in the wild-type vs 90% in the *Cd39*-null), but remained statistically insignificant (P=0.17).

Although preliminary, these results suggest that differences in the absolute production of MMPs by wild-type and *Cd39*-null endothelial cells do not account for the observed decrease in MMP activity in the *Cd39*-null myocardial infarct tissue. It is more likely that failure of inflammatory and endothelial cells to migrate into the infarcted tissue in *Cd39*-null mice resulted in a decrease in MMP production in the infarct and protection from cardiac rupture.

However, we do accept that modulation of the MMP system may still be responsible for some of the observed angiogenesis defects of the *Cd39*-null mouse. MMPs are often targeted at the leading edge of migrating cells in complex with integrins,[95,109] and this interaction at the cell surface could be affected by deletion of *Cd39*, without affecting the expression of MMPs in secreted membrane vesicles.

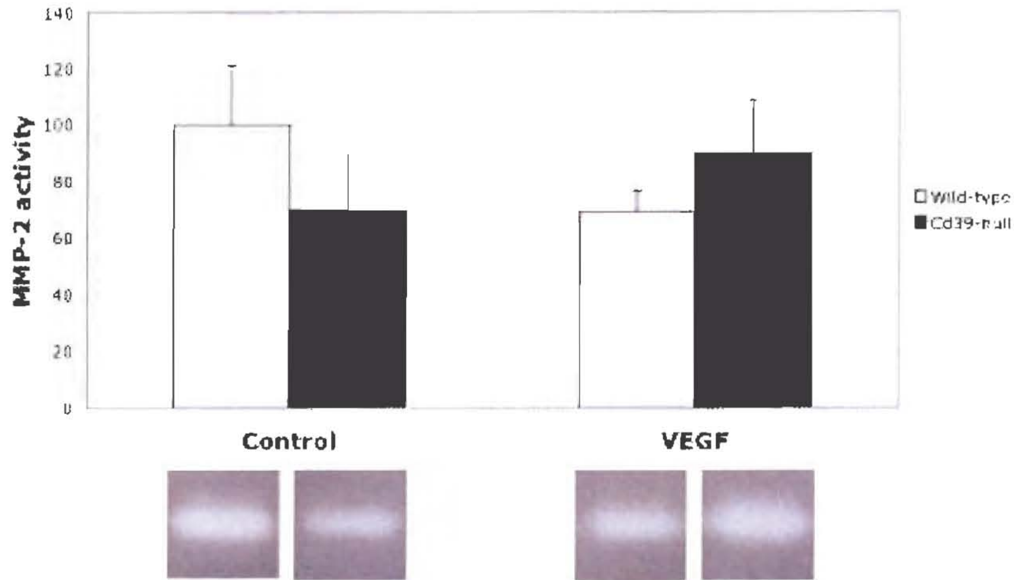


Figure 5.6.3 - Matrix metalloproteinase-2 (MMP-2) expression by wild-type and *Cd39*-null endothelial cell-derived vesicles. Control group (0.5% FBS), treatment group (0.5% FBS plus VEGF 10ng/ml). MMP-2 activity assessed by densitometry analysis of digitally inverted zymography gels. Results expressed as mean of three independent experiments ± s.t.d. Representative zymography gels are shown below.

5.7 HYPOTHESIS: DELETION OF *Cd39* AND DISORDERED P2-SIGNALING CAUSES VASCULAR INTEGRIN DYSFUNCTION

To summarize our initial studies of wild-type and *Cd39*-null endothelial cells we have shown, firstly, that defects in growth factor signaling and endothelial cell proliferation are not responsible for the observed angiogenesis defects in the *Cd39*-null mouse. Secondly, we demonstrated that *Cd39*-null endothelial cells do not undergo greatly increased apoptosis in response to nucleotide stimulation. Finally, we showed that there is no major decrease in the secretion of the gelatinase metalloproteinases by *Cd39*-null endothelial cells. These results all suggest an alternate molecular mechanism for the observed defects in *in vivo* angiogenesis in the *Cd39*-null mouse.

A marked decrease in the migration of endothelial cells into implanted Matrigel™ plugs was seen in *Cd39*-null mice. This suggests that a defect in integrin function could exist in *Cd39*-null endothelial cells.

This is an attractive hypothesis because P2-receptors have been shown to form complexes with integrins at the cell membrane level. For example, the purinoceptor $P2Y_2$ has an RGD sequence in its first extracellular loop, suggesting that it may bind to the RGD-binding motif of integrin $\alpha_V\beta_3/\beta_5$. Mutation of this sequence to RGE greatly decreases the colocalization of $P2Y_2$ and $\alpha_V\beta_3/\beta_5$ and also limits the downstream signaling of $P2Y_2$ to FAK following UTP stimulation.[195]

This suggests that the formation of P2-receptor and integrin complexes are important for normal P2-receptor signaling, but does not answer whether this complex formation is important for integrin function. Both ATP and UTP have been shown to be chemotactic to endothelial cells, which implies a link between P2-receptor signaling and integrin function.[204]

We hypothesize that stimulation of endothelial cells with nucleotides results in the colocalization of P2-receptors and integrins in large signaling complexes at the cell membrane and that this interaction is important for the activation and function of integrins (figure 5.7.1). Many integrins are not constitutively active, but rather, are expressed on the cell surface in an inactive state. For example, the extracellular domain of integrin $\alpha_V\beta_3$ is folded over in the inactive state, but, upon activation activation, straightens and allows interaction of the RGD-binding site with the extracellular matix.[116] The finding that P2-receptors and integrins interact at the cell surface implies that nucleotide-mediated signaling may have a role in promoting integrin activation.

We suggest that the deletion of *Cd39* results in increased nucleotide concentrations at the cell surface and, subsequently, causes differential P2-receptor desensitization. This may perturb the activation of various integrins by P2-receptors and result in endothelial integrin dysfunction (figure 5.7.2).

Alternatively, nucleotide-signaling may promote the expression of specific integrins on the cellular membrane (figure 5.7.3). Recent work by Kaczmarek et al., has shown that UTP treatment upregulates the expression

of the α_V-integrin subunit on the surface of human umbilical vein endothelial cells (HUVEC). *Cd39* deletion and subsequent in vivo P2-receptor desensitization may thus result in decreased expression of important vascular integrins in the absence of effects on integrin activation (figure 5.7.4).

Finally, *Cd39* itself could form part of signaling complexes involving P2-receptors, integrins and growth factor receptors (figure 5.7.5). We have recently published that the N-terminus of CD39 interacts with a scaffolding membrane phosphoprotein, RanBPM.[257] In addition to binding the HGF-receptor cMET and modulating a number of intracellular signaling cascades,[258] RanBPM also interacts with integrins and may effect integrin activation directly.[259] Deletion of the *Cd39* gene may disrupt integrin function independent of the loss of ectonucleotidase activity (figure 5.7.6).

In summary, the observed cellular migration defects in *Cd39*-null mice suggest that the function of various integrin receptors might be perturbed in vivo in these mice. We hypothesize that integrin function is disrupted secondary to P2-receptor desensitization and that this results in either decreased activation or decreased expression of integrins at the cell surface, Alternatively, important macromolecular cell surface complexes may be disrupted by *Cd39* deletion independent of the enzymatic function of Cd39.

To explore these hypotheses, we measured cellular integrin activity by adhesion assays and measured downstream signaling pathways following integrin ligation.

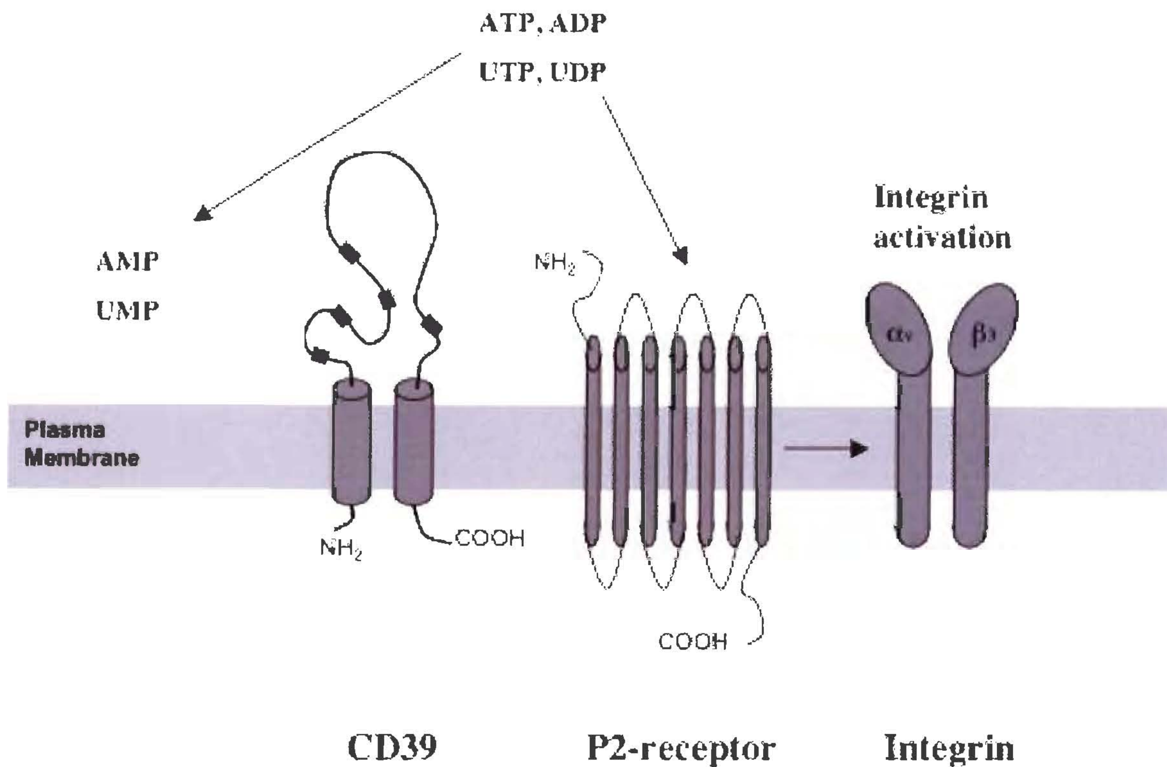


Figure 5.7.1 - Hypothesis No. 1

Nucleotide activation of P2-receptor promotes co-localization of purinergic receptors with integrins. This signaling complex results in activation of these important angiogenic integrins. In this setting, CD39 serves to protect P2-receptors from desensitization caused by low-level basal nucleotide levels.

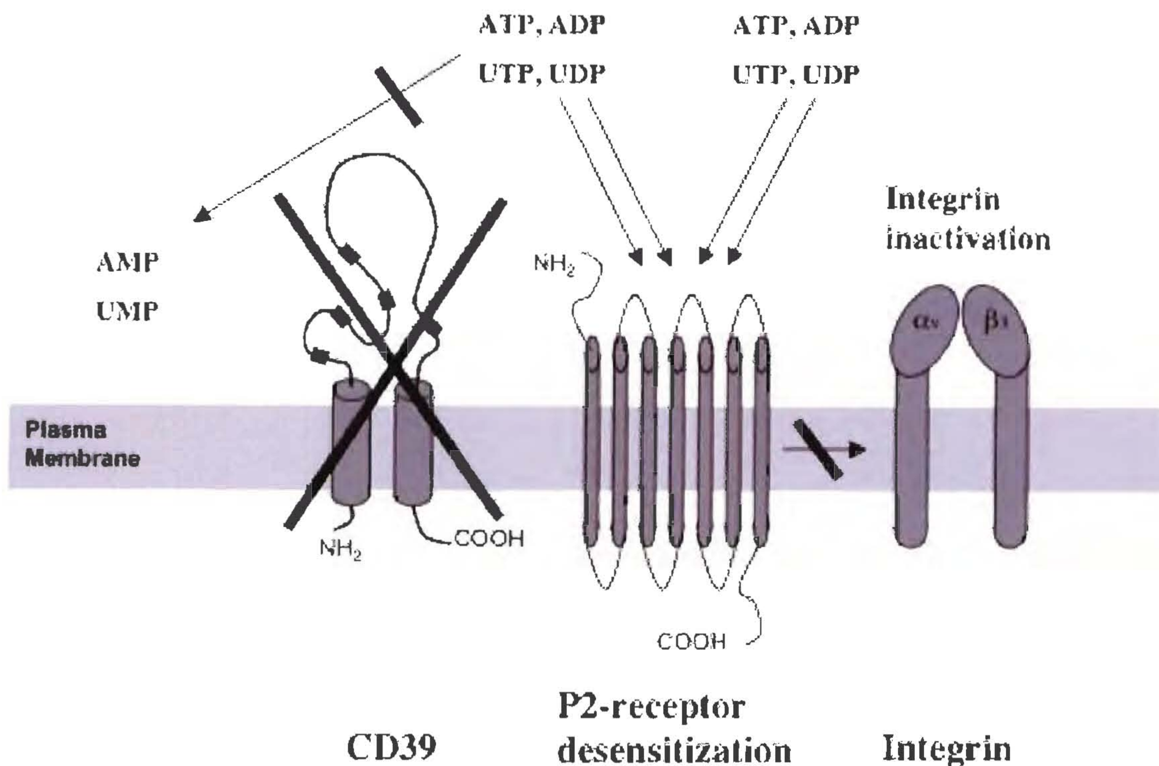


Figure 5.7.2 - Hypothesis No. 1

Deletion of *Cd39* results in P2-receptor desensitization because of increased background nucleotide levels. These desensitized P2-receptors can, therefore, not respond to further nucleotide signals, and are unable to activate angiogenic integrins.

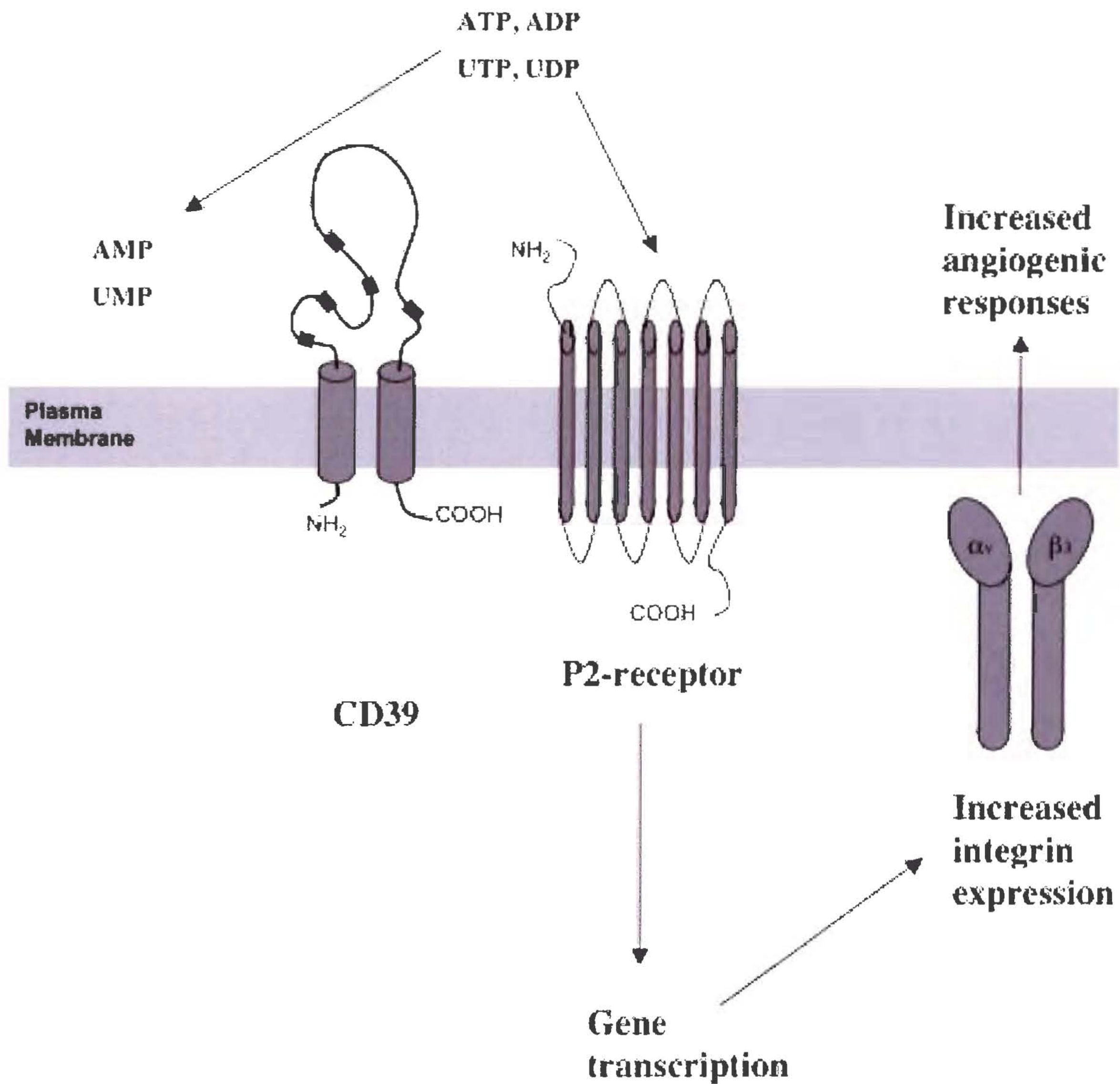


Figure 5.7.3 - Hypothesis No. 2

Rather than directly activating integrins, P2-receptors may respond to nucleotide-mediated signals by increasing the expression of specific angiogenic integrins. Once again, CD39 preserves these purinergic signals by preventing P2-receptor desensitization.

