Novel pathways in microvascular signalling

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ABSTRACT

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The regulation of microvascular survival impacts both developmental remodelling of the vasculature, and various pathologies. The aim of this thesis was to examine the role of a recently identified A20 binding inhibitor of NF-κB, a zinc finger protein termed ABIN-2 in endothelial protection. More specifically, looking at its effects on inflammation and apoptosis in endothelia both at the cellular level and *in vivo*. The involvement of ABIN-2 in the Tie2 receptor pathway was also examined. Tie2 is an endothelial receptor essential for blood vessel formation and promotes endothelial survival. A transfection protocol was established allowing expression ABIN-2 in up to 90% of endothelial cells. ABIN-2 was shown to reduce apoptosis in endothelial cells as well as improve cell survival following growth factor deprivation. This effect was inhibited by Wortmannin and LY294002 which are known inhibitors of phatidylinositol-3 kinase. Expression of the truncated form of ABIN-2 lacking the carboxy terminal of ABIN-2 did not protect cells from apoptosis. In addition, expression of the truncated form prevented cell rescue by angiopoeitin-1 from apoptosis.

The chick chorioallantoic membrane was used as an in vivo model for testing the role of ABIN-2 in vessel inflammation. It was possible to use this model to transfect plasmids into live microvessels using electroporation resulting in high yield expression of target protein. This model was adapted to look at microvessel inflammation and apoptosis. Expression of ABIN-2 in microvessels reduced leukocyte rolling following tumour necrosis factor-α and lipopolysaccharide induced inflammation. The truncated form of ABIN-2 lacking the carboxy terminal did not reduce microvessel inflammation.The NF-κB inhibitor PTDC was found to suppress leukocyte rolling. ABIN-2 expression also appeared to give limited protection against apoptosis *in vivo*.

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To God for giving me everything I need and more.

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Chapter 1

Introduction

Chapter 1

1. Introduction

The formation of an adequate and functional circulatory system is a truly remarkable process. It involves mechanisms so complex and so intricate that the majority of them still remain a mystery despite years of research. It goes without saying that blood vessel formation and function is essential for survival. This occurs via two processes: vasculogenesis, whereby a primitive vascular network is established during embryogenesis from multipotential mesenchymal progenitors, and angiogenesis, in which pre-existing vessels (both in embryo and adult) send out capillary sprouts to produce new vessels {Carmeliet P. 2000 19 /id}. These vessels are lined with endothelial cells which regulate all aspects of vascular behaviour.

Endothelial cells are centrally involved in each process: They migrate and proliferate and then assemble into tubes with tight cell-cell connections to contain the blood. Peri-endothelial support cells are recruited to encase the endothelial tubes, providing maintenance and modulatory functions to the vessels; such cells include pericytes for small capillaries, smooth muscle cells for larger vessels, and myocardial cells in the heart {Benjamin L.E. 2000}.

This is a complex process therefore involving activation, migration and proliferation of endothelial cells and their functional maturation. A balance between angiogenic and angiostatic molecules modulates these steps {Benjamin L.E. 2000 }{Davis S 1999}. Regulated growth of new capillary blood vessels occurs in adults during wound healing, development of the corpus luteum, and formation of blood vessel

collaterals. On the other hand, uncontrolled angiogenesis with reduced vessel regression is a feature of pathological processes such as diabetic retinopathy, psoriasis and tumour growth {Risau W. 1997}. The endothelial cells are also involved and play a central part in many inflammatory conditions such as rheumatoid arthritis, systemic lupus erythemaotus and disseminated intravascular coagulation {Zimmerman G, Kurt H, et al. 1999}. Vessel inflammation and regression is an important feature of these physiological and pathological processes, it is of clinical relevance to understand vascular morphogenesis, to identify factors that stimulate or inhibit these processes and to elucidate the mechanisms of drug action.

1.1 Vascular inflammation and regression in disease

The regulation of microvascular survival impacts both developmental remodelling of the vasculature, and various pathologies which involve either vessel inflammation or regression. Understanding the regulatory mechanisms controlling stability and regression of microvessels will allow therapeutic manipulation of these processes in pathologies in which excessive or defective inflammation have significant roles. For example, more efficient resolution of vascular trauma and leak in burns patients, could be accomplished by therapeutic induction of regression of microvessel within the zone of stasis as well as dampening down the overall post burn inflammatory response.

Similarly, the coordinated regulation of angiogenesis and vessel regression is essential in the normal development of the vascular system. The induction of apoptosis in endothelial cells dramatically disturbs the establishment of the primordial vascular

network in the embryo and leads to embryonal death. {Dimmeler S, Zeiher M, 2000, i.d. 151}.

In addition, a number of human disorders are associated with obliteration of pre-existing blood vessels. Microvessel rarefaction often takes place in the hypertensive lung, in the myocardium of patients with chronic renal failure, and in the elderly {Keshet E, 2003, i.d.152}. Conversely, a failure to eliminate transient embryonic vasculature destined for regression may lead to a disease, as exemplified by the common congenital developmental anomaly of the eye, persistent hyperplastic primary vitreous, in which hyaloid vessels fail to regress. A striking example of a disease caused by vessel regression is retinopathy of prematurity (ROP) {Dimmeler S, Zeiher M, 2000}. ROP is a blindness-causing neovascularizing disease that affects premature infants treated with high concentrations of oxygen. ROP develops following a hyperoxic insult which leads to obliteration of immature retinal vessels, thereby compromising retina perfusion. Clearly insight into the mechanisms controlling microvessel regression and specifically endothelial apoptosis will have great value in determining therapeutic consequences.

1.2 Mechanisms controlling vascular inflammation

A variety of pathophysiological situations that affect endothelial and smooth muscle cells, leads to the expression of genes such as chemokines and adhesion molecules that are dependent on members of the nuclear family (NF)-κB of transcription factors. The corresponding gene products mediate important biological functions such as immune and inflammatory reactions, smooth muscle proliferation and angiogenesis.

1.2.1 Inflammation & the leucocyte adhesion cascade

Inflammation is a defence reaction caused by tissue damage or injury from a variety of causes. It is defined as the local recruitment and activation of leucocytes. The primary objective of pathological inflammation is to localize and eradicate the irritant and repair the surrounding tissue. The inflammatory process consists of a series of events that hinge upon the mechanics of adhesion between leucocytes and endothelial cells. The inflammatory response has three distinct stages: Firstly dilation of the capillaries to increase blow flow; secondly microvascular structural changes and escape of plasma proteins from the blood stream; and thirdly leucocyte transmigration through the endothelium and accumulation at the site of injury. Leucocyte transmigration and extravasation out of the blood vessels occurs in a series of steps termed the adhesion cascade. At least 5 steps are known; Capture, rolling, slow rolling, firm adhesion and finally transmigration. Blocking of any of these steps can severely reduce the leucocytes' ability to accumulate in the tissue. At any given moment, all these steps maybe occurring simultaneously within the same microvessel involving different leucocytes {Evangelista VS; Manarini S, 1996, i.d. 153}.

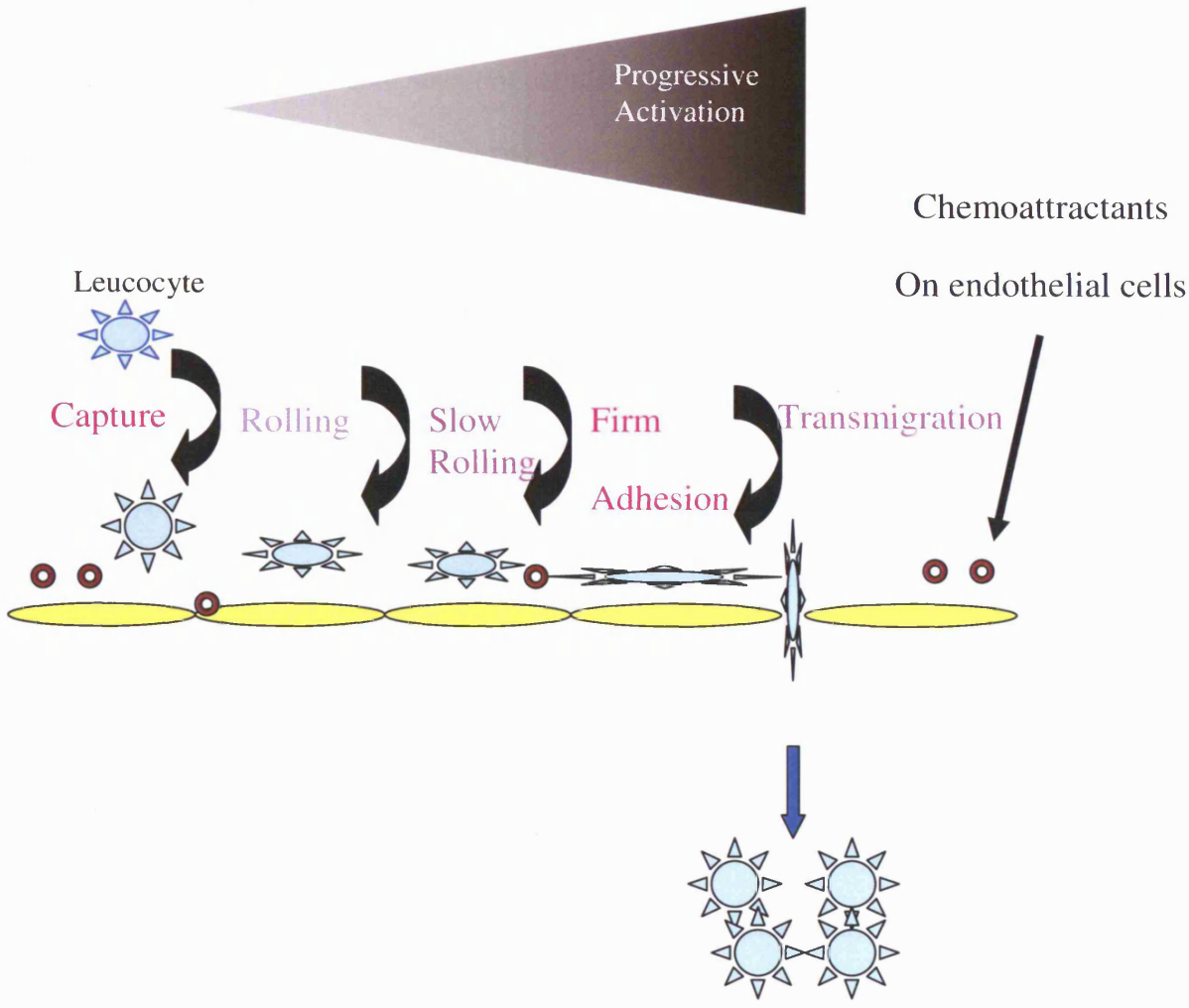

Figure 1.2. Endothelial activation leading to leucocyte triggering of the leucocyte adhesion cascade. Activation of endothelial cells leads to capture, rolling and eventual transmigration of leucocytes out of the intravascular compartment.

1.2.2 Endothelial activation

Endothelial injury is often identified in many diseases occurring in the clinical scenario such as inflammatory or infectious conditions (sepsis), burns trauma, Acute respiratory distress syndrome (ARDS), reperfusion injury, transplant graft rejection or disseminated intravascular coagulation . A major concept in vascular biology was the

discovery that endothelial cells can become activated and that this can occur independently of, or as a component of cellular injury {Jeonb, Khanday, et al. 2003}. Endothelial activation is now considered to be the principal initiating mechanism that triggers a chain of complex events leading to many inflammatory and infectious diseases {Ward & Fantone 1996}

There are many definitions of endothelial activation. It has been defined previously as altered synthesis of proteins that mediate functional characteristics of the cells in response to stimulation with cytokines. This definition was formed after in vivo observation of endothelial cells involved in type IV or delayed hypersensitivity reactions {Zimmerman G, Kurt H, et al. 1999}

There are a number of known stimuli that induce activation-dependent functional alterations in human as well as animal endothelial cells such as the cytokines including (TNF-α, IL-1β) which interact with cell surface receptors and pleiotropic signalling factors such as thrombin, bacterial endotoxin lipopolysaccharide (LPS) and other microbial products, haemodynamic perturbations, oxidants and radiation {Cines, Pollak, et al. 1998}.

Light microscopy over 100 years ago allowed Cohnheim to observe the inflammatory response to injured tissue with the discovery that leucocyte margination along vessel lumen preluded extravasation. It was not until the 1980's that Pober discovered that exposure of endothelial cells to various cytokines such as IL-1 or TNF, enabled the EC's to bind leucocytes 20-40 times more than untreated EC's {Pober J.S. 2002}. These endothelial cells were noted to have an altered adhesivity from the induction of

new surface proteins which bind counter receptor proteins on the surface of leucocytes termed endothelial leucocyte adhesion molecules (ELAMS). These include E-selectins such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) Figure 1.3. Furthermore, cytokine treated EC's themselves produce chemo attractant cytokines (Chemokines) which activate the ability of the leucocytes to bind to ELAMS. {hPober JS and Cotran 1990}.

These observations together with the emergence of new experimental models such as *in vivo* videomicroscopy led to the current multistep model for leucocyte binding outlined above. Currently it is believed that endothelial cells are in a non-interactive state, which has minimal interactions with leucocytes and minimal functional or phenotypical changes in the event of an encounter.

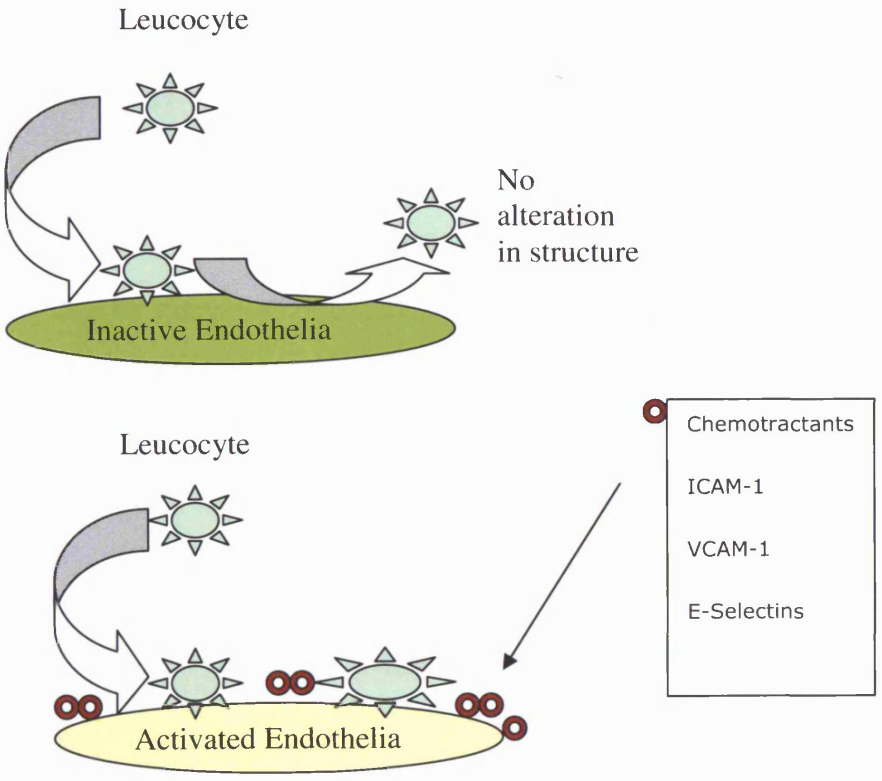

Figure 1.3. Activation of endothelial cells leads to structural alterations allowing them to bind leucocytes more effectively and express new surface proteins on their surface such as E-selectin, ICAM-1 and VCAM-1

1.2.3 Nuclear Factor KappaB (NF-κB)

Nuclear Factor kappa B is a transcription factor that plays a critical role in regulating cellular inflammatory responses {Ouaaz F; Ming Li, 1999, ref i.d. 154}.

Pro-inflammatory cytokines such as tumour necrosis factor (TNF-α) and Interlukin-1 initiate signalling pathways in endothelial cells activating NF-kB {Zen K, Stempien-Otero A, et al. 1999} leading to regulation of inflammatory response in endothelial cells by elevating the production of several adhesion molecules and chemokines which control docking and trans-endothelial migration of leucocytes.

This DNA binding protein is therefore interesting due to its widespread implication for the therapy of the substantial number of disease processes.

NF-kB is formed through the association of multiple subunits, either as a homodimer or heterodimer. NF-kB Subunits have been identified as p50 (NF-kB1), p65 (RelA), c-Rel, RelB and p52 (NF-kB2) {Baldwin 1996 }. The classic NF-kB form exists as a p50-p65 heterodimer and predominates in many cell types. This heterodimeric protein may also be composed of different combinations of members of the Rel family of transcription factors. The Rel/ NF-kB family of transcription factors are involved mainly in stress-induced, immune and inflammatory responses {Ghosh, S May, et al. 1998}.

Knock out mice with targeted deletions of the Rel family members p50, p52, c-rel, p65 (RelA), and RelB exhibit an impaired function of B cells, T cells, macrophages, or monocytes {Ghosh, S., May, M. J., Kopp, (1998)}

In addition to their role in the immune system, the ubiquitously expressed Rel family members p50 and p65 serve to maintain tissue integrity and protect against various forms of cellular stress. In this respect, the anti-apoptotic function of NF-B appears to be of central importance {Baichwal & Baeuerle 1997}.

More recently, NF-kB family members have been implicated in neoplastic precursors and the formation of neural synapses. As will be discussed later, NF-kB is also important in cell fate decisions such as programmed cell death (apoptosis) control and is critical in tumourgenesis {Baldwin 1996}

So how does NF- κB act? It is known to be sequestered in the cytoplasm of unstimulated cells via non-covalent interactions with a class of inhibitor proteins named IkBs {Zandi E & Karin M 1999}. To date 7 IkBs have been identified which all contain copies of a 30-33 amino acid sequence which mediate the association between IkB and NF-kB.

Signalling cascades triggered by cytokines induce NF-kB activity cause the phosphorylation of IkB as well as their dissociation and subsequent degradation. This allows the NF-kB complex to translocate into the nucleus where it binds DNA at kB binding motifs and induces gene expression {Beinke S; Ley B 2004 id 154}. The IkB kinase complex (IKK) is also responsible for phosphorylating IkB thereby allowing the degradation of the IkB protein and thus movement of the NF-kB molecule into the nucleus. Control of IkB phosphorylation is therefore a key element of NF-kB regulation.

NF-kB can be activated by exposure of the cells to lipopolysaccharides (LPS) or inflammatory cytokines such as TNF- a, interlukin-1, growth factors, lymphokines, oxidant free radicals, inhaled particles, viral infection or expression of certain or viral or bacterial gene products, ultraviolet radiation, B or T cell activation or by a multitude of physiological or pathological stimuli.

TNF-α is the best studied of these activators, binds to its receptor and triggers a protein cascade by initially recruiting a protein called TNF-Associated Receptor Domain Death (TRADD) which then binds to the TNF Receptor-Associated Factor-2 (TRAF2). TRAF-2 then recruits NF-kB- Inducible Kinase leading to IkB phosphorylation and NF-kB activation.

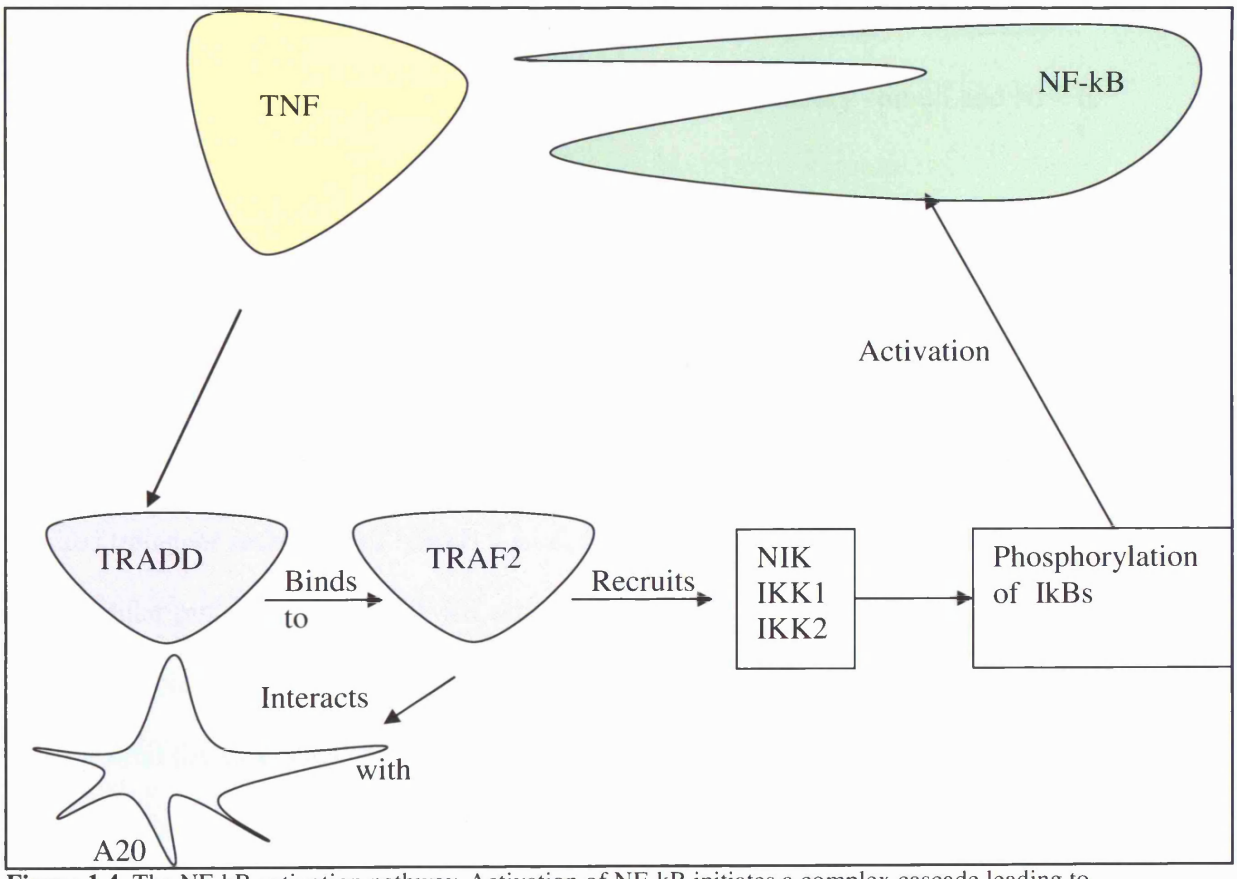

Figure 1.4. The NF-kB activation pathway. Activation of NF-kB initiates a complex cascade leading to the phosphorylation of IkBs as well as A20 activation.

It has recently been shown that the IkB kinases; IKKγ and IKKβ that are responsible for phosphorylation of IkBs are recruited to the TNF signalling receptor complex through association with the third IKK subunit, IKK gamma (NEMO). This acts as an adaptor molecule which in turn binds to receptor interacting protein (RIP). Localization of IKKs to the signalling complex may facilitate their activation. Similarly, NF-kB activation through IL-1 receptor requires several adaptor molecules including TNF receptor-associated factor 6 (TRAF-6). IL-1 and TNF-α signalling pathways converge during activation of the IkB kinase signalosome. The IKKβ and IKKγ (NEMO) subunits of the NF-kB- activating signalsome complex are known to be essential for activating NF-kB by TNF-α and other stress like stimuli.{Li Q, Van Antwwerp D, et al. 1999}, {Li ZW, Chu W, et al. 1999}. Surprisingly, IKKα also seems to play a critical role in IKK activation by pro inflammatory stimuli and NFκ B activation {Hu Y, Baud M, et al. 1999}.

1.2.4 Regulation of NFKB activity

NF-kB stimulates A20 expression and A20 feedback to inhibit NF-kB. This allows limited transient activation of NF-kB { Li Z, Chu W et al 1999}. A number of intracellular proteins regulate NF-kB activity. These include A20, which plays an important role in limiting the extent and duration of inflammation. A20 deficient mice cannot limit the extent and duration of NF-κB activity and develop severe inflammation {Lee EG, Boon L, et al. 2000 110 /id}. Studies in cultured cells have demonstrated that NF-κB activity is down-regulated by over-expression of the C-terminal portion of A20, which contains seven zinc fingers {Uta K, Jurgen F, et al.

2003 111 /id}. The mechanism utilized by A20 has not been fully elucidated. It has been shown recently {Wertz I, Orourke K, et al. 2003}, however, that the A20 zinc finger domain interacts with NEMO, and it is possible that this association provides an opportunity for A20 to exert an inhibitory effect on IkB kinase function. The N-terminal region of A20 (residues 1–386) binds to TNF receptor-associated factor 2 (TRAF-2) signal adaptors which are critical components of TNF-α and IL-1 signalling pathways {Heyninck K.., De Valck D, et al. 1999}. Thus A20 functions to block TRAF-2 mediated NF-κB activation.

1.2.5 A 2 0 bining inhibitor of Nuclear Factor- Kappa B, ABIN-2

The zinc finger molecule A20 is a TNF-α and Interlukin-1 (IL-1) inducible protein which is a crucial negative regulator of NF-κB activity. As NF-κB has an anti-apoptotic effect in many cell types, A20 is therefore important in apoptosis. Expression of A20 inhibits apoptosis induced by growth factor deprivation of Human umbilical vein (HUVE) cells {Varani J, Dame K, et al. 1995 },by TNF in HUVE cells {Opipari AW, Hu HM, et al. 1992} and lipopolysaccharides in human microvascular endothelial cells. {Hu X, Yee E, et al. 1998}

In an attempt to elucidate the mechanism of A20 action, studies were performed to identify A20 binding proteins {Bayaert et al 1999}. Amongst proteins discovered to interact with A20 are the ABINs (A20 binding inhibitors of NF-kB). The human ABIN-2, and the related ABIN-1, are A20-associating cytosolic proteins which block NF-kB activation induced by various stimuli {Heyninck K.., De Valck D, et al.

1999}. ABIN-2 has 429 amino acids. It was found to have a key role in limiting the extent and duration of inflammatory activation {Beyaert R, Heyninck K, et al. 2000}.

The mechanism by which ABIN-2 inhibits NF-kB is not known. Interestingly, ABIN-2 has been suggested to have a direct transcriptional activity independently of its interaction with NF-kB {BEINKE S; Ley B 2004 id 154}.

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ABIN-2 has the potential to enter the nucleus and plays a role in mediating transcriptional activation in both yeast and mammalian cells {Chien CY, Liu WK 2003 156 id}. It has been suggested that ABIN-2 functions as a transcriptional co-activator and facilitates transcription in yeast. In contrast to the activity in yeast, however, only the C-terminal fragment of ABIN-2 exerts the trans-activating activity in mammalian cells but not the full length ABIN-2 protein. This observation has led to the identification of the N-terminal 195 amino acids of ABIN-2 as a regulatory domain, which retains the full-length ABIN-2 in the cytoplasm of mammalian cells and thus cannot trans-activate {Chien CY, Liu WK 2003}. It has also been found that BAF60a, a component of chromatin- remodelling complex, interacts with ABIN-2 by yeast two-hybrid analysis. Together, this suggests that the nuclear ABIN-2 defines a novel transcriptional co-activator and acts presumably by recruiting a chromatin-remodelling complex to the site of the target gene.

This link between NF-kB and apoptosis has proven to be an interesting challenge in the clinical setting. As NF-kB has pro-inflammatory as well as anti-apoptotic activity, blocking NF-kB activity to reduce the inflammatory response will also induce endothelial cell death. This is illustrated in the TNF-induced survival pathway which is mediated by the transcription factor NF-κB where activation of NF-κB occurs via

phosphorylation of I-κB, resulting in the dissociation and subsequent nuclear localization of active NF-κB. Recent studies have demonstrated that cells in which the NF-κB signalling pathway is blocked are more likely to undergo apoptosis in response to TNF {Plumpe J, Malek NP., et al. 2000 124 /id}. Therefore development of compounds to reduce inflammation by blocking the NF-kB pathway may have disastrous apoptotic side-effect unless blockade of the NF-kB pathway can occur further downstream.

1.3 Control of endothelial survival

It is now well established that cellular suicide (apoptosis or programmed cell death) is central to a number of physiological cellular processes and is essential in the maintenance of homeostasis and survival of multicellular organisms. Equally, or perhaps even more important is the role of apoptosis in the pathogenesis of many human diseases.

Disruption of the apoptotic pathways may account for resistance to chemotherapy and treatment failures in human neoplastic disease. Such treatment failure is well exemplified by acute promyelocytic leukaemia {Wang z; Chen Z, 2000, id 157}. This cancer is only transiently responsive to both cytotoxic drugs that act through the stimulation of apoptosis as well as differentiating therapies such as retinoic acid. The development of therapeutic approaches that overcome drug resistance is therefore of primary importance. Approaching apoptosis from another angle; enhancement of

endothelial cell survival in microvessels would be advantageous in increasing the survival of free tissue transfers and transplant.

From a molecular point of view this field concentrated heavily on the caspases and endogenous inhibitors of apoptosis, predominantly Bcl-2 proteins. Over the past few years a considerable amount of research has been conducted and our view of apoptosis has changed dramatically. Major advances have included the emergence of the IAP ("**I**nhibitor of **A**poptosis **P**roteins") family. This field has grown exponentially since 1995 and continues to do so. Although XIAP and survivin remain the better known members of this family, 8 human IAPs have now been identified. Members of this family represent perhaps the most important regulators of apoptosis by virtue of the fact that they intercept and regulate two convergent apoptotic pathways - the extrinsic (receptor-mediated) and intrinsic (mitochondria-mediated) pathway.

The extrinsic pathway occurs by ligation of cell surface receptors of the TNF receptor family initiating the death pathway which activates adaptor molecules which in turn activate kinases, proteolytic enzymes such as caspase-8 and transcription factors. In the intrinsic pathway intracellular damage or loss of extracellular survival signals initiate the mitochondrial pathway leading to activation of proteolytic enzyme caspase-9 leading to cell death.

1.3.1 The role of the phosphatidylinositol 3' kinase (PI-3k)/Akt pathway in cell survival

There is a class of phosphate kinases that have been implicated in cell signalling pathways that affect cellular death and longevity as well as many other processes that have medically important implications. Phosphoinositide-3 kinase (PI-3K) is involved in a phosphorylation of phosphatidylinositol (Ptdlns) on the third carbon of the inositol ring . The phosphorylation of Ptdlns to Ptdlns-3-P, Ptdlns-3,4-P and Ptdlns-3,4,5-P is part of many signal cascades with in a cell. These include pathways regulating PI-3K dependent cell motility and adhesion and hence it is able to contribute to metastatic/ invasive phenotypes of cancer cells. Initial work on PI-3K was however more focused on its function in cell growth and transformation.

1.3.2 Structure of PI-3K

There have been three mammalian PI3K genes cloned and they share 42-58% amino acid sequence identity, these are designated p110α , p110γ and p110δ . Each of these proteins contains an N-terminal region that interacts with regulatory protein subunits, a domain that binds a small G protein Ras, a PIK domain and a C- terminal catalytic subunit. The regulatory unit is called p85 because the molecular weight of the first two isoforms purified and cloned, p85α and p85γ was 85 kDa. These subunits do not contain lipid kinase activity but contain domains that are modular, which can be separated functionally and spatially from the rest of the protein. {Fruman D; Meyers R 1998}.

There are three forms of PI3Ks and they differ in the composition of subunits. In general PI3K is heterodimeric, it is composed of a regulatory subunit and a catalytic

subunit. The three classes of PI3K kinases are differentiated based on their substrate specificity. Class I PI3K contains a catalytic subunit of 110 to 120 kDa which phosphorylates Ptdlns, Ptdlns4,5- bisphosphate and Ptdlns-4-P. Class I PI3Ks interact with the SH-2/SH-3 domain containing p85 proteins. The p85 subunit SH2/SH3 domains allow this regulatory subunit to interact with phosphorylated tyrosine residues in cell receptors and other molecules within the cell and change the binding affinity of the catalytic subunit in some instances. Class II PI3K phosphorylate Ptdlns and Ptdlns-4-P but not Ptdlns-4,5-bisphosphate. Class III PI3K are specific, they only react with Ptdlns and are structurally and chemically similar to yeast Vps34p, which is involved with trafficking of proteins from golgi to a vacuole in yeast {Vanhaesebroeck et al, 1997 id 158}.

1.3.3 Mechanism of the PI3K action

As well as playing a key role in endothelial cell proliferation, PI3-K is central to cell survival. Initial evidence was based on the ability of Wortmannin, a PI3K inhibitor, to cause apoptosis in PC12 cells {Papapetropoulos A, Fulton D, et al. 2000 }.
The mechanism by which PI3K protects from programmed cell death has been the subject of intensive research and involves the downstream activation of the protein kinase Akt. This phosphatidylinositol 3' kinase (PI3K)/Akt pathway is now acknowledged as a key component of cell survival.

Activated by numerous receptors including RTK's and several transmembrane receptors of the small GTPase Ras, the various PI3K isoforms phosphorylate inositol lipids to form second messenger phosphoinositides. These are able to bind Akt recruiting it to the plasma membrane where it is then phosphorylated to its active form

by 3-Phosphoinositide-dependent protein kinase-1 and -2 (PDK-1 & -2). This allows Akt to target proteins involved in cell death including members of the Bcl-2 family, caspase-9 and activation of transcription factors such as NF-kB leading to cell protection from apoptosis. In addition PI3K activation is able to increase the expression of members of the inhibitors of apoptosis family such as the c-IAPs {Gagnon V, St-Germain ME, et al. 2003}.

As well as regulating inflammatory gene expression, NF- κB has anti apoptotic activity in many cell types. In endothelial cells NF-kB is necessary for the prevention of apoptosis induced by TNF-α and growth factor deprivation {Levkau B, Scatena M, et al. 1999}.