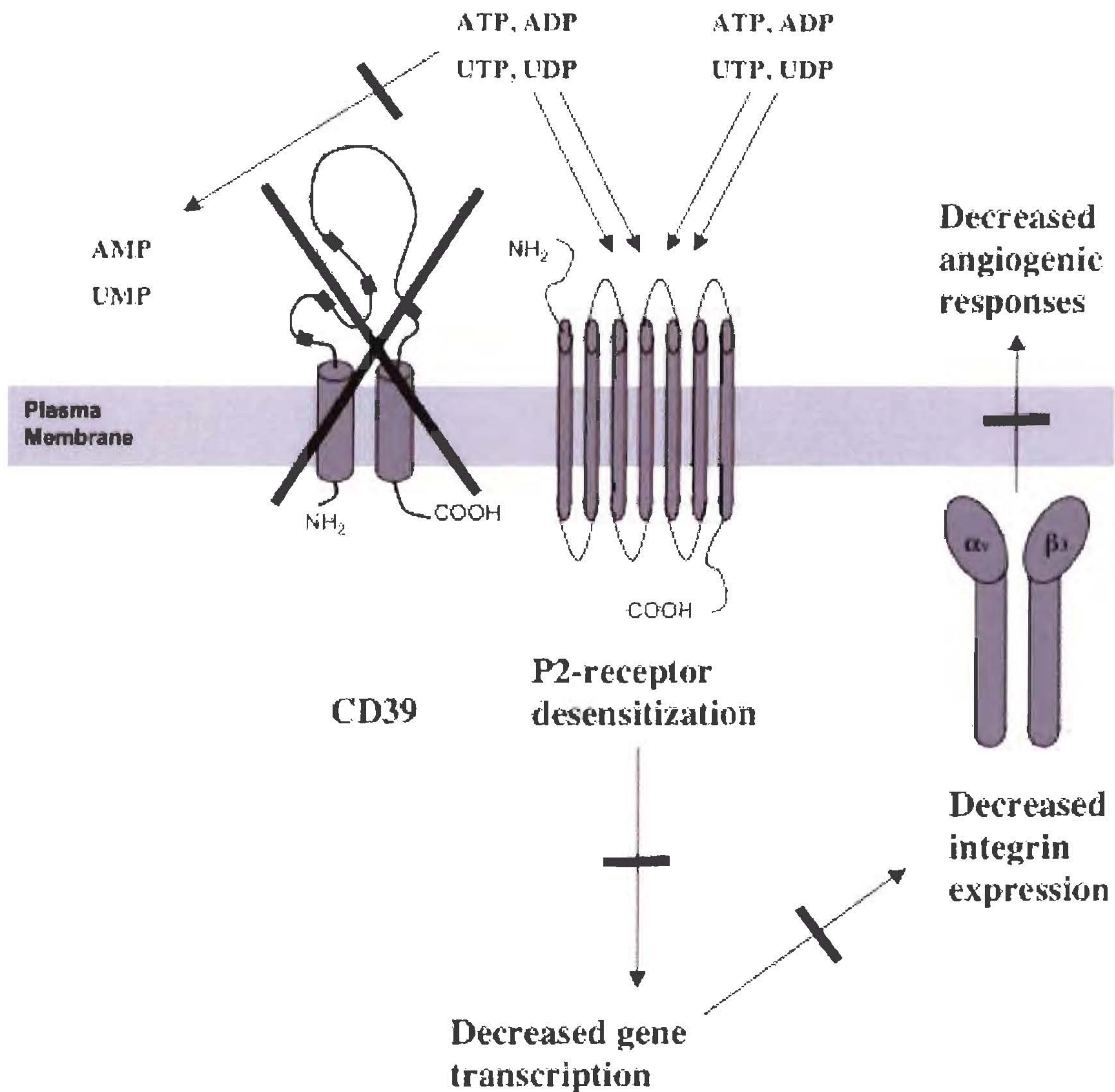
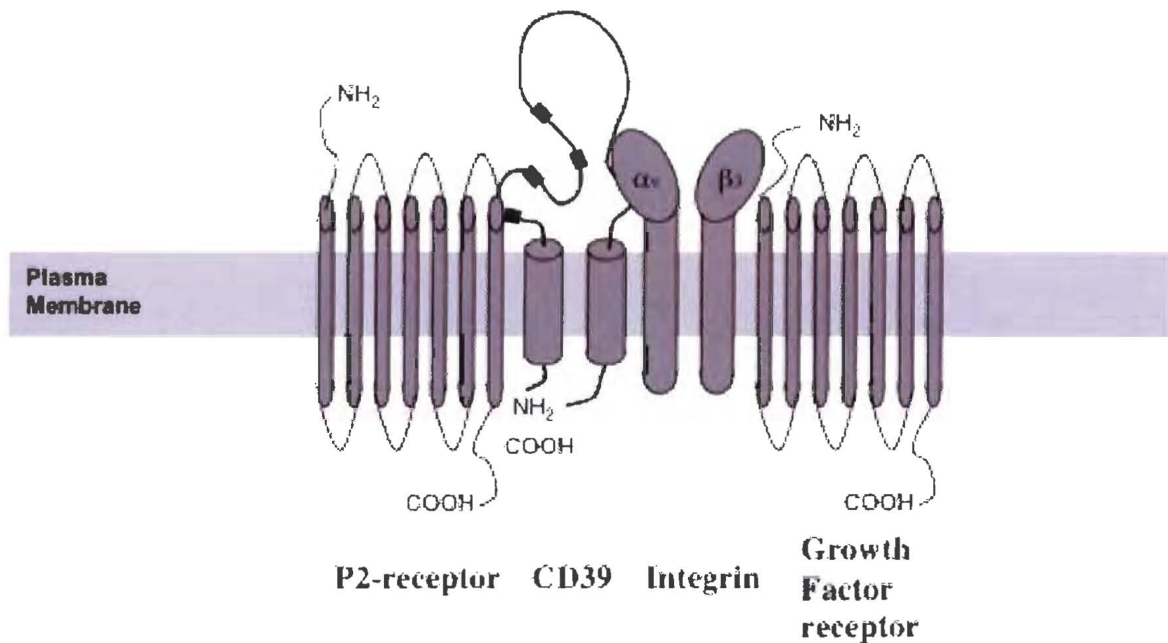


Figure 5.7.4 - Hypothesis No. 2

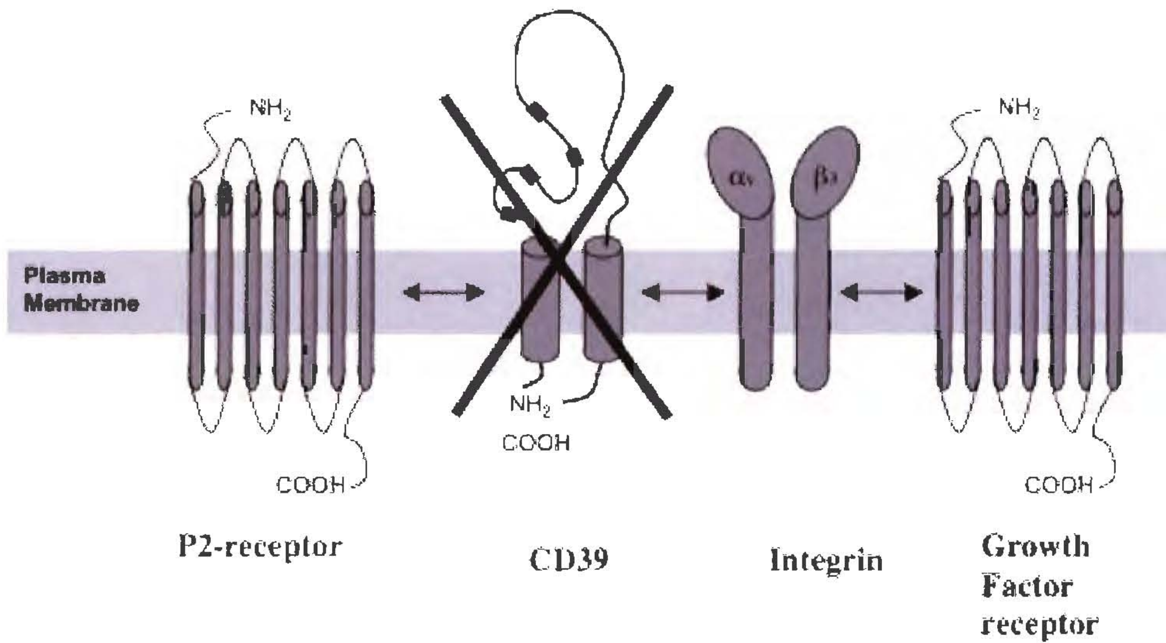
Once again, deletion of *Cd39* results in P2-receptor desensitization because of increased background nucleotide levels. These desensitized P2-receptors can, therefore, not respond to further nucleotide signals, and are unable to induce increased expression of angiogenic integrins.



CELL MEMBRANE SIGNALING COMPLEX

Figure 5.7.5 - Hypothesis No. 3

The final hypothesis posits that CD39 forms an integral structural of an important cell surface signaling complex involving integrins, P2-receptors and growth factor receptors.



DISRUPTED CELL MEMBRANE SIGNALING COMPLEX

Figure 5.7.6 - Hypothesis No. 3

Deletion of *Cd39* may then prevent the structural assembly of this putative signaling complex, with resulting disruption of growth factor signaling and integrin activation.

5.8 ADHESION ASSAYS

5.8.1 Introduction

Cell adhesion to extracellular matrix is a crucial process in a number of physiological and pathological conditions, including wound-healing, blood coagulation, tumour angiogenesis and tumour metastasis. The primary group of receptors regulating attachment to extracellular components is the integrin family of heterodimeric transmembrane receptors. In addition to providing structural support to cells, integrin-mediated cellular adhesion induces a range of intracellular signals that regulate cellular survival, proliferation and differentiation.[34]

As discussed above, the P2-receptor $P2Y_2$ co-localizes with integrin $\alpha_V\beta_3$ and this interaction has been shown to be important for the normal function of $P2Y_2$.[195] However, it was not reported whether this integrin-purinoceptor interaction is also required for normal $\alpha_V\beta_3$ activity.

We suggest that the binding of $P2Y_2$ to $\alpha_V\beta_3$ promotes the activation of integrin $\alpha_V\beta_3$ to its ligand-binding state. Deletion of *Cd39* and the consequent decrease in endothelial ectonucleotidase activity would lead to elevated nucleotide concentrations at the endothelial membrane. This may result in desensitization of $P2Y_2$, preventing the normal interaction of $P2Y_2$ with integrin $\alpha_V\beta_3$ and result in vascular integrin dysfunction.

Considering the heterogeneity of integrin subunit interactions and the multiple purinoceptors expressed by endothelial cells, it is entirely possible that several important integrin-purinoceptor interactions are disrupted by deletion of *Cd39*. Increased nucleotide concentrations at the endothelial cell surface could cause differential desensitization of various endothelial purinoceptors and lead to complex alterations in endothelial integrin activity. Thus, nucleotide-mediated integrin dysfunction may explain the cellular migration defects, and hence the angiogenesis defect, observed in *Cd39*-null mice.

During angiogenesis, a number of important angiogenic integrins are upregulated by the actions of various growth factors.[34] The finding that UTP promotes the expression of the α_V-subunit suggests that nucleotide-mediated signaling may have important roles in altering vascular integrin expression profiles during angiogenesis.[204]

The major UTP receptors on endothelial cells are $P2Y_2$ and $P2Y_4$.[247] $P2Y_4$, and to a lesser extent $P2Y_2$, rapidly desensitize following treatment with UTP,[180] which suggests that UTP-induced responses may be dampened in *Cd39*-null endothelial cells. If UTP treatment does indeed upregulate α_V-integrin expression in murine microvascular endothelial cells, one would expect decreased upregulation, or even decreased baseline expression, of α_V in *Cd39*-null endothelial cells.

Cd39 deletion and P2-receptor desensitization may thus result in decreased surface expression of important vascular integrins, including $\alpha_V\beta_3/\beta_5$, and cause the observed defects in endothelial cell migration.

In addition, the development of large signaling complexes on the surface of endothelial cells is thought to be important for the coordination of multiple signaling inputs. In this context, P2-receptors, integrins and growth-factor receptors form important cell-membrane signaling complexes.[195-198] Cd39 binds RanBPM, a scaffolding membrane phosphoprotein with known links to integrins, including LFA-1 (lymphocyte function-associated antigen-1).[257,259] In addition, Cd39 undergoes palmitoylation and targeting to lipid rafts, called caveolae, and may thus form part of important signaling complexes involving integrins.[198]

Deletion of *Cd39* could disrupt these signaling complexes independent of the loss of endothelial ectonucleotidase activity. The loss of this structural role for Cd39 in macromolecular signaling complexes may explain the observed cellular migration defects in *Cd39*-null mice.

The measurement of cellular adhesion to various matrix strata is considered one of the simplest, but most direct, assays of integrin function. In order to ascertain, firstly, whether integrin dysfunction occurs in *Cd39*-null endothelial cells, and, secondly, which integrin function is defective, we tested the adhesion of wild-type and *Cd39*-null endothelial cells to a number of matrix components, including gelatin, fibronectin and vitronectin.

Gelatin is a heterogeneous mix of high-molecular weight, water-soluble proteins derived from collagen.[260] The integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, in particular, bind to these collagens.[34]

Fibronectin is an extracellular matrix protein with a wide tissue distribution. It is present as a fibrillar network in the ECM and as soluble protomers in body fluids. In total, 10 different integrins have been shown to bind to fibronectin via either its RGD-sequences or to various secondary binding sites.[261] Because it contains a number of RGD-sequences, it binds the important angiogenic integrins $\alpha_5\beta_1$ and $\alpha_V\beta_3$.[34]

Vitronectin is a single-chain glycoprotein present both in plasma and bound to the extracellular matrix. Its structure is divided into a number of domains including the somatomedin B (SMB) domain, which binds the protease inhibitor PAI-1, and a connecting region with a single RGD-sequence. By binding PAI-1, matrix-associated vitronectin has a role in regulating extracellular proteolysis.[262] It also binds integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ by its single RGD-sequence.[263]

Therefore, by testing the adhesion of wild-type and *Cd39*-null to these important extracellular matrix components, we expect to test the function of a number of different vascular integrins.

5.8.2 Methods

a) Adhesion assays

- 96-well plates were coated overnight at 37°C with gelatin (1μg/ml), vitronectin (3μg/ml) or fibronection (40μg/ml). Prior to use, the wells were washed once with PBS then blocked with 1% bovine serum albumin (BSA) for 2 hours at 4°C.
- Wild-type and *Cd39*-null endothelial cells were grown to 70% confluence and serum starved in 0.5% FBS overnight before use.
- In the apyrase pre-treatment experiments, endothelial cells were pre-treated with Grade VII apyrase (5units/ml) for 6 hours.
- Endothelial cells were detached by trypsinization, the trypsin neutralized with soybean trypsin inhibitor and the cells resuspended in serum-free media. The cells were then spun down to a pellet at 1000 rpm for 5 minutes and the supernatant discarded.
- The endothelial cells were then counted and resuspended in a volume of serum-free media corresponding to 30 000 cells / 100μl.
- For integrin activation experiments, $MnCl_2$ (500μM) or PMA (100nM) was added to the samples and incubated with the endothelial cells at 37°C for 15 minutes prior to plating.
- 30 000 cells were added per well of the 96-well plate and incubated at 37°C for 1 hour.
- After 1 hour, the media was carefully removed from the plates and the adherent cells washed once with PBS.

- A percoll solution was then added to the cells to lift poorly attached cells by density gradient. The percoll solution consist 73ml percoll (density 1.13mg/ml. Amersham Pharmacia Biotech), 27ml distilled water and 900mg NaCl.
- The cells were then washed twice with PBS.
- The adherent cells were then fixed with methanol for 30 minutes at room temperature.
- After fixation, the adherent cells were stained with 0.5% crystal violet in 20% methanol for 30 minutes at room temperature.
- The plates were then washed thoroughly with distilled water and the absorbance measured at 560nm using a microtitre plate reader.
- Results are expressed an as average of three experiments ± standard deviation.

b) Integrin expression by immunoprecipitation and Western blot analysis

- Wild-type and *Cd39*-null endothelial cells were grown to 70% confluence and serum starved in 0.5% FBS overnight before use. The cultured endothelial cells were then treated with 100μM UTP for 0, 2, 4 or 6 hours.
- The adherent cells were collected in a 300μl solution of lysis buffer, centrifuged at 14000 rpm for 10 minutes, the supernatant conserved and the nuclear pellet discarded.
- Sample protein concentrations were determined using the Biorad DC Protein Assay.

- 20μL anti-α_V antibody was added to 300μg protein aliquots diluted in 400μL PBS, and incubated on ice for 30 minutes.
- 15μL protein A-coupled sepharose beads (Sigma-Aldrich Corp. St. Louis, MO) were added to the mixture and incubated on ice for 60 minutes with frequent mixing.
- The protein A-coupled sepharose beads were separated by pulse centrifugation and then washed in PBS to remove unbound antigen.
- The samples were boiled in SDS-PAGE Sample Buffer containing 2% β-mercaptoethanol.
- Western blot analysis was performed as described in 5.2.1(c), using anti-α_V antibody (1:1000 concentration) as the primary antibody.

5.8.3 Results and discussion

a) Decreased adhesion of *Cd39*-null endothelial cells to vitronectin.

We studied the adhesion of wild-type and *Cd39*-null endothelial cells to cell-culture plates coated with different extracellular matrix proteins. Figure 5.8.1 and Table 5.8.1 compare the adhesion of wild-type and *Cd39*-null endothelial cells to gelatin-, fibronectin- and vitronectin-coated cell culture plates.

There was no difference in the attachment of wild-type and *Cd39*-null endothelial cells to gelatin (wild-type 75.5±30.6% vs. *Cd39*-null 59±13.2%. P=0.22) or to fibronectin (wild-type 75.8±30.5% vs. *Cd39*-null 75.6±17.6%. P=0.50). However, there was a significant defect in the adhesion of *Cd39*-null endothelial cells to vitronectin (wild-type 100±12.3% vs. *Cd39*-null 45.2±24.9%. P=0.013).

This implies a specific defect in the function of the important angiogenic integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ in *Cd39*-null endothelial cells. The finding that *Cd39*-null endothelial cells bind normally to fibronectin may initially appear to contradict these results because $\alpha_V\beta_3/\beta_5$ binds to fibronectin via its multiple RGD-binding sites. However, fibronectin also binds a number of other integrins by its RGD-sequences and via various secondary binding site.[261] Therefore, *Cd39*-null endothelial cells may bind fibronectin with other normally-functioning integrins even though $\alpha_V\beta_3/\beta_5$ are dysfunctional.

Table 5.8.1: Endothelial cell adhesion to various extracellular matrix proteins. Wild-type and *Cd39*-null endothelial cells were allowed to attach to 96-well plates coated with gelatin (1μg/ml), fibronection (40μg/ml), or vitronectin (3μg/ml). Results expressed as a percentage of maximal adhesion ± standard deviation.

	Gelatin	**Fibronectin**	**Vitronectin**
Wild-type	75.5±30.6%	75.8±30.5%	100±12.3%
***Cd39*-null**	59±13.2%	75.6±17.6%	45.2±24.9%
P-values	0.22	0.50	0.013

Fig 5.8.1

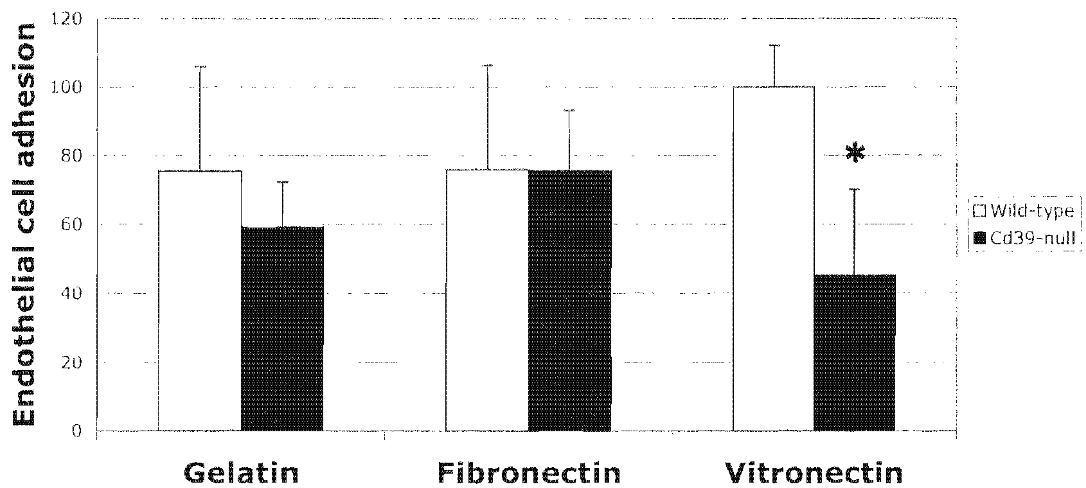


Figure 5.8.1 - Wild-type and *Cd39*-null endothelial adhesion to collagen, fibronectin and vitronectin. Results expressed as a percentage of wild-type adhesion to collagen ± s.e.m. *, $P<0.05$.

Indeed, β_3-null endothelial cells have been shown to adhere normally to fibronectin in the face of significantly decreased adhesion to vitronectin.[129] This emphasizes the crucial importance of integrins $\alpha_V\beta_3/\beta_5$ in mediating, specifically, cellular adhesion to vitronectin.

b) Decreased vitronectin adhesion of *Cd39*-null endothelial cells occurs because of decreased integrin activation, not decreased integrin expression.

Previous experimental evidence has demonstrated that purinergic signaling has important roles in promoting both integrin activation and integrin expression. The decreased adhesion of *Cd39*-null endothelial cells to vitronectin may, therefore, be caused by either decreased expression or decreased activation of $\alpha_V\beta_3/\beta_5$.

We measured the surface expression of the α_V-integrin subunit on the surface of wild-type and *Cd39*-null endothelial cells by immunoprecipitation and Western blot analysis. In addition, because UTP was shown to increase the expression of the α_V-subunit in HUVECs, we tested the effect of UTP treatment on wild-type and *Cd39*-null murine pulmonary endothelial cells.

Fig 5.8.2 shows the expression of the α_V-integrin subunit in wild-type and *Cd39*-null endothelial cells at baseline in culture and after 2, 4, or 6 hours treatment with 100μM UTP. These results demonstrate 2 important findings. Firstly, it can be seen that there is no difference in the expression of the α_V-integrin subunit in wild-type and *Cd39*-null endothelial cells at baseline.

This is important because the α_V-subunit is required for both $\alpha_V\beta_3$ and $\alpha_V\beta_5$ assembly and is crucial during the process of cellular adhesion to vitronectin. Although we have not confirmed that no difference exists in the endothelial expression of integrin β_3- and β_5-subunits between wild-type and *Cd39*-null endothelial cells, the finding that there is no difference in α_V expression raises the possibility that differences in integrin activation, rather than integrin expression, account for the observed defective adhesion of *Cd39*-null cells to vitronectin.

Secondly, it can be seen that treatment with UTP does not increase the expression of the α_V-integrin subunit in either wild-type or *Cd39*-null cells. This contradicts the finding of Kaczmarek et al. that UTP significantly upregulates the expression of α_V in HUVECs.[204] Possible explanations for this discrepancy include the use of a different population of endothelial cells (human umbilical vein endothelial cells vs. murine pulmonary endothelial cells), or different experimental protocols used. Kaczmarek et al. determined α_V-integrin expression by Western blot analysis of whole cell lysates, while we used immunoprecipitation to purify the α_V-integrin subunit prior to analysis. The α_V-integrin subunit was expressed at too low a level to be detected by Western blot analysis of whole cell lysates in our experiments (results not shown).

Alternatively, these findings could be represent experimental error because of saturation of the primary anti-α_V antibody. In future, we should repeat these experiments using titrations of the primary antibody to confirm that excess antibody has been added. In the meantime, we feel confident that our

data does represent a true finding in that: firstly, 20μL of anti-α_V antibody is likely sufficient given the low expression of integrin α_V *in vitro*; secondly, this data is consistent with our intial experiments showing no difference in expression via α_V immunofluorescence; and, finally, the data is consistent with the hypothesis of integrin desensitization presented below.

As discussed previously, because UTP receptors are thought to be desensitized in *Cd39*-null endothelial cells, the control of α_V-integrin expression by UTP would provide an important link between nucleotide-mediated signaling and integrin function. If the surface expression of the α_V-integrin subunit was promoted by UTP, we would expect increased surface expression of α_V in wild-type endothelial cells, compared with *Cd39*-null cells. However, we have shown, firstly, that there is no difference between the expression of the α_V-integrin subunit in wild-type and *Cd39*-null endothelial cells at baseline, and secondly, that UTP does not upregulate the expression of α_V in murine pulmonary endothelial cells.

We, therefore, propose that *Cd39*-null endothelial cells have defective attachment to vitronectin because of decreased activation of $\alpha_V\beta_3$ and/or $\alpha_V\beta_5$ integrins. Further proof of this hypothesis was obtained by the finding that non-specific integrin activation corrects the adhesion of *Cd39*-null endothelial cells to vitronectin.

$MnCl_2$ is a potent effector of a number of integrin-mediated adhesion events and is thought to activate integrins by binding to specific cation-binding sites and inducing conformational changes in the ligand-binding site.[264]

Table 5.8.2 and figure 5.8.3 shows the effect of $MnCl_2$ treatment on the adhesion of wild-type and *Cd39*-null endothelial cells to vitronectin. As shown previously, *Cd39*-null endothelial cell adhesion to vitronectin was significantly decreased compared with wild-type cells (wild-type 100±15.7% vs. *Cd39*-null 57.3±23.2%. P=0.011). In keeping with our hypothesis that $\alpha_V\beta_3/\beta_5$ activation is decreased in *Cd39*-null endothelial cells, $MnCl_2$ corrected the defective adhesion of *Cd39*-null endothelial cells to vitronectin (wild-type plus $MnCl_2$ 101.1±5.9% vs. *Cd39*-null plus $MnCl_2$ 95.4±8.6%. P=0.198). $MnCl_2$ treatment was associated with a statistically significant increase in the adhesion of *Cd39*-null endothelial cells to vitronectin (*Cd39*-null control 57.3±23.2% vs. *Cd39*-null plus $MnCl_2$ 95.4±8.6%. P=0.022), but had minimal effect on wild-type cells (wild-type 100±15.7% vs. wild-type plus $MnCl_2$ 101.1±5.9%. P=0.458).

This shows that normal integrins are expressed on the surface of *Cd39*-null endothelial cells, but that they are not fully functional because of decreased integrin activation. It also implies that P2-receptors have an important role in activating important angiogenic integrins.

As discussed in the literature review, extracellular nucleotides signal through type-2 purinergic (P2) receptors called P2Y (G—protein coupled) or P2X (ligand gated ion channels).[265] Most P2Y receptors activate phospholipase C (PLC), resulting in the formation of inositol 1,4,5-triphosphate (IP_3), diacylglycerol (DAG) and the mobilization of cytosolic Ca^{2+}. This results in the activation of an important intracellular signaling molecule protein kinase C (PKC) and has been implicated in integrin activation.[266,267] Phorbol 12-

myristate 13-acetate (PMA) is an analogue of DAG,[268] which has been used in numerous experimental settings to activate integrins by binding PKC.[269,270]

Our hypothesis is that excess nucleotides at the cellular surface in *Cd39*-null cells, results in desensitization of P2-receptors and in secondary defects in integrin activation, perhaps via defective PKC activation. For this reason, we studied whether we could correct the defective adhesion of *Cd39*-null endothelial cells to vitronectin by using PMA to activate protein kinase C (PKC).

Table 5.8.3 and figure 5.8.4 shows the effect of PMA on the adhesion of wild-type and *Cd39*-null endothelial cells to vitronectin. It can be seen that, while PMA increases the adhesion of *Cd39*-null endothelial cells to vitronectin, this increase is not as great as that induced by $MnCl_2$ treatment. PMA treatment increases the adhesion of *Cd39*-null endothelial cells from 57.3% to 81.5% of wild-type adhesion, but this increase is not statistically significant (P=0.078).

Despite repeating this experiment numerous times, we could not prove that PMA-induced activation of PKC corrected integrin dysfunction in *Cd39*-null endothelial cells. Decreased PKC activation in *Cd39*-null cells may still be involved in the defective adhesion of *Cd39*-null cells to vitronectin, but alternate intracellular signaling molecules must also play a role in nucleotide-mediated integrin activation in wild-type and *Cd39*-null endothelial cells. It is possible that several P2-receptors, each activating different intracellular signaling pathways, work in tandem to activate integrins during angiogenesis.

c) Defective integrin function in *Cd39*-null endothelial cells occurs because of decreased nucleotide hydrolysis.

As discussed above, *Cd39* deletion may result in integrin dysfunction secondary to either, loss of ectonucleotidase activity and P2-receptor desensitization, or by structural disruption of integrin function in macromolecular signaling complexes. For this reason, we replaced the ectoenzyme function of Cd39 by pre-treating wild-type and *Cd39*-null endothelial cells with Grade VII apyrase for 6 hours.