1.4 The role of receptor tyrosine kinases and growth factors in the regulation of vessel inflammation and survival

1.4.1 Modulators of Receptor Tyrosine Kinases

Several paracrine signals, many of which are protein ligands bind and modulate the activity of receptor tyrosine kinases (RTKs). These are transmembrane proteins that span the cell membrane and act to convey signals from the extracellular to the intracellular environment.

RTKs are coupled into the intracellular signal transduction cascade and are capable of inducing cell proliferation { Krymskaya et al, 2005}.

RTKs are composed of three domains, an extracellular and intracellular domain as well as a transmembrane region sandwiched in between. {Ulrich A & Schlesinger J 1990}. The specificity of the RTK for ligands and other receptors is conferred by the structure of the extracellular domain. The transmembrane portion stabilizes the receptor on the cell following receptor activation {Ulrich A & Schlesinger J 1990}, while the intracellular domain has a tyrosine kinase domain. Oligomerization occurs in response to ligand binding. {Huang L, Turck CW, et al. 1995}. This allows the kinases of individual RTK's to transphophorylate each other on tyrosine residues resulting in both increased kinase activities and generation of phosphotyrosines {Weiss A & Schlesinger J 1998} that can act as docking sites for intracellular proteins containing SH2 or PTB domains {Schenk PW & Snaar-Jagalska BE 1999}.

The number of growth factors that specifically act on the blood vasculature remains quite small and are limited to two families of factors known by their prototypical members: The vascular endothelial growth factor (VEGF) also called vascular family and the angiopoeitin family {Yancopoulos GD, Davis S, et al. 2000}, {Eriksson U & Ali talo K. 1999}. Members of these families achieve their specificity based on the limited distributions of their receptors which are expressed almost exclusively on the vascular endothelium. Both these families act primarily via RTKs. They share a similar transmembrane domain structure with variations between families in the extracellular and intracellular domains. There are three receptor tyrosine kinases that bind the VEGF's, these are designated VEGF receptors 1 to 3. The angiopoeitins bind to a distinct RTK called Tie2. The enormous interest in these two families results from the realization that they are important in vascular inflammation, cell survival and angiogenesis. The VEGFs play a critical role in protecting endothelial cells from

apoptosis during embryonal development, whereas the angiopoeitins seem to exert more effects on vessel survival and remodelling{Suri C, Jones N, et al. 1996}.

1.4.2 Vascular Endothelial Growth Factor Receptors

So far, in the VEGF family, five VEGF- related genes have been identified VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E. They display differential interactions with three related receptor tyrosine kinases (VEGFR-1-/FLT-1, VEGFR-2/FLK-1 and VEGFR-3/FLT-4) {Risau W. 1997}. VEGFR-1 and VEGFR-2 are restricted largely to vascular endothelium in their expression accounting for the specificity of action of this growth factor family, whereas VEGFR-3 may play a role in angiogenesis and lymphgenesis but appears to be restricted to the lymphatic endothelium {Kukk E, Lymboussaki A, et al. 1996}. Gene knockout studies using mice either lacking VEGFR-1, VEGFR-2 or VEGF die in the early embryonic days due to abnormal and undeveloped vasculature respectively. These 2 receptors and VEGF therefore appear essential at the start of vasculogenesis {Yancopoulos GD, Davis S, et al. 2000}.

Withdrawal of VEGF by targeted inactivation of the VEGF gene results in massive endothelial cell apoptosis, which leads to severe haemorrhage and is lethal in the mouse embryo {Ferrara N, Carver-Moore k, et al. 1996}, Similarly the VEGF receptor-2 is known to predominantly mediate the antiapoptotic effects of VEGF {Gerber HP, McMurtrey A, et al. 1998}.

Mechanistically, VEGF activates the PI3K/Akt pathway promoting cell survival{Kim I., Kim H.G., et al. 2000}. Activation of the serine/thereonine kinase Akt in turn stimulates the phosphorylation of proapoptotic proteins thereby inhibiting apoptosis

execution. Moreover, Akt activates the endothelial nitrous oxide (NO) system, leading to increased synthesis of NO {Kwaja A 1999}, which promotes endothelial cell survival by inhibiting the cysteine protease activity of caspases {Papapetropoulos A, Fulton D, et al. 2000}. VEGF also mediates upregulation of the antiapoptotic protein Bcl-2 {Gerber HP, Dixit V, et al. 1998}.

1.4.3 Tie1 Receptor Characterisation

As mentioned earlier, so far three tyrosine kinases participating in the regulation of Tie2 receptor signalling have been characterized. However, the Tie1 signal transduction has been difficult to study as no ligand for this receptor has been identified. However, recent studies indicate that Tie1 participates in ligand-independent signalling. Tie1 might also form heterodimers with Tie2 and therefore modulate Tie2 signalling {Fujikawa K 1999}.

1.4.4 The Angiopoietins

Four angiopoietins, Ang-1 to 4 have been described. They are ligands for the Tie2 receptor, they have either agonistic (Ang-1) {Davis S, Aldrich TH, et al. 1996} and (Ang4) or antagonistic (Ang3) actions regulating vascular expansion and survival. Ang2 can stimulate or inhibit angiogenesis in different contexts {Maisonpierre PC, Suri C, et al. 1997}. In addition, it seems to be important for the development of lymphatic vessels. Most research so far has focused on Ang-1 and Ang2.

The Tie2 ligands Ang-1 and Ang2 are both ~75-kD secreted proteins with considerable sequence homology; with similar structures. Both bind to the Tie2 receptor with similar affinity, and neither binds to the related receptor Tie1. Yet their effects on Tie2 are distinctive, as are their expression patterns.

Ang-1 is primarily expressed in pericytes, fibroblasts and smooth muscle cells, whereas Ang2 is selectively expressed in endothelial cells {Kim I, Kim JH, et al. 2000}.

Ang-1 activates its receptor Tie2. Ang-2 on the other hand seems to play an important role during blood vessel remodelling and possibly endothelial cell survival. Ang2-overexpressing transgenic mice die with vascular defects similar to Tie2- or Ang-1-knockout mice {Maisonpierre PC, Suri C, et al. 1997}.

These observations suggest that Ang2 acts as a natural antagonist of Tie2 by blocking receptor activation by Ang-1. So unlike Ang-1, Ang2 does not induce phosphorylation of Tie2, but rather inhibits the Ang-1-induced phosphorylation of Tie2 in vascular endothelial cells {Maisonpierre PC, Suri C, et al. 1997}. Thus Ang2 presents a negative signal to Tie2, an interesting observation given its high homology to Ang-1.

In vitro studies have confirmed that Ang2 is a natural antagonist for Ang-1 mediated Tie2 activation, moreover, this distinctive effect is apparently endothelial cell-specific. If a modified Tie2 is forcibly expressed in 3T3 fibroblasts, both Ang-1 and Ang2 induce receptor phosphorylation and yet do not stimulate fibroblast proliferation {Maisonpierre PC, Suri C, et al. 1997}.

The angiopoietin structure is divided into 3 domains: An N-terminal Coiled-coil domain, a short linker region and c terminal fibrinogen domain. Ang-1 and 2 are able to form tetramers, hexamers and multi order multimers via their coiled-coil domain.{Davis S, Papadopoulos N, et al. 2003 90 /id}, {Procopio WN, Pelavin PI, et al. 1999}.

The binding of Ang-1 and 2 to Tie2 is thought to occur via the fibrinogen like domain. {Procopio WN, Pelavin PI, et al. 1999}

Tie2 activation may occur with a minimum tetrameric Ang-1 structure in endothelial cells however Ang-1 dimers can only activate Tie2 in fibroblasts but not in endothelial cells suggesting a more complex mechanisms of Tie2 activation in the endothelium.

It has been shown that chimeras of the Ang-1 fibrinogen like domain was an agonist to endothelial Tie2 whereas that for Ang2 were antagonist.

It is therefore thought that the structural differences in the fibrinogen like domains confer the different activation properties of Ang-1 and 2 on Tie2. {Davis S, Papadopoulos N, et al. 2003}.

The angiopoietins bind Tie2, this RTK is a member of the Tie family of receptors which has two known members, Tie1 and Tie2. The Tie receptors are required for the angiogenic remodelling of vessels during embryonic development and for vessel stabilization in quiescent adult vasculature. Tie receptors also contribute to the abnormal vascular growth that is associated with many pathological conditions such as venous malformations, tumour growth and rheumatoid arthritis. The signal-transduction pathways controlled by the Tie receptors are overlapping but independent as Tie1 and Tie2 do not seem to functionally compensate for one another.

1.4.5 Angiopoietins regulate vascular inflammation and survival

In addition to roles in vessel formation, there is increasing evidence that Ang-1 has important roles in adult quiescent vasculature where it has been implicated in maintaining vessel integrity, suppressing inflammation and inhibiting apoptosis. Ang-1 has recently been shown to be a potent inhibitor of vascular leakage. Transgenic mice overexpressing Ang-1 inhibit the tissue oedema induced by vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF)-α and mustard oil, powerful stimulators of vascular leak. Ang-1 also suppresses expression of inflammatory genes including E-selectin, VCAM1 and ICAM1 in endothelial cells {Kim I, Moon SO, et al. 2001}. In-vivo anti-inflammatory action of Ang-1 is supported by trials of decreased leucocyte adhesion and extravasation in cardiac allograft arteriosclerosis in a rat model {Sihvola R; Pulkkinen VP, 2002, id 161}. The mechanism by which Tie2 regulates vessel inflammation is not known. However, it has recently been found that activated Tie2 recruits the NFκB inhibitor ABIN-2 in human endothelial cells {Hughes D, Marron M, et al. 2003}. Furthermore this recruitment was found to be required for the Ang-1/Tie2 inhibition of NFκB as Tie2 activation did not result in suppression of NFκB when a dominant negative form of ABIN-2 was expressed. It is not yet known whether ABIN-2 was inhibits NFκB or expression of inflammatory genes in endothelial cells in vivo.

In vitro experiments have shown that Ang-1 is a strong anti apoptotic factor in endothelial cells by stimulating Tie2 under conditions of serum deprivation,

irradiation and mannitol treatment {Papapetropoulos A, Yancopoulos GD, et al. 1999}, induces sprouting and chemotactic response but has little effect on proliferation.

It is now known that Ang-1 phosphorylates Tie2 in cultured endothelial cells leading to increased survival by facilitating phosphatidylinositol-3-OH kinase (PI3K) dependent endothelial cell survival through stimulation of the serine–threonine kinase Akt, leading to protection from apoptosis {Kim I., Kim H.G., et al. 2000}. This is thought to occur by upregulating the anti-apoptotic gene survivin which is a downstream effector of Akt. In a recent publication Ang-1 has been shown to upregulate survivin expression, furthermore this was shown to be blocked by the use of wortmanin, a PI3K inhibitor{Papapetropoulos A, Fulton D, et al. 2000}.
Ang-1 is also known to inhibit VEGF induction of I-CAMS and can block TNF-α and VEGF leucocyte adhesion {Metheney-Barlow L, Li L, 2003}

In vivo evidence of the Ang-1/ Tie2 system ability to reduce inflammation by suppressing leucocyte infiltration was provided by Nykanen's findings that Ang-1 protects against the development of cardiac allograft arteriosclerosis {Nykanen AI, Krebs R, et al. 2003 128 /id}. It also protects against endotoxic shock and diabetic retinopathy {Witzenbichler B, Westermann D, et al. 2005}.

1.5 Aims of this study

As discussed earlier Tie2 interacts with ABIN-2 in a ligand-dependent manner {Hughes D, Marron M, et al. 2003}. As the name suggests ABIN-2 is an inhibitor of

NF-κB {Van Huffel S, Delaei F, et al. 2001} and it appears to have a role in Ang-1 suppression of NF-κB activity {Hughes D, Marron M, et al. 2003}. It is not known whether ABIN-2 mediates the anti-inflammatory effects of Ang-1 via Tie2 in vivo. The effects of ABIN-2 on apoptosis and its role in Tie-2 mediated endothelial survival also remain unexplored. ABIN-2 could suppress inflammation by blocking NF-κB but this may induce apoptosis. However, as A20 inhibits NF-κB and apoptosis and since ABIN-2 has been implicated as a mediator of A20 it is possible therefore that ABIN-2, could both inhibit NF-κB and suppress cell death. This would make ABIN-2 an attractive target for promoting cytoprotection of the endothelium by being a key therapeutic target in endothelial cells for suppression of inflammation and blocking cell death in many pathologies. The aims of this study, therefore, are to

i) Determine whether ABIN-2 regulates endothelial apoptosis

ii) Define whether ABIN-2 is involved in the anti-apoptotic effects of Ang-1

iii) Establish gene transfer method for overexpressing ABIN-2 in blood vessels in vivo

iv) Determine the effects of ABIN-2 on vascular inflammation and apoptosis in vivo.

Chapter 2
Materials and methods

2. Materials and methods

2.1.1 General reagents

All chemicals were of analytical grade unless otherwise stated. All were obtained from Sigma Aldridge, (Poole, Dorset, UK) or Fischer Scientific (Loμghboroμgh, UK), unless otherwise stated. Cell culture media was obtained from Sigma or Gibco Life Technologies (Paisley, Uk).

2.1.2 Antibodies

Anti-PI3-Kinase p85α (Z-8) rabbit polyclonal antibodies was obtained from Santa Cruz Biotechnology (supplier: Autogen Bioclear UK LTD, Wiltshire, UK). Anti-mouse monoclonal antibodies Anti Phosphotyrosine (PY20), Anti-Phosphotyrosine:HRP (PY20) were obtained from Transduction Laboratories (Supplier: Affinity Research products Ltd, Exeter.). Secondary antibodies Anti-mouse IgG HRP, Anti-Rabbit IgG HRP, Anti-Goat IgG HRP were obtained from Amersham Pharmacia Biotech (Little Chalfont, UK). Anti-goat IgG HRP conjugate was obtained from DAKO (Ely, Cambridgeshire). The mouse monoclonal antibodies Anti-FLAG M2 affinity gel and Anti-FLAG M2-HRP conjugate were obtained from Sigma (Poole, Dorset, UK).

The antibodies recognizing Tie2 were obtained from R&D Systems and Santa Cruz Biotechnology Inc, and the monoclonal antibody against phosphotyrosine was from BD Transduction Laboratories.

2.1.3 Mammalian cell culture

For the most part cells were grown as a monolayer in 80 cm sterile tissue culture flasks at 37 °C in the presence of 5% CO_2. Stocks of cells were stored in liquid nitrogen in freezing vials containing cultured cells in appropriate growth media containing 10% DMSO.

2.1.4 Culture of bovine aortic endothelial cells

Bovine aortic endothelial (BAE) were cells were provided by Dr M. Boarder (Department of cell physiology, University of Leicester). Cells were maintained in culture until passage 2 or 3 and surplus cells stored in liquid nitrogen. BAE cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml penicillin, 100 µg/ml streptomycin (1% Pen/Strep) and 1% L-Glutamine. Cells were passaged when at 95 to 100% confluent at ratios of 1:3 and 1:10 for short term or longer-term maintenance, respectively. Cells used in experiments had not exceeded passage 12 (P12).

2.1.5 Culture of human umbilical vein cells

Human umbilical vein endothelial cells were provided by G. Williams or D. Crocston, (Department of Surgery, University of Leicester). Human umbilical cords were collected after ethical approval and patient approval from the department of Obstetrics at the Leicester Royal Infirmary. Tissue culture flasks were coated with 1% gelatin in phosphate buffered saline. HUVE cells cultured in M199 medium (with Earles salts) supplemented with 20% FCS, 1% Pencillin/Streptomycin, 1% L-glutamine, 5 IU/ml heparin as well as 0.5 mg/ml endothelial cell growth supplement (ECGS) which was added prior to use. For maintenance purposes cells were passaged at a ratio of 1:3 when almost 100% confluent.

2.1.6 Molecular biology reagents

Restriction endonucleases were obtained from Roche Diagnostics Ltd (Lewes, Sussex, UK) and Promega (Southampton, UK).

2.1.7 Bacterial strains

The supercompetent E.Coli XL-1 Blue MRF' Kan with extra permeable membranes were obtained from Stratagene (Cambridge, UK).

2.1.8 Plasmid vectors

The expression vector pc DNA3.1 was obtained from Invitrogen (CH Groningen, Netherlands). The expression vector pFlag-CMV-2 and p3xFLAG-CMV-7.1 were obtained from Sigma (Poole, Dorset).

2.2 Molecular biology techniques

2.2.1 Microbiological media

SOB medium

20g bact-tryptone, 5g bactoyeast extract, 0.5g NaCl and 10 ml of 250mM KCl solution per litre, pH 7.0. Prior to use 5 ml of sterile 2M $MgCl_2$ was added.

SOC medium

As SOB plus the addition of sterile glucose to a final concentration 20 mM prior to use.

All media was autoclaved at 120° C for 20 minutes at 15 lbs/sq. in. on a liquid cycle and allowed to cool sufficiently before the addition of antibiotic. Media-agar plates were prepared as above with the addition of 15g of Select agar (Gibco) per litre prior to autoclaving.

2.2.2 Bacterial growth and manipulation

Bacterial clones were cultured on LB plates supplemented with 100 μg/ml ampicillin. Bacterial cultures were gown in LB media unless otherwise stated, supplemented with ampicillin (100 μg/ml). For long term storage glycerol stocks of bacterial clones were stored at -85 C.

2.2.3 Isolation of plasmid DNA from bacteria

Plasmid DNA was isolated from 10 ml bacterial liquid cultures using two commercially available kits. These kits utilise the ability of DNA to bind to an anion exchange resin under appropriate low salt and pH conditions or to silica based membranes. For the purpose of transfection and DNA sequencing, the Plasmid Mini Kit (Qiagen) was used. For routine DNA restriction digests, to verify presence/ orientation of inserts within vectors, the Wizard Plus SV mini prep DNA Purification System (Promega) was used. Plasmid DNA was isolated according to manufacturers' instructions and eluted using 20/30 ul sterile double distilled water (ddH2O) and concentration determined using a spectrophotometer.

2.2.4 Spretrophotometric determination of DNA concentrations

DNA and RNA concentrations were determined using a Shimadzu UV-1601 UV-Visible spectrophotometer by taking sample readings at wavelengths of 260 and 280 nm. At 260 nm an optical density (O.D.) of 1 corresponds to approximately 50 μg/ml for double stranded DNA (ds DNA) and 40 μg/ml for single stranded DNA (ssDNA) and RNA (Sambrock et al 1989).

The ratio of O.D. 260:280 nm (O.D. 260/ O.D.280) provided an estimate of the purity of the nucleic acid. DNA and RNA having an O.D.260/O.D.280 values of 1.8 and 2.0 were used.

2.2.5 Restriction enzyme digests.

Restriction digests were performed in order to insert cDNA fragments into vectors and in some cases determine the orientation and presence of inserts within the vectors. Unless otherwise stated, in order to insert cDNA into a plasmid vector, 0.5 to 2.0 μg of DNA was digested with 10 units of restriction enzyme or enzymes, in a suitable buffer as recommended by the manufacturer, at 37 ^{0}C for 30- 90 minutes.

Digests were analysed on 1.5% agarose gels (section 2.2.6).

2.2.6 Analysis of DNA using agarose gel electrophoresis

Restriction Digested DNA fragments were resolved by agarose gel electrophoresis. Essentially 10 ul of restriction digest was analysed using 1.5% agarose gels made with 1 X Tris-acetate (TAE) buffer (40mM tris-acetate, 10 mM EDTA). To each sample an appropriate volume of 6x Type IV loading buffer (0.25% bromophenol blue, 40% (w/v) sucrose in water) was added prior to loading onto the gel. Electophoresis was performed at 150 mV in TAE buffer, using 0.5 μg of a 1 Kb DNA Ladder (Gibco) as a standard for DNA size comparison as well as a crude estimate of DNA concentration. DNA was stained by adding ethidium bromide to the gel and TAE buffer at a concentration of 0.5 μg/ml. Agarose gels were visualised under U.V. light. Photographs were then taken using an Alpha Imager 1220 (Alpha Innotech Corp, supplied by Flowgen, Shenston, U.K.)

2.2.7 Purification of DNA from agarose gel

DNA fragments were purified from agarose gels using a QIAquick Gel Extraction Kit (Qiagen) following the manufacturers instructions. Briefly, the desired gel fragment was cut from the gel and weighed. 3 volumes of buffer QG were added to each volume of gel and incubated at 50 °C for one hour. The sample was then added to a Q1A quick column, washed with buffer PE and centrifuged for 1 minute at 13,000 rpm. The DNA was then eluted with 30 μl of nuclease free H_2O. The amount of DNA extracted was estimated by comparing the intensity of 2 μl of target DNA to that of 5 μl of DNA ladder loaded onto electrophoresis gel. DNA fragments were visualised on an agarose gel under ultraviolet light and excised using a sterile scalpel. DNA was

eluted from the spin column with 30ul of ddH2O (pH 7.0-8.5) and a small volume ran on a 1.5% agarose gel for recovery estimates against a 1 Kb DNA ladder (Gibco) standard.

2.2.8 Phenol: chloroform extraction

Phenol: chloroform extractions to recover DNA from reaction solutions containing protein contaminants. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the DNA containing solution and contents mixed by vortexing to create an emulsion. The mixture was centrifuged at 6 G for 5 minutes resulting in an aqueous phase (top) and an organic phase (bottom). The aqueous phase was collected and DNA concentrated by ethanol precipitation.

2.2.9 Ethanol precipitation

A DNA solution; one tenth volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol were added and mixed well. DNA was allowed to precipitate at -20C for 1 hour then centrifuged at 6 G for 10 minutes. The supernatant was aspirated carefully and DNA pellet washed with 1ml of 70% ethanol and sedimented as before. The resulting pellet was air dried at room temperature and resuspended in 20 ul of ddH2O.

2.2.10 Dephosphorylation of 5' cDNA ends

In order to prevent the self ligation of restriction endonuclease linearised vector DNA, calf intestinal alkaline phosphates (CIAP) was used to dephosphorylate 5' phosphorylated ends of DNA. Essentially, linearised DNA was incubated with 1 unit of CIAP (Boehringer) per mol of 5' terminal phosphorylated DNA at 37 ^{0}C for 1

hour. The alkaline phosphatase was then inactivated by the addition of one tenth volume of 200 mM EGTA and heating at 65 ^{0}C for 10 minutes. Dephosphorylated DNA was recovered by phenol: chloroform extraction followed by ethanol precipitation.

2.2.11 Ligation reactions

For Ligation reactions either a 1:1 or a 3:1 molar ratio of insert to vector was used. The ratio used was in most part determined by the amount of insert available. The amount of insert required was determined using the following equation:

$$\text{ng of insert} = \frac{\text{ng of vector} \times \text{Kb size of insert}}{\text{Kb size of vector}} \times \text{molar ratio (of insert/vector)}$$

Ligation reactions were performed using the desired concentrations of insert and vector DNA, 3 units of T4 ligase (Promega) in 1x ligase buffer (30 mM Tris-HCl pH 7.8, 10 mM MgCl2, 10 mM DTT, 1 mM ATP) and dd H2O to a final volume of 20 ul. Ligation reactions were incubated at 14 ^{0}C for 14-24 hours. Control reactions were set up as above with the omission of the insert DNA. Ligated DNA was used to transform supercompetent bacterial cells.

2.2.12 Transformation of competent and supercompetent cells

Supercompetent E. Coli cells were gently thawed and ice and 100ul of cells per transformation was aliquoted into prechilled Falcon 2059 polypropylene tubes. 100-500 ng of DNA was added to the cells and swirled gently before further incubation on ice for 30 minutes. Tubes were heat pulsed at 42C for 45 seconds the returned to the ice for a further 2 minutes. 400ul of pre-heated SOC media to 42 ^{0}C was added to the

tubes. The mixture was then incubated in an orbital incubator at 37 ^{0}C for 1 hour at 0.5 G. For each transformation, 50, 100, 150 and 200ul aliquots of cells were plated out onto LB agarose plates containing the appropriate selection antibiotic. After air-drying, plates were incubated at 37 ^{0}C overnight.

2.3 Protein expression, analysis and immunodetection

2.3.1 Transfection of HUVE cells

HUVE cell transfections were carried out using the Targefect F-2 transfection reagent (Targeting systems, Santee, California). The Targefect F-2 transfection reagent consists of non-lipid cationic polymers with DNA condensing properties that can enhance cellular uptake of DNA.

HUVE cells were grown as a monolayer in 35mm tissue culture plates until approximately 80% confluent. A transfection complex consisting of 1μg of plasmid DNA was made up to 1 ml in serum free media. 5ul of Targefect F-2 (1 mg/ml) was added to the diluted DNA and mixed by gently swirling.

Targefect F-2/DNA mix was incubated at room temperature for 30 minutes. Following incubation HUVE cells were washed once with serum free media and the Targefect F-2/DNA complex added to the cells. Cells were then incubated at 37 ^{0}C for 2 hours, the transfection mixture was then replaced with HUVE cell growth medium supplemented with ECGS (10 μg/ml), transfected cells were allowed to recover by incubation at 37 ^{0}C overnight.

2.3.2 Agonist treatment of HUVE cells

Prior to stimulation with agonists, the media was removed and the cells washed twice with Optimem 1 (Gibco), incubated for 16 hours in Optimem with one medium change 2 hours before the start of the experiment. For Angiopoeitin, cells were stimulated with 400 ng/ml Ang-1 for 4 hours unless otherwise stated. For Challenge with Tumour necrosis factor (TNF), cells were treated with 100ng/ml of TNF for a period of 3 hours unless otherwise stated.

All control cells were treated with appropriate volumes of sterile water or other appropriate solvent as described for each individual experiment.

2.3.3 Resolving proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were routinely resolved using SDS-Page and mini Protean II cell (Bio-Rad) electrophoresis units. Separation of proteins was performed under denaturing conditions using either a 10% or 12% resolving gel (Table)

Resolving gel was cast within the mini-gel assembly between two glass plates and a layer of water added to the top to exclude oxygen. Once polymerized, the water layer was removed and a 6% stacking gel cast on top. A comb was inserted to form wells and the stacking gel allowed to polymerize.

Table1.1: Components of Gels for SDS-Page. Acryl/ Bis: 30% acrylamide/ 0.8% bis-acrylamide solution (National Diagnostics), APS (Fisons): Ammonium persulphate (made up of fresh prior to use), SDS: sodium dodecyl sulphate (Sigma), TEMED N,N,N', N'- Tetramethylethylenediamine (Sigma).

Reagent	12% resolving gel	10% resolving gel	6% stacking gel
30% Acryl/Bis	8.0 ml	6.7 ml	3.3 ml

2M Tris pH 8.8	3.7 ml	3.7 ml	
1M Tris pH 6.8			2.5 ml
dd H2O	7.9 ml	9.6 ml	13.7 ml
10% SDS	200 ul	200 ul	200 ul
10% APS	134 ul	134 ul	200 ul
TEMED	14 ul	14 ul	20 ul

Protein samples were heated to 105 ^{0}C in 1x reducing buffer (62.5mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophemol blue, 5mM EDTA, 100mM dithioreitol (DTT), centrifuged at 13000 xg for 5 minutes, and loaded onto the gel using a Hamilton syringe (Sigma). Proteins were separated by electrophoresis at 200 volts in running buffer (200 mM glycine, 25 nM Tris pH 8.3, 0.1% SDS).

2.3.4 Immunuprecipitation

Immunoprecipitation experiments were performed using cells grown as a monolayer in 80 cm tissue culture flasks. Cells were lysed with 500 µl lysis buffer (50 mM Tris pH 7.4, 50mM Na Cl, 1mM NaF, 1mM EGTA) supplemented with 0.1mM AEBSF and 1% (v/v) Triton ® X-100 prior to use, and incubated at 4 ^{0}C for 10 minutes on a plate rocker. Cells were scraped from the flasks using a cell scraper and vortexed for 30 seconds. Lysates were cleared by centrifgation and a 25µl sample collected to represent whole cell lysates (WCL). Added to the remaining lysate was an appropriate concentration of target antibody and complex incubated for 2 hours at 4 ^{0}C on a spiral mixer. 40µl Protein G sepharose beads (50% slurry in 20% ethanol (Sigma), prewashed in 400 ul lysis buffer, was added and further incubated as above for a

minimum of 2 hours. Sepharose beads were collected by a brief centrifugation and washed four times with 1 ml of lysis buffer. An equal volume of 2x reducing sample buffer (125 mM Tris pH 6.8, 20% glycerol, 4% SDS, 0.2% bromophenol blue, 10 mM EDTA, 200 mM dithioreitol (DTT) was added and beads heated to 105 ^{0}C followed by centrifugation at 13,000 xg for 5 minutes and supernatant taken for SDS/PAGE For Immunoprecipitation of target proteins using antibodies conjugated to agarose beads the protein G step was omitted.

2.3.5 Western blotting reagents

Buffers:

Tris-Buffered saline (TBS): 25 mM Tris, 144 mM $NaHPO_4$, 1.8 mM KH_2PO, pH 7.3

Blocking Buffers:

Anti-phosphotyrosine antibodies: 5% (w/v) Bovine serum albumin in TBS containing 0.1% Tween 20 (TBS-T + 5% BSA)

Non Anti-phosphotyrosine antibodies: 5% (w/v) non fat dried milk in TBS containing 0.1% Tween 20 (TBS-T + 5% Milk)

Wash Buffer: TBS containing 0.1% Tween 20

2.3.6 Western blotting

Proteins resolved by SDS-Page were electrophoretically transferred at room temperature onto a nitrocellulose membrane (Hybond ECL, Amersham) in transfer buffer (192 mM glycine, 25 mM tris base, 20% methanol) at a constant current of 0.1 amps for 16-18 hours in mini TRANS BLOT cells (Bio-Rad).

Following transfer, non-specific binding sites were blocked by incubating the nitrocellulose membrane in blocking buffer for one hour at room temperature or

overnight at 4 ^{0}C with constant agitation. Primary antibodies were diluted in wash buffer, except for anti-phosphtyrosine antibodies which were diluted in the appropriate blocking buffer and incubated with the membrane at room temperature for one hour with agitation. Membranes were washed twice with TBS-T, 5 minutes per wash and incubated with an appropriate secondary antibody horseradish peroxidase conjugate for 1 hour at room temperature. Membranes were washed in TBS-T at least three times, 15 minutes per wash, prior to developing using ECL, chemiluminescence kit (Amersham) and Kodak imaging film (Sigma).

2.3.7 Preparation of HUVE cell lysates

Endothelial cells were washed in PBS and lysed at 4 °C in 0.5 ml of lysis buffer (50 mM Tris, pH 7.4, containing 50 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EGTA) and Complete^{TM} protease inhibitor mixture. For examination of tyrosine phosphorylation in total cell extracts, aliquots of lysate were diluted with Laemmli sample buffer containing 100 mM dithiothreitol

2.3.8 Stripping and reprobing of nitrocellulose membrane

Multiple reprobing of membranes with antibodies involved stripping the membranes of all previously attached antibodies. The membranes were submerged in stripping buffer (62.5 mM Tris- HCL, pH 6.7, 2% SDS and 100 mM β-mercaptoethanol) and incubating at 65 ^{0}C for one hour with constant agitation. Membrane was washed three

times with TBS-T, 5 minutes/ wash, and blocked in suitable blocking buffer prior to probing with the desired antibody.

cDNAs encoding human Ang-1 and Tie2 were obtained from the American Tissue Culture Collection and the latter subcloned into the expression vector pCR3 (Invitrogen Life Technologies). Ang-1* and Ang-1 were kindly provided by Dr B Dunmore. Department of Surgery, Leicester University.

2.3.8 Treatment of serum starved cells with inhibitors

Cells were treated with 100 nM Wortmannin (Sigma Aldrige, Poole, Dorset, UK). Wortmannin was dissolved in ethanol to produce a 1mM stock which was stored at 4^0C. All control cells were treated with relevant amount of ethanol as described.

2.3.9 Expression in HUVE cells

All expression work on HUVE cells was kindly carried out by Mrs Gweneth Williams (Department of Surgery, University of Leicester). For expression of full-length and deleted versions of ABIN-2, the relevant cDNA sequences were subcloned into pFLAG-CMV2 expression vectors (Sigma) in-frame with the amino-terminal FLAG-epitope tag and were kindly provided by Dr D. Hughes. Expression vectors were transfected into CHO and endothelial cells using Superfect transfection reagent (Qiagen) or Targefect F-2 (Targeting Systems).

2.4 Apoptosis and inflammation

2.4.1 HUVE cell survival assays

The expression vector encoding green fluorescent protein (GFP) was obtained from BD Biosciences Clontech. Endothelial cells were transfected using Targefect F2 transfection reagent (Targeting Systems). For examination of endothelial survival, HUVEC were grown to 80-90% confluence on gridded tissue culture dishes and transfected with control or test plasmids, together with green fluorescent protein. 24 h post transfection cells were washed and incubated in growth factor- and serum-free medium for 18 h before analysis of the transfected cells, assessed by expression of GFP, for cell survival, the numbers of viable transfected HUVEC were counted at the end of the serum-free incubation period and calculated as percentage of starting cell number in the same 5 grids in each dish. Cell viability was routinely determined by exclusion of 0.2% trypan blue.

2.4.2 Detection of HUVE cell apoptosis using 4'-6-Diamidino-2-phenylindole (DAPI) staining

4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, showing a fluorescence specificity for AT, AU and IC clusters{ Fuh R. 1997}. Because of this property, and because when DAPI binds to DNA, its fluorescence is strongly enhanced, DAPI has become a useful tool in various cellular investigations such as apoptosis.

For examination of endothelial apoptosis, HUVE cells were grown to 80-90% confluence on gridded tissue culture dishes and transfected with control or test plasmids, together with green fluorescent protein. 24 h post transfection cells were washed and incubated in growth factor- and serum-free medium for 18 h before analysis of the transfected cells, assessed by expression of GFP, for apoptotic index or cell survival. Apoptotic index was determined essentially as described by Mejillano 24. Briefly, after growth factor deprivation media was removed and cells fixed for 10 min at room temperature in 4% formaldehyde in PBS. Nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI), (Sigma) at 10μg/ml in 10mM Tris, pH 7, 10mM EDTA, 100mM NaCl, for 5 min at room temperature.