Grade VII apyrase is a soluble enzyme with significant adenosine 5'-triphosphatase and adenosine 5'-diphosphatase activity, which was purified from potato (S. tuberosum). Different apyrase isoenzymes with differing ATPase/ADPase ratios have been isolated from potato.[271] Grade VII apyrase was used because, since it has a low ATPase/ADPase ratio, it approximates the enzymatic function of CD39.

Pretreating *Cd39*-null cells with soluble Grade VII apyrase would replace the ectonucleotidase function of Cd39, limit the pericellular accumulation of high nucleotide concentrations and, thus, allow desensitized P2-receptors to resensitize. If P2-receptor desensitization is responsible to the integrin dysfunction of *Cd39*-null endothelial, this treatment should correct that dysfunction.

Table 5.8.4 and figure 5.8.5 and show the effect of apyrase pre-treatment on the adhesion of wild-type and *Cd39*-null endothelial cells to vitronection. It can be seen that, without apyrase pretreatment, the adhesion of *Cd39*-null endothelial cells to vitronectin was less than 60% of wild-type adhesion (wild-type 100±15.7% vs. *Cd39*-null 57.3±23.2%. P=0.011). However, apyrase pre-treatment corrected the vitronectin adhesion of *Cd39*-null endothelial cells to wild-type levels (wild-type 95.3±15.6% vs. *Cd39*-null 98.9±20.3%. P=0.40). Apyrase pretreatment had no effect on wild-type endothelial cells (control wild-type 100±15.7% vs. apyrase-pretreated wild-type 95.3±15.6%. P=0.34)

This suggests that integrin dysfunction in *Cd39*-null endothelial cells occurs because of loss of vascular ectonucleotidase activity, and not because of the structural absence of Cd39

Table 5.8.2: Endothelial cell adhesion to vitronectin following treatment with $MnCl_2$. Wild-type and *Cd39*-null endothelial cells were allowed to attach to 96-well plates coated with vitronectin (3μg/ml) in the presence or absence of 500μM $MnCl_2$.

	Control	**$MnCl_2$**
Wild-type	100±15.7%	101.1±5.9%
***Cd39*-null**	57.3±23.2%	95.4±8.6%
P-values	0.01	0.20

Table 5.8.3: Endothelial cell adhesion to vitronectin following treatment with PMA. Wild-type and *Cd39*-null endothelial cells were allowed to attach to 96-well plates coated with vitronectin (3μg/ml) in the presence or absence of 250μM PMA.

	Control	**PMA**
Wild-type	100±15.7%	96.9±10.1%
***Cd39*-null**	57.3±23.2%	81.5±10.1%
P-values	0.01	0.07

Table 5.8.4: Endothelial cell adhesion to vitronectin following pre-treatment with apyrase. Prior to the adhesion assay, wild-type and *Cd39*-null endothelial cells were treated with grade VII apyrase (5units/ml) for 6 hours.

	Control	**Apyrase**
Wild-type	100±15.7%	95.3±15.6%
***Cd39*-null**	57.3±23.2%	98.9±20.3%
P-values	0.01	0.40

Figure 5.8.2

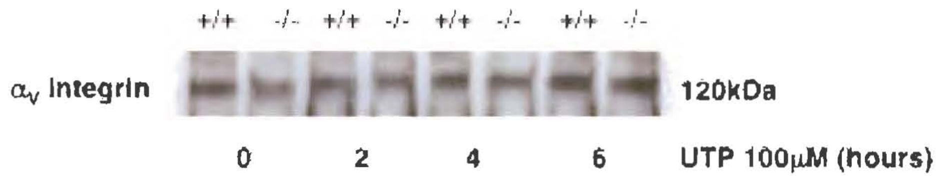


Figure 5.8.2 - α_v-integrin subunit expression in wild-type (+/+) and *Cd39*-null (-/-) endothelial cells by immunoprecipitation and Western-blot analysis. Cells were treated with UTP (100μM) for 0, 2, 4, and 6 hours.

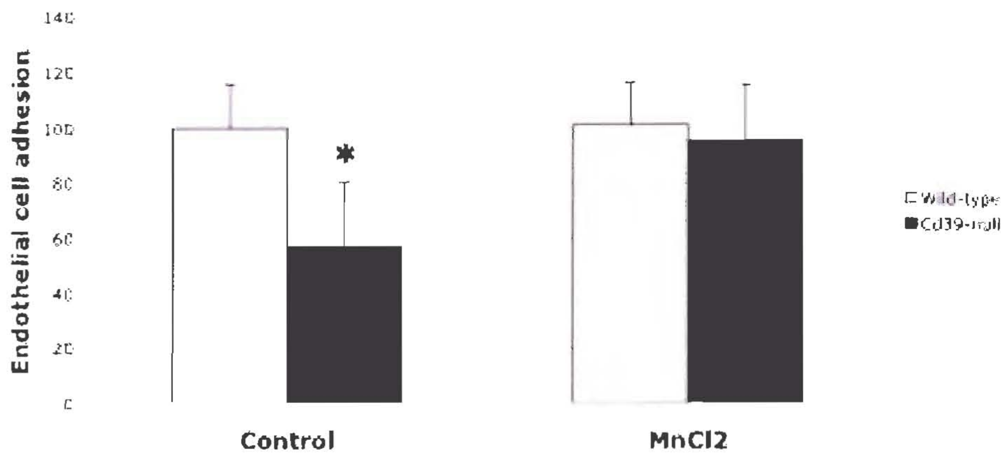


Figure 5.8.3 - Wild-type and *Cd39*-null endothelial cell adhesion to vitronectin following treatment with $MnCl_2$ (500μM). Results expressed as a percentage of wild type adhesion to vitronectin ± s.e.m. *, $P<0.05$. (Wild-type control vs. *Cd39*-null control).

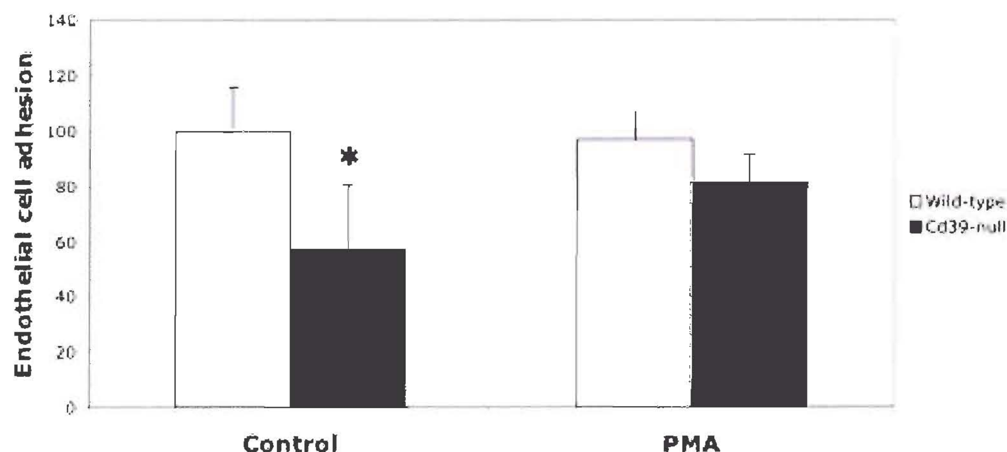


Figure 5.8.4 - Wild-type and *Cd39*-null endothelial cell adhesion to vitronectin following treatment with PMA (100nM), showing partial correction of the defective adhesion of *Cd39*-null endothelial cells to vitronectin. Results expressed as a percentage of wild type adhesion to vitronectin ± s.e.m. *, $P<0.05$. (Wild-type control vs. *Cd39*-null control).

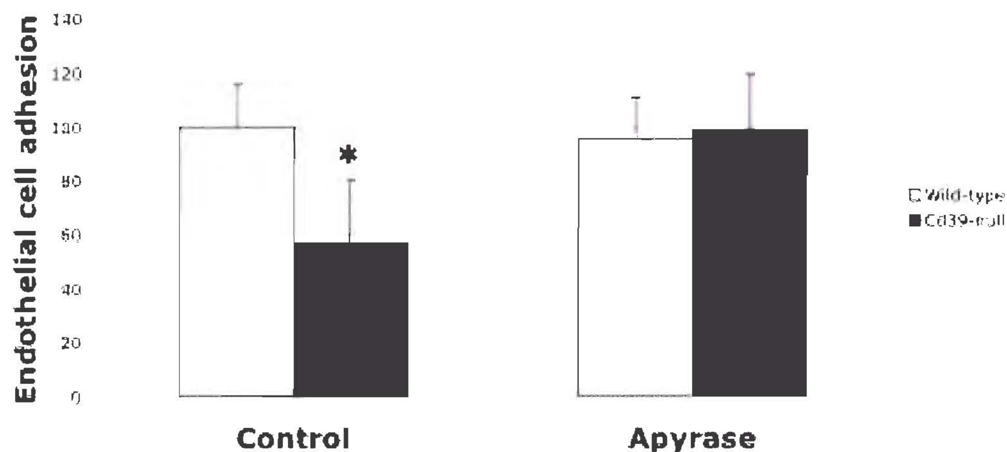


Figure 5.8.5 - Wild-type and *Cd39*-null endothelial cell adhesion to vitronectin following 6-hour pre-treatment with soluble Grade VII apyrase (5units/ml). Results expressed as a percentage of wild type adhesion to vitronectin ± s.e.m. *, $P<0.05$. (Wild-type control vs. *Cd39*-null control).

5.9 INTEGRIN-MEDIATED INTRACELLULAR SIGNALING

5.9.1 Introduction

Integrins mediate cell-cell and cell-matrix interactions, and transduce signals from the extracellular matrix that modulate cellular growth, differentiation and migration. However, integrins have very short intracytoplasmic domains devoid of enzymatic activity and thus transmit extracellular signals by recruiting intracellular signaling molecules into signaling complexes. These complexes, known collectively as focal adhesions,[115] connect integrins to both intracellular kinases and to the cytoskeleton, in a process known as "outside-in" signaling (Figure 5.9.1).We have demonstrated specific integrin defects in *Cd39*-null endothelial cells, implying defective activation of a number of important intracellular mediators with specific actions during angiogenesis.

One of the most important intracellular signaling kinases with a crucial role in focal adhesion assembly and, hence, integrin-mediated signaling is focal adhesion kinase (FAK). After being recruited to focal adhesions, FAK autophosphorylates Tyr397, creating a binding site for the Src homology 2 (SH2) domain of Src or Fyn. The Src kinase then phosphorylates a number of focal adhesion components, including the cytoskeletal proteins paxillin and tensin.[272]

The importance of FAK in cellular migration is demonstrated using FAK-/- fibroblasts, which migrate poorly and have an increased number of prominent focal adhesion complexes.[273] FAK functions as an integrin scaffold which, by recruiting Src to focal adhesions, positions this kinase close to cytoskeletal proteins and intracytoplasmic signaling molecules that are crucial for cellular migration.[274] For example, activation of FAK and Src results in the activation of Rac, a molecule vital for lammelipodial extension.[275]

FAK also activates phosphoinositide 3-OH kinase (PI 3-kinase) and Akt, either directly or through Src. Although numerous signaling pathways activate Akt, each with differing cellular functions, the activation of Akt following integrin ligation is thought to be important for the induction of cellular survival pathways and prevention of anoikis (cellular death occurring in the absence of binding to the ECM).[276] The excess endothelial cell apoptosis noted in *Cd39*-null mice following liver regeneration could possibly be caused by a defect in integrin signaling to Akt.

Finally, FAK has a role in the activation of mitogen activated protein kinases (MAPK). The MAPKs are a family of serine-threonine protein kinases, which are activated by a range of growth hormones, cytokines and extracellular matrix components.[277] The p42/44 MAPK (also known as ERK1/2) modulates angiogenesis on several levels. Firstly, activation of ERK1/2 protects endothelial cells from apoptosis and promotes proliferation by promoting entry into the cell cycle.[242] Secondly, ERK1/2 has been shown to promote VEGF expression by increased VEGF gene transcription.[278] In this context ERK1/2 also phosphorylates HIF-1α, suggesting a link between

growth factor-induced and hypoxia-induced upregulation of pro-angiogenic factors.[279] Thirdly, in addition to modifying gene expression, ERK1/2 may also regulate endothelial cell migration by phosphorylating cytoskeletal proteins.[280]

Integrin ligation and activation of FAK is vital for ERK1/2 signaling. Placing cells in suspension prevents growth-factor mediated activation of the MAPK cascade, while adhesion of cells to extracellular matrix in the absence of growth factors stimulates MAPK activity. However, the nature of integrin signaling to MAPK is not entirely understood and probably differs in different circumstances.

One suggested mechanism is that cell adhesion stimulates the translocation of Rac to the cell membrane and results in the subsequent activation of PAK. PAK phosphorylation of MEK then leads to downstream MAPK activation.[281] Another possible link is that Src phosphorylates FAK at Tyr925, creating a binding site for the adaptor Grb2 and mSOS. This results in stimulation of the MAPK cascade via the activation of Ras.[282]

We have shown that defective endothelial cell migration in *Cd39*-null mice during angiogenesis is associated with specific defects in integrin function. However, we have not demonstrated which downstream intracellular mediators are affected by this integrin dysfunction. Therefore, we measure the activation of FAK, Akt and ERK1/2 following vitronectin-mediated integrin ligation in wild-type and *Cd39*-null endothelial cells. Each of these intracellular mediators has important roles in endothelial behaviours during angiogenesis – whether by promotion of focal adhesion assembly, by

protection from apoptosis or by promotion of endothelial migration and differentiation.

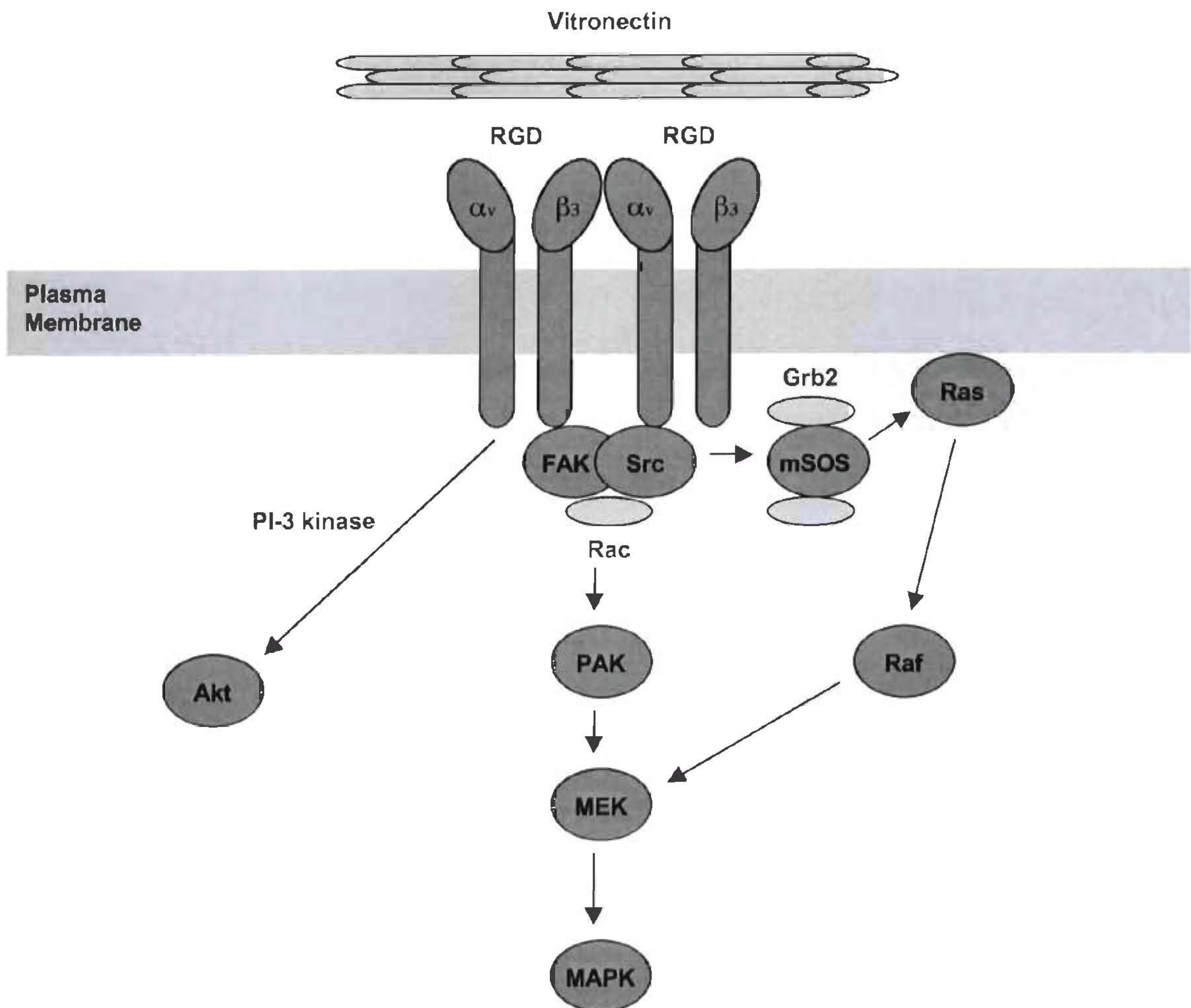


Figure 5.9.1 - FAK signaling

Integrin binding to vitronectin induces integrin clustering and targeting of FAK to focal adhesions. FAK autophosphorylates at Tyr397, resulting in binding of Src via its SH2 domain. This results in activation of Akt via PI-3 kinase. MAPKs (including ERK1/2) are activated either by Rac translocation to the cell membrane and downstream PAK activation or by mSOS-induced activation of Ras.