The fraction of GFP-expressing cells with condensed and fragmented nuclei were determined by fluorescence microscopy. For cell survival assays the numbers of viable transfected HUVEC were counted at the end of the serum-free incubation period and calculated as percentage of starting cell number in the same 5 grids in each dish. Cell viability was routinely determined by exclusion of 0.2% trypan blue.

2.4.3 Determination of apoptosis by annexin V- PE staining

Annexin V-PE Apoptosis Detection is based on the observation that soon after initiating apoptosis, most cell types translocate the membrane phospholipid phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface {Huppertz B; ,Frank HG 1999}.. Once on the cell surface, PS can be easily detected by staining with a fluorescent conjugate of Annexin V, a protein that has a strong natural affinity for PS. The assay can be directly performed on live cells, without the need for fixation. Annexin V-PE contain a fluorescent dye that produces

an intense signal in the far-red region of the spectrum (max emission = 670 nm) and therefore it is very useful for double labeling of cells with green as in the case of GFP fluorescence and red coloured fluorescent probes.

HUVE cell staining with AnnexinV-PE was performed using a detection kit (MBL, Nagoya, Japan). HUVE cells were grown to 80-90% confluence on gridded 24 well tissue culture dishes and transfected with GFP as previously described. After18 hours in serum free media, HUVE cells were washed with PBS and 500ul of 1X Annexin V Binding Buffer added. 5µl of Annexin V-PE was added and the mixture incubated at room temperature for 5 minutes in the dark. The HUVE cell were then analysed under direct microscopy for evidence of apoptosis indicated by red membrane staining.

2.4.4 Determination of apoptosis by cleaved caspase-3

Caspase activation (cleavage of procaspase to active caspase) is a hallmark of almost all apoptotic systems (Green 2000). Caspase-3 is a central effector caspase in many cells and mediates the cleavage of itself, other downstream caspases, and other caspase substrates. In non-apoptotic cells, caspase-3 should exist as a procaspase in which the potential cleavage site is intact. Once cleaved through the activation of the apoptotic cascade, the peptide end of this active caspase represents a novel epitope not present in normal cells. Therefore, the detection of this epitope is a unique and sensitive indicator of apoptosis.

Cleaved active caspase-3 was detected in lysates from adherent and floating HUVE cells transfected with either ABIN-2 or control vector after a period of 18 hours in serum free media by Western Blotting using antibodies from Cell Signalling

Technology. Preparation of cell lysates and Western Blotting were performed as previously described. For Western Blots, a nitrocelulose membrane was immersed with cleaved caspase-3 (Asp 175) Antibody in 5% w/v non-fat dry milk, 1x TBS, 0.1% Tween-20 at 4C with gentle, shaking overnight. Similar protein loading was confirmed by probing for B-actin (Sigma) and the presence of expressed ABIN-2 confirmed by probing for the Flag-epitope tag present on the N-terminus of expressed ABIN-2.

2.4.5 Determination of apoptosis by flow cytometry

The flow cytometric assay uses a DNA specific, fluorescent dye to measure the DNA content of individual cells. Normal populations of growing cells have either a single compliment of DNA (G_0 and G_1 phase), a double complement (G_2 and M phase cells) or an intermediate amount of DNA (S phase cells). This can be seen in the flow cytometer when the cells are labelled with a DNA-specific dye such as propidium iodide (PI). Apoptotic cells will therefore contain fragmented DNA which will appear smaller than the G_0 population {Lamm G, Steinlein P, et al. 1997 }.

Apoptosis was detected using a method described by Gabor in 1997 for the flow cytometric determination of apoptosis in a population of cells transiently transfected with GFP alone or co-transfected with a plasmid carrying the gene of interest. The assay combines GFP as a marker to detect the transfected cells (green fluorescence) and the reduced fluorescence of the DNA binding dye PI in the apoptotic sub population as a marker for apoptosis (red fluorescence). The reduced fluorescence of PI in apoptosing cells results in the appearance of a characteristic sub-2N fluorescence peak with respect to the G_0/G_1 cell cycle region.

Approximately 2.5 x 10^6 GFP transfected HUVE cells in a volume of 500µl of PBS were washed with PBS, to this 2ml of 70% ethanol were added and left on ice for 60 minutes then left at 4°C overnight, after which the mixture was centrifuged at 12000 xg for 5 minutes then washed with PBS. The cells were pelleted then resuspended in 800µl PBS. 100ul of RNAse and 100µl PI [100 ng/ml] was added to the suspension which was then incubated at 37C for 30 minutes. 2ml of the mixture was then analysed using a Beckman Coulter Diagnostics Flow Cytometer (High Wycombe, model epicsXL-MCL). The data was then analysed using expo32ADC XL software (Beckman, High Wycombe).

2.5 The chick chorioallantoic membrane

2.5.1 Preparation of the chick Chorioallantoic membrane as a model for studying angiogenesis

Fertilized eggs of the White Leghorn Chick (Gallus Gallus) were incubated at 37.8 ^{0}C and 80% humidity. Chicks were obtained from the Biosciences laboratories at Leicester University. At day 2 incubation, 2 vertical slits were made on either axes of the egg with extraction of 3ml of yolk. After 10 min a window was made in the eggshell using a Dremmel 125 W electrical Saw to expose the CAM. No ethical approval was needed as eggs were terminated prior to day 10.

2.5.2 Stages of development using time lapse photography

CAMs were filmed using an Olympus BH2 microscope, zeiss lens (10x

magnification) connected to a Panasonic AG 6730 video recorder with recording period set for 48 hour continuous loop. After a window was made in day 2 eggs were maintained incubated at temperature range 37.1 to 38 ^{0}C, Humidity was maintained at 30- 40 %. Time lapse images were taken at 1 image/ second for the first 2 days and at 0.5 images/sec for the subsequent 6 days. All eggs were terminated by day 10.

2.5.3 Optimising the CAM injection methods

A window was made in day 3 CAMs (see above). Initial injections of ink into the CAM vessels using pre-made microinjection needles fitting onto a Pneumatic microinjection system (Intracel PV820) and a micropipette beveller Model BV-10 was not efficient as the microinjection system did not allow for the variation between diastolic and systolic pressure in the cardiac cycle of the CAM. An alternative method using Microinjection needles which were created using a 1.0 mm and a 1.2 mm internal diameter internal diameter microcapillary tubes pulled off on a Narishige (Japan) vertical pipette puller model 720 with settings 17.4 heat and 2 solenoid. The ink was loaded into the capillary tube using a micropipette delivery system (eppendorf). Pneumatic pressure was achieved by connecting the capillary tube to a 10 ml syringe via a 10 cm length rubber tubing of 0.9 mm diameter.

2.5.4 Intravital fluorescence microscopy

Intravital Fluorescence microscopy was performed using a modified Olympus microscope with a 100 watt Mercury lamp and a 10X water immersion Olympus objective. The microscopic images were recorded using a CCD digital camera at 3

images/ second (Digital Pixel) and transferred directly onto computer (Viglen) using a specially designed program (IP-lab) for off-line evaluation.

2.5.5 Analysis of microcirculation using intravital microscopy

CAM microcirculation was investigated prior to and in response to cautery, TNF-α and IL-1B stimulation. The temperature of the CAM under the microscope was maintained at 37.8C using a Solent Scientific Heater, Germany and a specially modified incubator. Digital images were recorded prior to scalding/stimulation with TNF-α and IL-1B at 1 min, 5min, 10 min, 15 min, 30 min, 45 min and 1 hour respectively. Cautery was carried out on each CAM using a fine point cautery device for a period of 30 seconds (Oasis, ref 3501-A). TNF-α (1 μl, 100 μl/ml) or IL-1 (1μl) was added to the surface of each CAM microvessels of similar size. Single unbranched vessels (10-20 μm in diameter) were typically selected.

Following the experiment, all CAMs were killed before day 7 by freezing to –20C.

2.5.6 Quantification of microcirculatory parameters.

Quantification assessment of microcirculatory parameters was performed off-line by frame-to-frame analysis of the digitally recorded images. The number of freely flowing leucocytes, of leucocytes rolling along the vascular endothelium was determined off-line by digital video image analysis. Leucocyte velocity measured with the aid of a Graticule (1 mm, 100X 0.001). Leucocyte-endothelial interactions in microvessels diameter (10-20 μm) were quantified by counting the number of firmly adherent leucocytes that were not moving during the observation period of 20 sec.

2.5.7 Visualizing Leucocyte-Endothelial Cell Interactions.

To visualize leucocyte-endothelial cell interactions within CAM microvessels, Rhodamine 6G (0.75ul, 0.05%, Mol. Wt. 479; Sigma Aldrich) was given for contrast enhancement of leucocytes in the microcirculation. This was carried out by direct injection into one of the branches of the vitelline artery using a microinjection needle (Eppendorf Femotips, Intracel). For each CAM the same microvessel was scanned for a period of 300 seconds at different time intervals outlined above.

2.5.8 Induction of Endothelial Cell inflammation and Apoptosis in CAM Vessels

Endothelial cell apoptosis was induced by using 10/0 prolene suture (Ethicon) as sling around one of the branches of the vitelline artery to act as a clamp for a period of 4 hours. At the end of this period of ischaemia the sling was removed to allow full reperfusion of the clamped vessel.

2.5.9 Visualizing Apoptotic Endothelial Cells in CAM Vessels

Visualizing apoptotic endothelial cells was achieved by direct injection of 1μl Annexin V-PI (immunotech) at a concentration of 100 ng/ml or Hoechst solution into a reperfused microvessel following the 4-hour period of clamping. The digital images (Nikon Coolpix 3500) were stored in computer for off-line analysis.

2.510 Electroporation of Chick Chorioallantoic Membranes Vessels

Fertilised chick eggs were placed on their side and incubated in a 37.8 ^{0}C (Dynal, Sweden) with 30- 35% humidity for 48 hours. The eggs were wiped and sprayed with 70% ethanol, using a 16 gaμge needle 3ml of albumin were extracted from the small of the egg and a window made in the superior aspect of the egg as described previously. A sharp tungsten needle was used to tease away the vitelline membrane from around the segment of vessel to be electroporated. DNA encoding either GFP or GFP with ABIN-2 or A20 was loaded into the micro capillary needle at a concentration 0f 2 μg/ml. A suitable artery was then visualized under microscope and DNA injected directly into the lumen of the vitelline vessel using an Eppendorf microinjector. 5ul of DNA solution is injected into the vessels unless otherwise stated. 5ul of ice cold PBS were placed on the vessel. Electroporation probes were placed 2 mm on either side of the vessel. Electroporation is then initiated at 8-10 volts, 3 pulses and at LV 99 ms/500V The CAM is then taped and replaced in the incubator on its side.

Evaluation of transfection was carried out after 10-12 hours following electroporation.

2.5.11 Chick chorioallantoic membrane angiogenesis assay

Chick chorioallantoic membrane (CAM) assays were performed as described. Saline or Ang-1 -stimulated CAMs were treated for 24 hours with 20 % Rhodamine , or control Ab's and then injected intravenously with 50 μl Annexin V-FITC. CAMs were harvested after 2 hours, and then analyzed by microscopy. For all treatment groups, CAMs were fixed with 3.7% paraformaldehyde prior to excision. CAMs were also

stained by the TUNEL method to detect fragmented DNA.

2.5.12 Grafting of human skin onto the surface of the CAM

Skin samples were grafted onto the surface of day 3 CAMs prepared as previously described. Fresh human skin was obtained from the plastic surgery department within 30 minutes of excision. (Ethical approval obtained by the department of Surgery Leicester Royal Infirmary). The subcutaneous fat and part of the dermis was excised to leave only epidermis and partial dermis. This was then divided into segments measuring about 5mm by 5 mm epidermal surface area. A single segment was applied directly onto the surface of a single CAM with the dermis directly in contact with a well vascularised section on CAM. The chick CAM was then re-incubated as above. At day 10, the CAM was injected with 0.3 ul of blue dye which was allowed to circulate in the CAM vaculature. The grafted skin was then removed and the vessels analysed microscopically and photographed digitally. The egg CAM was terminated as above.

2.5.13 Evaluation of Evans blue extravasation

Microvascular permeability was evaluated using Evans blue dye 2% w/v ,(Sigma). 0.5-1 μl of Evans blue was injected into one of the branches of the vitelline vein. The CAM was then scaled in several areas and the dye extravasation assessed using digital photography.

2.5.14 Statistical analysis

Statistical tests were carried out using Prism program and with the kind assistance of Nick Brindle. Unless otherwise stated Anova paired tests were used.

CHAPTER 3

ABIN-2 PROTECTS ENDOTHELIAL CELLS FROM DEATH AND HAS A ROLE IN THE ANTI-APOPTOTIC EFFECTS OF ANGIOPOIETIN-1

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ABIN-2 PROTECTS ENDOTHELIAL CELLS FROM DEATH AND HAS A ROLE IN THE ANTI-APOPTOTIC EFFECT OF ANGIOPOIETIN-1

Introduction

The receptor tyrosine kinase Tie2 is expressed primarily in endothelial cells and is essential for blood vessel formation . A family of ligands, the angiopoietins, have been identified for Tie2. Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang2) are the best characterized members of this ligand family. Ang-1 activates Tie2 whereas Ang2 can antagonize this effect. Activation of Tie2 stimulates endothelial cell migration and sprouting and the receptor is required for correct organization and integrity of vascular system during development. In addition, Ang-1 stimulation of Tie2 inhibits vascular leakage in the adult and suppresses vascular inflammation. These effects point to an important vascular protective activity of the Ang-1/Tie2 system. In accordance with this, angiopoietin stimulation of Tie2 also inhibits endothelial cell apoptosis in response to growth factor deprivation, irradiation and mannitol treatment. Recently Tie2 receptor was found to interact with the protein A20 binding inhibitor of NF-κB activation 2 (ABIN-2) in a ligand dependent manner. As the name suggests ABIN-2 is an inhibitor of NF-κB and it may have a role in Ang-1 suppression of NFkB activity. As well as regulating inflammatory gene expression, NF-κB has antiapoptotic activity in many cell types. In endothelial cells NF-κB activity is

necessary for prevention of apoptosis induced by tumour necrosis factor-α (TNF) and growth factor-deprivation. Although ABIN-2 inhibits NF-κB its effects on apoptosis are not known and its involvement in Tie2-mediated endothelial survival are unexplored.

As mentioned earlier, ABIN-2, and the related protein ABIN-1, were originally discovered as binding partners for the inducible protein A20. This zinc finger protein is a potent inhibitor of NF-κB activity with a key role in limiting the extent and duration of inflammatory activation . ABIN-2 has been postulated as one of the mediators of the inhibitory effect of A20 on NF-κB. A20 inhibits apoptosis in endothelial cells and some other cell types. Expression of A20 inhibits apoptosis induced by growth factor-deprivation of HUVE cells and by lipopolysacharide in human microvascular endothelial cells. TNF-induced apoptosis in HUVEC, as well as MCF7 breast carcinoma cells, NIH 3T3, Fibroblasts and some other cell types are also inhibited by A20. It is possible therefore, that ABIN-2, like A20, could both inhibit NF-κB and suppress cell death. Such activity would make ABIN-2 an attractive target for promoting cytoprotection of the endothelium . In part of this study I examined the effects of ABIN-2 on apoptosis of endothelial cells and its potential involvement in Tie-2 mediated endothelial survival as well as its involvement in the effects of A20 activity.

3.1 Transfection of HUVE Cells

In order to effectively assess the role of target protein expression on HUVE cell survival, a reliable method was needed that will produce a high transfection efficiency

to give significant results. Several transfection methods utilizing plasmids encoding green fluorescent protein (GFP) were assessed and transfection efficiency monitored by counting the percentage of cells expressing GFP. Two reagents; the Qiagen SuperFect Transfection Reagent and the Targefect F2 Transfection Reagent were found to give adequate transfection rates of 45-50% and 65 -75% respectively. Once transfected however, HUVE cells appeared fragile with death occurring within 36 hours of transfection. As the primary aim of transfecting the HUVE cells was to assess apoptosis using a variety of methods such as serum starvation, it was essential that transfection produced minimal effects on cell viability. Transfections were carried out using varying monolayer confluency ranging from 30 to 95%, variable fetal calf serum supplement as well as endothelial growth factor levels in the culture medium in order to optimize for best transfection efficiency and minimal effects on endothelial viability. Table 3.1 summarizes the transfection conditions tested.

In the eventual protocol adopted using 80% confluence rates of monolayer HUVE cells, it was possible to achieve GFP/ Target DNA co-transfection rates of up to 90% with almost all cells surviving beyond 48 hours in culture medium.

Table 3.1. Conditions used to maximize HUVE cell transfection using Suprefect. Washing with serum free media, addition of FCS 10% and growth factor 10 μg/ml appeared to give best transfection results.

Alteration	Transfection efficiency	%Survival 12 hours post transfection	%Survival 18 hours post transfection	%Survival 24 hours post transfection	%Survival 48 hours post transfection
PBS washing	64%	74%	32%	12%	5%
Serum free media washing	72%	93%	93%	93%	71%
FCS 2%	76%	90%	91%	91%	63%
FCS 5%	78%	89%	87%	86%	58%
FCS 10%	84%	93%	90%	89%	71%
Growth Factor 5 μg/ml	82%	85%	76%	62%	43%
Growth Factor 10 μg/ml	91%	95%	93%	93%	69%

3.2 Time course of serum starvation on cell survival

The optimal serum starvation period for assessing survival of HUVE cells was tested. Growth factor deprivation of HUVE cells were carried out at periods of 3, 6, 10, 12, 14, 18, 24, 30 and 36 hours respectively following transfection with target plasmid and GFP. Three independent experiments were carried out for each serum deprivation period. The optimum serum deprivation interval following transfection with target plasmid was 18 hours (Figure 3.1). Serum deprivation less than 18 hours resulted in minimal cell death, conversely HUVE cell serum starvation of 30 hours or more resulted in virtually no surviving cells.

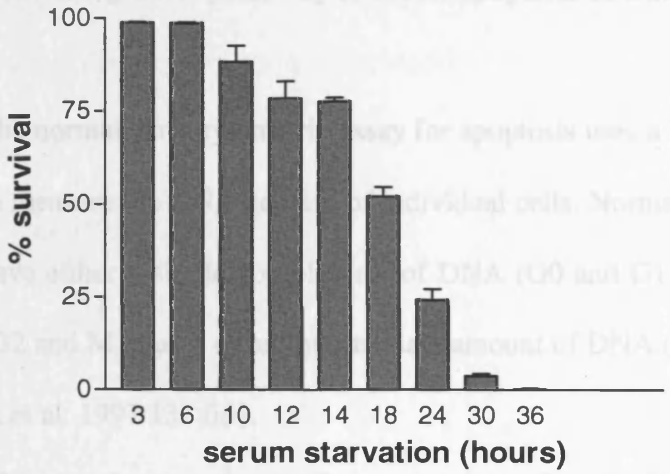


Figure 3.1. The effect of serum starvation on HUVE cell survival. Almost 50% of HUVE cells have survived following 18 hours in serum free media. Beyond 30 hours in serum free media virtually all cells have not survived. Survival was assessed by the number of live GFP labelled cells visualized under microscopy.

3.3 Establishment of method to assess endothelial cell apoptosis

The effects of ABIN-2 on endothelial survival and apoptosis were examined. It was therefore necessary to establish appropriate assays. As the transfection methods I used transfected less than one hundred percent of the HUVE cells, it was also required that the assays used would allow assessment of only the transfected cells. To accomplish this, cells were transfected with plasmids encoding the protein of interest together with a plasmid encoding GFP. Apoptosis assays were tested for their ability to detect apoptosis in GFP expressing cells.

A number of different methods were tested including flow cytometry, binding of Annexin V and assessment of nuclear morphology.

3.3.1 Using flow cytometry to detect apoptosis in transfected endothelial cells

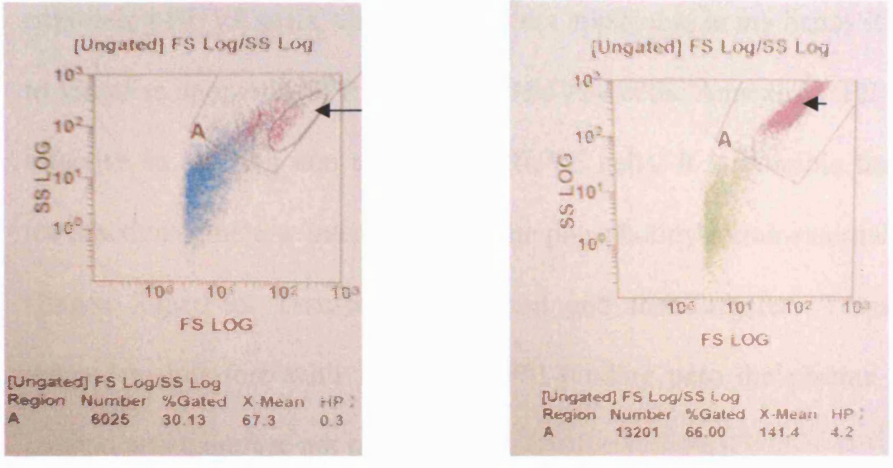
The normal flow cytometric assay for apoptosis uses a DNA specific, fluorescent dye to measure the DNA content of individual cells. Normal populations of growing cells have either a single complement of DNA (G0 and G1 phase), a double complement (G2 and M phase) or an intermediate amount of DNA (S phase). {Lamm G, Steinlein P, et al. 1997 132 /id}.

This can be seen in the flow cytometer when the cells are labelled with a DNA specific dye such as propidium iodide (PI). This method should be capable of detecting apoptosis in cells co transfected with a plasmid encoding GFP as well a plasmid carrying the gene of interest using Fluorescence Activated Cell Sorter (FACS) to detect transiently transfected cells and the reduced fluorescence of the DNA-binding dye PI in the apoptotic subpopulation as a marker for apoptosis. The reduced fluorescence of PI in apoptosing cells results in the appearance of a characteristic sub-2 N fluorescence peak with respect to the G0/G1 cell cycle region. It was thus hoped not only to pick apoptosis rates but also detect the cell cycle phase of the HUVE cells in the assay.

This assay was attempted in HUVE cells transiently transfected with GFP. Analysis was attempted at time: 0, 6 hours, 12 hours , 18 hours and 24 hours and 48 hours of serum starvation. Early results appeared promising. The flow cytometer was apparently capable of detecting apoptosis levels in the transfected HUVE cells. However it became evident that the results were not consistent.

A number of experiments were performed varying RNAse, ethanol and PI concentrations, however this did not improve the sensitivity nor the specificity of the

technique. As the overall consistency of the data did not improve, it was decided not to pursue flow cytometry as a method for detecting apoptosis due to the unreliability of this method.



a b

Figure 3.2. FACS assisted detection of apoptosis. a) HUVE cell transfected with control empty vector survival (arrow) following 18 hours in serum free medium. The number and percentage of live cells is shown in the table below. b) ABIN-2 expressing HUVE cells after 18 hours in serum free medium showing a greater proportion of live cells (arrow head) when compared to HUVE cells expressing control vector.

3.3.2 Using Annexin V to detect apoptosis in transfected endothelial cells

One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phophatidylserine (PS) from the inner to the outer leaflet of the plasma membrane {Huppertz B; ,Frank HG 1999 i.d. 151}. Once exposed to the extracellular environment, binding sites on PS become available for Annexin-V, a 35-36 kDa, calcium dependent phospholipids binding protein with a high affinity for PS.

The translocation of PS precedes other apoptotic processes such as loss of plasma membrane integrity, DNA fragmentation and chromatin condensation. Annexin-V can

be conjugated to a variety of fluochromes such as fluorescein and Phycoerythrin (PE) for identification of cells in the early stages of apoptosis.

Despite several attempts to utilize Annexin V PE as a marker for apoptotic GFP expressing HUVE cells, this assay was not applicable in my hands it was not possible to visualize apoptotic GFP transfected HUVEc cells. Annexin V PE nevertheless was effective in staining non transfected HUVE cells. It is possible that the process of transfection somehow interfered with the phosphatidylserine externalization. Both the Qiagen SuperFect Transfection Reagent and the Targefect Transfection Reagent seemed to interfere with Annexin V PE binding onto the plasma membrane. This method was therefore not pursued.

3.3.3 Detection of nuclear morphological changes as a marker for apoptosis in transfected endothelial cells

4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, showing a fluorescence specificity for AT, AU and IC clusters. Because of this property DAPI is a useful tool in various cytochemical investigations. When DAPI binds to DNA, its fluorescence is strongly enhanced, what has been interpreted in terms of a highly energetic and intercalative type of interaction, but there is also evidence that DAPI binds to the minor groove, stabilized by hydrogen bonds beween DAPI and acceptor groups of AT, AU and IC base pairs.

Cells undergoing apoptosis display typical features, namely cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation. {Kerr A, Wylie A. 1972}. Dramatic changes occur within the nucleus during apoptotic death. It is

commonly thought that the nuclear changes are due to activation of endogenous nuclease(s) which cleaves DNA into oligonucleosomal fragments. This is associated with the appearance of dense, crescent-shaped chromatin aggregates which line nuclear membrane. Later, the nucleus disintegrates, nuclear membrane develops deep invaginations and, ultimately, the nucleus fragments into dense granular particles (apoptotic bodies) {Kerr A, Morris D. 1984}.

Chromatin condensation, nuclear shrinkage and formation of apoptotic bodies can easily be observed under fluorescence microscopy, after appropriate staining of nuclei with DNA-specific fluorochromes such as DAPI.

This method as described in section 2.4.2 was found to produce clear and consistent results when used for assessing apoptosis of GFP expressing HUVE cells (figure 3.3).

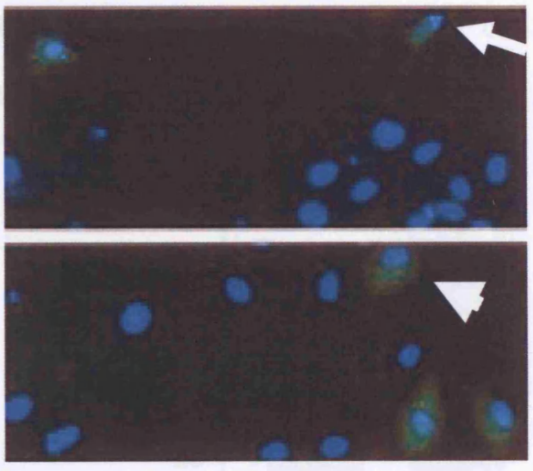


Figure 3.3. Representative fluorescence photomicrograph showing nuclear morphology of GFP expressing apoptotic and non apoptotic HUVE cells using DAPI staining. HUVECs were cotransfected with GFP and control vector (CV) (upper panel) or with GFP and vector encoding human ABIN-2 (lower panel). Twenty-four hours after transfection, cells were subject to growth-factor deprivation for 18 hours. An example of an apoptotic (arrow) and nonapoptotic (arrowhead) transfected cell is indicated. Original magnification, X200.

3.4 Effects of ABIN-2 on endothelial cell survival

The effects of ABIN-2 on endothelial cell survival were tested. To do this, subconfluent HUVE cells in gridded dishes were transfected with empty control vector (CV) or vector encoding ABIN-2, together with GFP encoding plasmid. Following transfection, cells were washed and transferred to serum free medium. The number of GFP-expressing cells were counted in each grid in the dish at the beginning of the serum deprivation period. After 18 hours cells were washed with serum free medium and the remaining GFP expressing cells in each grid were counted accordingly. The percentage of surviving but transfected HUVE cells was calculated. As shown in Figure 3.4 HUVE cells transfected with empty vector exhibited 33.6% survival. In contrast ABIN-2 expressing cells showed a significant increase in survival (67.3%). This data suggests that ABIN-2 inhibits cell death. HUVE cell transfection with ABIN-2 had a significantly increased cell survival compared with cells transfected with empty control vector $P < 0.01$.

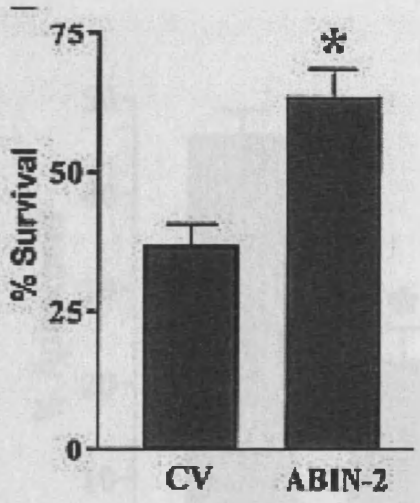


Figure 3.4. Survival of HUVE cells expressing ABIN-2 compared to control vector. Data are presented as means and SEM for 5 independent experiments, $*P < 0.01$ versus CV.

3.5 Effects of ABIN-2 on endothelial cell apoptosis

The role of ABIN-2 in endothelial cell apoptosis was assessed. As in section 3.3 HUVE cells were either cotransfected with either GFP and ABIN-2 or GFP and CV in gridded dishes and subjected to serum starvation. 18 hours following serum starvation, the cells in the dish were washed with serum free medium. DAPI staining was then carried out as described in section 2.4.2 in order to differentiate apoptotic HUVE cells from non apoptotic cell. Apoptotic cells were characterised by nuclear condensation and fragmentation as shown in Figure 3.3. The number of apoptotic transfected cells in each grid was then counted. The apoptotic index was then calculated which is the number of apoptotic cells following serum starvation divided by the total number of transfected cells. HUVE cells expressing ABIN-2 were found to exhibit a significant two-fold decrease in apoptotic index (Figure 3.5) with HUVE cells transfected with control vector showing a 46.3% apoptosis compared with 20.6% apoptosis in cells transfected with ABIN-2. ABIN-2 therefore reduces HUVE cell apoptosis.

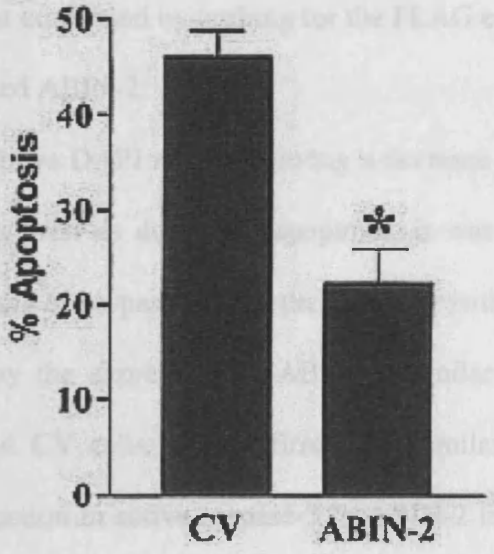


Figure 3.5. The effect of ABIN-2 expression on HUVE cell apoptosis. Apoptotic index of cells expressing ABIN-2 was significantly lower than for those expressing control vector (CV). Data are presented as means and SEM for 3 independent experiments, *P < .01 versus CV.

3.6 Effects of ABIN-2 on activation of Caspase-3

Diverse groups of molecules are involved in the apoptosis pathway. One set of mediators implicated in apoptosis belong to the asparate-specific cysteinyl proteases or caspases. A member of this family, caspase-3 (CPP32, apopain, YAMA) has been identified as being a key mediator of apoptosis of mammalian cells {Kothakota S 1997 147 /id}. The effects of co-transfection on HUVE cells with ABIN-2 and GFP on caspase-3 activation were examined to provide an additional test of the effects of ABIN-2 on apoptosis. Subconfluent HUVE cells were either transfected with GFP and CV or ABIN-2 and GFP. After 18 hours of serum deprivation, all lysate from adherent cells on the dish as well as floating cells was collected, western blotting with Cleaved Caspase-3 antibody then carried out as described in section 2.4.5. At the same time, β-actin was used to demonstrate similar protein loading. The presence of expressed ABIN-2 was confirmed by probing for the FLAG epitope tag present on the N-terminus of expressed ABIN-2.

Consistent with the above DAPI results showing a decrease in HUVE cell apoptosis expressing ABIN-2 as well as decreased apoptosis, it was found that the cleaved (17/19kDa) active forms of caspase-3 were present in growth factor-deprived HUVE cells and decreased by the expression of ABIN-2. Similar loading of transfection truncated ABIN-2 and CV cells was confirmed by similar intensity β-actin blots (Figure 3.5). The reduction in active caspase-3 in ABIN-2 transfected cells provides further evidence that ABIN-2 expression reduced HUVE cell apoptosis.

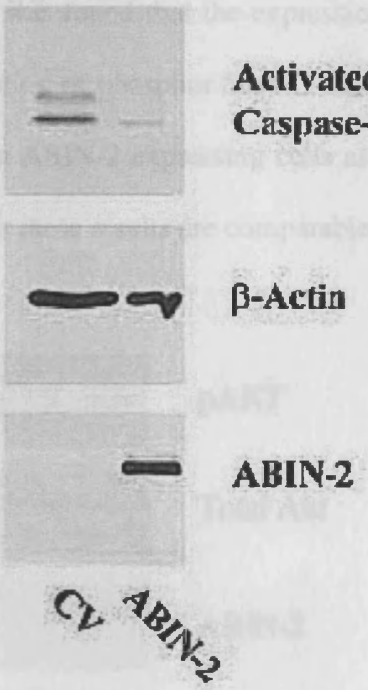


Figure 3.6. Activation of caspase-3 in HUVE cells. Activation of p17/19 caspase-3 was detected by western blotting in HUVE cells expressing either ABIN-2 or control vector as indicated. B-actin probing was used to confirm equal protein loading. The presence of expressed ABIN-2 was confirmed by probing for the flag-epitope tag present on the N-terminus of expressed ABIN-2.