5.9.2 Methods

- Wild-type and *Cd39*-null mouse lung endothelial cells were cultured to 70% confluence in normal endothelial media. Prior to experimentation, the cells were starved overnight in a basal media containing 0.5% fetal bovine serum (FBS) and no endothelial mitogen.
- For the apyrase pre-treatment experiments, the cells were treated with Grade VII apyrase (5units/ml) for 6 hours prior to experimentation.
- 6-well plates were pre-coated with 0.5μg/ml vitronectin overnight at 37°C.
- The following day, wild-type and *Cd39*-null endothelial cells were detached using a 0.05% trypsin solution. After detachment the trypsin was neutralized with soybean trypsin inhibitor and the cells resuspended in serum-free media. $3x10^5$ cells were added to each vitronectin-coated plate.
- The zero minute control samples were spun down at 1000rpm for 5 minutes and the pellet immediately placed on ice.
- At 15, 30 or 60 minutes the media in the 6-well plates were removed by suction and the adherent cells collected in a 120μl solution of lysis buffer, centrifuged at 14000 rpm for 10 minutes, the supernatant conserved and the nuclear pellet discarded.
- Western blot analysis was then performed as described in 5.2.1(c) using anti-phosphoFAK (phosphoTyr397), anti-phosphoERK1/2 (phosphoThr202/Tyr204) or anti-phosphoAkt (Ser473) as primary antibodies (1:1000 concentration).

- After development, the membranes were then stripped using the Restore Stripping Buffer (Pierce Biotechnology, Inc. Rockford, IL.). Prior to further probing with antibody, the stripped membrane was developed with Supersignal chemiluminescent substrate (Pierce Biotechnology, Inc. Rockford, IL.) to confirm that no residual signal remained.
- The membrane was then reprobed with antibodies to FAK, ERK1/2 or Akt to measure the total protein expression of these signaling molecules in each sample.
- Results are expressed as the ratio of phosphorylated FAK, ERK1/2 or Akt, to total FAK, ERK1/2 or Akt at each time point for three independent experiments.

5.9.3 Results

The finding that *Cd39*-null endothelial cells have defective adhesion to vitronectin, implies specific integrin dysfunction and suggests disordered integrin-mediated intracellular signaling. Using phosphospecific antibodies, we studied the activation of the focal adhesion kinase (FAK), extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt following vitronectin-induced integrin ligation in wild-type and *Cd39*-null endothelial cells. In each experimental group, results are expressed as the ratio of phosphorylated (activated) mediator to total mediator at 0, 15, 30 and 60 minutes after plating onto vitronectin.

In keeping with our finding that there is defective adhesion of *Cd39*-null endothelial cells to vitronectin, we have shown that plating of *Cd39*-null endothelial cells onto vitronectin results in defective activation of FAK and downstream ERK1/2.

Figure 5.9.2 shows the phosphorylation of FAK at tyrosine 397 at various time points after plating wild-type and *Cd39*-null endothelial cells onto vitronectin. In wild-type endothelial cells, FAK is strongly activated by 15 minutes and this phosphorylation is sustained for 60 minutes. In contrast, *Cd39*-null cells have poor and delayed activation of FAK. *Cd39*-null endothelial cells have a statistically significant decrease in activation of FAK at 15 minutes (wild-type 100% vs. *Cd39*-null 58%. P=0.013), and at 30 minutes (wild-type 74% vs. *Cd39*-null 36%. P=0.017) after vitronectin-plating. By 60 minutes after plating, phosphorylation of FAK in *Cd39*-null cells remains decreased compared with wild-type levels, although this result fails to achieve statistical significance. (wild-type 64% vs. *Cd39*-null 42%. P=0.06).

Cd39-null endothelial cells also have defective activation of downstream ERK1/2 after plating on vitronectin (Figure 5.9.3). In wild-type endothelial cells, ERK1/2 has little phosphorylation after 15 minutes, but becomes strongly phosphorylated after 30 minutes and remains phosphorylated until 60 minutes. This delay in activation relative to FAK is in keeping with ERK1/2's downstream position in the signaling cascade.

Cd39-null endothelial cells show decreased phosphorylation of ERK1/2 after plating onto vitronectin. Activation of ERK1/2 at 30 minutes was

approximately half as efficient in *Cd39*-null compared with wild-type endothelial cells (wild-type 100% vs. *Cd39*-null 51%. P=0.0015). By 60 minutes, *Cd39*-null cells achieved almost equal activation of ERK1/2 relative to the wild-type cells (wild-type 91% vs. *Cd39*-null 80%. P=0.28), indicating that a kinetic delay exists in $\alpha_V\beta_3/\beta_5$ integrin-mediated signaling in *Cd39*-null endothelial cells.

Marked differences in FAK and ERK1/2 activation are noted at baseline in wild-type and *Cd39*-null endothelial cells. FAK has increased activation in wild-type cells vs *Cd39*-null cells at baseline (wild-type 24.8% vs. *Cd39*-null 0.3%. P=0.04). In contrast, ERK1/2 is increased in unbound *Cd39*-null cells. (wild-type 2.7% vs. *Cd39*-null 26.4%. P=0.01). A possible explanation for this is that FAK is activated in wild-type cells by the paracrine release of extracellular nucleotides, acting via P2-receptors, which have been desensitized in *Cd39*-null cells. In contrast, ERK1/2 may be activated at baseline by a P2-receptor, which is not readily desensitized, like $P2Y_6$. Stimulation of this receptor would be greater in *Cd39*-null cells because of deletion of the bulk of cellular ectonucleotidase activity.

Further work is required to determine the mechanisms underlying these differences in unstimulated cells, however, it is worth noting that the addition of soluble apyrase abolishes these differences *in vitro* (vide infra). This suggests that deletion of the ectonucleotidase effect of Cd39, rather than any structural effects of the protein, are responsible for the observed differences in *Cd39*-null cells.

Figure 5.9.2

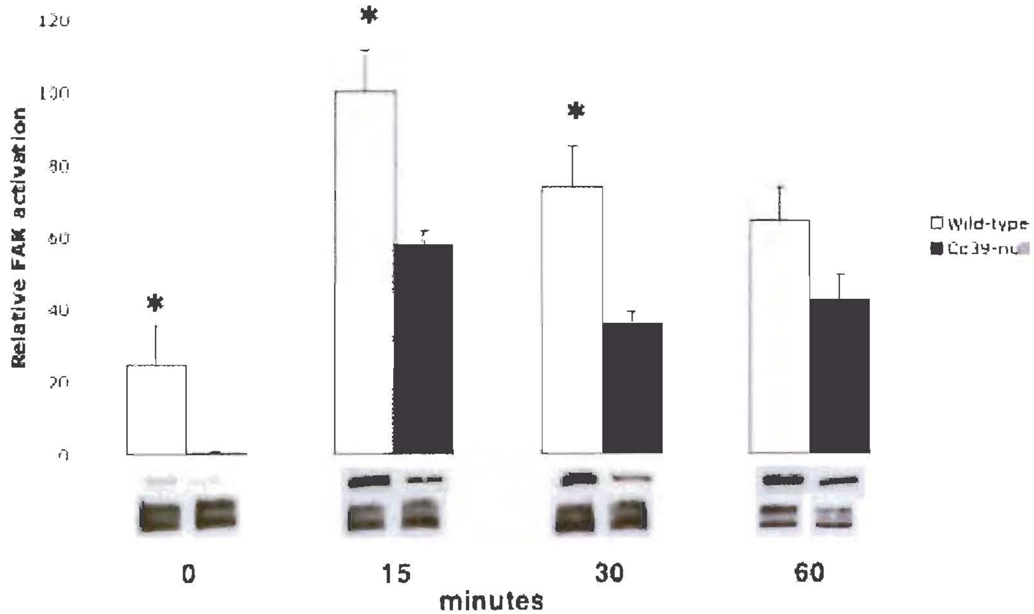
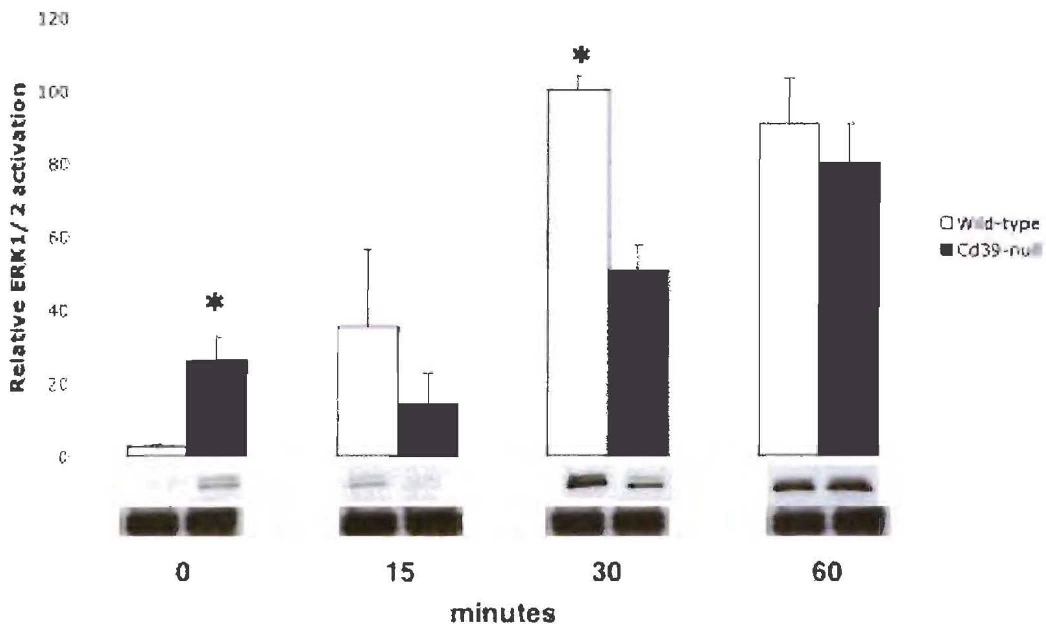


Figure 5.9.3



Figures 5.9.2 and 5.9.3 - Graphs represent phosphorylation of FAK (*Fig 5.9.2*) and ERK1/2 (*Fig 5.9.3*) in wild-type and *Cd39*-null endothelial cells at 0, 15, 30 and 60 minutes after plating onto vitronectin. Bar charts represent densitometry results (means ± s.e.m) of phosphorylated FAK or ERK1/2 divided by total FAK or ERK1/2, respectively. Representative blots are shown below graphs. *, $P<0.05$.

We had previously hypothesized that defective activation of Akt may be responsible for the increased endothelial cell apoptosis in regenerating livers of *Cd39*-null mice. However, following ligation of the $\alpha_V\beta_3/\beta_5$ integrin, wild-type and *Cd39*-null endothelial cells showed similar phosphorylation of Akt (Figure 5.9.4). Both wild-type and *Cd39*-null cells had initial submaximal activation of Akt at 15 minutes (wild-type 76% vs. *Cd39*-null 65%. P=0.44), and then sustained activation at 30 minutes (wild-type100% vs. *Cd39*-null 96%. P=0.45), and 60 minutes (wild-type 96% vs. *Cd39*-null 83%. P=0.36).

The fact that there is no significant difference in the phosphorylation of Akt in wild-type and *Cd39*-null cells implies, firstly, that defective activation of Akt is not responsible for the endothelial cell apoptosis during angiogenesis in regenerating mouse livers in *Cd39*-null animals. Secondly, it suggests an added complexity to the defective integrin function we have noted in *Cd39*-null endothelial cells. There appears to be a sustained defect in the activation of the FAK and downstream ERK1/2 pathway in *Cd39*-null endothelial cells, while the FAK, PI-3 kinase, Akt pathway remains unaffected.

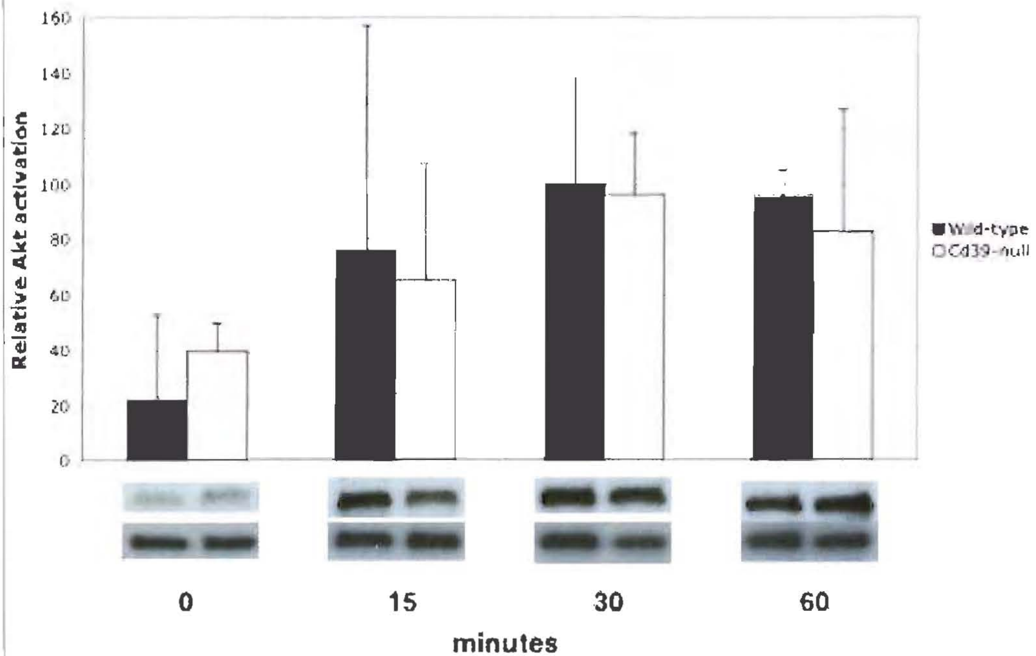


Figure 5.9.4 - Graphic representation of Akt phosphorylation in wild-type and *Cd39*-null endothelial cells at 0, 15, 30 and 60 minutes after plating onto vitronectin. Bar charts represent densitometry results (means ± s.e.m) of phosphorylated Akt divided by total Akt. Representative blots are shown below graphs.