3.7 The effects of ABIN-2 on phospho Akt

The activation of Akt is a common upstream event in suppression of apoptosis {Madrid LV; Wang CY, 2000}. It was of interest therefore to determine whether expression of ABIN-2 was associated with Akt activation. Akt phosphorylated on Ser473 is commonly used as an index of Akt activation. In these experiments therefore, the effects of ABIN-2 on Ser473-Akt was tested. After a period of 18 hours serum deprivation floating and adherent HUVE cells were collected.

Western blotting was used to detect the activated form of Akt (phospho-Ser473) in ABIN-2 expressing HUVE cells as well as CV expressing cells. Blotting with antibody for total Akt was also carried out to ensure similar protein loading.

As shown in Figure 3.5, it was found that the expression of ABIN-2 in HUVE cells resulted in increased activation of phosphor Ser473-Akt compared to control vector. The total Akt levels in both ABIN-2 expressing cells as well as CV expressing cells were similar confirming that these results are comparable (Figure 3.7).

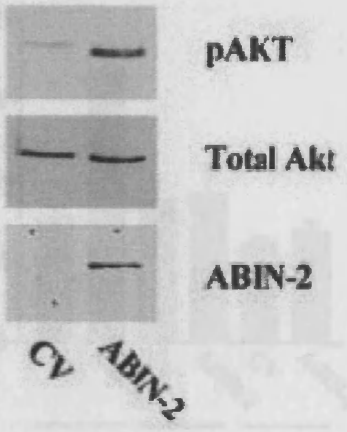


Figure 3.7. The effect of ABIN-2 on phosphor Akt. Phospho-Ser473 Akt and total Akt were detected by Western blotting in HUVECs transfected with CV or ABIN-2 as indicated. The presence of expressed ABIN-2 confirmed by probing for the FLAG-epitope tag.

3.8 Role of PI3 Kinase on pro-survival activity of ABIN-2

The finding that Akt was activated by ABIN-2 suggested the possible involvement of upstream kinase PI3K. This was tested by examining whether the cell survival and anti-apoptotic activities of ABIN-2 were sensitive to PI3K inhibitors. The effects of the PI3 kinase inhibitors Wortmannin and LY294002 on the survival of ABIN-2 expressing cells was therefore assessed. HUVE cells expressing ABIN-2 or CV were cultured on a gridded dish and the number of GFP expressing cells in each grid was counted. The cells were then subjected to 18 hours of growth factor deprivation with the addition of 10 μm LY294002 or 30 mM Wortmannin respectively. Cell survival was assessed as in section 3.4.

Addition of these inhibitors was found to completely counteract the anti-apoptotic effects of ABIN-2 on HUVE cell survival. In three independent experiments the increase in cell survival resulting from ABIN-2 expression was significantly inhibited by LY294002 and Wortmannin (Figure 3.8). This suggests a role for the PI3 Kinase/ Akt pathway in the pro-survival activity of ABIN-2.

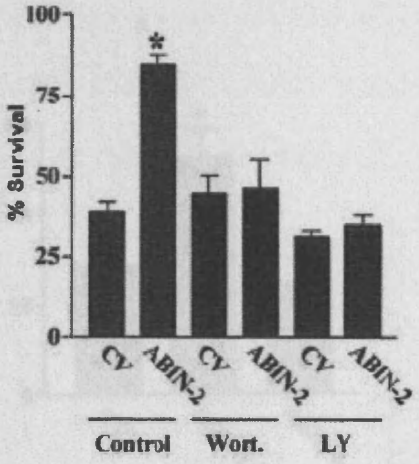


Figure 3.8. The role of PI3kinase on ABIN-2 activity.Survival of HUVEC-transfected CV or ABIN-2 and incubated with control vehicle, 30 nM wortmannin, or 10 μm LY294002 as indicated during growth factor deprivation. Data are presented as means and SEM for at least 3 independent experiments. *P < .01

3.9 The effects of ABIN-2 deletions on HUVE cell apoptosis

In order to identify the region on ABIN-2 responsible for its prosurvival activity in HUVE cells, various deletions of ABIN-2 were examined. HUVE cells were transfected with full length ABIN-2 wild type (ABIN-2-WT) as well as 2 forms of ABIN-2 deletions (figure 3.9); ABIN-2 (1-345) which contains the first 345 amino acids from the N terminus and ABIN-2-ΔC (deletion of the carboxy terminal 85 amino acid residues). HUVE cells were then subjected to growth factor deprivation and the percentage cell survival counted as in section 3.4. It was shown that ABIN-2-

ΔC expression in HUVE cell did not confer protection from apoptosis when compared to ABIN-2 (1-345) or ABIN-2 wild type (data not shown).

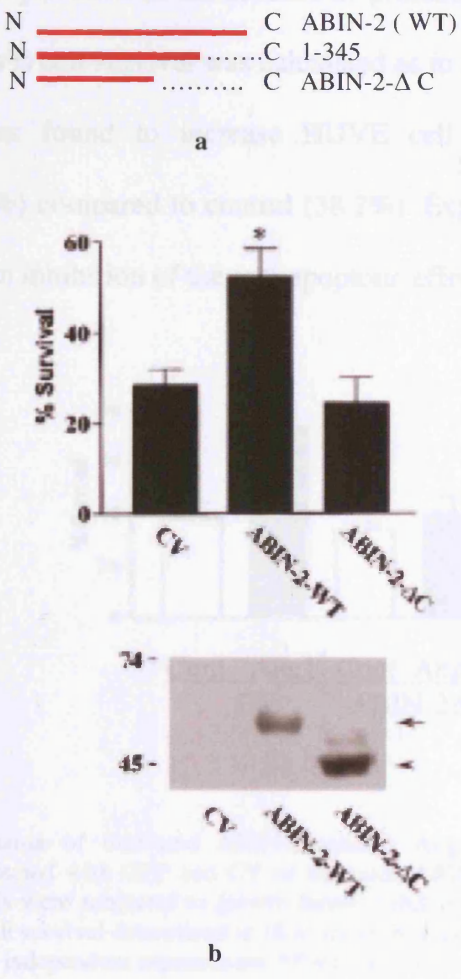


Figure 3.9. The effects of ABIN-2 deletions on HUVE cell apoptosis .a) Various forms of ABIN-2 used to transfect HUVE cells. Wild type (WT) ABIN-2 as well as 2 deletions of ABIN-2 were used. 1-345 ABIN-2 contained the first 345 amino acids from the N terminus. ABIN-2-ΔC lacking the last 85 amino acids from the C terminus. b) HUVE cells were transfected with GFP and control vector (CV) or with vector encoding wild-type ABIN-2 (ABIN-2-WT) or ABIN-2 lacking 85 amino acids of the carboxy-terminus (ABIN-2ΔC), as indicated. Cell survival of transfected cells was determined as described in section 3.4. The lower part of panel A depicts a Western blot showing expression of wild-typeABIN-2 (ABIN-2-WT) (arrow) and ABIN-2ΔC (arrowhead) in HUVECs transfected with CV, ABIN-2-WT, or ABIN-2ΔC as indicated. The presence of expressed forms of ABIN-2 was detected by probing for the N-terminal FLAG-epitope tag. Relative mobility of molecular mass markers is shown in kilodaltons. Data are presented as means and SEM for 5 independent experiments. *$P < 0.01$ vs CV.

3.10 Involvement of ABIN-2 in the anti-apoptotic effect of Ang-1*

Possible involvement of ABIN-2 in the anti-apoptotic effects of Ang-1* were tested. HUVE cells were transfected with either control vector or truncated forms of ABIN-2 as shown in section 3.9. Twenty four hours post transfection the cells were subjected to growth factor deprivation in the absence or presence of Ang-1* at a concentration of 400ng/ml. HUVE cell survival was calculated as in section 3.4. As shown in figure 3.10, Ang-1* was found to increase HUVE cell survival after growth factor deprivation (72.6%) compared to control (38.2%). Expression of a truncated form of ABIN-2 resulted in inhibition of the anti-apoptotic effects of Ang-1*.

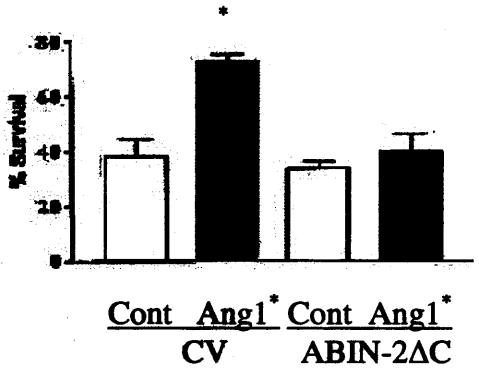


Figure 3.10. Expression of truncated ABIN-2 inhibits Ang-1*-stimulated endothelial survival. HUVECs were transfected with GFP and CV or truncated ABIN-2 as indicated. Twenty-four hours after transfection, cells were subjected to growth factor withdrawal in the absence or presence of 400 ng/mL Ang-1* and cell survival determined at 18 hours as detailed in section 3.4. Data are presented as means and SEM for 3 independent experiments. *$P < 0.01$ vs CV.

3.11 Verification of A20 as a pro-survival agent in HUVE Cells

The role of A20 as a pro-survival agent was tested. HUVE cells were transfected with CV and A20. The percentage of surviving cells after 18 hours of growth factor deprivation was calculated as in section 3.4.

The results confirmed that A20 increases HUVE cell survival (34.9%), compared to control vector survival rates of 23.2%.Interestingly, addition of the dominant negative form of ABIN-2 did not significantly reduce the pro-survival activity of A20 (27.1%). A20 therefore appears to be important in HUVE cell survival. The fact that there was a small decrease in survival rates when A20 was co-expressed with the dominant negative form of ABIN-2 suggests that ABIN-2 may play a role in the anti-apoptotic activity of A20.

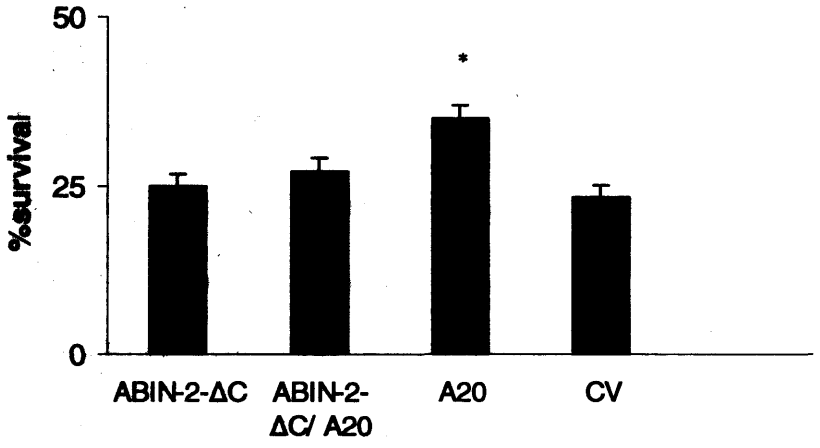


Figure 3.11. The effects of A20 expression on HUVE cell survival. Expression of truncated ABIN-2 inhibits A20 stimulated endothelial survival. HUVECs were transfected with A20/GFP and CV or truncated ABIN-2 as indicated. Twenty-four hours after transfection, cells were subjected to growth factor withdrawal and cell survival determined at 18 hours as detailed in section 3.4. Data are presented as means and SEM for 3 independent experiments. $*P < 0.01$ vs CV.

Discussion

At the start of this study very little was known about the role ABIN-2 in endothelial cell function. In this chapter a role of ABIN-2 in endothelial cell apoptosis as well as a possible mechanism for this effect was demonstrated.

An important factor allowing a more detailed analysis of the role of ABIN-2 was the establishment of optimal transfection conditions for the fragile HUVE cells. Transfection rates as high as 90% of HUVE cells expressing target protein were obtained.

It is known that growth factor deprivation induces substantial apoptotic HUVE cell death {Kim I., Kim H.G., et al. 2000}. Mejillano's method was used for apoptotic index measurements {Mejillano M, Yamamoto M, et al. 2001}. Induction of apoptosis by growth factor deprivation for 18 hours in transfected HUVE cells as well as establishing methods for measurement of endothelial cells apoptosis and survival rates set in place an adequate, efficient and reproducible method for studying the effects of ABIN-2 expression on endothelial cells.

The discovery that ABIN-2 reduced endothelial cell apoptosis was demonstrated, for the first time a potential role for this newly discovered protein. Three independent assays were performed to assess the effects of ABIN-2 on endothelial death, measurements were directly performed on endothelial survival and the apoptotic index was assessed by nuclear morphology and the activation of Caspase-3 determined.

Caspase-3 is one of the key executioners of apoptosis, being partially or totally responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP). Activation of caspase-3 requires

proteolytic processing of its inactive zymogen into activated p17 and p12 subunits cleaved caspase-3 antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to (Asp 175). This antibody does not recognize full length caspase-3 or other cleaved caspases. It was shown that ABIN-2 transfection does not activate caspase-3. This provided further evidence of ABIN-2 inhibition or at least reduction of the apoptotic pathways.

The role of ABIN-2 in activation of the PI3-kinase and Akt signalling pathway was then tested. The PI3-kinase/Akt pathway is known to have a central role in promoting endothelial cell survival{Kim I, Kim H.G., et al. 2000}. As ABIN-2 has been shown to interact with Tie-2 {Hughes D; Marron M., et al 2003, id 127 ,the next step was to investigate whether ABIN-2 reduces endothelial cell apoptosis by a similar mechanism as that of Tie2 namely the PI3-kinase/Akt pathway. Phosphorylation of Akt was demonstrated in ABIN-2 expressing HUVE cells suggesting that this maybe a possible mechanism by which ABIN-2 exerts its anti-apoptotic effects.

In order to identify the region on ABIN-2 responsible for the prosurvival activity, various deletions of ABIN-2 were examined. This was based on the discovery by Van Huffel that deletions of the carboxy-terminus of ABIN-2 prevents it from inhibiting stimulated NFκB activity when expressed in human embryonic kidney cells {Van Huffel S, Delaei F, et al. 2001}. It was shown that truncated form of ABIN-2 , ABIN-2 ΔC which lacks the terminal carboxy terminal 85 amino acids did not have the pro-survival effects noted with ABIN-2. This truncated form of ABIN-2 is therefore required for ABIN-2 pro-survival activity.

ABIN-2 ΔC was used to shed more light on the link between ABIN-2 with Tie-2. Ang 1 is one of the agonist for Tie2 which in turn has been shown to inhibit endothelial

cell death. Ang-1* was shown to cause a significant increase in the survival of growth-factor deprived endothelial cells expressing control vector. In contrast, the protective effects of Ang-1* on HUVE cells expressing the truncated form of ABIN-2 was not observed. This gives an indication that ABIN-2 is not only important but crucial in the role of Tie-2 in endothelial cell survival. The fact that addition of PI3K inhibitors seemed to inhit the effects of ABIN-2 implies that the PI3K/Akt pathway is important in ABIN-2 function.

Finally, attention was turned to the assessment of the interaction between A20 and ABIN-2. It has been demonstrated that A20 inhibits NF-κB activation. A20 has also been shown to inhibit cell death in a number of cell types, including growth factor-deprived endothelial cells, however, the effects of the A20 binding protein and putative NF-κB effector ABIN-2 on apoptosis have not been explored. In addition whether this inhibition has an effect on endothelial cell survival is not known. In a series of independent experiments, expression of A20 in HUVE cells significantly increased survival and reduced the apoptotic index compared to cells expressing control vector.

I did not show that co-expression of the truncated form of ABIN-2 alongside A20 interfered with its endothelial protective role to a statistically significant extent. More work is needed to before any conclusions about the interactions between A20 and ABIN-2 can be made.

Chapter 4

Establishing a novel in vivo system to look at inflammation and apoptosis

Chapter 4
Establishing a novel in vivo system to look at inflammation and apoptosis

Introduction

Currently, microvessel regression as well as microvessel responses to a variety of insults can be evaluated in vivo usually using a variety of well established mammalian models such as mice, rats and dogs. In addition to ethical issues mammalian models are both time and labour intensive as well as expensive. Furthermore, most experiments do not allow real time monitoring with continuous evaluation of the mammalian model but rather tissue procurement usually via animal sacrifice. These procedures become even more laborious with the testing of multiple reagents at different time points. For these reasons an alternative in vivo approach for the analysis of the microvascular system was explored.

Cancer biologists, developmental biologists, and ophthalmologists have described the chick chorioallantoic membrane (CAM) as a model system for studying vascular development {Fortune JE, Cushman RA, et al. 2000}, cancer behaviour {Vacca A, Ribatti D, et al. 2001} angiogenesis {D'Amore PA, Glaser BM, et al. 2005}, and photodynamic therapy{Lange N, Ballini JP, et al. 2001}

The chick CAM, a part of the extraembryonic tissue, begins to develop 3 days after initial incubation from the fusion of the chorion and the allantois {Fancsi T. & Feher G. 1979}. The fused CAM develops and eventually covers the entire surface of the inner shell membrane. The chick normally hatches at day 21. The CAM serves as a support for the extraembryonic respiratory capillaries, actively transports sodium and chloride from the allantoic sac and calcium from the eggshell into the embryonic

vasculature, and forms part of the wall of the allantoic sac, which collects excretory products.

This structure forms a supportive matrix for the extensive vascular network that courses through the CAM, analogous to the retina and its vasculature which makes it valuable in studying vasculature.

The accessibility of the CAM microvessels would allow the possibility of intravital microscopy and the visualisation of leucocyte: vessel wall interactions. Furthermore, it may be possible to transfect the endothelial cells of CAM microvessels in order to analyse the effects of putative modulations of inflammatory gene expression. In this chapter I describe work aimed at establishing the chick CAM as a model system in which to examine the potential in vivo effects of ABIN-2.

4.1 Phases of chick egg growth

Time lapse videophotography was used to confirm and monitor the CAM growth phases. At day 3 a window was made in the flat surface of the egg outer shell, the events occurring on the CAM surface were recorded using an Olympus BH2 microscope, the images were recorded directly on a video recorder as as described in section 2.5.2. The maximum continuous videophotography monitoring period was 62 hours. It was not possible to video a single CAM from day 3 to day 10 as the CAM did not tolerate the strong lighting conditions needed for videophotography. The CAM development was therefore monitored from day 3 to day 10 using multiple eggs at different phases of development for 12 hour periods.

License rules did not allow monitoring of the egg CAMs beyond day 10 incubation.

The first visible sign of development occurred between 90 to 96 hours of incubation. The fused CAM develops and covers the entire surface of the inner shell membrane by day 9 of incubation. Angiogenesis of CAM vessels occurred in a predictable manner after the formation of the 2 vitteline vessels (figure 4.1). Several vessels appeared to undergo natural regression however the site and timing of this could not be predicted.

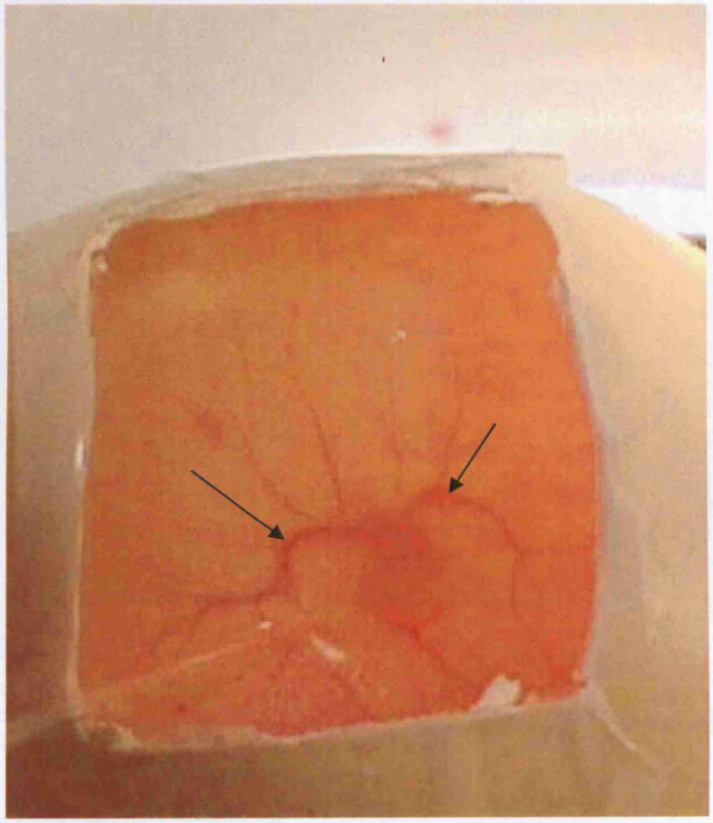


Figure 4.1. A window is made in the Chick egg at day 3 as shown. CAM microvasculature is visible showing the 2 main vitteline arteries (arrows) bifurcating from the embroynal heart.

4.2 Injection of chick CAM

Testing the feasibility of injecting a desired substance into the vascular system of the chick CAM was attempted using India blue ink dye. Commercially available microinjection needles (Eppendorf) were prepared to the desired bore (0.1-0.2 mm) using a special grinding plate. Once prepared, the dye was loaded and the needle connected to an Eppendorf injection pump (figure 4.2).

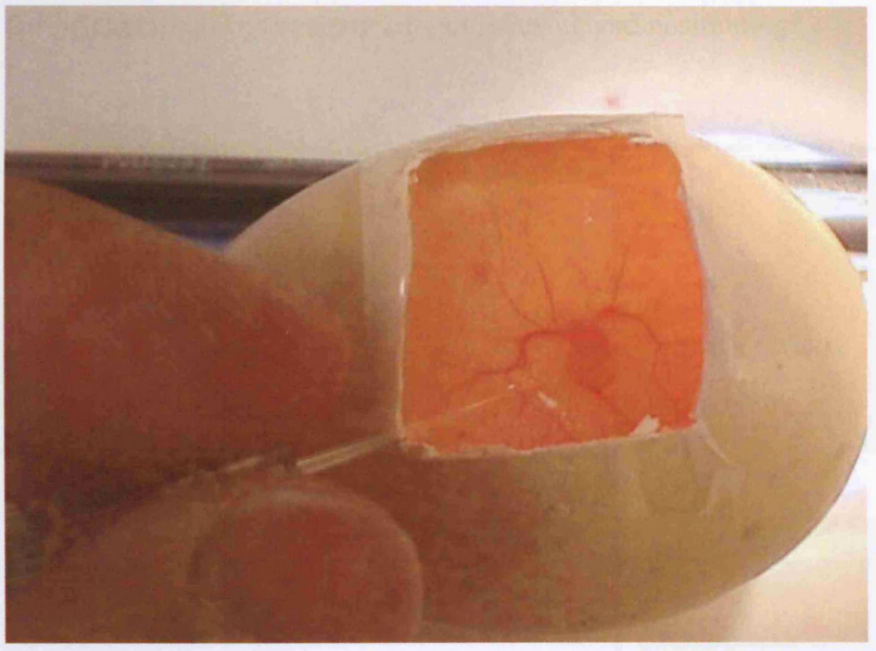


Figure 4.2. Injection of the chick CAM vitteline vessel side branches using the Eppendorf microinjection system.

Apart from the high cost as well as time consumption preparing the needles. A reliable injection method could not be achieved with these needles due to problems encountered with the reliability of the needles as well as excessive pressure and volume of the ink injection using the Eppendorf pump. In addition, the curved sloping inner edges of the shell made it difficult to achieve an acute angle of injection using the long straight Eppendorf needles. Almost all the injections were therefore carried

Special Note

Page 80 missing from the original

will allow constant access to the chick CAM without a substantial reduction in temperature and at the same time maintaining the required humidity levels.

One of the main problems encountered in setting up this system was ensuring that the microscope remained constantly focus on the area of interest. The loss of focus was due to the micro vibrations arising from the chick circulation which were magnified by the oval shape of the egg as well as general back ground vibrations present in the building. This problem was overcome by regular checks on the CAM every 5 minutes during experiments, to ensure correct focusing and positioning.

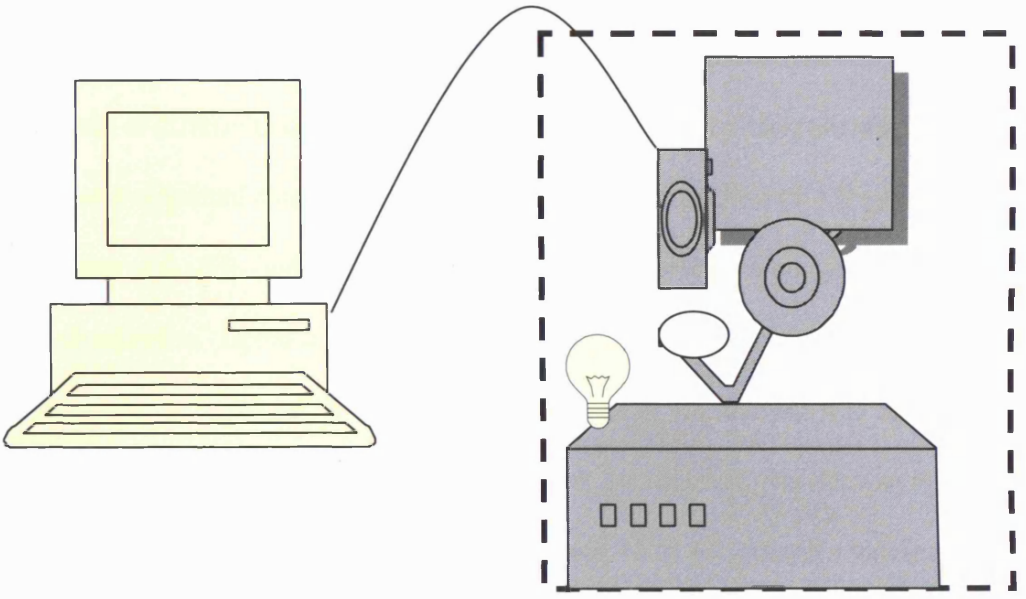


Figure 4.3. Monitoring CAM microcirculation using intravital microscopy. A clear Perspex incubator was constructed around the video microscopy camera and lighting.

Another problem was loss of humidity on the CAM surface over prolonged periods due to the lighting conditions which resulted in microvessel thrombosis and eventually in chick death. This was overcome in part by adequate hydration of the incubator prior to placing the chick, in addition to spraying the surface of the CAM with PBS. Despite these measures chick CAMs would begin to dry up by 2 hours

under the microscope. A compromise was reached by marking the area of interest with a ring of internal shell membrane and returning the CAMS to the main incubator after 90 minutes of monitoring for at least 15 minutes. Using this method it was possible to monitor CAMs up to day 10.

4.4 Using intravital Microscopy to Outline Chick CAM leucocytes

Previous studies have used Rhodamine 6G as a marker for highlighting leucocytes in intravital microscopes {H Baatz, U Pleyer, et al. 1995 141 /id}.. As Rhodamine 6G has never been used on chick CAMs previously, analysis of leucocyte contrast enhancement at different concentrations of Rhodamine 6G at different time intervals was necessary. Optimal fluorescence was achieved after injection of 1-2 µl at a concentration of 0.05% (w/v). The highest intensity fluorescence occurred within 4 minutes of injection (figure 4.3). After 15 minutes post injection this intensity had deceased markedly. Injection of higher concentrations of Rhodamine 6 G resulted in fluorescence of endothelial cells as well leucocytes. In addition, Rhodamine 6G leaching into the vasculature was occurring between 15 to 30 minutes reducing the contrast between the stained leucocytes and the background. Injection of Rhodamine 6 G into CAMs for studying leucocyte dynamics was optimum between 1 and 15 minutes post injection.

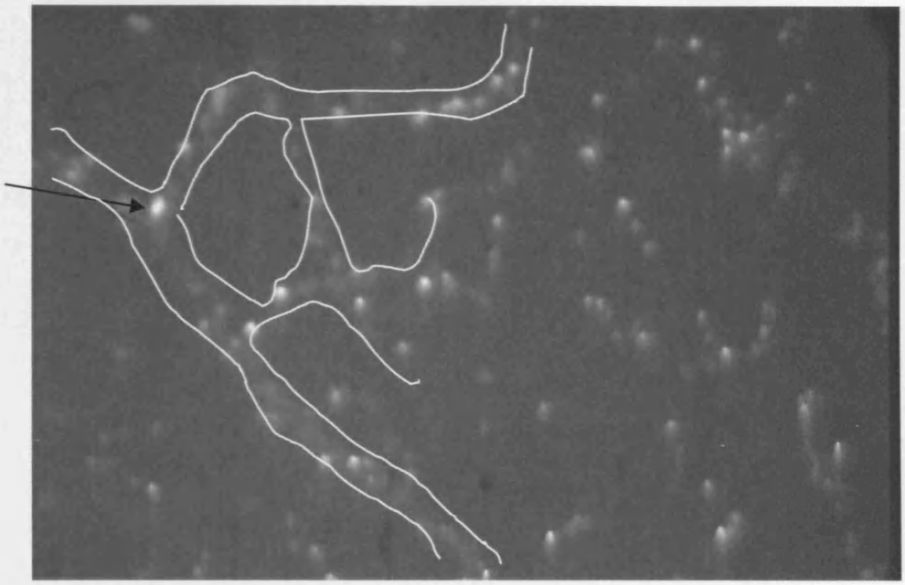


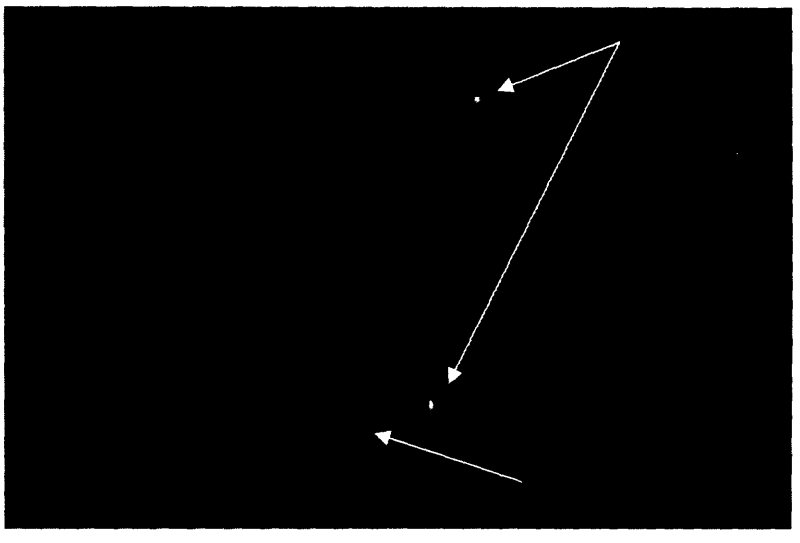
Figure 4.4. Rhodamine labelling of leucocytes in CAM microvessels. Injection of 1-2µl at a concentration of 0.05% (w.v) for 4 minutes produced optimal leucocyte fluorescence (arrow)

4.5 The use of intravital microscopy to monitor CAM inflammation

A variety of methods were used in order to induce CAM microvessel inflammatory response and these will be discussed in more detail in the next chapter. Inflammation was considered to be induced if there was evidence of increased vascularity as well as activation of the leucocyte adhesion cascade using intravital microscopy namely; capture, firm adhesion, and transmigration. Each of these steps is necessary for effective leucocyte recruitment.

Microscopy was successful in outlining each of the steps involved in the leucocyte adhesion cascade using Rhodamine G6. Leucocyte extravasation was demonstrated (figure4.5), however this phenomenon did not occur frequently over the time course of observation. Leucocyte rolling and adherence occurred with much greater frequency and were therefore deemed to be more reliable signs of inflammation. This

is the first report of using the Chick CAM to investigate microvascular inflammation with intravital microscopy.



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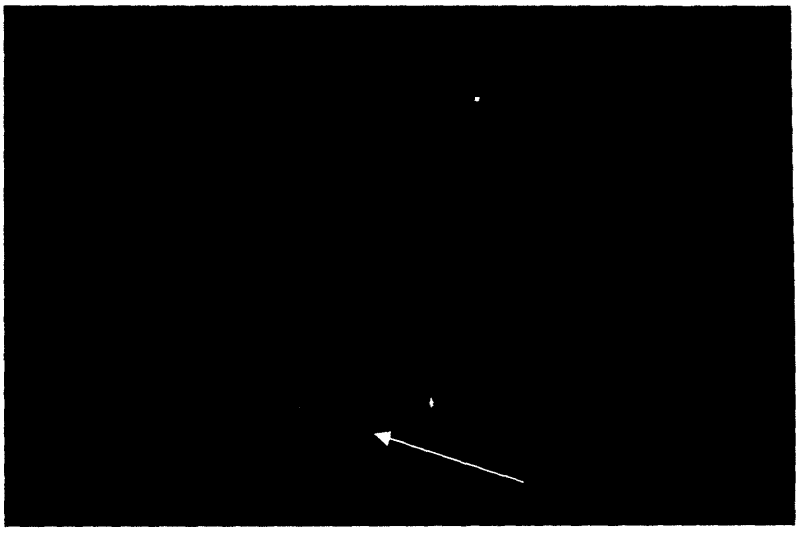

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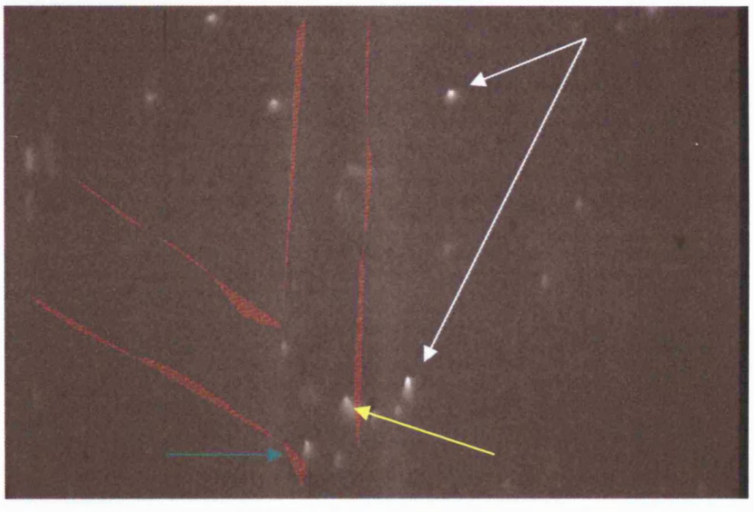
is the first report of using the Chick CAM to investigate microvascular inflammation with intravital microscopy.