We have hypothesized that purinergic receptors may form complexes with and assist in the activation of cell-surface integrins. This has been shown to occur with $P2Y_2$ and $\alpha_V\beta_3$, but may also occur with other purinergic receptors and integrin combinations. CD39 may modulate this interaction in several ways. Firstly, the absence of CD39 may result in P2-receptor desensitization because of excess nucleotide concentrations at the cell surface. This may alter the interaction between purinergic receptor and integrin and result in integrin inactivation. We have shown previously that *Cd39*-null platelets fail to activate integrin $\alpha_{IIb}\beta_3$ in response to ADP because of desensitization of the platelet ADP receptor, $P2Y_1$.[184]

Secondly, CD39 is targeted to caveolae on the cell surface and may associate directly with integrins and purinergic receptors in a signaling complex.[197,198] Deletion of *Cd39* may result in integrin inactivation because of absence of this protein in the signaling complex rather than by loss of nucleotide hydrolysis at the cell surface.

In order to resolve this question, we pre-treated the wild-type and *Cd39*-null endothelial cells with soluble Grade VII apyrase. Grade VII apyrase is a soluble enzyme, prepared from potato, which efficiently hydrolyzes ATP and ADP down to AMP. It thus acts as a soluble surrogate for CD39. Pre-treatment of *Cd39*-null cells with apyrase would correct nucleotide-induced P2-receptor desensitization, but would not replace the deleted CD39 protein in the putative integrin-purinoceptor-ectonucleotidase signaling complex.

Figure 5.9.5 shows the vitronectin-induced activation of FAK in wild-type and *Cd39*-null endothelial cells after pre-treatment with apyrase for 6 hours. In keeping with our finding that apyrase pretreatment corrects *Cd39*-null endothelial cell adhesion to vitronectin, treatment with soluble apyrase also corrects defective vitronectin-induced signaling to FAK and ERK1/2 in *Cd39*-null cells.

Plating wild-type endothelial cells onto vitronectin induces rapid and sustained phosphorylation of FAK from 15 minutes through 60 minutes. This FAK activation is unaffected by apyrase pre-treatment, an expected finding because wild-type endothelial cells express functional CD39 in culture.[184] In *Cd39*-null endothelial cells, pre-treatment with apyrase completely corrects the defect in signaling through FAK (15 minutes, wild-type 100% vs. *Cd39*-null 78.6%. P=0.14. 30 minutes, wild-type 73.2% vs. *Cd39*-null 81.2%. P=0.27. 60 minutes, wild-type 113.5% vs. *Cd39*-null 89.2%. P=0.29).

Similarly in the ERK1/2 activation experiments, pre-treatment with apyrase completely corrected the defective vitronectin-induced activation of ERK1/2 in *Cd39*-null endothelial cells. As shown in figure 5.9.6, there was no significant difference in the activation of ERK1/2 at 30 minutes (wild-type 93.8% vs. *Cd39*-null 90.7%. P=0.34) and 60 minutes (wild-type 100% vs. *Cd39*-null 96%. P=0.07) after plating onto vitronectin. At 15 minutes, ERK1/2 activation was actually higher in *Cd39*-null cells relative to the wild-type (wild-type 41.1% vs. *Cd39*-null 68%. P=0.01). However, this is probably just a consequence of rapid activation of MAPK occurring at this time in both wild-type and *Cd39*-null cells, which renders the experiment

exquisitely sensitive to variations in timing. Therefore, we feel that the increased activation of MAPK at 15 minutes in *Cd39*-null cells is not a result of enhanced integrin signaling in *Cd39*-null cells following apyrase pre-treatment.

In conclusion, we have shown that there is defective activation of FAK and ERK1/2 in *Cd39*-null endothelial cells after ligation of integrin $\alpha_V\beta_3/\beta_5$ by vitronectin. In addition, we have shown that this defective integrin-mediated signaling in *Cd39*-null cells is completely corrected by reconstitution of the ectonucleotidase function of CD39 by soluble apyrase. This implies that the defective "outside-in" integrin signaling in *Cd39*-null cells occurs because of P2-receptor desensitization, and not because of CD39's structural role in cell-surface signaling complex assembly. It also suggests that reconstitution of desensitized purine receptors is sufficient for normal $\alpha_V\beta_3/\beta_5$ activity in *Cd39*-null endothelial cells.

Figure 5.9.5

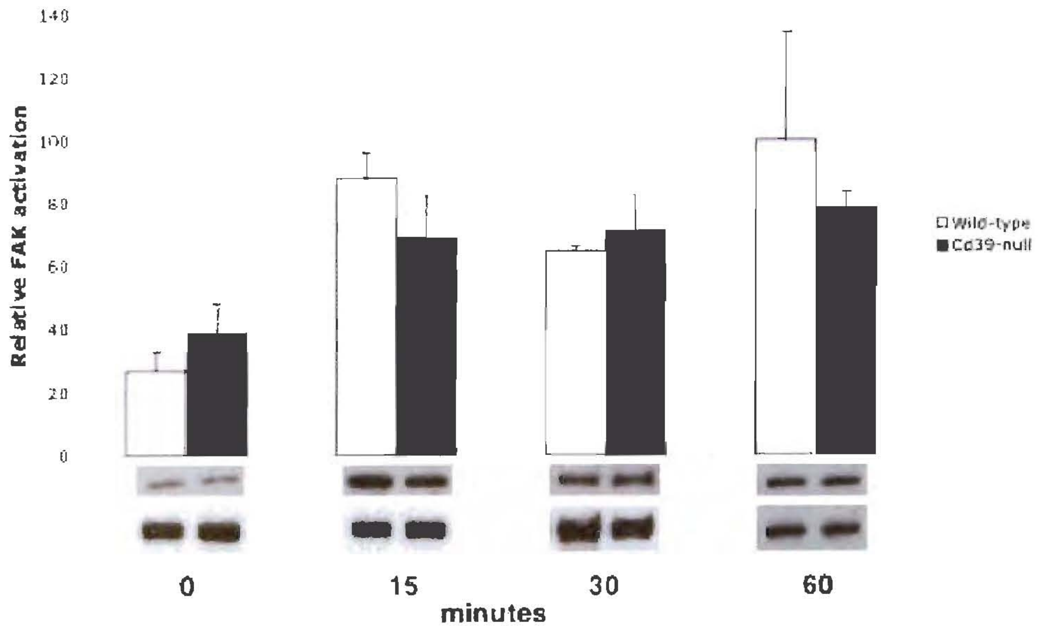
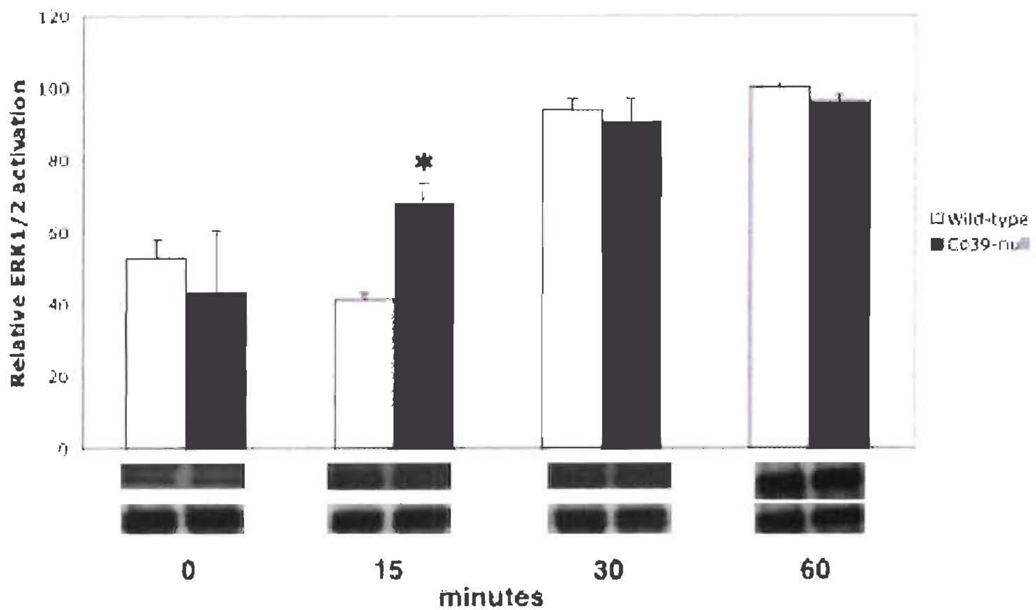


Figure 5.9.6



Figures 5.9.5 and 5.9.6 - Phosphorylation of FAK (*Fig 5.9.4*) and ERK1/2 (*Fig 5.9.5*) in wild-type and *Cd39*-null endothelial cells pretreated for 6 hours with soluble apyrase (5units/ml). Bar charts represent densitometry results (means ± s.e.m) of phosphorylated FAK or ERK1/2 divided by total FAK or ERK1/2, respectively. Representative blots are shown below graphs.

5.10 DIFFERENTIAL EXPRESSION OF ACTIVATED "LIGAND-RECEPTIVE" $\alpha_V\beta_3$ BY IMMUNO-CYTOCHEMISTRY

5.10.1 INTRODUCTION

In order to test our hypothesis that there is defective integrin activation in *Cd39*-null mouse lung endothelial cells, we analyzed the expression of activate integrin $\alpha_V\beta_3$ relative to total $\alpha_V\beta_3$ using the synthetic antibody WOW-1.

WOW-1 is novel synthetic ligand specific for activated integrin $\alpha_V\beta_3$, which was developed by a group at the Schripps Research Institute in California. It was determined that adenovirus enters eukaryotic cells by binding only to activated $\alpha_V\beta_3$. The adenovirus penton base that binds $\alpha_V\beta_3$ was isolated and using a technique called patch engineering was grafted into a monovalent Fab antibody PAC1 that is specific for activated $\alpha_{IIb}\beta_3$. This created a new monovalent Fab antibody that was shown to be specific for activated $\alpha_V\beta_3$, and have no affinity for $\alpha_{IIb}\beta_3$.[283] The antibody has been shown to have activity against transfected human $\alpha_V\beta_3$ and against $\alpha_V\beta_3$ expressed on bovine aortic endothelial cells.[283,284] Although never tested on murine cells, we hypothesized that, since WOW-1 was developed as a chimeric protein with specificity for the $\alpha_V\beta_3$-binding domain, we could show positive staining on murine endothelial cells.

A kind gift of WOW-1 antibody from Sanford Shattil, then at the Scripps Institute, afforded us the opportunity of analyzing the activation status of $\alpha_V\beta_3$ on wild-type and *Cd39*-null mouse lung endothelial cells directly, without having to use surrogate markers like cellular adhesion and intracellular signaling.

5.10.2 METHODS

a) FACS analysis

- Wild-type and *Cd39*-null mouse lung endothelial cells were grown to 70% confluence in normal endothelial media, then detached by trypsinization and resuspended in FACS buffer at 0.5×10^5 cells/ experiment.
- 50μL anti-α_V or WOW-1 antibody (1μg/mL) was added and incubated on ice for 15 minutes.
- After washing, 50μL FITC-conjugated fluorescent secondary antibody was added and incubated on ice for 15 minutes.
- Expression of total α_V integrin and activated $\alpha_V\beta_3$ on wild-type and *Cd39*-null endothelial cells was measured by fluorescence-activated cell sorter (FACS) analysis with excitation at 488nm (blue) and detection at 530nm (green).

b) Immunocytochemistry

- Immunofluorescent staining of endothelial cells performed as described in 5.2.1(b) using anti-α_V (1:200) and WOW-1 (1:100) as primary antibodies.

5.10.3 RESULTS

We have demonstrated that *Cd39*-null endothelial cells have defective integrin $\alpha_V\beta_3/\beta_5$ function on the basis of defective vitronectin-induced cellular adhesion and intracellular signaling. However, the expression of the α_V-integrin subunit is equal on wild-type and *Cd39*-null endothelial cells and the non-specific integrin activator $MnCl_2$ can correct the defective adhesion of *Cd39*-null endothelial cells. This suggests that $\alpha_V\beta_3$ integrin is expressed by *Cd39*-null endothelial cells, but that the integrin is expressed in an inactive state.

In order to prove that $\alpha_V\beta_3$ is expressed but inactive on *Cd39*-null cells we analyzed wild-type and *Cd39*-null cells by flow cytometry and immunocytochemistry using a synthetic antibody specific for active $\alpha_V\beta_3$, namely WOW-1.

In their initial publication of the development of WOW-1,[283] the authors demonstrated its specificity for activated $\alpha_V\beta_3$ using flow cytometry analysis of CHO cells stably transfected with $\alpha_V\beta_3$, or $\alpha_{IIb}\beta_3$. They showed that WOW-1 bound to $\alpha_V\beta_3$-, but not $\alpha_{IIb}\beta_3$-transfected cells. Also, activation of

the integrins using phorbol 12-myristate 13-acetate (PMA) and $MnCl_2$, significantly increased the binding of WOW-1 to the $\alpha_V\beta_3$-transfected cells. This demonstrated that WOW-1 was not only specific for $\alpha_V\beta_3$, but only bound to the integrin in its activated status.

Unfortunately, when we analyzed wild-type and *Cd39*-null endothelial cells using anti-α_V integrin and WOW-1 antibodies, we failed to get a positive shift in fluorescent intensity in either wild-type or *Cd39*-null cells with either the anti-α_V integrin or WOW-1 antibodies. In contrast, probing with an anti-CD31 antibody resulted in a strong signal in both wild-type and *Cd39*-null cells (results not shown).

There are a number of possible explanations for this finding. Firstly, trypsinization may have damaged the cells prior to flow cytometry analysis. Although it is possible that trypsin could have a greater effect on integrins expressed on the cell surface than it does on CD31, the fact that robust integrin-activated signaling through FAK and MAPK occurs 15 minutes after trypsinization implies that the $\alpha_V\beta_3$ integrin is not significantly damaged in this process.

Alternatively, $\alpha_V\beta_3$ may be expressed in an inactive state in solution prior to flow cytometry analysis. However, treatment with $MnCl_2$ failed to increase the binding of WOW-1 in either the wild-type or *Cd39*-null cells (results not shown). In addition, we also failed to show a signal using a polyclonal antibody specific for the α_V integrin subunit. The binding of this antibody is unaffected by integrin activation status.

The most likely explanation is that the expression levels of α_V integrin on both wild-type and *Cd39*-null mouse lung endothelial cells is too low for analysis by flow cytometry. The initial study describing WOW-1 used cells, which had been transfected with integrin $\alpha_V\beta_3$ prior to flow cytometry. In fact, untransfected control CHO cells had poor binding of WOW-1 in this study.[284]

We know that $\alpha_V\beta_3$ is up-regulated in proliferating angiogenic endothelium in vivo.[285] The endothelial cells we used in this experiment may approximate this condition in that they are derived from proliferating cells in culture, which have been exposed to a number of endothelial growth factors. However, the expression of $\alpha_V\beta_3$ was still not elevated to a level, which we could detect by flow cytometry.

Therefore, we decided to analyze the expression of activated integrin $\alpha_V\beta_3$ by immunocytochemistry. In a second publication by the group that developed the WOW-1 antibody, it was demonstrated that, after plating of bovine aortic endothelial cells onto fibrinogen, activated $\alpha_V\beta_3$ is recruited to lammellipodia at the protrusive edges of endothelial cells.[284] Because they had achieved a usable WOW-1 signal in a non-transfected cell line, we decided to test the WOW-1 antibody on wild-type and *Cd39*-null endothelial cells plated on a glass slide.

We first coated the glass slides with gelatin to increase the attachment of the endothelial cells prior to staining. We specifically chose gelatin, which is not

a ligand for $\alpha_V\beta_3$, because the WOW-1 antibody is specific for activated, *unbound* $\alpha_V\beta_3$ integrin.

As expected from the flow cytometry experiments the expression of $\alpha_V\beta_3$ was very low on both wild-type and *Cd39*-null endothelial cells. However, in this experimental model we were able to show a high fidelity signal using the anti-α_V integrin subunit antibody.

Figure 5.10.1 shows the expression of the α_V-integrin subunit on wild-type and *Cd39*-null mouse lung endothelial cells. It can be seen that the α_V-integrin subunit is expressed on both wild-type and *Cd39*-null endothelial cells, and that there is no significant difference in the expression levels between each cell type. This is consistent with our previous Western blot analyses of α_V-integrin expression, which showed no difference in the expression of α_V-integrin on wild-type and *Cd39*-null endothelial cells.

Unfortunately, we could not show similar results using the WOW-1 antibody. Repeated attempts at achieving a positive signal using this antibody failed in spite of $MnCl_2$-induced activation of surface integrins. Since we were able to demonstrate α_V-integrin expression by immunofluorescence using the anti-α_V-integrin subunit antibody, we concluded that the WOW-1 antibody was either not specific for murine $\alpha_V\beta_3$, or the batch of antibody we had been given was inactive. Since WOW-1 is not a commercially available antibody, we could not confirm species specificity or purchase more antibody. We were therefore unable to confirm the nature of $\alpha_V\beta_3$ activation on the surface of *Cd39*-null endothelial cells.

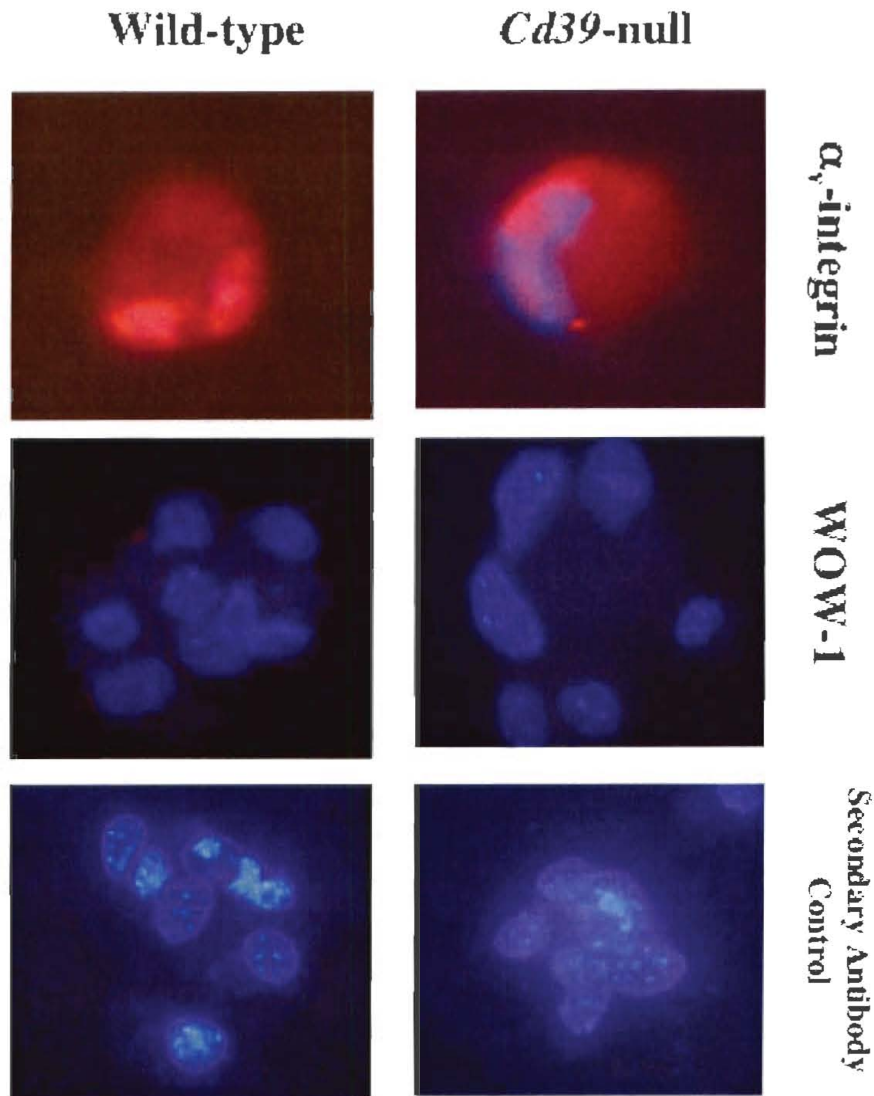


Figure 5.10.1 - Immunofluorescent staining (red) of α_v-integrin (upper panel) and WOW-1 (lower panel) on wild-type (left) and *Cd39*-null (right) endothelial cells. Note qualitatively equal α_v-integrin staining, but absence of WOW-1 staining. Negative control with only secondary antibody (Fluorescein-conugated rabbit anti-rat IgG) shown below. Cell nuclei are stained blue (Hoescht).

CHAPTER 6: DISCUSSION

We have shown that abnormal purinergic signaling in the *Cd39*-null vasculature results in profound defects in both physiological and pathological angiogenesis. Deletion of the majority of vascular ectonucleotidase activity results in defective Matrigel™ plug-induced angiogenesis,[214] abnormal liver regeneration following hepatic resection and disordered cardiac remodeling following experimental myocardial infarction. In addition, decreased angiogenic responses in the *Cd39*-null mouse led to markedly decreased growth of implanted tumours and of tumour metastases.

Angiogenesis is characterized initially by vessel dilation, increased permeability and fibrin deposition. Maturation of newly formed blood vessels requires the formation of endothelial tubes surrounded by new basement membrane and the recruitment of pericytes and smooth muscle cells around the new endothelium.[286]

Cd39-null mice exhibit normal initial phases of angiogenesis, including mother vessel formation, increased vascular permeability and fibrin deposition. However, there is a profound defect in the migration of endothelial and inflammatory cells into tumours in *Cd39*-null animals. There is also abnormal resolution of angiogenesis in *Cd39*-null mice characterized by poor recruitment of surrounding pericytes and smooth muscle cells to new blood vessels. As embryonic vascular development is normal in *Cd39*-null mice,[184] our data suggests that purinergic signaling has differential roles

in physiological blood vessel development as opposed to pathological angiogenesis.

Despite this defect in tumour angiogenesis, deletion of *Cd39* is associated with the development of spontaneous hepatocellular carcinomas in aged mice (Sun Xiaofeng, unpublished observation). These carcinomas are characterized by the development of large sinusoidal blood vessels, rather than true angiogenic microvasculature. This implies that alternate mechanisms of tumour vascularization, perhaps co-opting of existing vasculature, compensated for the observed defects in endothelial cell migration and angiogenesis.

Our initial *in vitro* analyses demonstrated that the defective angiogenesis in *Cd39*-null mice did not occur because of defects in endothelial cell proliferation, apoptosis or matrix metalloproteinase expression. Rather, we showed that the cellular migration defect in *Cd39*-null mice occurs because of abnormal vitronectin receptor ($\alpha_V\beta_{3/5}$) function in *Cd39*-null endothelial cells.

Although several members of the integrin family have been implicated, the largest body of data has linked the vitronectin receptor $\alpha_V\beta_3$ with the promotion of neovascularization. Numerous $\alpha_V\beta_3$ antagonists, including blocking antibodies directed at the extracellular domain of $\alpha_v\beta_3$ (LM609), inhibit angiogenesis in experimental animal models[34] and are currently undergoing human clinical trials.[229] Matrix metalloproteinase-2 (MMP-2) also co-localizes with $\alpha_V\beta_3$ and may direct proteolytic activity at the leading

edge of migrating endothelial cells[95]. Inhibition of this interaction, without blocking either $\alpha_V\beta_3$ or MMP-2 function, inhibits angiogenesis *in vivo*[109,287].

Despite the efficacy of these $\alpha_V\beta_3$ inhibitors, genetic deletion of $\alpha_V\beta_3$ results in increased tumour angiogenesis.[129] It has been proposed that integrins might provide positive and/or negative signals to cells in the presence of appropriate extracellular ligands.[130] Absence of these interactions could result in loss of putative negative signal(s) and enhanced angiogenesis. These observations imply that $\alpha_V\beta_3$ could be involved in the fine-tuning of angiogenesis responses *in vivo*.

We have shown that normal purinergic signaling is also required for $\alpha_V\beta_3$ function and for angiogenesis. Cellular signaling by G protein–coupled receptors (GPCRs), such as P2Y-receptors, involves not only the coupling of the receptors to G proteins, but also the formation of large protein complexes that assist in transmitting an extracellular signal to an intracellular response[208,209]. $P2Y_2$ contains an integrin-binding, arginine-glycine-aspartic acid (RGD) domain in its first extracellular loop, suggesting interactions with integrin $\alpha_V\beta_3$; mutation of the RGD sequence to RGE, decreased the co-localization of α_V-integrins with $P2Y_2$ ten-fold and greatly impaired UTP-induced phosphorylation of FAK.[195]

Direct association between $P2Y_2$ and α_V-integrins appears to also be necessary for UTP-induced endothelial cell migration, implying an important link between purinergic signaling, integrin function and pro-angiogenic cellular behaviors[204]. We hypothesize that deletion of *Cd39*

results in differential P2-receptor desensitization, which subsequently modulates the activity of $\alpha_V\beta_3$. In keeping with this supposition, $MnCl_2$ and apyrase pretreatment of *Cd39*-null endothelial cells was able to normalize function of $\alpha_V\beta_3$, implying that P2-receptor desensitization and secondary integrin inactivation is responsible for these findings.

We have recently published that the N-terminus of CD39 interacts with the multi-adaptor scaffolding membrane phosphoprotein, RanBPM.[257] RanBPM binds the HGF-receptor cMET and modulates a number of intracellular signaling cascades, in particular the Ras-ERK-SRE pathway.[288] In addition, RanBPM interacts with LFA-1 (lymphocyte function-associated antigen-1) and integrins and may affect integrin activation and signaling pathways directly.[289] These results suggest that CD39 may regulate cellular signal transduction pathways and integrin function by two separate mechanisms. Firstly, by modulating P2 receptor function following hydrolysis of extracellular nucleotides, and secondly, by interacting with other signaling proteins, possibly using RanBPM as a bridge.

An important future direction for study would be to determine which P2-receptors are important for the proposed $\alpha_V\beta_3$ activation/inactivation. This study is limited by the lack of truly specific P2-receptor agonists and antagonists.[180] Additionally, P2-receptor desensitization likely involves receptor phosphorylation or uncoupling of the receptor from its associated G-protein. This suggests that simple RT-PCR analysis of P2-receptor expression in wild-type and *Cd39*-null endothelial cells would unlikely identify the involved P2-receptors.

The most rational approach would be to target those P2-receptors with known links to $\alpha_V\beta_3$, for example $P2Y_2$.[195] $P2Y_2$ desensitization has been linked to phosphorylation sites on the C-terminus of the receptor.[290] An approach would be to use C-terminal truncation mutants of $P2Y_2$ in systems involving Cd39, to determine whether mutation of $P2Y_2$ reverses the $\alpha_V\beta_3$ inactivation noted in *Cd39*-null cells. Alternatively, $P2Y_1$, $P2Y_2$ and $P2Y_4$ knock-out animals have already been developed, and these could be used to develop double knock-out animals with *Cd39*-null mice to test this hypothesis further.[291-293]

Two Src homology-3-binding sites on $P2Y_2$ have been shown to be responsible for UTP-induced transactivation of vascular endothelial growth factor receptor (VEGFR)-2 by $P2Y_2$.[294] This implies that $P2Y_2$ desensitization may result in VEGF-signaling defects in *Cd39*-null animals. Our data suggest that deletion of *Cd39* is not associated with defects in VEGF signaling *in vitro*, but further work is required to examine this possibility in settings involving activation of $P2Y_2$ by UTP.

An additional hypothesis exists for the development of integrin dysfunction following *Cd39* deletion. In addition to modulating P2-receptor signaling by nucleotide hydrolysis, CD39 also generates adenosine by hydrolyzing ATP and ADP to AMP, which is subsequently converted to adenosine by the action of ecto-5'-nucleotidase (CD73). Adenosine has also been shown to have important pro-angiogenic functions. For example, adenosine, acting via the A_{2A}-receptor, promotes wound healing by stimulating angiogenesis.[201]

It is certainly possible that deletion of *Cd39* results in decreased production of adenosine and that it is the lack of this pro-angiogenic factor, rather than P2-receptor desensitization, which leads to defective angiogenesis in *Cd39*-null mice.

Further work is required to examine of this possibility. For example, we have recently studied the effect of the non-selective adenosine agonist 5'-N-ethylcarboxamidoadenosine (NECA) on intracellular signaling events following $\alpha_V\beta_3$ ligation. Preliminary results suggest that NECA does not correct defective FAK and ERK1/2 activation in *Cd39*-null endothelial cells (preliminary data, not shown). This implies that P2-receptor desensitization, rather than lack of adenosine effects is responsible for the defective function of $\alpha_V\beta_3$. We still need to study the effects of NECA on endothelial cell adhesion, as well as confirm our findings with other synthetic adenosine analogues.

The *Cd73*-null mouse would provide another avenue to study the role of adenosine in angiogenesis *in vivo* and *in vitro*. Deletion of ecto-5'-nucleotidase (CD73) would result in decreased production of adenosine from purine monophosphates and potentially defective angiogenesis. It would also be interesting to examine whether *Cd73*-null endothelial cells have similar defective $\alpha_V\beta_3$ function *in vitro*, implying that our findings in *Cd39*-null cells are secondary to decreased adenosine generation.

A final important avenue to study would be the role of anti-tumour immune mechanisms on tumour growth in wild-type and *Cd39*-null animals. Ohta et al. have recently reported that A_{2A}-receptor signaling inhibits anti-tumour lymphocytes and promotes tumour growth *in vivo*.[295] They propose targeting the hypoxia → adenosine → A_{2A}-receptor pathway in order to prevent the inhibition of anti-tumour immune cells within the hypoxic tumour microenvironment.[295] This finding has important implications for our work as *Cd39*-null animals may have decreased inhibition of anti-tumour lymphocytes because of limited generation of adenosine. In this regard, we have evidence that CD39 forms an important part of the immunosuppressive apparatus of regulatory T cells (Treg), at least in part via the generation of adenosine (S. Deaglio, K. Dwyer, manuscript submitted).

The most exciting aspect of our work on CD39 and angiogenesis is that we may be able to modulate purinergic signals therapeutically in the future. Soluble derivatives of CD39 have already been proposed as effective anti-thrombotic or anti-inflammatory therapies.[234] Our findings raise the possibility of new anti-angiogenesis therapies based on modulating extracellular nucleotide-mediated signals. These goals could be achieved by either blocking the action of CD39 directly, or by targeting those nucleotide (P2) receptors that are important in activating integrins during angiogenesis. It is our hope that purinergic signaling systems may become an important target of anti-angiogenic cancer therapies.