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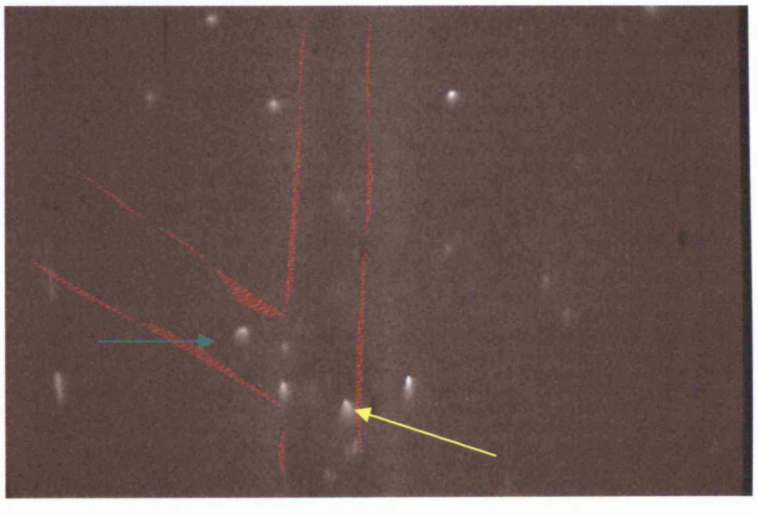

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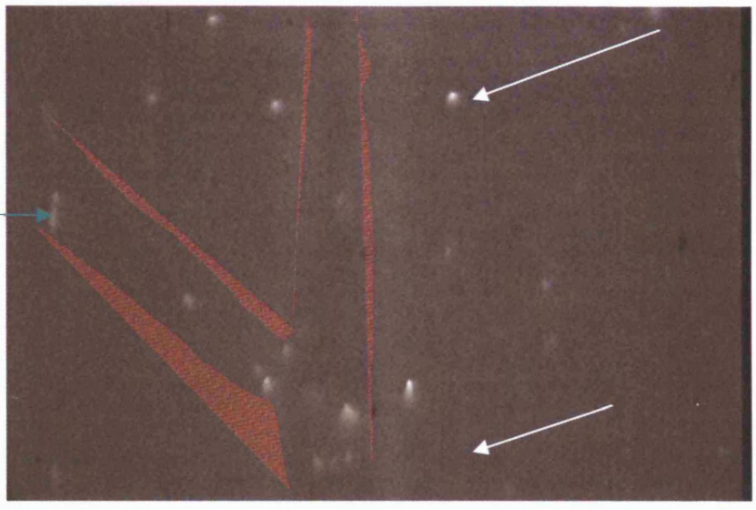

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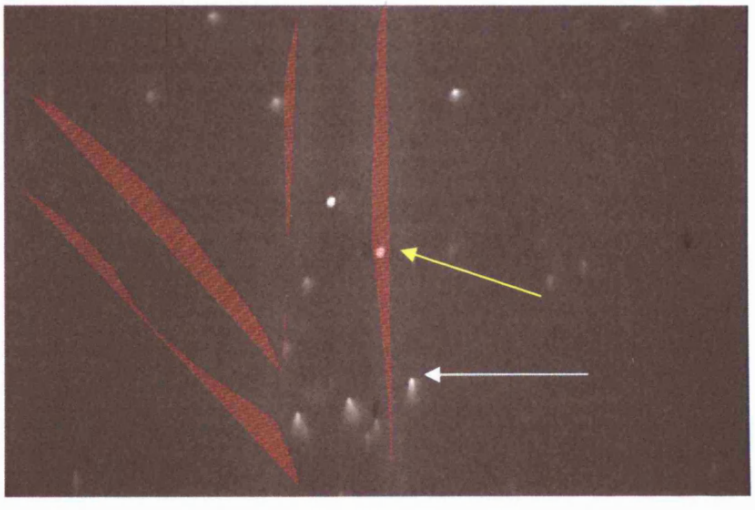


d

Figure 4.5. Steps in leucocytes activation. Still images from digital video sequences of LPS activated CAM microvessels 4 minutes following injection of Rhodamine 6G. The sequences were taken at 1 second intervals (figure a, b and c) showing Leucocyte rolling (green arrow) within the microvessel (outlined in red). d) Leucocyte extravasation (white arrows) outside microvessels and Leucocyte adherence (yellow arrow) were also observed following Rhodamine 6G staining. Under direct microscope vision, leucocytes have a deep red fluorescence.

4.6 Induction of CAM microvessel apoptosis

In order to investigate the effects of ABIN-2 on endothelial cell survival in vivo, a reliable method was needed to induce segmental microvessel apoptosis in Chick CAMs. The microvessel needed to remain patent once apoptosis was established in

order to allow for the uptake of the apoptotic markers as they circulate into the area of apoptosis.

Cessation of blood flow has been shown to result in microvessel regression and apoptosis of endothelial cells {A Woywodt & M Haubitz 2002 142 /id} .This approach was tested in the CAM. Areas of the CAM supplied by end arteries were chosen with the aim of disrupting the blood supply to these segments thereby producing apoptosis in the endothelial cells lining these microvessels. A number of methods were tested for stopping blood flow in CAM vessels.

Cauterisation of the feeding vessel to the segment of interest was successful in stopping the microcirculation, unfortunately despite multiple repeated attempts, the microcautery device also perforated the CAM surface leading to the sinking of the CAM below the surface of the chorionic fluid effectively, making further investigation impossible.

Eventually apoptosis was induced by temporary constriction of the end vessel using a 10/0 prolene suture under microscope magnification loosely tied around the end vessel and anchored to the outer shell membrane of the egg. Mild tension on the suture ensured vessel closure, tension on the suture was simply relaxed in order to regain patency. Transfixion sutures were applied for 24 and 36 hours. It was possible to inject the microvessel with Evans Blue ink up 36 hours post suturing showing that the vessel was still patent (figure 5.6). Following cessation of blood flow, detection of any vessel apoptosis was attempted using several methods described below.

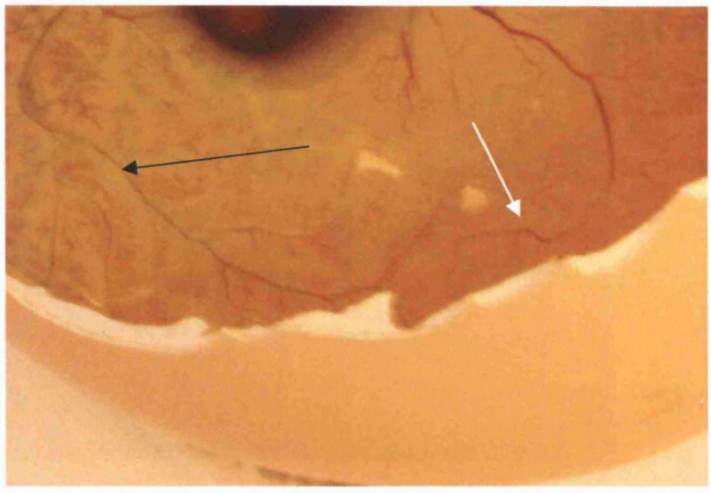
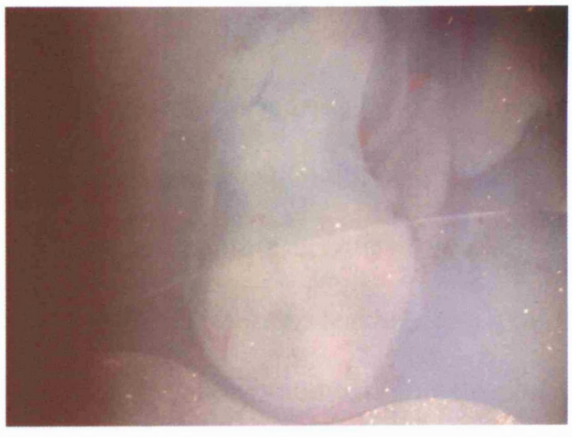


Figure 4.6. Evans blue injection of microvessel. Transfixion suture was removed following a period of 36 hours ligation, the vessel was injected with Evans blue dye showing the distal vessels to be still patent (black arrow). The non ligated vessel is also shown (white arrow).

4.7 Monitoring natural apoptotic processes in the CAM

It is also known that a natural process of microvessel apoptosis occurs during chick limb development between the web spaces resulting in digit development. Monitoring this natural apoptotic phenomenon was attempted for potential research on the apoptotic pathway. By injecting Evans blue dye directly into the CAM vessels, It was observed that apoptosis and differentiation of the vascular lakes in the chick limb buds occurred between day 4 and 5 with formation of distinct digits (figure 4.7)



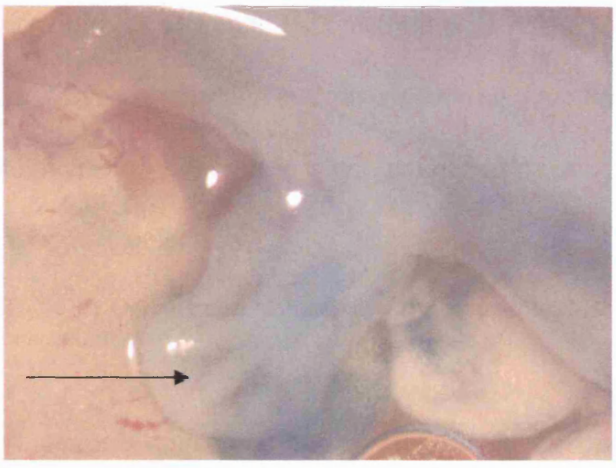


Figure 4.7. Natural endothelial apoptosis in the chick CAM limb. a) Day 3 chick limb injected with Evans blue dye showing vascular lakes. b) Day 4 chick limb showing specific differentiation and apoptosis of endothelial cells resulting in the formation of web spaces.

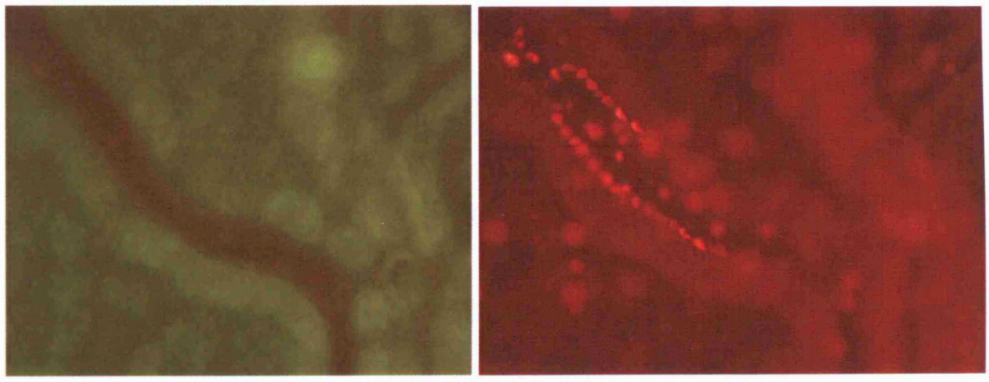
4.8 The use of Annexin V to examine chick CAM apoptosis

The use of Annexin V PE in detecting apoptotic endothelial cells within regressing microvascular lumens on the chick CAMs was investigated. Apoptotic cells express actively PS in the outer leaflet of the plasma membrane during execution of apoptosis. PS then functions as an "eat me" signal for phagocytes. Annexin V binds onto apoptotic cells displaying PS thereby playing a key role in apoptosis detection.
As the Annexin has never been used to detect apoptosis in a chick CAM, the concentration and volume of Annexin V injected directly into the CAM was adjusted in multiple experiments in order to achieve optimal fluorescence (Table 4.1). Injection of 5 µl of Annexin V at a concentration of 5mg/ml appeared to enhance apoptotic cells adequately. Increasing the concentration of Annexin V beyond 5mg/ml did not improve apoptotic cell outlining. No staining with Annexin V was apparent in the non apoptotic control sections of the CAM in the same chick, however it appeared that Annexin V was also leaking from gaps in the endothelium of apoptotic vessels into the surroundings and outlining the peri-vascular apoptotic cells. This made isolation of true apoptotic endothelial cells from a background of interstitial apoptosis

possible but difficult. This method is therefore suitable for detection of apoptosis in CAM vasculature but is time consuming. One further drawback to this method is the high cost of Annexin V which may limit its use as a commonly used method for apoptosis detection.

Table 4.1 Optimisation of Annexin V concentrations. Maximum fluorescence was achieved at a concentration of 5 mg/ml.

Annexin V concentration	Apoptotic cell detection
1mg/ml	No fluorescence
2mg/ml	+
3mg/ml	+
4mg/ml	++
5mg/ml	+++
10mg/ml	+++



a b

Figure 4.8. AnnexinV staining. a) Digital image of section of cam following induction of apoptosis by vessel ligation followed by AnnexinV staining. a) Section of CAM under 40X magnification with no filter. b) Same segment of CAM showing AnnexinV stained apoptotic endothelial cells (arrow) lining the microvessel.

4.9 The use of DAPI for detection of apoptotic endothelial cells.

DAPI was used in the previous chapter to detect apoptotic HUVE cells on culture plates. Due to the relative inefficiency and expense of using the Annexin V staining method, DAPI was tested as an alternative method for detection of endothelial cell apoptosis in live chick CAMs. A segment of vessel on the chick CAM was ligated for a period of 24 hours in order to induce apoptosis as described in section 4.6. DAPI was then injected into the apoptosing segment of vessel as well as non apoptosing vessels. DAPI injection caused extensive denaturation of the CAM surface upon injection which limited its usefulness to outline the underlying endothelial cells under the microscope. Modification of the protocols used in section 2.4.2 on HUVE cells was needed in order to limit denaturation of the Chick CAM and to ensure adequate fluorescence of DAPI stained nuclei. DAPI staining was capable of outlining nuclear changes in apoptosing endothelia however it was not specific to the endothelial cells. Leaking of the DAPI solution from the vascular puncture site following injection resulted in staining of part of the CAM surface making correct calculation of endothelial apoptotic rates difficult. Results therefore were not of sufficient clarity to use this method reliably.

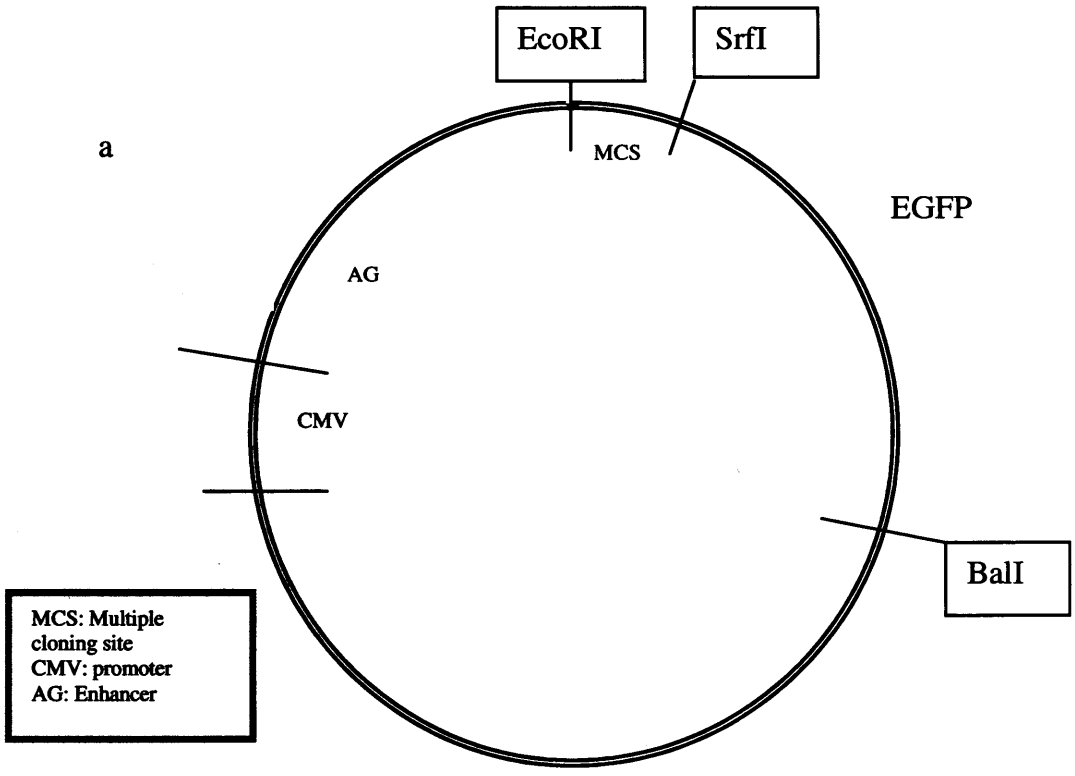
4.10 Sub Cloning into GFP/IRES vector

The next step was to identify a plasmid capable of transfecting the CAM. An attempt was made to produce a plasmid co-expressing GFP and the protein of choice (ABIN-2 or A20). This plasmid could then be used to transfect the exceptionally delicate chick CAM vessels in one procedure. A pIRES2-EGFP plasmid containing an MCS-IRES_EGFP sequence which is an internal ribosome entry site next to a multiple

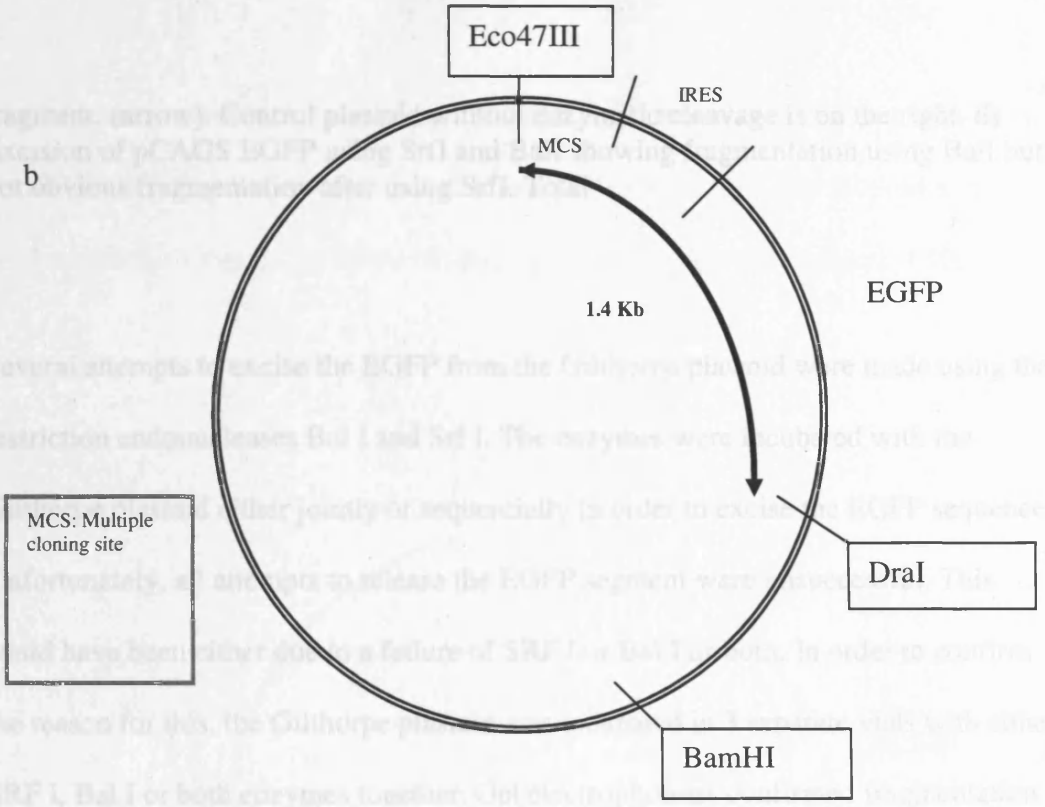
cloning site was chosen which would then be inserted into a pCAGS EGFP plasmid near the promoter and enhancer site after removing the EGFP from the pCAGS EGFP (Gilthorpe plasmid figure 4.9) to produce the desired plasmid. The IRES2-EGFP would be inserted into the pCAGS EGFP vector as it has proven to be a better promoter in the chick system {Yaneza M, Gilthorpe JD, et al. 2002 143 /id}

Blunt ended MCS-IRES_EGFP was then successfully removed from p-IRES2-EGFP by using the enzyme Eco47III initially followed by further cutting using DRAI. Agarose gel electrophoresis confirmed the presence of the expected 1.4 Kb (Figure 4.9)

a



pCAGS EGFP



pIRES2EGFP

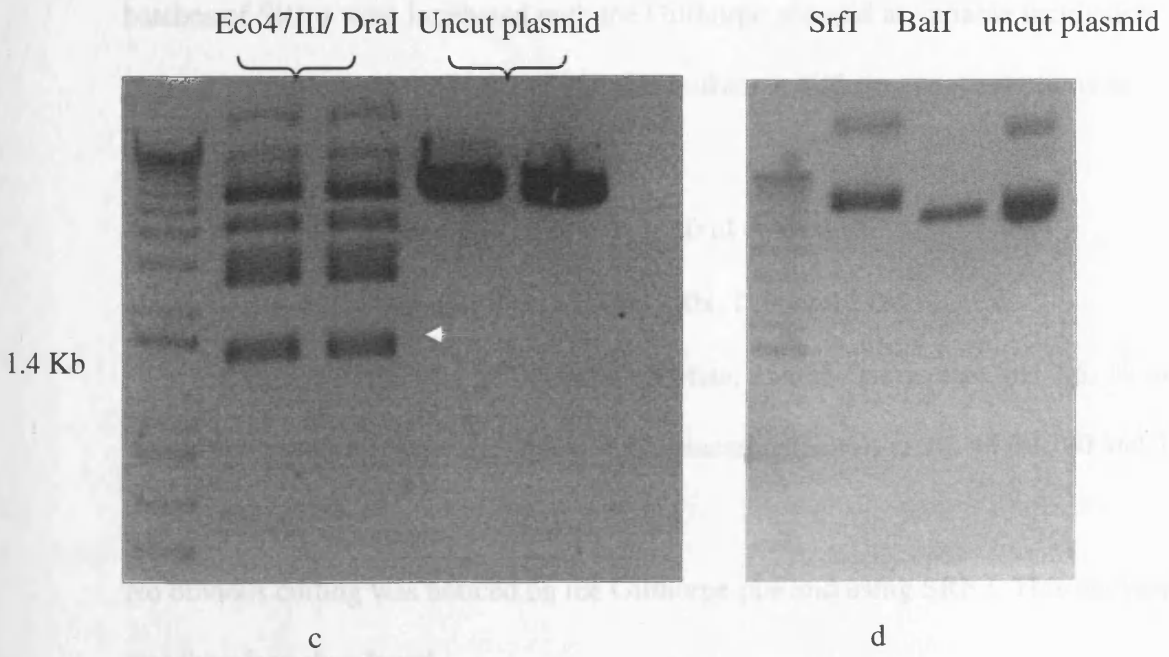


Figure 4.9. Subcloning into GFP-IRES vector. a) pCAGS EGFP plasmid showing sites of cleavage using SrfI and Bal I to excise the EGFP segment b) Excision of 1.4 Kb fragment containing the internal ribosome entry site (IRES) from the pIRES2-EGFP plasmid. c) Gel electrophoresis confirming cleavage of pIRES2-EGFP using Eco47III and DraI with the production of 1.4 Kb

fragment. (arrow). Control plasmid without enzymatic cleavage is on the right. d) Excision of pCAGS EGFP using SrfI and BalI showing fragmentation using BalI but not obvious fragmentation after using SrfI. Total

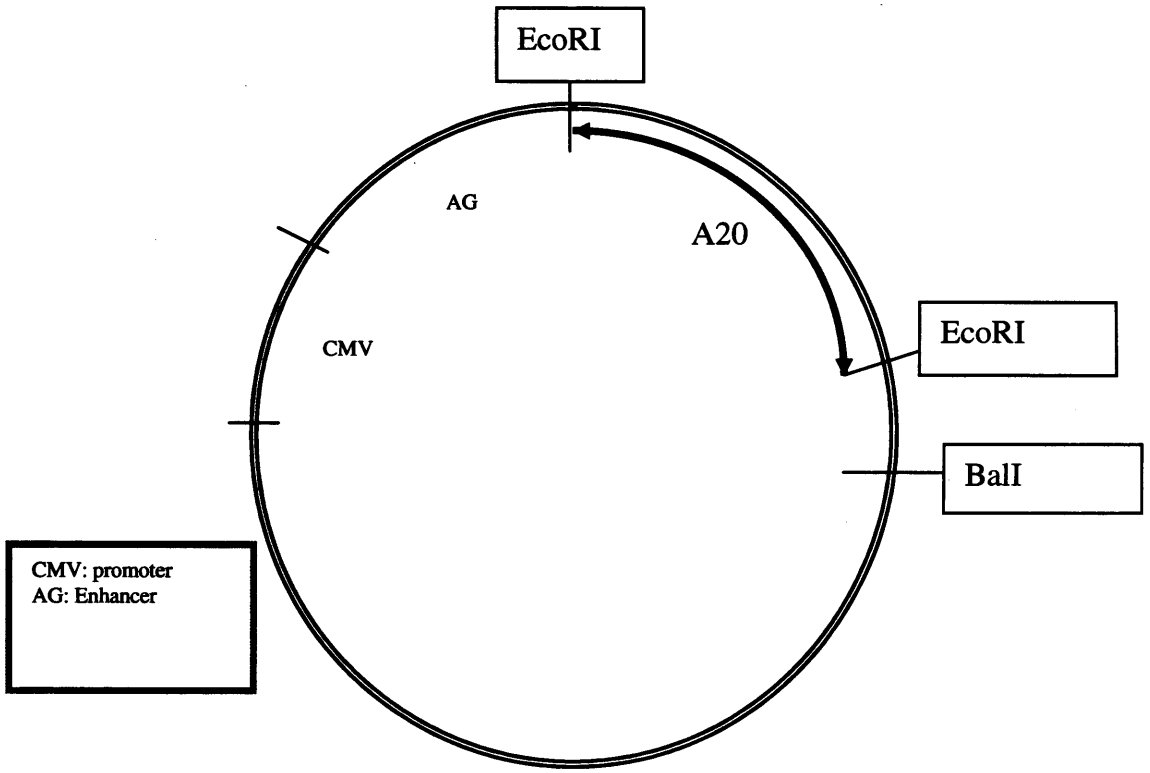
Several attempts to excise the EGFP from the Gilthorpe plasmid were made using the restriction endonucleases Bal I and Srf I. The enzymes were incubated with the Gilthorpe plasmid either jointly or sequentially in order to excise the EGFP sequence. Unfortunately, all attempts to release the EGFP segment were unsuccessful. This could have been either due to a failure of SRF I or Bal I or both. In order to confirm the reason for this, the Gilthorpe plasmid was incubated in 3 separate vials with either SRF I, Bal I or both enzymes together. Gel electrophoresis confirmed fragmentation of the Gilthorpe plasmid using Bal I but no change using SRF I (figure 4.9).

In order to rule out insufficient incubation times as a cause for this failure, different batches of SRF I were incubated with the Gilthorpe plasmid at variable incubation periods, variable temperatures and variable buffers at differing concentrations as shown below:

- Enzyme volumes 0.5, 1, 1.5, 2.0 µl/ 10 µl of mixture.
- Universal buffer dilutions: of 0.5x, 1.0x, 1.5x and 2.0x.
- Reaction buffer (0.1 M potassium acetate, 25mM Tris acetate, pH 7.6, 10 mM. magnesium acetate and 0.5 mM 2-metacarpoethanol) at 30, 45 60 , 90 and 120 minutes.

No obvious cutting was noticed on the Gilthorpe plasmid using SRF I. This enzyme was therefore abandoned.

An Alternative strategy was used attempting to ligate an IRES EGFP fragment from the IRES2-EGFP plasmid onto a fragment of pCAGGSEhA20 which contains a promoter and enhancer site similar to that of the Gilthorpe vector (figure 4.10).



PCAGGSEhA20

Figure 4.10. PCAGGSEhA20 plasmid, showing restriction endonuclease sites EcoRI and BalI.

Initially restriction endonucleases Eco47III and Dra I were used to precipitate the EGFP fragment on pIRES2EGFP .The enzymes were incubated simultaneously at a temperature of 37 °C. The digest was successful with the production of the desired 1.4 kb fragment described earlier.

EcoR1 and BalI were used to cut the plasmid pCAGGSEhA20 at the sites shown in figure 4.10. BalI (1 µl) was incubated at first I with 1 µl of DNA at 37 °C for 1 hour followed by the addition of EcoRI 1 µl for a further hour. Bal I was incubated first as

its cleavage site was very close to the EcoR I site. Gel electrophoresis confirmed the presence of the desired 4.8 kb fragment containing the CMV (promoter) and AG (Enhancer site). (figure 4.11)

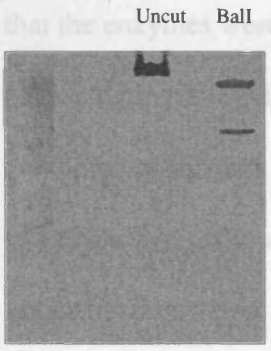


Figure 4.11. Gel elocrophoresis plate showing restriction endonuclease cleavage of the PCAGGSEhA20 plasmid using BalI producing the desired 4.8 Kb fragment.

4.11 Insertion of 1.4 kb EGFP fragment into pCAGGSEhA20 digest

The 1.4 kb segment of interest from the pIRES2EGFP was extracted from the gel in section 4.8.3 using the Q1A quick gel extraction Kit as described in section 2.2.7.

A similar method was used to extract the 4.8 kb fragment from pCAGGSEhA20 plasmid.

Ligation of the pCAGGSEhA20 vector and the 1.4 kb pIRES2EGFP insert was attempted using the standard formula as described in section 2.2.11.

After leaving the ligation mixture overnight at 16 °C, the resulting plasmid was used to transform supercompetent XL1 Ecoli cells as described in section 2.2.12.

After a 24 hour period of incubation no colonies were visible on the LB plate.

The experiment was repeated several times with the same results. No obvious reason for the failure to transform Ecoli cells was discovered.

In order to test if the 1.4 kb fragment was incorporated into the vector, digestion of the ligation mixture with the endonuclease Xba 1 was attempted in 10 experiments using variable concentrations of enzyme. No cleavage products were detected.
More detailed analysis of Bal I and EcoRI cleavage of pCAGGSEhA20 confirmed that the enzymes were cleaving at the expected sites producing 2 fragments 2.5 kb and 4.8 kb in length respectively. The efficiency of the ligation process was then tested by attempted re-ligation using a rapid ligation protocol as described in section 2.2.11.
Gel electrophoresis confirmed that re-ligation did not occur. A similar result was also obtained when using a different slow ligation protocol as described in section 2.2.11.
Again no growth of the plasmid was observed on the LB media plate.
After many attempts, construction of a PCAGS-IRE- EGFP plasmid was abandoned.

4.12 Transfection of chick vessels using electroporation

Previous work has shown that it is possible to transfect cells into the neural tube of the Chick by electroporation {Yaneza M, Gilthorpe JD, et al. 2002 143 /id}.The possibility of transfecting chick CAM vessels with GFP or GFP with a vector of choice using electroporation was therefore assessed. As similar methods had never been studied before, plasmid introduction into the prepared CAM vasculature was tested initially by simply applying equal volumes of the target plasmid at a concentration of 1,2, 6, 8 or 10 μg/μl directly onto the surface of each CAM. Electroporation was then carried out at a different setting as described in section 2.5.9 to each CAM with variation in electroporation space, width, voltage, number of pulses and electrode distance from vessels (Table4.2). Following an incubation period on 6, 12, 18, 24 and 48 hours the chick CAM were analysed under fluorescence

microscopy to check for GFP fluorescence signalling successful transfection. No transfection was noted under any of the above conditions.

Table 4.2. Electroporator settings. Variations in voltage, pulse numbers, pulse width and pulse space were tested to achieve microvessel GFP transfection in the CAM. all attempts proved unsuccessful at achieving transfection. Egg death occurred above 14 volts or pulse width above 30 ms.

volts	pulses	Width	Space	Transfection
1-15 increment of 1 volt	1-5 increments of 1	5 ms- 50 ms increments of 5 ms	5ms 50 ms increments of 5 ms	0%

Transfection was then attempted by direct intravascular injection of the target GFP containing plasmid at a concentration of 1, 2, 6, 8 or 10 μg/ μl using variable Electroporator settings as before on each egg. No transfection was noted despite extensive variation in transfection conditions such as addition of ice cold PBS, injection of plasmid at temperatures below 4 °C to reduce the blood flow rates in the CAM or ligation of the target vessel with 9/0 ethilon sutures to allow the DNA more contact time with the endothelial cells prior to electroporation.

The same GFP used for transfection of HUVE cells was used in attempting to transfecting the chick CAM. It was therefore decided to analyse a retroviral GFP plasmid (donated by D Hughes. Leicester). A similar protocol as above was used with target plasmid application either directly on the CAM surface or by intravascular injection at the vessel of choice. CAM vessels were analysed as before at 6, 12, 18, 24 and 48 hours to check for GFP fluorescence.