Our results also demonstrate a caveat to the use of soluble CD39 derivatives as anti-thrombotic and anti-inflammatory therapies. We have shown that single doses of apyrase increases the development of pulmonary metastases

in both wild type and *Cd39*-null mice. This suggests that ectonucleotidases might promote tumour cell seeding and consequent survival in the pulmonary vasculature. The implication is that CD39-based therapies may promote tumour metastasis and will require careful monitoring with human use.

In conclusion, we have provided evidence for the importance of purinergic angiogenesis in disease and described novel links between purinergic signaling and integrin activation. These findings raise the possibility of new anti-angiogenesis therapies based on modulating extracellular nucleotide-mediated signals.

CHAPTER 7: APPENDICES

7.1 ABBREVIATIONS

α-SMA	α-smooth muscle actin
β-HCG	β-human chorionic gonadotrophin
Akt	protein kinase B
AMP, ADP, ATP	adenosine 5'-mono, di, triphosphate
Ang-1, -2	angiopoietin-1, -2
ATCC	American Type Culture Collection
BrdU	5-bromo-2-deoxyuridine
CAM	chick chorioallantoic membrane
CFTR	cystic fibrosis transmembrane conductance regulator
dNTP	deoxyribonucleoside triphosphate
DAB	3,3-diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
ECM	extracellular matrix
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm
ELISA	enzyme-linked immunosorbent assay
E-NTPDase	ecto-nucleoside triphosphate diphosphohydrolase
EC	endothelial cell

EDTA	ethylenediaminetetraacetic acid
ERBB2	v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2
ERK1/2	extracellular signal-regulated kinase 1/2
ES	embryonic stem
E-NPP	ectonucleotide pyrophosphatase/phosphodiesterase
FACS	Fluorescence Activated Cell Sorting
FAK	focal adhesion kinase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GDP	guanosine 5'-diphosphate
GPI	glycosylphosphatidylinositol
Grb2	growth factor receptor-bound protein 2
HSPG	heparin-sulphate proteoglycan
HGF	hepatocyte growth factor
HIF-1α	hypoxia-inducible transcription factor 1α
HUVEC	human umbilical vein endothelial cells
ICAM-2	intracellular adhesion molecule 2
IFN-α	interferon-α
ILF	irintecan, fluorouracil and leucovorin
ILK	integrin-linked kinase
IGF-1	insulin-like growth factor
IP_3	inositol 1,4,5-triphosphate
LCA	leukocyte common antigen (CD45)

LLC	Lewis lung carcinoma
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
MMP	matrix metalloproteinase
MT-MMP	membrane-type metalloproteinases
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
NAD	nicotinamide adenine dinucleotide
NEJM	New England Journal of Medicine
NF-κB	nuclear factor-κB
NK	natural killer
NRP-1	neuropilin-1
NTPDase	nucleoside triphosphate diphosphohydrolase
PAI-1	plasminogen activator inhibitor-1
PAK	p21-activated kinases
PBS	phosphate-buffered saline
PDGF-β	platelet-derived growth factor-β
PCR	polymerase chain reaction
PECAM-1	platelet/endothelial cell adhesion molecule 1
PGI2	prostaglandin I2 (prostacyclin)
PlGF	placenta growth factor
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonyl fluoride
PI 3-kinase	phosphoinositide 3-OH kinase
RGD	arginine-glycine-asparagine

RGE	arginine-glycine-glutamic acid
RIPA	radioimmunoprecipitation assay
RNAse	ribonuclease A
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2/SH3	Src homology 2/3 domain
SMB	somatomedin B
SOS	son of sevenless
TAF	tumour angiogenesis factor
TCA	trichloroacetic acid
TdT	terminal deoxynucleotidyl transferase
TGF-1	transforming growth factor-1
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
TIMP	tissue inhibitors of matrix metalloproteinases
TNF-α	tumour necrosis factor-α
t-PA	tissue-type plasminogen activator
TSP-1	thrombospondin-1
Tunel	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
UMP, UDP, UTP	uridine 5'-mono, di, triphosphate
U-PA	urokinase-type plasminogen activator
u-PAR	urokinase-type plasminogen activator receptor
VEGF	vascular endothelial growth factor

VEGFR-1,-2	vascular endothelial growth factor receptor-1 (Flt-1), -2 (Flk-1/KDR)
vWF	von Willebrand factor
VPF	vascular permeability factor
VE-cadherin	vascular endothelial-cadherin

7.2 MATERIALS AND SUPPLIERS

7.2.1 ANTIBODIES

- **α-smooth muscle actin**
- Sigma-Aldrich Corp. St. Louis, MO — Cat: A5228
- **Akt**
- Cell Signaling Technology, Inc. Danvers, MA — Cat: 9272
- **CD16/CD32 (FcγIII/II)**
- BD Biosciences Pharmingen. San Jose, CA — Cat: 553142
- **CD31 (PECAM-1)**
- BD Biosciences Pharmingen. San Jose, CA — Cat: 553371
- **CD61 (β3 integrin subunit)**
- BD Biosciences Pharmingen. San Jose, CA — Cat: 550541
- **CD144 (VE cadherin)**
- BD Biosciences Pharmingen. San Jose, CA — Cat: 550548
- **F4/80 (macrophage marker)**
- Serotec, Inc. Raleigh, NC — Cat: MCA497GA
- **FAK**
- Cell Signaling Technology, Inc. Danvers, MA — Cat: 328
- **Fluorescein-conjugated Goat anti-Rabbit IgG**
- Vector Laboratories. Burlingame, CA — Cat: FI-1000
- **Fluorescein-conugated Rabbit Anti-Rat IgG**
- Vector Laboratories. Burlingame, CA — Cat: FI-4000
- **Heparan Sulphate Proteoglycan**
- CHEMICON International, Inc. Temecula, CA — Cat: MAB1948

- **ICAM-2 antibody**
- BD Biosciences Pharmingen. San Jose, CA Cat: 550544
- **Integrin α_v chain**
- BD Biosciences Pharmingen. San Jose, CA Cat: 550024
- **p44/42 MAP Kinase**
- Cell Signaling Technology, Inc. Danvers, MA Cat: 9102
- **Phospho-Akt (Ser473)**
- Cell Signaling Technology, Inc. Danvers, MA Cat: 4058
- **Phospho-FAK (Tyr925)**
- Cell Signaling Technology, Inc. Danvers, MA Cat: 3284
- **Phospho-p44/42 MAP Kinase (Thr202/Tyr204)**
- Cell Signaling Technology, Inc. Danvers, MA Cat: 9101
- **Pig anti-Rabbit $F(ab')_2$**
- Dako North America, Inc. Carpinteria, CA Cat: E0431
- **Platelet derived growth factor (PDGFR-β) receptor**
- Santa Cruz Biotechnology, Inc. Santa Cruz, CA Cat: sc-339
- **Rabbit anti-Mouse $F(ab')_2$**
- Dako North America, Inc. Carpinteria, CA Cat: E0413
- **Rabbit Anti-Rat IgG**
- Vector Laboratories. Burlingame, CA Cat: BA-4000
- **Vascular endothelial growth factor receptor-2 (VEGFR-2)**
- Kind gift from R. Brekken and P. Thorpe
- University of Texas Southwestern Medical Center, Dallas
- **von Willebrand Factor**
- BD Biosciences Pharmingen. San Jose, CA Cat: 555849

7.2.2 REAGENTS

- **Adenosine**
  - Sigma-Aldrich Corp. St. Louis, MO — Cat: A4036
- **Adenosine 5'-triphosphate (ATP)**
  - Sigma-Aldrich Corp. St. Louis, MO — Cat: A6559
- **ApopTag® Peroxidase In Situ Apoptosis Detection Kit**
  - CHEMICON International, Inc. Temecula, CA — Cat: S7100
- **Apyrase from potato (grade VII)**
  - Sigma-Aldrich Corp. St. Louis, MO — Cat: A6535
- **BenchMark™ Pre-stained Protein Ladder**
  - Invitrogen Corp. Carlsbad, California — Cat: 10748-010
- **Bovine Serum Albumin**
  - Sigma-Aldrich Corp. St. Louis, MO — Cat: A6003
- **5-Bromo-2-deoxyuridine (BrdU)**
  - Sigma-Aldrich Corp. St. Louis, MO — Cat: 858811
- **BrdU Labeling and Detection Kit III**
  - Roche Diagnostics Corp. Indianapolis, IN — Cat: 1444 611
- **Collagenase, Type 1**
  - Worthington Biochemical Corp. Lakewood, NJ — Cat: LS004194
- **Coomassie Blue**
  - Bio-Rad Laboratories, Inc. Hercules, CA — Cat: 161-0400
- **Creatinine Assay Kit**
  - Sigma diagnostics, Inc. St. Louis, MO — Cat: 555A

- **Crystal Violet**
- Sigma-Aldrich Corp. St. Louis, MO Cat: HT90132
- **DC Protein Assay Kit**
- Bio-Rad Laboratories, Inc. Hercules, CA Cat: 500-0112
- **Dithiothreitol (DTT)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: 43815
- **DNeasy® tissue kit**
- Qiagen, Inc. Valencia, CA Cat: 69504
- **Dulbecco's Modified Eagle Medium (DMEM) (low glucose, plus GLUTAMAX™)**
- Gibco (Invitrogen Corp) Carlsbad, CA Cat: 10567-014
- **Dynabeads (sheep anti-rat IgG)**
- Dynal (Invitrogen Corp) Carlsbad, CA Cat: 110-35
- **Endothelial Mitogen**
- Biomedical Technologies, Inc. Stoughton, MA Cat: BT-203
- **F-12 Nutrient Mixture (Ham) (plus GLUTAMAX™)**
- Gibco (Invitrogen Corp) Carlsbad, CA Cat: 31765-035
- **Fetal Bovine Serum**
- Gibco (Invitrogen Corp) Carlsbad, CA Cat: 16000-044
- **Fibroblast Growth Factor (FGF)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: F3133
- **Fibronection from rat plasma**
- Sigma-Aldrich Corp. St. Louis, MO Cat: F0635
- **Gelatin (type B)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: G9391
- **Glycine**
- Sigma-Aldrich Corp. St. Louis, MO Cat: 8898

- **Goat serum**
- Sigma-Aldrich Corp. St. Louis, MO Cat: G9023
- **Heparin sodium salt**
- Sigma-Aldrich Corp. St. Louis, MO Cat: H4784
- **Hepatocyte Growth Factor (HGF)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: H1404
- **Human Chorionic Gonadotropin (HCG) ELISA Kit**
- Alpha Diagnostics Intl. Inc. San Antonio, TX Cat: 0400
- **Insulin (Human lisproinsulin – humalog)**
- Eli Lilly and Co. Indianapolis, IN
- **Insulin-like Growth Factor-I (IGF-1)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: I3769
- **L-Glutamine**
- Gibco (Invitrogen Corp) Carlsbad, CA Cat: 21051-024
- **Laemmli (SDS) reducing buffer**
- Boston BioProducts, Inc. Worcester, MA Cat: BP-110R
- **Liquid scintillation mixture**
- Sigma-Aldrich Corp. St. Louis, MO Cat: 327123
- **Manganese chloride ($MnCl_2$)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: M8054
- **Matrigel™ (Growth Factor Reduced)**
- BD Biosciences. San Jose, CA Cat: 356231
- **β-Mercaptoethanol**
- Sigma-Aldrich Corp. St. Louis, MO Cat: M7154
- **2-methyl-2-butanol**
- Sigma-Aldrich Corp. St. Louis, MO Cat: 240486
- **Mouse serum**

- Sigma-Aldrich Corp. St. Louis, MO Cat: M5905
- **Nonimmune Mouse IgG**
- Sigma-Aldrich Corp. St. Louis, MO Cat: I5381
- **Nonimmune Rabbit IgG**
- Sigma-Aldrich Corp. St. Louis, MO Cat no: I5006
- **Nonimmune Rat IgG**
- Sigma-Aldrich Corp. St. Louis, MO Cat: I4131
- **PCR primers (D3M, NEOP2, MEC8P)**
- Gibco (Invitrogen Corp) Carlsbad, CA
- **Penicillin-Streptomycin (5000units Penicillin, 5000μg Streptomycin)**
- Gibco (Invitrogen Corp) Carlsbad, CA Cat: 15070-063
- **Percoll (density 1.13mg/ml)**
- Amersham Biosciences. Piscataway, NJ Cat: 17-089102
- **Phorbol 12-myristate 13-acetate (PMA)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: P1585
- **Phosphate-buffered saline**
- Gibco (Invitrogen Corp) Carlsbad, CA Cat: 70011-044
- **Phosphate-buffered saline (no calcium or magnesium)**
- Gibco (Invitrogen Corp) Carlsbad, CA Cat: 14200-075
- **Porcine serum**
- Sigma-Aldrich Corp. St. Louis, MO Cat: P9783
- **Precast polyacrylamide gels (4-15% linear gradient)**
- Bio-Rad Laboratories, Inc. Hercules, CA Cat: 161-1104
- **Proliferating Cell Nuclear Antigen (PCNA) Staining Kit**
- Gibco (Invitrogen Corp) Carlsbad, CA Cat: 93-1143
- **Protein A-Sepharose®**

- Sigma-Aldrich Corp. St. Louis, MO Cat: P3391
- **Protease Inhibitor Cocktail Tablets**
- Roche Diagnostics Corp. Indianapolis, IN Cat: 1836153
- **PVDF membrane**
- Bio-Rad Laboratories, Inc. Hercules, CA Cat: 162-0177
- **Rabbit serum**
- Sigma-Aldrich Corp. St. Louis, MO Cat: R9133
- **Restore Western Blot Stripping Buffer**
- Pierce Biotechnology, Inc. Rockford, IL. Cat: 21059
- **Saponin**
- Sigma-Aldrich Corp. St. Louis, MO Cat: 47036
- **Sodium dodecyl sulphate (SDS)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: L3771
- **Sphingosine-1-phosphate**
- BIOMOL Int. L.P. Plymouth Meeting, PA Cat: SL140
- **SuperSignal (Femto) Chemiluminescent Substrate**
- Pierce Biotechnology, Inc. Rockford, IL. Cat: 34095
- **SuperSignal (Pico) Chemiluminescent Substrate**
- Pierce Biotechnology, Inc. Rockford, IL. Cat: 34080
- **TACS XL-Basic (In Situ Apoptosis Detection Kit)**
- R&D Systems, Inc. Minneapolis, MN Cat: TA100
- **Taq DNA Polymerase, Hot-Start Version Kit**
- Takara Bio Inc. Shiga, Japan Cat: R007A
- **Texas Red Avidin D**
- Vector Laboratories. Burlingame, CA Cat: A-2006
- **[methyl-^{3}H] Thymidine (47 Ci/mmol)**
- Amersham Biosciences. Piscataway, NJ Cat: TRK637

- **2,2,2-Tribromoethanol**
- Sigma-Aldrich Corp. St. Louis, MO Cat: T48402
- **Trichloroacetic acid**
- Sigma-Aldrich Corp. St. Louis, MO Cat: 45670
- **Triton® X-100**
- Sigma-Aldrich Corp. St. Louis, MO Cat: T8787
- **Trizma® base (tris base)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: T1503
- **Trypsin-EDTA solution**
- Sigma-Aldrich Corp. St. Louis, MO Cat: T4174
- **Trypsin inhibitor from *Glycine max* (soybean)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: T6414
- **TWEEN® 20**
- Sigma-Aldrich Corp. St. Louis, MO Cat: P5927
- **Uridine 5'-triphosphate (UTP)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: U6875
- **Vascular Endothelial Growth Factor (VEGF)**
- Sigma-Aldrich Corp. St. Louis, MO Cat: V4512
- **Vitronectin from rat plasma**
- Sigma-Aldrich Corp. St. Louis, MO Cat: V0132

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