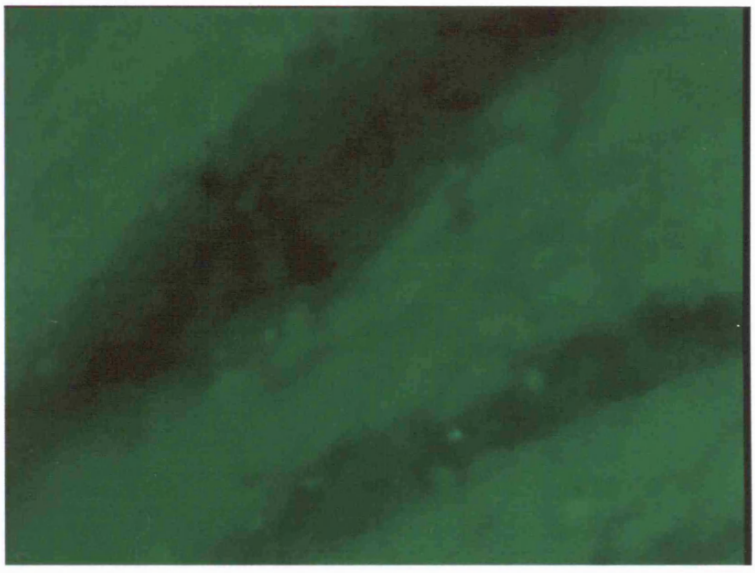
After 12 hours incubation chick CAMs which were injected directly with intravascular plasmid contained exceptionally sparse but viable green fluorescent cells indication successful transfection in these endothelial cells.

4.13 Establishing optimal chick transfection protocols

Transfection efficiency was improved in the chick CAM vasculature by extensive investigation into optimal chick CAM age, electroporator settings, electrode shape, electrode distance and orientation from the target vessel as well as other parameters to enhance the plasmid delivery to the target vessel. Eventually it was possible to achieve transfection of target vessels with plasmid with transfection rates varying between 80 to 95%. The GFP expressing endothelial cells lining the vessel lumen were clearly identified by their characteristic shape and size. The optimum values are list below (table 4.3).

Table 4.3. Electroporator settings used to optimise transfection rates in the Chick CAM.

voltage	pulse	Width	Space	Electrode distance	Ice Cooling	Transfection Maximum %
10	3	30 ms	10 ms	3 mm	yes	95



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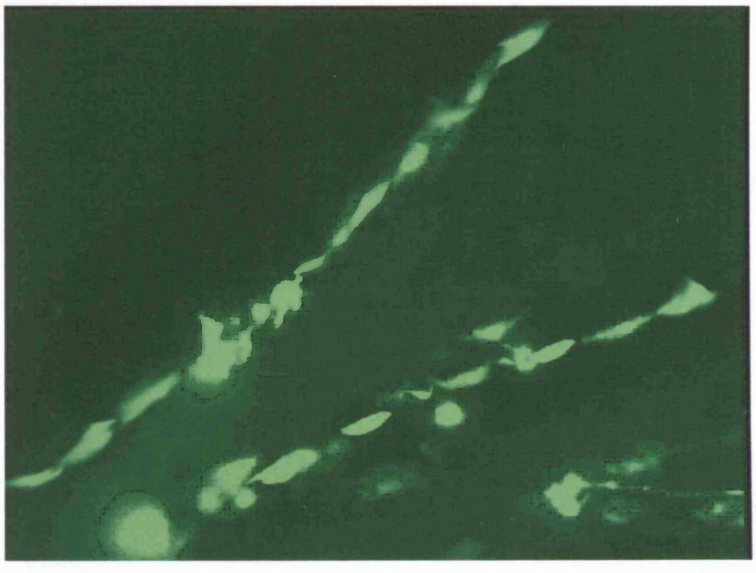

b

Figure 4.12. GFP transfection of CAM vessels. a) GFP Transfected patent vessels 40X magnification no filter. b) Same GFP transfected vessels using FITC lamp showing GFP green fluorescence in endothelial cells. Blood flow within the microvessel was obvious in real time.

4.14 Co-localisation of Rhodamine labelled leucocyte and GFP expressing vessels

In order to confirm that chick CAMs expression of GFP (along with the protein of interest) will not interfere with Rhodamine 6G outlining of leucocytes, CAM were

transfected with GFP plasmid as described in section 4.10, chick CAMs were incubated for 24 hours then injected with Rhodamine 6 G as in section 4.5. Leucocytes were visible along the entire course of the GFP expressing vessels. Furthermore, no significant differences in the leucocyte rolling rates along GFP expressing vessels and control vessels not expressing target transfected vectors

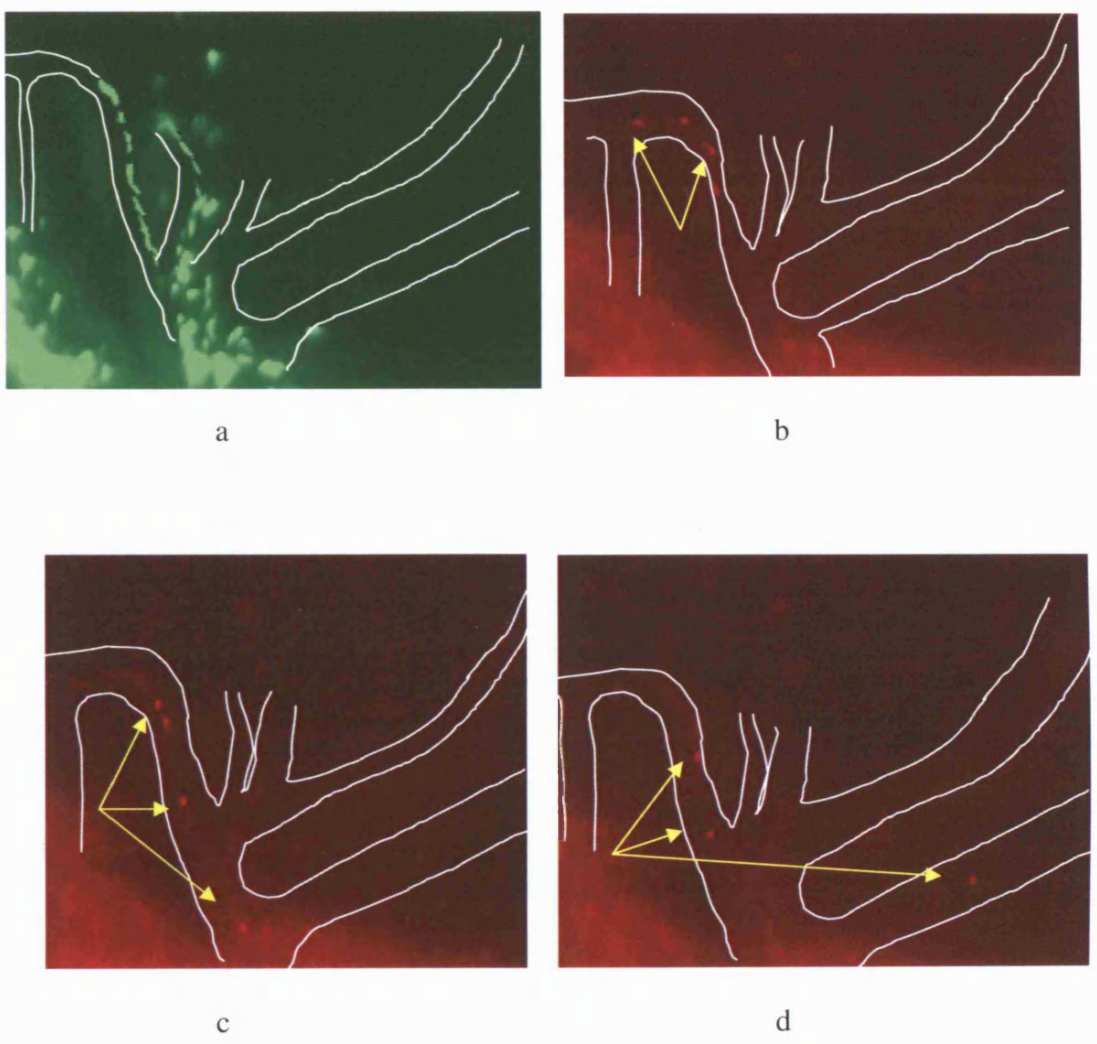

Figure 4.13. Leucocyte rolling in GFP transfected vessels. a) GFP transfected endothelial cells along section of CAM microvessels (Vessel course enhanced using white line). b-d) Still images from digital video showing course of Rhodamine 6G labelled leucocytes (arrows) along same section of microvessels at 1 second intervals at 40X magnification.

Discussion

There are three widely used assays for studying angiogenesis. The rabbit corneal micropocket, subcutaneous implants and the CAM. The avian chorioallantoic membrane is the outermost extraembryonic membrane lining the non-cellular eggshell membrane. Much research has been published documenting the role of the Chick CAM in studying angiogenesis as it is so accessible. The aim of this study was to attempt to establish a model for in vivo expression of ABIN-2, detection of apoptosis using this model and to investigate leucocyte dynamics in the transfected vessels of the CAM.

Up to now investigation of the CAM involved manipulation of the vasculature only from the surface. This is the only report of manipulation of microvessels directly by intravascular injection. The difficulty of this technique was not in injection of the vessels but in preventing excessive haemorrhage from the puncture site leading to CAM death. Once this problem was solved by using appropriate angulated microneedles a reliable method was established for introducing substances of choice into the vessels. Excellent transfection rates in the microvascular endothelia were eventually made possible by using the appropriate Gilthorpe GFP vector. GFP expressing endothelial cells were identified along the vessel lumen by their characteristic shape, size and site in the intima. Interestingly other cell types either in the media or the adventitia did not seem to express GFP.

As mentioned previously, one of the key components to the inflammatory response involves leucocyte transmigration through endothelium and accumulation at the site of injury. The leucocyte adhesion cascade is a sequence of adhesion and activation events that ends with extravasation of the leucocyte.

Using intravital microscopy and Rhodamine 6 G to assess microvessel inflammation on the CAM surface as well as successful transfection specifically of intravascular endothelial cells with target plasmid are 2 new techniques developed during the course of this project in order to first study leucocyte dynamics and secondly to assess whether transfection of the CAM vessels with ABIN-2 was possible. The work carried out so far demonstrated that these methods can be used for this purpose. The methods established may then be used in further to investigate the role of ABIN-2 in vivo using the Chick CAM generally in the continuous studying of drug and DNA delivery in vivo efficiently in a cost effective manner and specifically in later research on microvessel inflammation, apoptosis or regression.

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Chapter 5

ABIN-2 has an anti-inflammatory effect on CAM microvessels

Chapter 5

ABIN-2 has an anti-inflammatory effect on CAM microvessels

Introduction

The chick CAM mainly mediates gaseous exchange with the extraembryonic environment until hatching, it is well perfused with a thick capillary network that forms a continuous surface which is easily accessible. In the previous Chapter, the value of the chick CAM as method for studying inflammation and apoptosis was assessed. It was shown that this model could be used to assess apoptosis and inflammation in sections of the living CAM. Transfection of target plasmids was also demonstrated for the first time on the CAM microvessels.

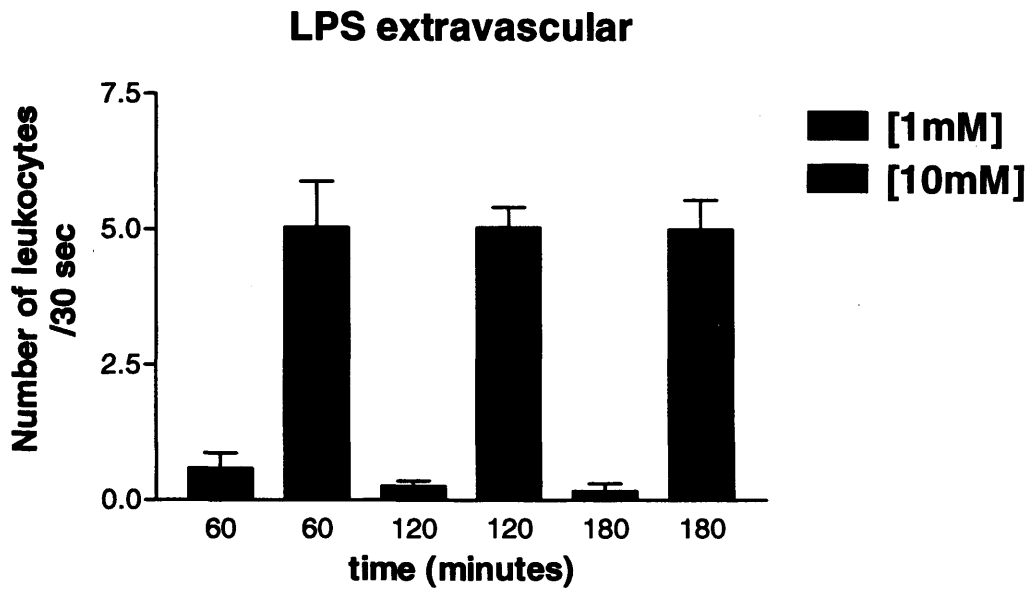
Apoptosis and inflammations have been studied extensively *in vitro* however the number of *in vivo* studies has been limited by comparison, possibly due to the difficulty in achieving continuous real time analysis of the effects of various apoptotic and inflammatory mediators. The work in this chapter therefore aims to examine the process of inflammation and apoptosis in vivo using the chick CAM. In addition, as it has been shown that expression of ABIN-2 and A20 reduce apoptosis in HUVE cells, this method was used to assess the effects of expressing these proteins *in vivo.*

5.1 Concentration course of LPS induced inflammation of CAM microvessels

The effects of Lipopolysaccharide (LPS) on the chick CAM vasculature was assessed. LPS is a potent inflammatory agent, which is known to increase leucocyte rolling, adhesion and extravasation on the endothelia. 5 µl of 1, 10, 100 mM concentration of

LPS were applied to a marked area of the chick CAM either topically directly over the microvessels or by intravascular injection.

Leucocyte rolling number was defined as the number of slowly moving leucocytes relative to normal leucocytes identified in a marked 5mm segment of CAM vessel within 2 x 30 second periods. Leucocyte adhesion was defined as the number of stationary leucocytes identified in a 10 μm section of CAM within 2x 30 second periods. Leucocyte extravasation was defined more generally as the number of extravasated leucocytes observed within 1 cm of the marked vessel. Direct measurement as well as digital video recording of the number of leucocyte rolling, adherence and extravasation was carried out after 60, 120 and 180 minutes of exposure to LPS. The CAMs were replaced into the incubator at the end of each period as it was not possible to monitor the chick CAM using intravital microscopy for the full 3 hour period. Data for leucocyte extravasation and adherence did not prove useful as the results were not reproducible. Only leucocyte rolling rates seemed to be reproducible. Figure 5.1 shows that no significant difference was observed between topical and intravascular application of LPS to induce inflammation. As expected increasing the LPS concentration increased endothelial activation resulting in increased leucocyte rolling numbers detected. This effect was maximal at a concentration of 10 mM. At 100 mM LPS no leucocyte rolling was detected at any of the time points due to severe reduction in blood flow or frank thrombosis within the microvessels. This was assumed to be due to the severe inflammatory response propagated by the high LPS concentration.



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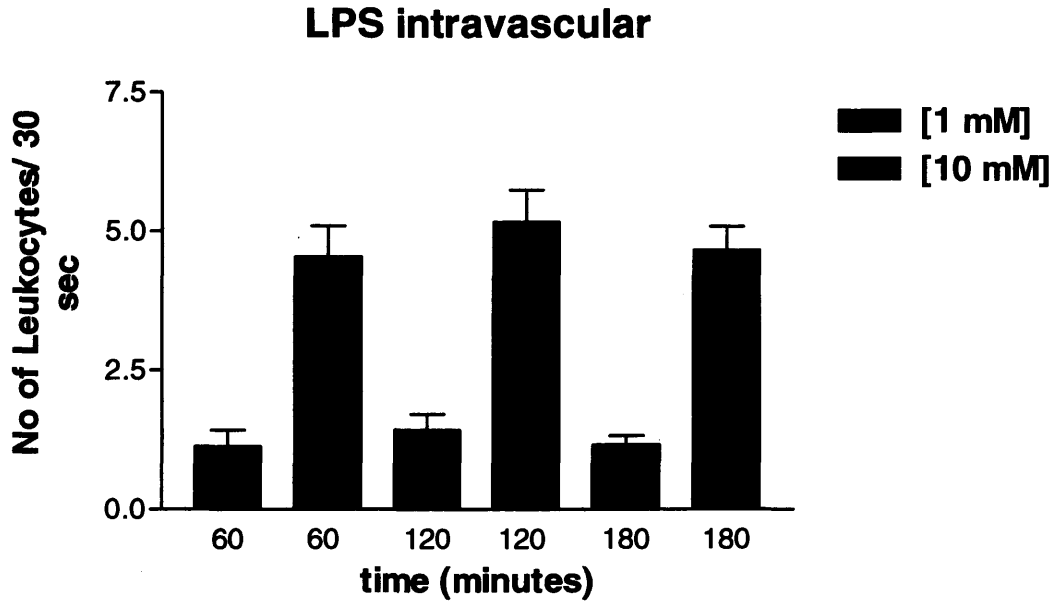

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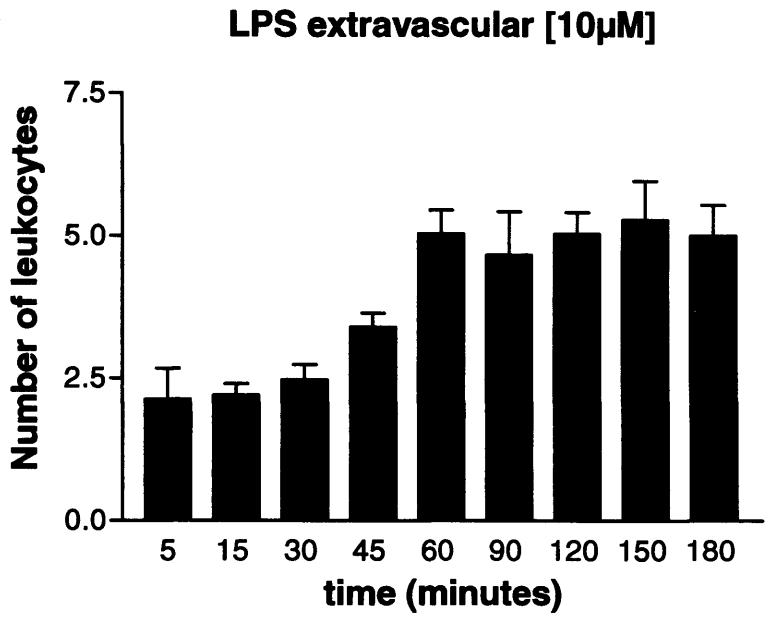
Fig 5.1: The effect of LPS concentration on leucocyte rolling. a) 10 mM extravascular LPS application significantly increases microvascular endothelial activation compared to 1 mM concentration of LPS. Data is presented as mean and SEM for at least 3 independent experiments, $P < 0.0001$ vs 1 mM. b) Similar differences between 1 mM and 10 mM concentrations are observed after induction of inflammation using intravascular LPS. At 1 mM LPS seems to induce more endothelial inflammation when injected directly into the microvessels rather than extravascular topical application. Data is presented as mean and SEM for at least 3 independent experiments, $P < 0.0001$ vs 1 mM

5.2 Time course of LPS induced leucocyte rolling of CAM microvessels

The effectiveness of Lipopolysaccharide (LPS) at induction of leucocyte rolling in microvessels was assessed at additional time points in order to detect the optimal endothelial activation time. 10 mM LPS was either injected or added topically on the surface of a marked area of the CAM using intravital microscopy as outlined in section 4. Measurement of leucocyte adhesion, rolling and extravasation were taken for 30 seconds every 15 minutes for a period of 3 hours. The effect of LPS 100 mM application on endothelial activation was also assessed in the same way every 15 minutes for a period of 1 hour. The chick CAMs were replaced into the incubator after each measurement to ensure adequate hydration and survival of the CAM. These data are shown in figure 5.2. Extravasation of leucocytes was difficult to assess as marker of inflammation in the chick CAM and was therefore not assessed in future LPS induced inflammation studies.

LPS induction of inflammation was not immediate. Maximal LPS action on leucocyte rolling occurring after 60 minutes of LPS application (figure 5.1). This time point was therefore used as a standard reference point in future CAM inflammation studies.



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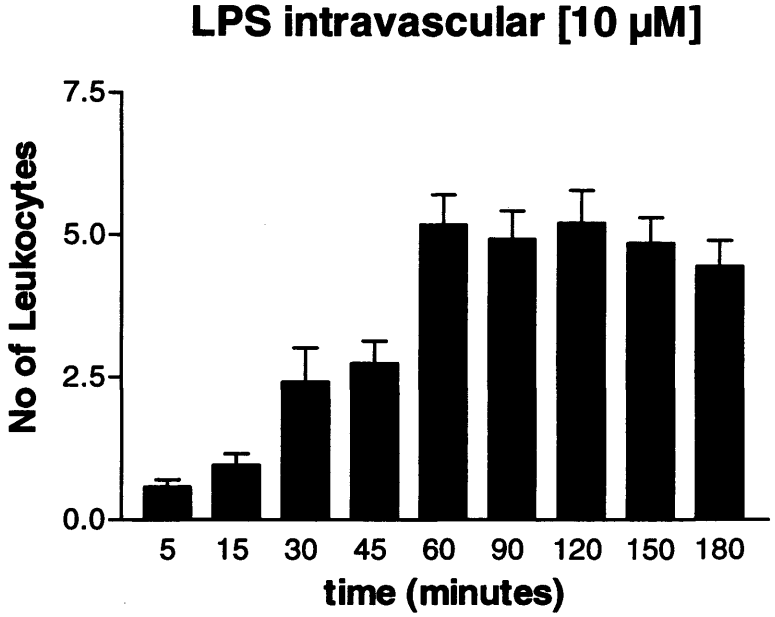

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Graph 5.2. a) The effect of extravascular LPS 10 μM on leucocyte rolling on CAM microvessels over the course of 3 hours. 2 measurements of leucocyte rolling were taken for each time interval in a 10 μm section of CAM vessel over a 30 second time period. Maximal leucocyte rolling was detected after 60 minutes of LPS activation. No significant difference in leucocyte rolling numbers was detected after 60, 90, 120 or 150 minutes of LPS addition. Data is presented as mean and SEM for at least 3 separate experiments with P> 0.05. b) Intravascular effects of LPS 10 μM application on leucocyte rolling. No significant difference in leucocyte rolling numbers was detected after application of LPS after 60, 90, 120 and 150 minutes. Data is presented as mean and SEM for at least 3 separate experiments with P> 0.05.. In addition no significant difference was detected between intravascular and extravascular LPS application on CAM leucocyte rolling numbers (data not shown).

5.3 Concentration dependent TNF-α induced inflammation of CAM microvessels

The effects of TNF-α in microvessel inflammation was assessed as in section 5.1. TNF-α was applied either topically or via intravascular injection to induce inflammation in the CAM microvessel at a concentration of 0.25, 2.5, 5.0, 25 or 100 ng/ml as outlined in section 5.1. Maximal induction of leucocyte rolling occurred at a concentration of 2.5, 5 and 25 ng/ml (fig 5.3). At a concentration of 25 ng/ml, TNF-α did not induce significant microvessel endothelial cell death. This was encountered however after addition of TNF-α at a concentration of 100 ng/ml. As observed with LPS no significant difference in inflammatory activation was detected between intravascular or extra vascular TNF-α application (data not shown).

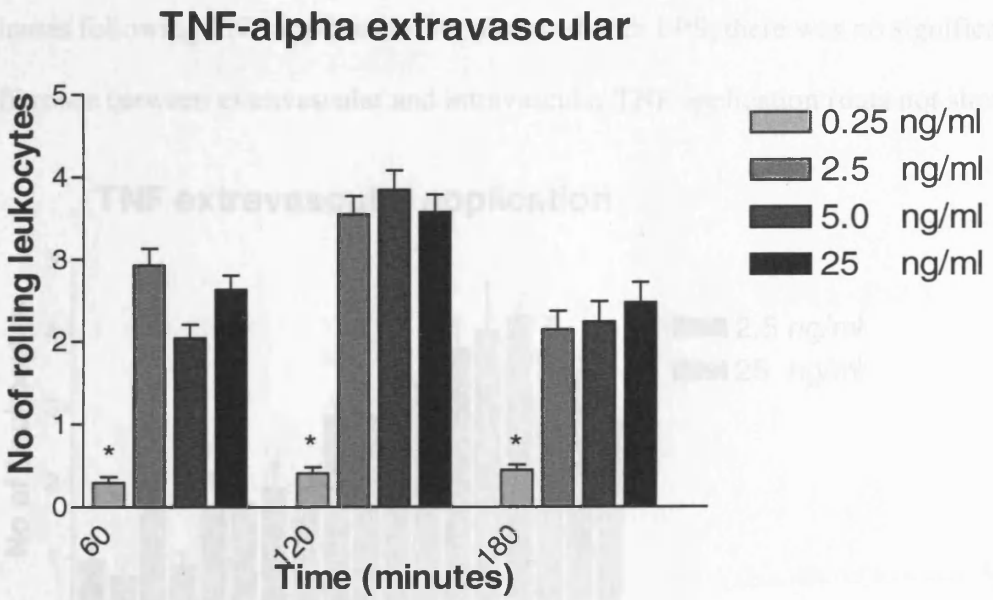


Figure 5.3. Concentration dependence of TNF-α induced inflammation of CAM microvessels. Minimal activation of leucocytes occurred with TNF-α 0.25 ng/ml after topical application for 60, 120 and 80 minutes. Significant increase in leucocyte rolling was observed with TNF-α 2.5, 5 and 25 ng/ml. Data is presented as mean and SEM for at least 3 independent experiments, $P < 0.0001$ vs 0.25 ng/ml (*). The optimal effect was observed between 120 and 180 minutes. No Data for [TNF-α] 100 ng/ml as vessel thrombosis occurred within 60 minutes of topical application.

5.4 Time course of TNF-α induced inflammation of CAM microvessels

Since TNF-α concentrations of 2.5, 5 and 25 ng/ml appeared to activate endothelial cells equally as observed in section 5.3. The time course in which TNF-α at a concentration of 2.5 and 25 ng/ml induced chick CAM inflammation was evaluated. TNF-α was applied either topically onto the surface of the chick CAM or by intravascular injection. Direct measurements of leucocyte rolling numbers were taken for 30 seconds every 15 minutes over a period of 3 hours as described in section 5.1. All images were stored digitally for future analysis and confirmation of values. As shown in figure 5.4, no difference was detected in leucocyte rolling numbers between concentrations of 2.5 and 25 ng/ml of TNF-α at any of the time points. At both concentration values, maximal endothelial activation occurred between 120 and 150 minutes following TNF application. As observed with LPS, there was no significant difference between extravascular and intravascular TNF application (data not shown).

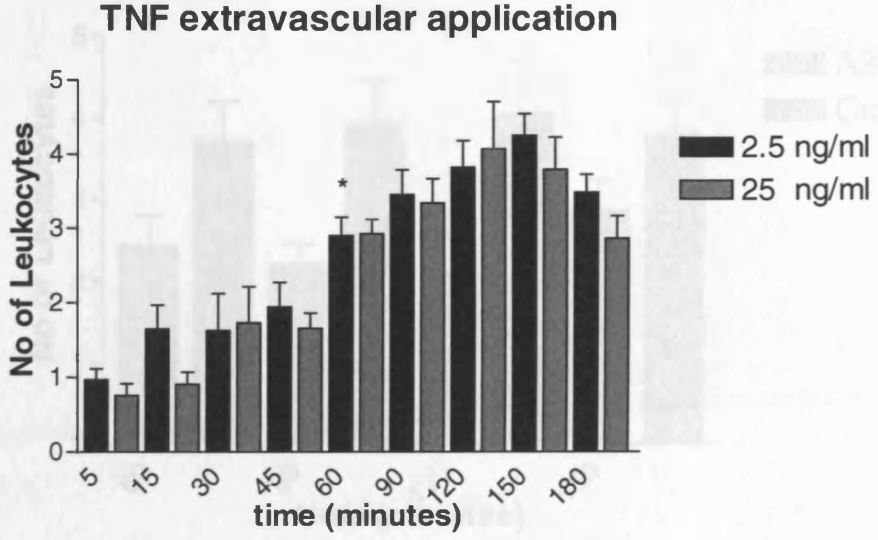


Figure 5.4. Time course of TNF induced inflammation of CAM microvessels. [TNF-α] induced maximal leucocyte rolling after 120 to 150 minutes following topical application. No significant difference was detected between TNF-α of 2.5 and 25 ng/ml. Data presented as mean +/- SD for 3 independent experiments, $P < 0.0001$ 25 ng/ml vs 2.5 ng/ml (*).

5.5 Transfection of A20 reduces LPS induced inflammation in CAM vessels

A20 was shown to reduce HUVE cell apoptosis and inflammation in chapter 3. Its role in reducing endothelial cell inflammation as determined by leucocyte rolling in the chick CAM was evaluated. A20 and GFP or GFP and empty vector were transfected into chick CAM microvessels using electroporation as described in section 4.10. Twenty four hours following transfection, LPS [10 mM] (fig 5.5) was applied topically. Measurements of rolling leucocyte numbers were taken in CAMs at 60, 90,120 and 150 respectively to measure endothelial activation. A20 was shown to reduce CAM endothelial activation at all time points when compared to control vector, showing that A20 appears to have a role in the control of microvascular inflammation in the chick CAM.

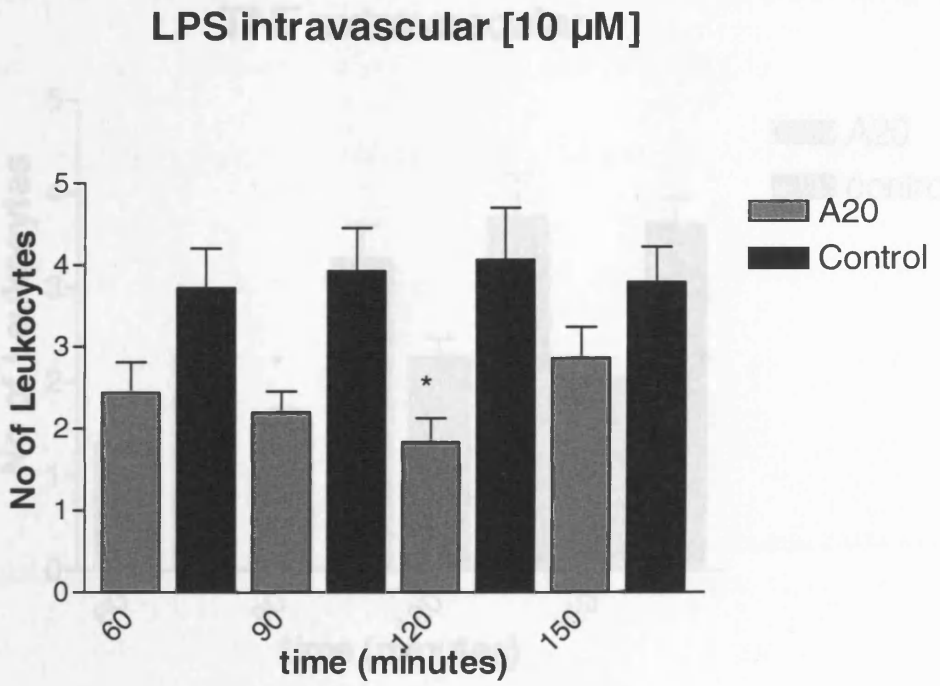


Fig 5.5. The effect of A20 expression on endothelial cell activation in the CAM. Expression of A20 in the CAM vessels significantly reduces leucocyte rolling numbers compared to empty control vector expression after application of LPS [10 mM]. It was observed that A20 expression significantly reduces

leucocyte rolling between 60 and 150 minutes. Data is presented as mean and SEM for at least 3 independent experiments, $P < 0.005$ vs A20 (*).

5.6 Transfection of A20 reduces TNF-α inflammation in CAM vessels

The effects of TNF-α induced inflammation on A20 transfected chick CAM was assessed. TNF-α at a concentration of 2.5 ng/ml was applied topically onto CAM microvessels 24 hours after transfection with either GFP and A20 or GFP and control vector. Leucocyte rolling was measured at 60, 90, 120 and 150 minutes (fig 5.6). Leucocyte rolling was reduced in A20 expressing CAM microvessels compared with controls, providing further evidence of the important role of A20 in reduction of inflammation *in vivo*.

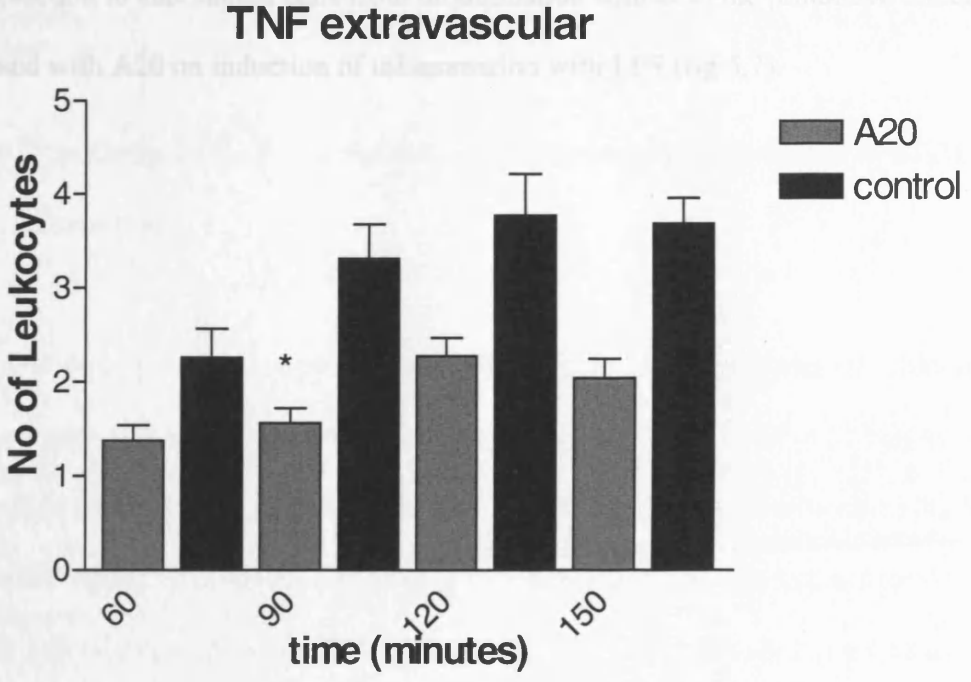


Fig 5.6. The effect of A20 expression on endothelial cell activation in the CAM. Expression of A20 in the CAM vessels significantly reduces leucocyte rolling numbers compared to empty control vector expression after TNF [2.5 ng/ml] extravascular application. Data is presented as mean and SEM for at least 3 independent experiments, $P < 0.002$ vs A20 (*).

5.7 Transfection of ABIN-2 reduces LPS induced inflammation in CAM Microvessels

ABIN-2 was originally identified as a potential mediator of the effects of A20 on NFκB activity. The effect of ABIN-2 expression on CAM endothelial cell inflammation was therefore analysed. Previously in section 3.5 it was shown that ABIN-2 expression in HUVE cells significantly reduces apoptosis and improves cell survival. ABIN-2 was transfected using the protocol established in section 4.10 along with GFP into CAM microvessels. GFP as well as empty vector were transfected in the control CAMs. Endothelial cells were activated using LPS [10 μM] applied topically. Measurement of rolling leucocytes numbers was taken after 60, 90, 120 and 150 minutes following LPS application. For each time point at least 3 separate CAMs were used to measure leucocyte numbers. ABIN-2 was found to significantly confer protection to endothelial cells from inflammation similar to the protective effects noted with A20 on induction of inflammation with LPS (fig 5.7).

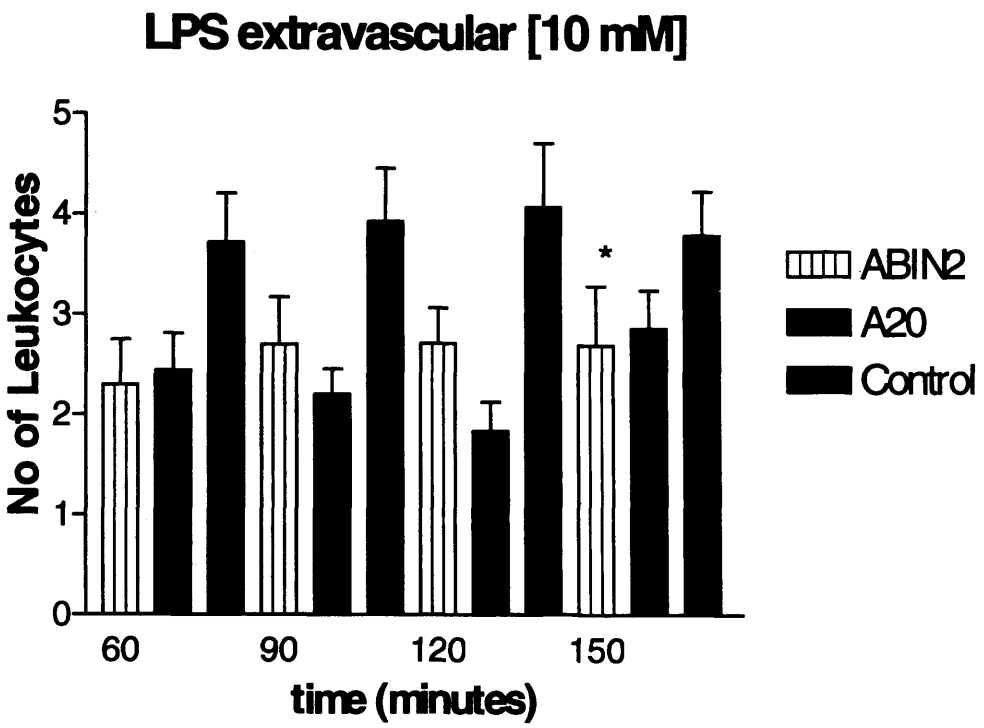


Figure 5.7. Transfection of ABIN-2 reduces LPS induced inflammation in CAM microvessels. A reduction in leucocyte rolling numbers was observed in ABIN-2 and A20 expressing CAM microvessels after 60, 90, 120 and 150 minutes of topical application of [LPS] 10 mM. Data is presented as mean and SEM for at least 3 independent experiments, $P < 0.005$ vs ABIN-2 (*).

5.8 Transfection of ABIN-2 reduces TNF-α induced inflammation in CAM microvessels

The effects of ABIN-2 expression on TNF-α induced CAM endothelial inflammation were studied in a similar fashion as described in section 5.7. TNF-α [2.5ng/ml] was applied topically to segments of CAM microvessels transfected with either ABIN-2 or control vector. Measurement of rolling leucocytes numbers was taken after 90, 120 and 150 minutes following TNF-α application (fig 5.8). The data suggests that ABIN-2 reduces the numbers of rolling leucocytes in the chick CAM following TNF-α induced inflammation as it does in LPS induced inflammation however, optimal inhibition was observed between 120 and 150 minutes.

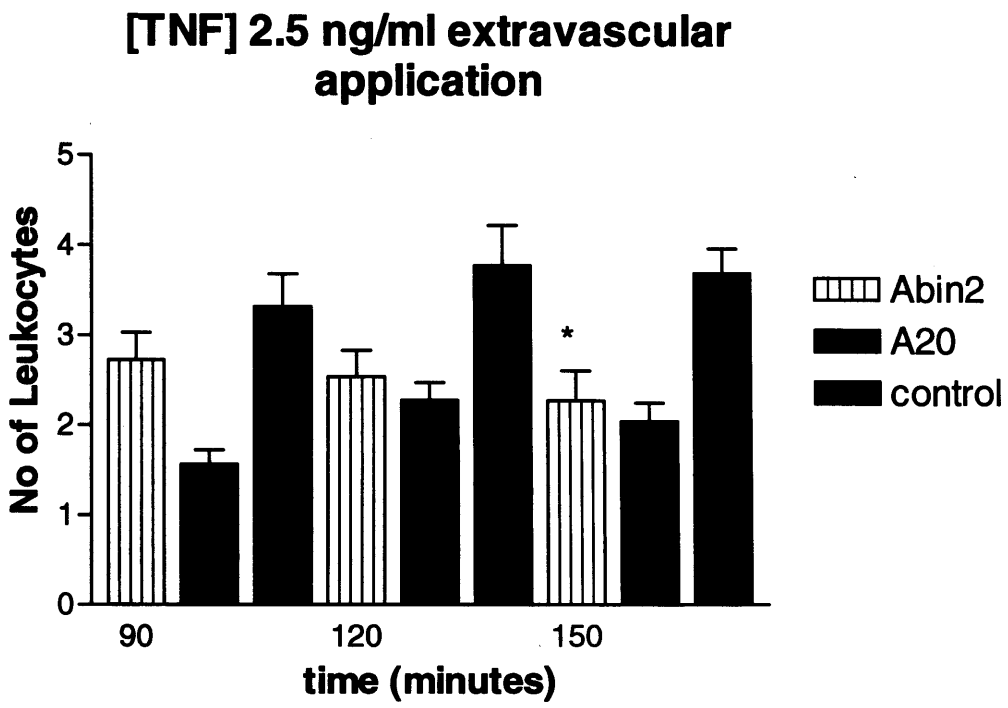


Figure 5.8. Transfection of ABIN-2 reduces TNF-α induced inflammation in CAM microvessels Topical application of TNF-α also induced significantly less endothelial activation in cells expressing A20 or ABIN-2 when compared to those expressing empty control vector. Data is presented as mean and SEM for at least 3 independent experiments, $P < 0.005$ vs ABIN-2 (*). This inhibition of activation was optimal between 120 and 150 minutes following TNF-α application

5.9 The effects of a truncated form of ABIN-2 plasmid transfection on LPS induced CAM inflammation

The C- terminus of ABIN-2 contains the A20 binding site and is thought to be important for the inhibition of the proinflammatory transcription factor NFκB {Beyaert R, Heyninck K, et al. 2000 102 /id}. Therefore the effects of expressing a truncated form of ABIN-2 lacking this domain were tested in the chick CAM. The dominant negative form of ABIN-2 (ΔC) used previously in section 3.9 as well as a control empty vector were transfected into CAM microvessels. LPS was used to activate the CAM endothelia and leucocyte activity monitored as before. The data in

figure 5.9 shows that leucocyte rolling numbers and therefore endothelial activation were similar between the dominant negative form of ABIN-2 and the control vector for each time point. The data suggests that the inhibitory effect of ABIN-2 on LPS induced leucocyte rolling is lost when the C-terminus is truncated.

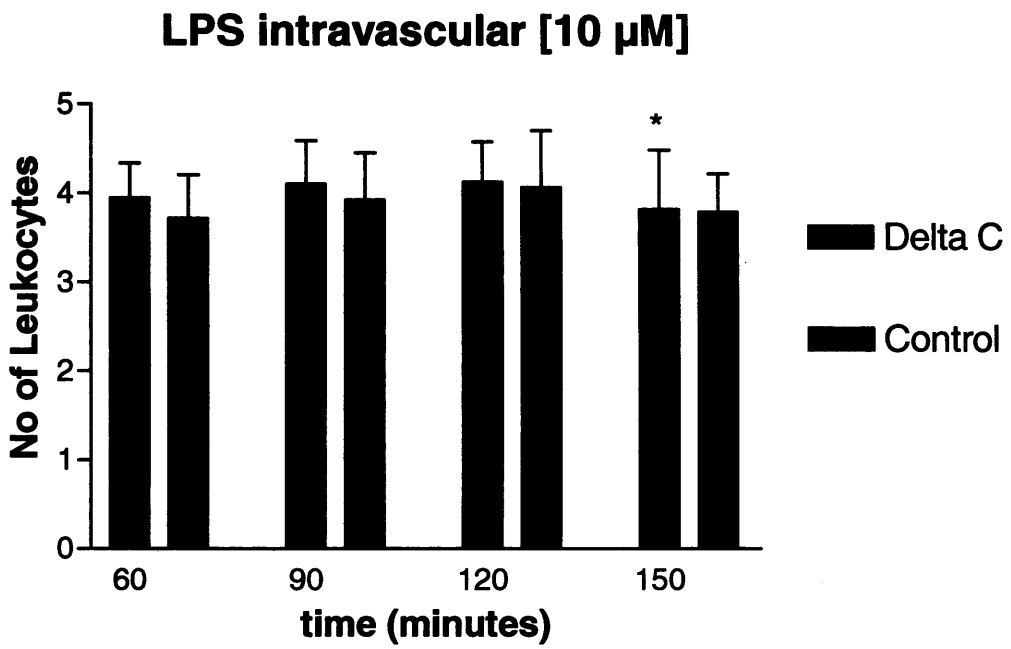


Figure 5.9. The effect of dominant negative ABIN-2-ΔC expression on endothelial inflammation in the presence of LPS showing no reduction in leucocyte rolling with ABIN-2-ΔC expression at all time intervals. Data is presented as mean and SEM for at least 3 independent experiments, $P > 0.05$ vs ABIN-2-ΔC

5.10 The effects of a truncated form of ABIN-2 plasmid transfection on TNF-α induced CAM inflammation

TNF-α [2.5ng/ml] was used to induce inflammation in the CAM following transfection of either a truncated form of ABIN-2 or control vector as described in section 5.8. Measurement of leucocyte rolling numbers were taken at 90. 120 and 150 minutes (Fig 5.9). The data obtained suggests that CAM endothelia expressing the

truncated form of ABIN-2 have lost their ability to reduce TNF-α induced leucocyte rolling.

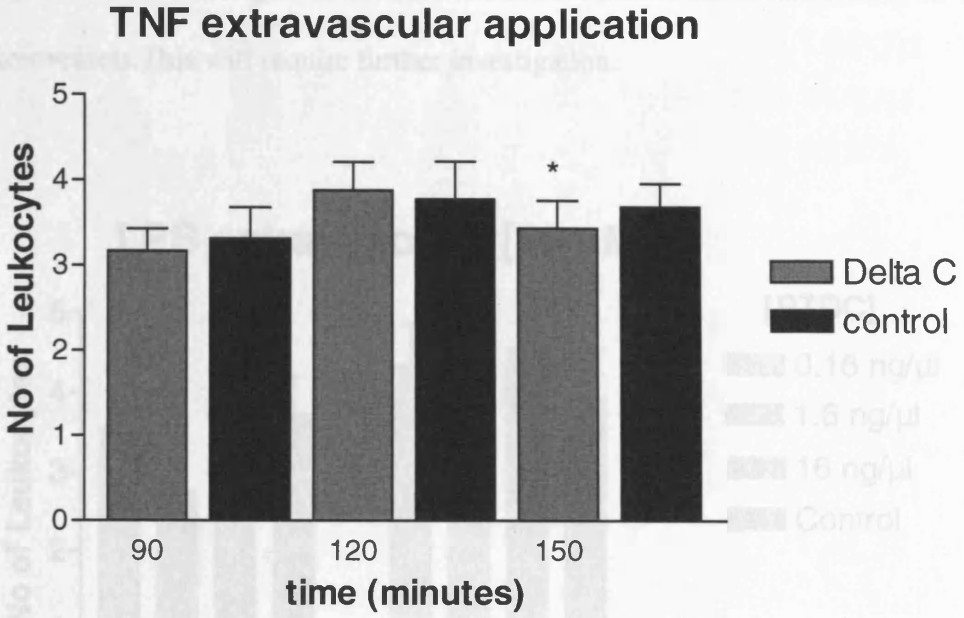
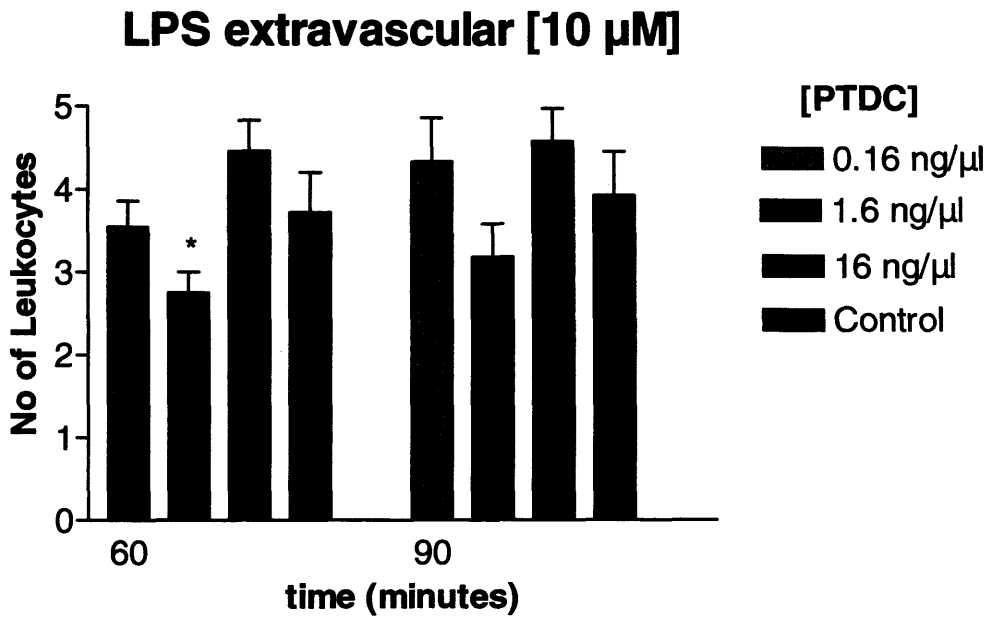


Figure 5.10. The effect of dominant negative ABIN-2-ΔC expression on endothelial inflammation after topical application of TNF-α [2.5ng/ml]. No significant difference was observed in leucocyte rolling numbers between truncated ABIN-2 expression and control vector. Data is presented as mean and SEM for at least 3 independent experiments, $P > 0.05$ vs ABIN-2-ΔC (*).

5.11 NFkB inhibitor PTDC reduces inflammation in the Chick CAM

Pyrolidine dithiocarbamate (PDTC), has been shown to be a potent nuclear factor (NF)-κB inhibitor {Wahl C, Liptay S, et al. 1998 144 /id}. To further examine leucocyte rolling in the CAM model therefore, the effects of PTDC on CAM endothelial cells were assessed. CAM inflammation was induced as described previously using LPS and TNF [10 Mm or 2.5 ng/ml] respectively. PDTC was applied to the CAM surface at variable concentration 0.16 ng/µl, 1.6 ng/µl and 16 ng/µl. PDTC application reduced leucocyte rolling rates in the CAM. Surprisingly this effect only occurred with lower concentrations of PDTC [1.6 ng/µl], increasing the PDTC concentration to [16 ng/µl] increased rather than reduced CAM vessel

inflammation. These results suggest a potential role of NF-κB in the inflammatory process of the Chick CAM which is inhibited by PDTC at lower concentrations. It maybe the case that higher concentrations of PDTC have additional effects on CAM microvessels.This will require further investigation.



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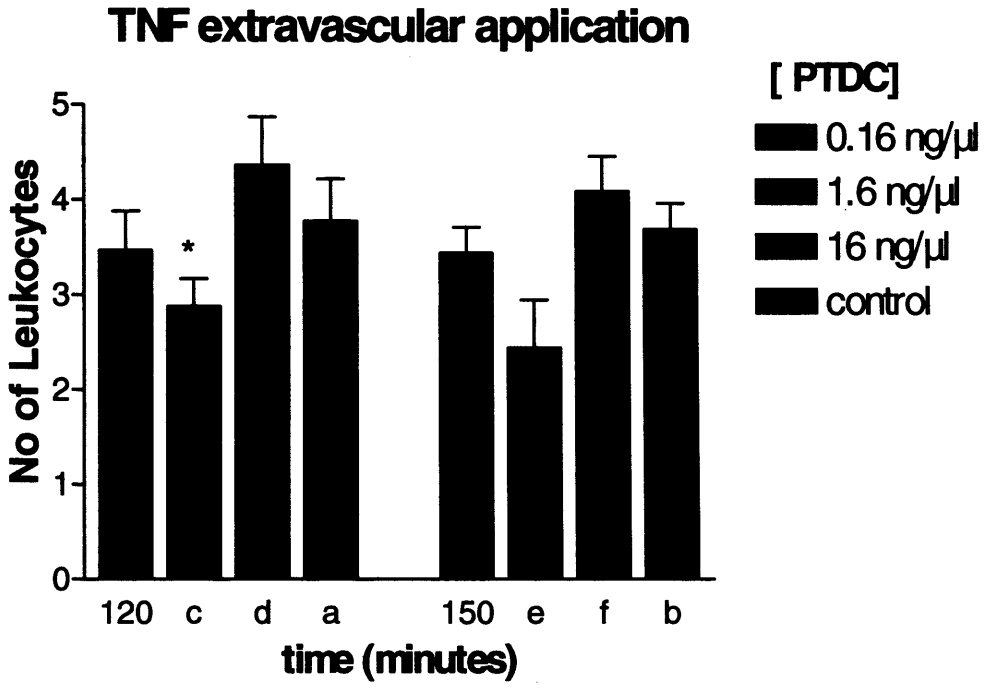

b

Fig 5.11. Pyrolidine dithiocarbamate (PDTC) dampening of the CAM inflammatory response induced by a) LPS extravascular application and b) extravascular TNF application. At 0.16 ng/µl in both cases no significant change was observed in leucocyte rolling rate was observed when compared to the control (CAM vessels no PTDC addition), endothelial activation was significantly reduced compared to control CAM vessels at [1.6 ng/µl] suggesting an inhibition of (NF)-κB. Data is presented as mean and SEM for at least 3 independent experiments, $P < 0.05$ vs 1.6 ng/ µl (*). At [16 ng/µl] PTDC however, this inhibition appears to be reversed with leucocyte rolling rates actually higher than control.

5.12 The effects of ABIN-2 transfection on CAM apoptosis

In order to test the role of ABIN-2 on apoptosis in the living CAM system, microvessels were transfected with either ABIN-2 or Empty vector. Transfection efficiency was monitored by GFP expression in both groups. 24 hours after transfection apoptosis was induced on the cell surface by 3 methods: Ligation of a segment of microvessel using 9/0 prolene sutures, topical extravascular TNF 100ng/ml or LPS 100 µM application. After 6 hours, apoptosing endothelial cells were quantified using DAPI staining as described in section 4.8. Overall, no significant differences were detected between microvascular endothelial cells

expressing ABIN-2 or control vector in apoptosis rates, however if the results from the induction of apoptosis by microvessel ligation are analysed separately, ABIN-2 appears to give some protection from apoptosis but the results are not statistically significant (Fig 5.12).

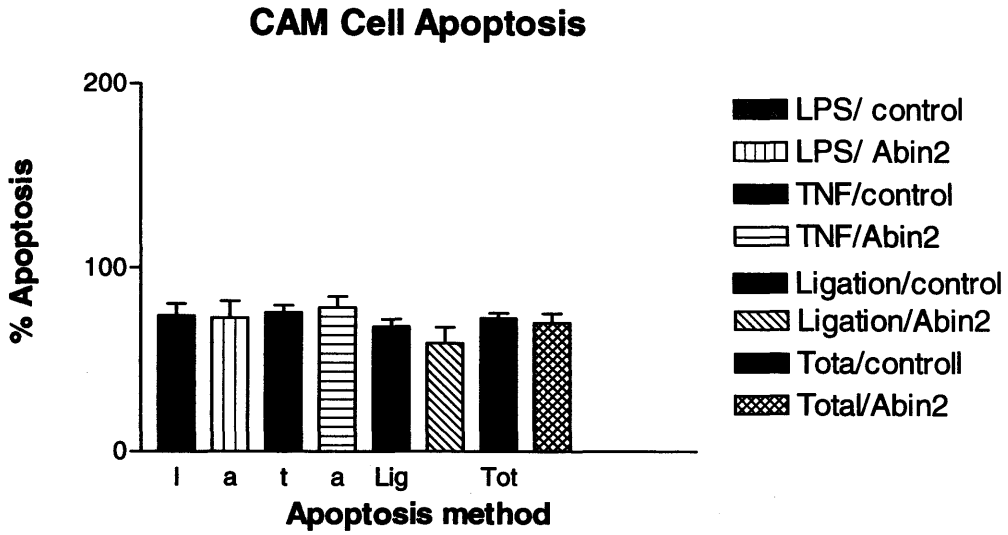

Figure 5.12. The impact of ABIN-2 expression on CAM endothelial apoptosis. Endothelial apoptosis was induced by either ligation, application of LPS 100 uM or TNF-α 100 ng/ml of CAM microvessels expressing either control vector or ABIN-2. No differences in the total apoptotic ratios were detected between cells expressing ABIN-2 and those expressing control vector.

5.13 The effects of ABIN-2 transfection on cell survival

Endothelial cell survival was assessed by a similar method used in HUVE cells as described in section 2.4.3. CAM vessels were transfected with GFP and ABIN-2 or GFP and empty vector as described above. After 24 hours the number of GFP expressing cells in a specific segment of CAM vessel measuring 1 cm was counted. Apoptosis was then induced either by vessel ligation, by the addition TNF 100ng/ml or LPS 100 μM (120 μg/ml) for a period of 24 hours. The number of GFP expressing endothelial cells in the same segment were counted. ABIN-2 expression by CAM

endothelial cells did not increase survival rates when compared to control protein expression. In order to confirm that these results are accurate and not simply due to excessive apoptosis, other agents which are known to improve cell survival needed to be transfected into the CAM vessels to see if increased cell survival is observed.

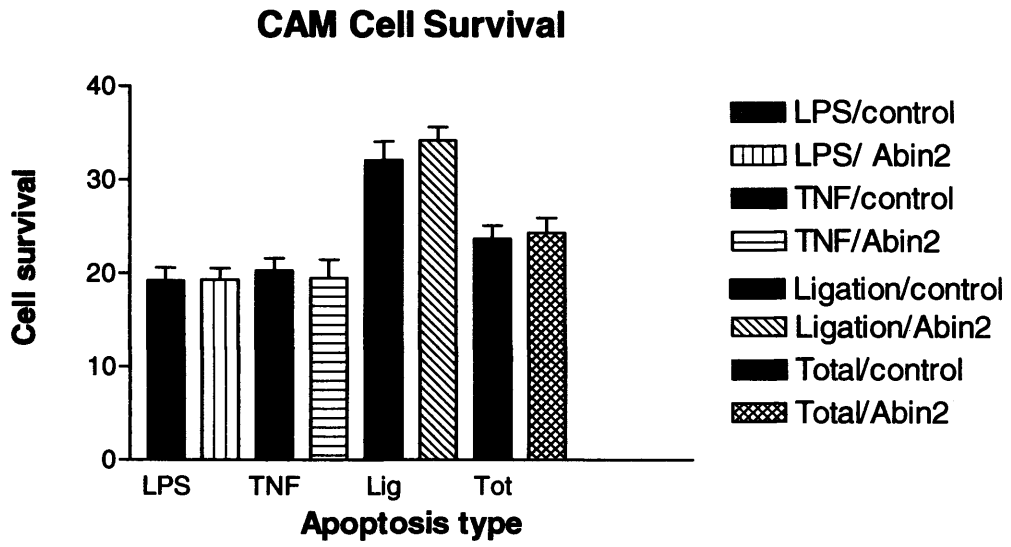

Figure 5.13. The effect of ABIN-2 expression on microvascular endothelial cell survival. Endothelial apoptosis was induced by either topical application of LPS [100 mM], TNF [100 ng/ml] or microvessel ligation for a period of 24 hours. Cell survival was calculated by measuring the ratio of ABIN-2/ GFP expressing cells prior to induction of apoptosis over the number of surviving cells after the apoptotic insult. Overall, ABIN-2 expression did not confer improvement in survival rates of CAM endothelia, however increased survival was detected if only vessel ligation was used to induce apoptosis.

5.14 The effects of A20 transfection on CAM endothelial apoptosis

A20 is known to suppress cell death by LPS in human microvascular cells and by TNF in HUVE cells. CAM endothelial cells were transfected using electroporation as described in section 4.10 to express either A20 and GFP or empty control vector and GFP. Transfection efficiency was estimated by GFP expression rates. Apoptosis was induced by ligation of vessels to prevent blood flow, LPS or TNF topical application

as described above in section 5.12. The effects on the chick CAM following A20 expression were analysed using DAPI staining as described above to monitor apoptosis. Surprisingly, no significant difference in apoptotic rates were detected between cells expressing A20 and control vector (fig 5.14).

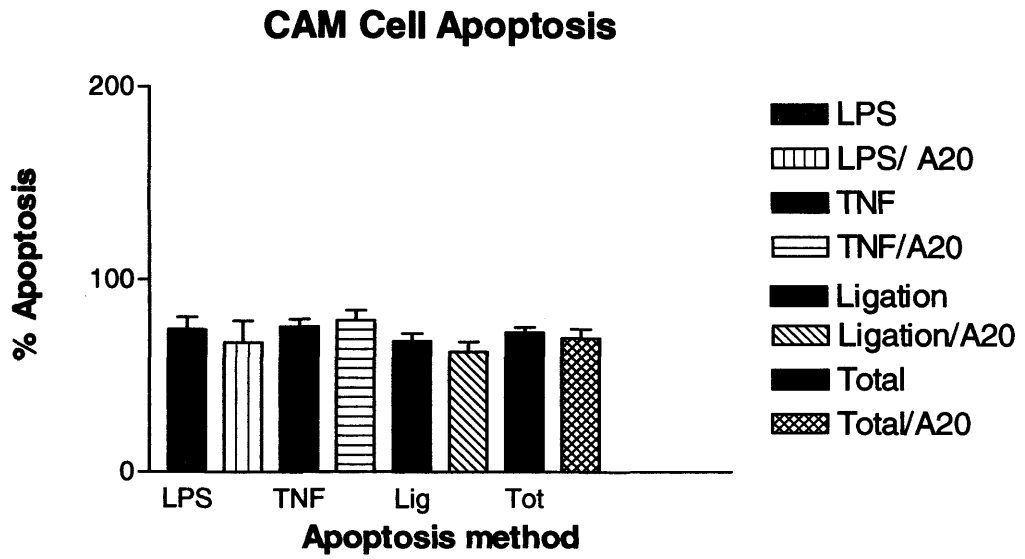

Figure 5.14. The effect of ABIN-2 expression on microvascular endothelial cell apoptosis. Endothelial apoptosis was induced by either topical application of LPS, TNF or microvessel ligation for a period of 24 hours as described in section 5.12. No overall significant difference in apoptotic ratios between cell expressing A20 and those expressing empty control vector. A significant difference was detected however if the apoptotic ratio was calculated using vessel ligation only.

5.15 The effects of A20 transfection on CAM endothelial cell survival

In order to test the effects of A20 expression on CAM microvessels survival, Apoptosis was induced by the three methods described above. Cell survival assays were then measured essentially as described earlier in section 3.4 by measuring the ratio of surviving GFP expressing cells over total GFP expressing cells prior to induction of apoptosis. No difference in endothelial survival was detected between cells expressing A20 and control vector. In addition, as A20 expression did not seem to reduce endothelial cell death, no conclusion can be made about the role of A20

expression on endothelial protection in the CAM. This lack of protection by A20 may have been due to an overly aggressive apoptosis protocol or simply due to A20 inactivity in the chick CAM.

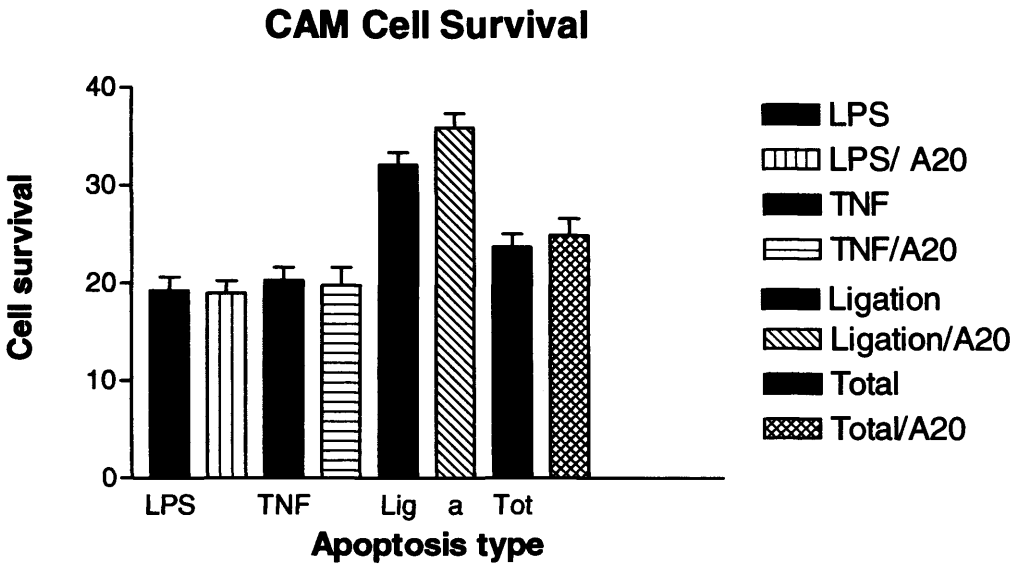

Figure 5.15. The effect of ABIN-2 expression on microvascular endothelial cell survival. Endothelial apoptosis was induced for a period of 24 hours as described in section 5.12. Expression of A20 in CAM endothelial cells only confers increased survival from apoptosis by the ligation method when compared to CAM cells expressing empty control vector. There was no difference in total survival rates between ABIN-2 expression and control vector expression in CAM endothelial cells.

Discussion

The work described in this chapter was to test the effect of ABIN-2 *in vivo*, in particular examining the effects of ABIN-2 expression on microvessel inflammation and apoptosis. Lipopolysaccharide has been shown to play an important role in both inflammation and apoptosis of microvessels by several authors.{Hayes J, Dmytro H 2003, Davenpeck KL, Zagorski J 1998}.

Leucocyte movement and migration is an essential step in any inflammatory process. Recruitment and transmigration of leucocytes through the vascular endothelium and eventual accumulation into inflamed tissue requires that highly coordinated adhesive events take place between neutrophils and endothelial cells in a process known as the adhesion cascade. In this study, I have examined aspects of leucocyte dynamics such as rolling, sticking and extravasation to investigate the early processes in vascular inflammation. With the exception of leucocyte rolling, numerous attempts failed to show that all three parameters in leucocyte dynamics can demonstrate the presence of microvascular inflammation in a reliable, reproducible manner. This observation was also noted when using TNF to induce microvessel inflammation. The reason for this is not clear. Both leucocyte adhesion and extravasation should follow automatically once rolling is demonstrated as this implies that the intimal endothelial cells have been activated following initiation of the adhesion cascade, eventually leading to disruption of the cell-cell interactions. It was still possible to establish a time course for optimal LPS and TNF induction of microvessel inflammation using leucocyte rolling data.

As mentioned previously, NF-κB activation represents an essential pathway for the induction of numerous pro-inflammatory genes in the vascular wall including CAMs

and Cytokines. This method for monitoring inflammation *in vivo* has shown that microvessel expression of the NF-κB inhibitor A20 was capable of reducing leucocyte rolling caused by TNF-α and LPS stimulation. These results validate recent studies looking at the mechanism of A20 inhibition of inflammation {Heyninck K & Beyaert R 2005} .

In a similar manner ABIN-2 was found to inhibit leucocyte rolling and subsequent inflammation, possibly by inhibiting NFkB activity. ABIN-2 is known to inhibit NF-kB by blocking the interaction of RIP (receptor-interacting protein) with the downstream effector IKKγ , a non-kinase component of the IκB (inhibitory κB) kinase complex {Darnay BG, Haridas V, et al. 1998}.When overexpressed in cells, ABIN-2 binds to IKKγ and prevents the association of IKKγ with RIP {Wei-Kuang LIU, Pei-Fen YEN, et al. 2004}. Furthermore, it was shown in chapter 3 that the truncated form of ABIN-2, ABIN-2-ΔC which lacks the terminal carboxy terminal 85 amino acids did not provide any protection from apoptosis as noted with ABIN-2. This truncated form of ABIN-2 is therefore of great value as a dominant negative tool to aid in the unravelling of the mechanism by which ABIN-2 exerts its effects. It seems that these 85 amino acids define a novel structural domain in mediating a key step in the NF-κB signalling pathway as ABIN-2 ΔC expression in microvascular endothelia did not confer any significant protection from TNF or LPS induced inflammation.

Further data suggesting a role for NF-κB in leucocyte rolling in the CAM microvessel was my observation that the NF-κB inhibitor PTDC also suppressed leucocyte rolling. High concentrations of PTDC did not seem to have a more pronounced effect on inflammation possibly by blocking the activity of apoptotic inhibitory proteins resulting in increased rather than decreased inflammation.

Finally, apart from its effects on inflammation, it seems ABIN-2 may play a role in reducing microvessel apoptosis *in vivo*. It was already shown in chapter 3 that ABIN-2 has a prosurvival effect on HUVE cells. In this chapter it was found that ABIN-2 reduced microvessel endothelial apoptotic rates *in vivo*. Again the terminal carboxy 85 amino acids seem to play a crucial role in this process as expression of the truncated form of ABIN-2 reduced endothelial apoptosis in chapter 3, this could not be detected in the CAM but this maybe due to vigorous apoptotic stimulation which may need to be altered in future work if any effects are to be obsereved. It may be that the CAM is not sensitive to these PI3 kinase inhibitors or more intriguingly, it could be that ABIN-2 is protecting endothelial cells via another mechanism. A great deal of work is needed to unravel the complex mechanisms involved in endothelial cell protection.

Chapter 6

General Discussion

Chapter 6

General Discussion

The general aim of this thesis was to investigate the role of ABIN-2, the A20 binding inhibitor of NF-κB in the regulation of inflammation and apoptosis in endothelial cells. More specifically, the results presented in this thesis are intended to shed some light on the effects of ABIN-2 on the apoptotic pathway, looking at its role in the Tie-2 mediated endothelial survival and exploring possible links to anti-apoptotic mediators such as Ang-1 which at the beginning of my research had been relatively unknown. This was due to the fact that ABIN-2 was identified only very recently with only very limited published data about the mechanisms by which it interacts and inhibits NF-κB.

Assessing the effects of ABIN-2 on endothelial inflammation and apoptosis, required establishing a transfection protocol that is capable of reliably optimising expression of target proteins in HUVE cells. This was achieved by refining a method capable of optimising transfection of ABIN-2 into HUVE cells at rates of up to 90%. This greatly facilitated later work looking at the effects of ABIN-2 expression on HUVE cell apoptosis.

Methods for assessing HUVE apoptosis and cell survival following periods of growth factor deprivation were also established which relied on changes in nuclear morphology and caspase-3 activation during apoptosis and cell survival assays were established which proved to be both reliable and efficient.

In order to assess the antiapoptotic effects of ABIN-2 *in vivo*, the chick CAM assay was used. By combining this known assay with already established techniques such

electroporation and intravital microscopy, I was able to adapt the chick CAM into an assay which was a cheap, reliable and a relatively easily accessible method for transfecting target vector into live microvessel endothelia resulting in high yields of cells expressing target protein. The effects of these proteins on the microvessels could then be analysed in a variety of ways such as examination under direct vision in real time or sectioned and stained for histological examination. The Chick CAM model was also adapted successfully to examine microvessel inflammation. Initially by intravascular injections of India Blue dye, microvascular leakage and interstitial oedema was outlined clearly following thermal injury to the CAM. More complex analysis of TNF-α and LPS induced microvessel inflammation using both intravital microscopy and Rhodamine 6G staining was also possible using the chick CAM in order to measure the extent of microvessel rolling which can be a marker for inflammation.

The data in this thesis demonstrated for the first time that ABIN-2 has an anti-apoptotic effect on endothelial cells. The nuclear morphology studies discussed earlier demonstrated that endothelial cells expressing ABIN-2 were less likely to either undergo apoptosis or activate caspase-3 after 18 hours of growth factor deprivation. This gave a possible clue to the mechanism of ABIN-2 action since TIE2 and ABIN-2 are known to interact (however the effects of this interaction were not known) and that TIE2 is known to have a prosurvival activity which is exerted by its activation of the PI3-kinase and Akt signalling pathway. ABIN-2 was also demonstrated to phophorylate Akt, this not only suggested a possible mechanism for the prosurvival activity of ABIN-2 but also since ABIN-2 is known to interact with TIE2, it raises the possibility that ABIN-2 is acting either upstream or downstream of the TIE2/ Akt

prosurvival pathway. Evidence that ABIN-2 was acting downstream in this pathway was identified by the demonstration that Ang-1*, a potent TIE2 agonist with a widely acknowledged role in cell survival, did not exhibit any cytoprotective activity in endothelial cells expressing the inactive truncated form of ABIN-2, termed ABIN-2 ΔC which lacks 85 amino acids from the carboxy terminal but did cause a significant increase in the survival HUVE cells expressing control vector after growth factor deprivation.

It is known that deletions of the carboxy terminus of ABIN-2 prevents NFκB inhibition{Van Huffel S, Delaei F, et al. 2001}. The data in this thesis suggests that the carboxy terminus is also essential for the pro-survival activity of ABIN-2 as HUVE cells expressing ABIN-2 ΔC did not have survival advantage over those expressing empty control vector. In addition to shedding more light onto the mechanism of ABIN-2 action, this cellular work has also shown that ABIN-2 ΔC can be used as a dominant negative vector for future work in understanding microvessel apoptosis.

The prosurvival activities of ABIN-2 could not be demonstrated *in vivo* using the chick CAM, however further work is needed to optimise apoptotic conditions in this novel system before the effects of ABIN-2 on chick microvessel survival can be fully assessed. ABIN-2 expression in the chick CAM microvessels however was found to markedly reduce leucocyte rolling after induction of inflammation using TNF-α and LPS when compared to empty control vector expression. The mechanism for this reduction maybe due to NFκB inhibition as PTDC which is known to inhibit NFκB also decreased leucocyte rolling.

The data in this work therefore demonstrates for the first time that ABIN-2 has antiapoptotic properties on endothelial cells via the TIE-2 and Akt/PI3k pathways. This maybe directly related to NFκB inhibition by ABIN-2. This inhibition seems to be also important in the anti-inflammatory properties demonstrated of ABIN-2 on microvascular endothelial cells *in vivo*. This dual role of ABIN-2 may therefore be of great benefit clinically in many scenarios such as free tissue transfer, tissue engineering, burns trauma or acute respiratory distress syndrome in order to stabilize and protect endothelial microvascular cells from apoptosis while at the same time dampening down any associated inflammatory response leading to improved vessel integrity and reduction in the destructive effects of interstitial oedema.

Bibliography

Reference List

A Woywodt,FBKdGHH, M Haubitz. Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer . Nephrol Dial Transplant 17:1728-1730.

Abbitt KB, Nash GB. The influence of haematological and rheological properties of the flow on leukocyte adhesion, studied in a whole blood, in vitro flow system. Journal of Physiology 523:145.

Baichwal,VR, Baeuerle,PA. Activate NF-kappaB or die? Curr Biol 7:94-96.

Baldwin,AS. The NF-kappa B and I kappa B proteins: new discoveries and insights. Ann.Rev.Immunol 14:649-681.

Barinaga M. Apoptosis: Life-Death balance within the cell. Science 274:724-730.

Barnes PJ. Nuclear factor kappa B. Int J Biochem Cell Biology 29:867-870.

Bates D, Hillman N, Neal C, Pocock T. Regulation of microvascular permiability by vascular endothelial growth factors. J.Anat. 200:581-597.

Beck H, Acker T, Wiessner C, Plate K. Expression of Angiopoietin 1, Angiopoietin 2 and Tie receptors after middle cerebral artery occlusion in the rat. Am J Pathol 157:1483.

Behrends M., Schulz R., Heusch G. Inconsistent relation of MAPK activation to infarct size reduction by ischaemic preconditioning in pigs. Heart and Circulatory Physiology 279:1111-1119.

BEINKE S, Ley B. Functions of NF-kB1 and NF-kB2 in immune cell biology. Biochemical Journal 382:393-409.

Benjamin L.E. The control of microvascular survival. Cancer Metastasis Rev 19:75-81.

Benjamin L.E. The control of microvascular survival. Cancer and metastasis review 19:75-81.

Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone Jr. Interleukin-1 acts on cultured human vascular endothelial cells to increase the adhesion of polymorphonuclear leukocytes, monocytes and related leukocyte cell lines. J Clin Invest 76:2003-2011.

Beyaert R, Heyninck K, Van Huffel S. A20 and A20-binding proteins as cellular inhibitors of nuclear factor-Kappa B-dependent gene expression and apoptosis. Biochem Pharmacol 60:1143-1151.

Burg J., Kirkpatrick CJ. GM-CSF expression by human lung microvascular endothelial cells: in votro and in vivo findings. Am J Physiol Lung 283:460-470.

Calvert JT, Riney TJ, Peters KG, Marchul T. Allelic and locus heterogenecity in inherited venous malformation. Hum Mol Genet 8:1279-1289.

Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 6:389-395.

Cheng G.Yu Z, Zhoo D., Mattson M.P. PI3 kinase-Akt kinase and p42/44 mitogen activated protein kinases mediate neutrophic and excitoprotective actions on a secreted form of amyloid precursor protein. Exp Neurol 175:407-414.

Chien CY, Liu WK, ,SJ. The A20-binding protein ABIN-2 exerts unexpected function in mediating transcriptional coactivation. FEBS 543:55-60.

Cines,D, Pollak,E, Buck,C. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 91:3527-3561b.

Coppo P., Veyradier A., Scobohaci M.L., Bussel A. Pathophysiology of thrombotic microangiopathies. Ann Med Internne 153:153-166.

D'Amore PA, Glaser BM, Brunson SK. Angiogenic activity from bovine retina: Partial purification and characterization. Proc Natl Acad Sci U S A 78:3068-3076.

Daniel S., Arvelo M., Ferran C. Overexpression of A20 in endothelial cells of vascularized grafts creates a protective barrier against TNF and FAS mediated apoptosis. Transplant Proc 33:225.

Darnay BG, Haridas V, Ni J, Moore PA, Aggarwal BB. Characterization Of The Intracellular Domain Of Receptor Activator Of NFkB (RANK): Interaction With Tumor Necrosis Factor Receptor-Associated Factors And Activation Of NFkB and c-Jun N-Terminal Kinase. J Biol Chem 273:20551-20555.

Davis S, Aldrich TH, Jones P, Acheson A,CD, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD. Isolation of angiopoeitin-1 a ligand for Tie2 receptor, by secretion trap expression cloning. Cell 87:1169.

Davis S, Papadopoulos N, Aldrich TH, Maisonpierre PC, Huang T, Kovac L, Xu A, Leidich R, Radziejewski C, Rafique A, Goldberg J, Jain V, Bailey K, Karow M, Fandl J, Samulason SJ, Daly TJ, Yancopoulos GD. Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering. Nat Struct Biol 10:38-44.

Davis S, Yancopoulos G.D. The angiotensins: Ying and Yang in angiogenesis. Curr Top Microbiol Immunol 237:173-185.

De Valck D, Jeang KT, Beyaert R. The zinc finger protein A20 interacts with a novel anti-apoptotic protein which is cleaved by specific capases. Oncogene 18:82-90.

Dickinson M.E., Waters C.W., Murray B.A., Haynes S.M., Longmuir K. Using electroporation and lipid-mediated transfection of GI expressing plasmids to label embryonic avian cells for vital confocal and two-photon microscopy. Differentiation 70:172-180.

Dimmeler S, Zeiher A. Endothelial Cell Apoptosis in Angiogenesis and Vessel Regression. Circulation Research 87:434-440.

Dube A, Akbarali Y, Sato T, Towia A, Oettgen P. Role of the ETs transcription factors in the regulation of the vascular-specific Tie2 Gene. Circulation Research 84:1177-1185.

Eberhard D, Karns L, Vandenberg S, Creutz C. Control of nuclear cytoplasmic partitioning of annexin II by a nuclear export signal and by p11 binding. JCS 114:3155-3166.

Eriksson U, Ali talo K. Structure, expression and receptor binding properties of novel vascular endothelial factors. Curr Top Microbiol Immunol 237:41-57.

Evangelista VS, Manarini S, Rotondo S, Cerletti S. Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the beta 2 integrin CD11b/CD18 . Blood 88:4183-4194.

Fancsi T., Feher G. Ultrastructural studies of chicken embryo chorioallantoic membrane during incubation. Anat HistolEmbryol 8:151-159.

Ferran C, Stroka D, Barichani A, Cooper J, Bach F. A20 inhibits NF kB activation in endothelial cells without sensitizing to tumour necrosis factor mediated apoptosis. Blood 91:2249-2258.

Ferrara N, Carver-Moore k, Chen H, Dowd M, Lu L, o'shea KS, Moore MW. Hetrozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439-442.

Foong LC, Beard RW. Microvascular changes in peripheral circulation of women with chronic pain due to plevic inflammation. BJOG 109:867-873.

Fortune JE, Cushman RA, Wahl C, Kito S. The primordial to primary follicle transition. Mol Cell Endocrinol 163:53-60.

Fruman D, Meyers R, Cantley C. Phosphoinositide Kinases. Annu.Rev.Biochem. 67:481.

Fujikawa K, Presman E, Varticovski L. Expression of tie1 and Tie 2 proteins during reendothelialization after ballon injured rat carotid artery. J Vasc Res 36:272-281.

Gabor M. Eur J Immunol 27:2986-2993.

Gagnon V, St-Germain ME, Parent S, Asselin E. Akt activity in endometrial cancer cells: regulation of cell survival through cIAP-1. J Int Oncol 23:803-810.

Gale N, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Wiegand S, Yancopoulos GD. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. Cell 3:411-423.

Gerber HP, McMurtrey A, Kowalski J, Yan M, Dixit V, Ferrara N. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 273:30336-30343.

Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem 273:13313-13316.

Ghosh, S May,M, JKopp,EB. NF-B and rel proteins: evolutionarily conserved mediators of immune responses. Annu.Rev.Immunol 16:225-260.

Grey ST, Arvelo MB, Back FH, Ferran C. A20 inhibits cytokine induced apoptosis and nuclear factor dependent gene activation in islets. J Exp Med 190:1135-1146.

H Baatz, U Pleyer, HJ Thiel, C Hammer. In vivo study of leukocyte-endothelium interaction in endotoxin-induced uveitis. Investigative Ophthalmology & Visual Science 36:1960-1967.

Hattori K, Diad S, Hackett NR, Lyden D, Rafii S. Vascular endothelial growth factor and angiopoietin 1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 193:1005-1014.

He K, Ting A. A20 inhibits tumour necrosis factor alpha induced apoptosis by disrupting recruitment of TRADD and RIP to the TNF receptor 1 complex in Jurkat T cells. Mol Cel Biol 22:6034-6045.

Heissmeyer V, Krappman D, Wulczyn F, Scheidereit C. NF-kB p105 is a target of IkB kinases and controls signal induction of Bcl-3-p50 complexes. Embo J 18:4766-4778.

Heyninck K, Beyaert R. A20 inhibits NF-kappaB activation by dual ubiquitin-editing functions. Trends Biochem Sci 1:1-4.

Heyninck K.., De Valck D, Beyaert R. The zinc finger protein A20 inhibits TNF- alpha induced NFkB dependent Gene expression by interfering with an RIP or TRAF2 mediated transactivation signal and directly binds to a novel NFkB inhibiting protein Abin. Cell biology 145:1471-1482.

Heyninck K.Beyert R. The zinc finger protein A20 inhibits TNF induced NF-KB- dependent Gene expression by interfering with a RIP or TRAF2 mediated transactivation signal and directly binds to a novel NFkB inbiting protein ABIN. PNAS 145:1471-1486.

Honma T., Hamasaki T. Association between hemosiderin deposition and blood vessel regression during the involution of forgeign body granuloma. J Submicrosc Cytol Pathol 33:173-186.

Hu X, Yee E, Harlan JM, Wong F, Karsan A. Lipopolysaccharide induces the antiapoptotic molecules, A1 and A20, in microvascular endothelial cells. Blood 92:2759-2765.

Hu Y, Baud M, Delhase M, Zhang T, Deerinck T, Karin M. Abnormal Morphogenesis but intact IKK activation in mice lacking the IKKa subunit of the IkB kinase. Science 284:316-320.

Huang L, Turck CW, Rao P, Peters K.G. GRB2 and SH-PTP2: potentially important endothelial signalling molecules downstream of the TEK/Tie2 receptor tyrosine kinase. Oncogene 11:2097-2103.

Hughes D, Marron M, Brindle N. The antiinflammatory endothelial Tyrosine Kinase Tie2 interacts with a novel nuclear factor-kB inhibitor Abin-2. Circulation Research 92:630-636.

Hughes D, Marron M, Brindle N. The antiinflammatory endothelial tyrosine kinase Tie2 interacts with a novel nuclear factor-{kappa}B inhibitor ABIN-2. Circulation Research 92:630-636.

Injune K, Ji-Hye K, Young S, Mingzhu L, Koh G.Y. Tumour necrosis factor upregulates angiopoietin human umbilical vein endothelial cells. Bio Bio Res Comm 269:361-365.

Jeon BH, Khanady F, Deshpande S, Haile A, Ozaki M, Irani K. Tieing the antiinflammatory effect of angiopoietin-1 to inhibition of NF kB. Circulation Research 92:586-588.

Jones N, Master Z, Bouchard D, Gunji Y, Dumont DJ. Identification of TEK / Tie 2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration. J Biol Chem 274:30896-30905.

Kakkainen M, Petrova T. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. Oncogene 19:5598-5605.

Kerr,JFR, Wyllie,AH, Currie,AR. Changes in cell morphology in cell undergoing apoptosis. Br.J.Cancer 26:239-257.

Keshet E.
Preventing pathological regression of microvessels. J Clin Invest 112:27-29.

Khandoga A, Biberthaler,P, Enders,G, Axmann,S, Hutter J, Messmer,K, Krombach,F. Platelet adhesion mediated by fibrinogen-intercelllular adhesion molecule-1 binding induces tissue injury in the postischemic liver in vivo1. Transplantation 74:681-688.

Kim I, Kim JH, Moon SO, Kwak HJ, Kim NG, Koh G.Y. Angiopoeitin-2 at high concentration can enhance endothelial cell survival through the phosphtilinositol3- kinase/Akt signal transduction pathway. Oncogene 19:4549-4552.

Kim I, Kim H.G., So JN, Kim JH, Kwak HJ, Koh GY. Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'- kinase/ AKT signal transduction pathway. Circulation Research 86:24-29.

Kim I, Moon SO, Klm SH, Kim H.G., Koh YS, Koh G.Y. Vascular Endothelial Growth Factor Expression of Intracellular Ahesion Molecule 1(ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1), and E-selectin Through Nuclear Factor-Kappa B activation in Endothelial Cells. J Biol Chem 276:7614-7620.

Kim I., Kim H.G., Koh G.Y. Angiopeitin-1 regulates endothelial cell survival through the phosphatidynositol 3'-kinase/ AKT signal pathway. Circulation Research 86:24-35.

Kim I., Moon SO, Pak YK, Koh G.Y. The angiopoietin Tie 2 system in coronary artery endothelium prevents oxidized low density lipoprotein- induced apoptosis. Cardiovasc Res 49:872-877.

Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U, Risau W. Angiopoietin-1 induces sprouting angiogenesis in vitro. Curr Biol 8:529-532.

Konak L.C, Resnick N. Transcriptional and post-translation regulation of the Tie 1 receptor by fluid shear stress changes in vascular endothelial cells. FASEB 10:104-119.

Kontos C., Stauffer T, Peters K.G. Tyrosine 1101 of Tie 2 is the major site of association of p85 units required for activation of phosatidylinositol 3-Kinase. Mol Cell Biolo 18:4131-4140.

Kontos C., Peters K.G. The endothelial receptor tyrosine kinase Tie1 activates phosphatidylinositol 3- kinase and Akt to inhibit apoptosis. MCB 22:1704-1781.

Kothakota S. Caspase 3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science 278:294-298.

Krymskaya et al. Src is necessary and sufficient for human airway smooth muscle cell proliferation. FASEB 19:428-430.

Kukk E, Lymboussaki A, Taira S, Kaipainen A, Atilalo K. VEGF-Creceptor binding and and pattern of expression suggests a role in lymphatic vascular development. Development 122:3829-3837.

Kunzi -Rapp K, Krahn G. Increased levels of Human S100 in melanoma Chick embryo Xenografts' circulation. Pigmant Cell Res 14:9-13.

Kuroda K, Sapadin A, Shoji T, Lebwohl M. Altered expression of angiopietins and Tie 2 endothelium in psoriasis. J Invest Dermatol 116:713-720.

Kusumawati A, Liatutard JP, Sri Widada J. Implication of annexin 1 in phagocytosis: effects of n terminal domain deletions and point mutations of the phosphorylation site Ser-27. Cell Biol Int 25:809-813.

Kwaja A. Akt is more than just a BAD kinase. Nature 401:33-34.

Laherty CD, Dixit VM. Human T cell leukaemia virus type I tax and phorbol 12-my13-acetate induce expression of the A20 zinc finger protein with distinct mechanism involving NF kappa B. J Biol Chem 268:5032-5039.

Lamm G, Steinlein P, Cotten M, Christofori G. A rapid quantitative and inexpensive method for detecting apoptosis by flow cytometry. Nucleic acid research 25:4855-4857.

Lange N, Ballini JP, Wagnieres G, van den Bergh H. A new drug-screening procedure for photosensitizing agents used in photodynamic therapy for CNV. Invest Ophthalmol Vis Sci 42:38-46.

Lee EG, Boon L, Chai S, Libby SL, Chien M, Ma A. Failure to regulate TNF-induced NF-kappa-B and cell death responses in A20- deficient mice. Science 289:2350-2354.

Lemus D., Dabancens A., Lopez C. Antiangiogenic effects of betamethasone on the chick cam stimulated by TA3 tumour supernatant. Biol Res 34:227-236.

Levkau B, Scatena M, Giachelli CM, Ross R, Raines EW. Apoptosis overrides survival signals through a caspase mediated dominant negative NF-kappa B loop. Nat Cell Biol 1:227-233.

Li Q, Van Antwwerp D, Mercurio F, Lee K, Verma I. Severe Liver degenration in mice lacking the IkB Kinase 2 Gene. Science 284:321-325.

Li Y, Goa F. Experimental study of therapy effects of anti VEGF antibody on osteosarcoma. ZWKZZ 12:241-249.

Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Karin M. The IKKb subunit is essential for nuclear factor kB activation and prevention of apoptosis. J Exp Med 189:1839-1845.

Madrid LV, Wang CY, Guttridge DC, Mayo MW. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. Mol Cel Biol 20:1626-1638.

Maisonpierre PC, Suri C, Jones P, Sato T, Yancopoulos GD. Angiopoietin 2, a natural antagonist for tie2 that disrupts angiogenesis. Science 277:48-50.

Maja Cemazar, Ian Wilson, Gabi U Dachs, Gillian M Tozer, Gregor Sersa. Direct visualization of electroporation-assisted in vivo gene delivery to tumors using intravital microscopy – spatial and time dependent distribution. Cancer 4:81-86.

Makitie T., Kivela T. Ezrin as a prognostic indicator and its relationship to tumour characteristics in uveal malignant melanoma. Invest Opthalmol 42:2442-2449.

Mejillano M, Yamamoto M, Rozelle AL, Sun HQ, Wang X, Yin HL. Regulation of apoptosis by phosphatidylinositol 4,5- bisphosphate inhibition of caspase inactivation of phosphtidylinositol phosphate 5-kinases. J Biol Chem 276:1865-1872.

METHENY-BARLOW L, LI L. The enigmatic role of angiopoietin-1 in tumor angiogenesis. cell research 13:309-317.

Mitsuhashi N., Miyazaki M. Angiopoeitins and Tie 2 expression in angiogenesis and proliferation of human hepatocellular carcinoma. Hepatology 37:1105-1113.

Mocanu M.M., Bell R.M., Yellon D.M. PI3 kinase and not p42/p44 appears to be implicated in the protection conferred by iscemic preconditioning. J Mol Cell Cardiol 34:661-668.

Momose t., Tonegawa A., Ogawa H., Yasud H. Efficient targeting of gene expression in chick embryos by microelectroporation. Dev Growth Differ 41:335-344.

Mustonen T., Ali talo K. Endothelial receptor tyrosine kinases involved in angiogeneis. J Cell Biol 129:895-898.

Nicholson K.M., Anderson N.G. The protein Kinase B/AKT signalling pathway in human malignancy. Cell Signal 14:381-385.

Nykanen AI, Krebs R, Saaristo A, Turunen P, Alitalo K, Lemstrom KB. Angiopoeitin-1 protects against the development of cardiac allograft arteriosclerosis. Circulation 107:1308-1314.

Ogura T. In vivo electroporation: a new frontier for gene delivery and embryology. Differentiation 70:163-71.

Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y. Hypoxia and vascular endothelial growth factor selectively upregulate angiopoietin-2 in bovine microvascular endothelial cells. J Biol Chem 274:15732-15739.

Opipari AW, Hu HM, Yabbowitz R, Dixit VM. The A20 zinc finger protein protects from tumour necrosis factor toxicity. J Biol Chem 267:12424-12427.

Ouaaz F, Ming Li, Amer A. A Critical Role for the RelA Subunit of Nuclear Factor B in Regulation of Multiple Immune-response Genes and in Fas-induced Cell Death. J Exp Med 189:999-1004.

Papapetropoulos A, Yancopoulos GD, Sessa WC. Direct action of angiopietin 1 on human endothelium. evidence for network stabilization, cell survival and interaction with angiogenic growth factors. Lab Invest 79:213-223.

Papapetropoulos A, Fulton D, Altieri D, Sessa WC. Angiopoietin 1 inhibits endothelial cell survival via the akt/ survivin pathway. JBC 275:9102-9105.

Plumpe J, Malek NP., Bock CT., Rakemann T, Manns MP, Trautwein C. NF-κB determines between apoptosis and proliferation in hepatocytes during liver regeneration. Am J Physiol.Gastrointestin Liver physiol 278:G173-G183.

Pober J.S. Endothelial Activation: intracellular signalling pathways. Arthritis Res 4:s109-s116.

Procopio WN, Pelavin PI, Lee WM, Yielding NM. Angiopoeitin -1 and -2 coiled coil domains mediate distinct homo- oligomerization patterns but fibrinogen like domains mediate ligand activity. J Biol Chem 274:30196-30201.

Puri MC, Rossant J, Ali talo K., Partanan J. The receptor tyrosine kinase Tie is required for the integrity and survival of vascular endothelial cells. Embo J 14:5884-5891.

Ribatti D, Vacca A, Presta M. The discovery of angiogenic factors: A historical review. General Pharmacology 35:227-231.

Risau W. Mechanisms of angiogenesis. Nature 386:671-674.

Risau W. Mechanisms of angiogenesis. Nature 386:671-674.

Sarma V, Lin Z, Tewari M, Dixit VM. Activation of the B cell surface receptor CD40 induces A20 a zinc finger protein that inhibits apoptosis. J Biol Chem 270:12343-12346.

Schenk PW, Snaar-Jagalska BE. Signal perception and transduction: the roles of protein kinases. Biochimica et Biophysica Acta 1449:1-24.

Shin J, Kim I, Lee S, Kim HH. A novel zinc finger protein that inhibits osteoclastogenesis and the function of tumour necrosis factor receptor-associated factor 6. JBC 277:8346-8353.

Sihvola R, Pulkkinen VP, Koskinen PK, Lemstrom KB. Crosstalk of endothelin-1 and platelet-derived growth factor in cardiac allograft arteriosclerosis. J Am Coll Cardiol 39:710-717.

Sizuki K., Murtuza B., Heslop L., Yacoub M.H. Single fibres of skeletal muscle as a novel graft for cell transplantation to the heart. J Thoracic Cardiovasc Surg 123:984-992.

Steff AM, Fortin M, Arguin C, Hugo P. Detection of a decrease in green fluorescent protein fluorescence for the monitoring of cell death: an assay amenable to high throughput screening technologies. Cytometry 45:237-243.

Strebel A, Harr T, Bachmann F, Erb P. Green fluorescent protein as a novel tool to measure apoptosis and necrosis. Cytometry 43:126-133.

Suri C, Jones N, Patan S, Maisonpierre PC, Davis S, Sato T, Yancopoulos GD. Requisite role of angiopoietin-1 a ligand for the Tie-2 receptor during embryonic angiogenesis. Cell 87:1171-1180.

Takahashi T, Daniel T. Kidney microvascular assembly and repair: power and promotion of molecular definition. Kidney Int 53:826-835.

Takakura N, Dumont DJ, Suda T. Critical role of the Tie 2 endothelial cell receptor in the development of definitive hematopoiesis. Immunity 9:677-686.

Teichert-Kulisszewska K, Maisonpierre PC, Jones N, Campbell AI, Bendeck MP, Ali talo K., Dumont DJ, Yancopoulos GD, Stewart DJ. Biological action of angiopietin-2 in a fibrin matrix model in angiogenesis is associated with activation of Tie 2. Cardiovasc Res 49:659-670.

Tran J.Kerbel R.S. A role for survivin in chemoresistance of endothelial cells mediated by VEGF. PNAS 99:4349-4354.

Tymi K., Matthew Costello O. Structural and functional changes in the microvasculature of disused skeletal muscle. Front Biosci 1:D45-D52.

Ulrich A, Schlessiger J. Signal transduction by receptors with tyrosine kinase activity. Cell 61:212.

Uta K, Jurgen F, Grone H, Ferran C. Expression of A20 in the vessel wall of rat kidney allografts collerates with protection from transplant arteriosclerosis. Transplantation 75:547-587.

Vacca A, Ribatti D, Roccaro AM. Bone marrow angiogenesis in patients with active multiple myeloma. SeminOncol 28:543-550.

Van Huffel S, Delaei F, Heyninck K, De Valck D, Beyaert R. Identification of a novel A20 binding inhibitor of nuclear factor B activation termed Abin2. J Biol Chem 276:30216-30223.

Vanhaesebroeck B, Higashi K, Raven C, Waterfield M. Embo J 18:1292-1302.

Varani J, Dame K, Taylor CG, Sarma V, Merino R, Kunkel RG, Nunez G, Dixit VM. Age dependent injury in human umbilical vein cells: relationship to apoptosis and correlation with lack of A20 expression. Lab Invest 73:851-858.

Vikkula M, Boon L, Mulliken J, Olsen B. Vascular dysmorphogenesis caused by activating mutations in receptor tyrosine kinase Tie 2. Cell 87:1181-1187.

Wahl C, Liptay S, Adler G, Schmid PM. a potent and specific inhibitor of nuclear factor kappa B. B.J Clin Invest 101:1163-1174.

Wang z, Chen Z. Differentiation and apoptosis induction therapy in acute promyelocytic leukaemia. Lancet oncol 1:101-106.

WANG,JH, BARTLETT,JD, DUNN,AC, SMALL,S, WILLIS,SL, DRIVER,MJ, LEWIS,AL. The use of rhodamine 6G and fluorescence microscopy in the evaluation of phospholipid-based polymeric biomaterials. Journal of Microscopy 217:216-224.

Ward,P, Fantone,J. *Adhesions molecules and the lung*. Marcel Dekker1-404.

Wazecha Z, Dembiski A, Konturek SJ. Deleterious effects of helicobacter pylori infection on the course of acute pancreatitis in rats. Pancreatology 2:386-395.

Wei-Kuang LIU, Pei-Fen YEN, Chia-Yi CHIEN, Ming-Ji FANN, Jin-Yuan SU, Chen-Kung CHOU. The inhibitor ABIN-2 disrupts the interaction of receptor-interacting protein with the kinase subunit IKK*a* to block activation of the transcription factor NF-*k*B and potentiate apoptosis. Biochem.J **378**: 867-876.

Weiss A, Schlessinger J. Switching signals on and off by receptor dimerization. Cell 94:277-280.

Wertz I, Orourke K, Zhou H, Eby M, Wu P, Wiesman C, Baker R, Boone D, Ma A, Koonin E, Dixit V. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kB signallimg. Nature 430:694-699.

Witzenbichler B, Westermann D, Knueppel S, Schultheiss HP, Tschope C. Protective role of Angiopoeitin-1 in endotoxic shock. Circulation 111:97-105.

Wyllie,AHMRG, Smith,AL, Dunlop,D. Chromatin cleavage in apoptosis. J.Pathol. 142:66-77.

Xu Y, Yu Q. Angiopoietin 1 unlike angiopoietin 2 is incorporated into the extracellular matrix via its linker peptide region. JBC 276:34990-34998.

Yancopoulos GD, Davis S, Gale N, Rugge J, Wiegand S, Holash J. Vascular specific growth factors and blood vessel formation. Nature 407:242-248.

Yancopoulos GD, Davis S, Gale N, Rudge J, Holash J. Vascular specific growth factors and blood vessel formation. Nature 407:242-248.

Yancopoulos GD, Davis S, Gale N, Rudge J, Holash J. Vascular specific growth factors and blood vessel formation. Nature 407:242-248.

Yaneza M, Gilthorpe JD, Lumsden A, Tucker AS. No evidence for ventrally migrating neural tube cells from the mid- and hindbrain. Dev Dyn 223:163-167.

Zandi E, Karin M. Bridging the gap: Composition, regulation and physiological function of the IkB kinase complex. Mol Cel Biol 19:4547-4551.

Zen K,KA, Stempien-Otero A, Yee E, Tupper J, Li x, Eunson T, Kay MA, Wilson CB, Winn RK, Harlan JM. NF-kappa B activation is required for human endothelial survival during exposure to tumor necrosis factor-alpha but not to interlukin-1 beta or lipopolysaccharides. J Biol Chem 274:28808-28815.

Zimmerman G, Kurt H, Holly J, Topham M. Endothelial activation and ARDS. Chest 116:18s-24s.