Micro-Thrombotic and Micro-Embolic Potential of Endothelial Cells During Apoptosis

By

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Table of Contents

CHAPTER 4:

Cellular Fragmentation and Shape Change During Endothelial Apoptosis

Declaration

The work of this thesis is wholly the work of Wei XU and was undertaken at the Cellular and Molecular Pathology Research Unit, the Department of Oral Medicine and Oral Pathology at Westmead Hospital Dental Clinical School, the Faculty of Dentistry of the University of Sydney, between March 1998 and March 2002 towards the degree of Doctor of Philosophy (University of Sydney). To the best knowledge of the student, this work is original and has not been published or presented elsewhere by others unless stated.

Wei XU

Publications of Work Described in This Thesis

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List of Figures

List of Tables

Abbreviations

AIF	Apoptosis-Inducing Factor
Apaf-1	Apoptotic Protease Activating Factor-1
APC	Activated Protein C
ATPase	Adenosinetriphosphatase
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanosine Monophosphate
DAB	Diamino-Benzo-Tetrahydrochloride
DMSO	Dimethyl Sulfoxide
DTS	Dense Tubular System
ECGS	Endothelial Cell Growth Supplement
EDTA	Ethlylene Diamine Tetraacetic Acid
FACS	Fluorescence Activated Cell Scanning
FCS	Foetal Calf Serum
FITC	Fluoroscein Isothiocyanate
HBSS	Hanks Balance Salt Solution
HUVEC	Human Umbilical Vein Endothelial Cells
IAPs	Inhibitor of Apoptosis Proteins
IFN-γ	Interferon-γ
ISEL	In Situ End-Labeling
L-NAME	L-N^G-Nitroarginine Methyl Ester
LPS	Lipopolysaccharide
M&SDA-EC	Matrix and Serum Deprived Apoptotic Endothelial Cells

M199	Medium 199
Mac-1	Leukocyte Adhesion Receptor
MDA-EC	Matrix Deprived Apoptotic Endothelial Cells
NGS	Normal Goat Serum
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OCS	Open Canalicular System
PA	Plasminogen Activator
PAI	Plasminogen Activator Inhibitor
PAR	Protease-Activated Receptor
PBS	Phosphate Buffered Saline
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H Synthase
PGI_2	Prostaglandin I_2 or Prostacyclin
PI	Propidium Iodide
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
RER	Rough Endoplasmic Reticulum
scu-PA	Precursor Single-Chain Form of u-PA
SDDA-EC	Serum Deprived Detached Apoptotic Endothelial Cells
SDnA-EC	Serum Deprived non-Apoptotic Endothelial Cells
SEM	Scanning Electron Microscopy
TdT	Terminal Deoxynucleotidyl Transferase
TEM	Transmission Electron Microscopy

TFPI	Tissue Factor Pathway Inhibitor
TGF-β1	Transforming Growth Factor β1
TM	Thrombomodulin
TNF-α	Tumour Necrosis Factor-α
t-PA	Tissue-Type Plasminogen Activator
TUNEL	TdT - mediated dUTP Nick End-Labelling
TXA_2	Thromboxane A_2
UEA-1	*Ulex Europeaus* Lectin Type 1
UnA-EC	Untreated non-Apoptotic Endothelial Cells
u-PA	Urokinase Plasminogen Activator
u-PAR	u-PA Receptor
VWF	von Willebrand Factor

Summary

This thesis describes work investigating the micro-thrombotic and micro-embolic potential of endothelial cells during apoptosis. In Chapter 1, aspects of blood clotting, endothelial biology and apoptosis are reviewed, providing background information relevant to the experimental work presented in later chapters. Where appropriate, further relevant literature is briefly discussed in the introductions to individual experimental chapters.

There appear to be two main biological mechanisms through which apoptotic endothelial cells are able to enter the circulation. The first of these is during microvascular remodeling, when apoptotic endothelial particles are shed into the blood stream. The second mechanism is when endothelium is traumatically detached and then becomes apoptotic due to the lack of matrix adhesion. Chapter 2 describes the establishment and exploitation of experimental cell culture models for investigating the effect upon platelet aggregation of apoptotic endothelial cells similar to those generated during either microvascular remodeling or trauma. Briefly, cultured human umbilical vein endothelial cells (HUVEC) were identified by their characteristic morphological, antigenic and lectin binding properties. Serum or matrix deprivation were used to generate apoptotic HUVEC, suggested as similar to those seen in microvascular remodeling and traumatic detachment respectively. Both apoptotic and non-apoptotic endothelium had strong anti-aggregatory activity for platelets stimulated by either thrombin or ADP. The possibility that the effects of HUVEC seen upon platelet aggregation were due to mechanical interference with platelets was excluded in experiments using latex beads of a similar size to apoptotic and non-apoptotic HUVEC. No difference was noted in these experiments between

apoptotic endothelial cells derived by either serum or matrix deprivation with regard to their anti-aggregatory activity. Collagen was also used as a platelet agonist in several experiments, however, it was found that the effects of apoptotic endothelium upon collagen induced platelet aggregation could be largely accounted for on the basis of agonist depletion by collagen binding to endothelium. The data suggest that apoptotic endothelial cells inhibit platelet aggregation and that this is similar regardless of the biological setting from which the apoptotic endothelial cells are derived.

In Chapter 3, further experiments are described investigating possible mechanisms mediating anti-platelet aggregatory activity by apoptotic endothelium. Prostacyclin and nitric oxide are both known and important anti-aggregatory factors released by endothelium. This was confirmed in this study while importantly, the anti-aggregatory activity of apoptotic HUVEC was also found to be mediated by prostacyclin and nitric oxide. This was determined firstly in experiments using the metabolic inhibitors indomethacin and L-NAME, and secondly through detection of the stable degradation products of both prostacyclin and nitric oxide in culture medium supernatants from apoptotic HUVEC. Finally, a role for endothelial surface ADPase (CD39) in the anti-aggregatory activity of HUVEC was excluded by fluorescence activated cell sorting analysis for CD39.

While Chapters 2 and 3 investigate aspects of the possible micro-thrombotic activity of apoptotic endothelial cells, the biological significance of such micro-thrombotic masses lies primarily in their micro-embolic potential. Apoptosis in endothelium seems unique as compared with apoptosis in other cell types in that apoptotic

endothelial cells internalize their plasma membranes to produce complex canalicular structures. This process has been termed canalicular fragmentation, as earlier work suggested a role for these canaliculi in increasing the mechanical fragility of apoptotic endothelium and thus reducing the micro-embolic potential. However, despite the anti-embolic role attributed to canalicular fragmentation, there are no published reports detailing changes in endothelial cell size during apoptosis and mechanical stress. Chapter 4 describes experiments in which scanning and transmission electron microscopy were used to characterize changes in HUVEC size and shape during apoptosis in conditions of both static culture and low mechanical stress. Apoptotic HL-60 cells were compared with apoptotic HUVEC in these experiments to help place observations in endothelium in context with non-endothelial cells. Apoptosis in HUVEC resulted in a greater relative reduction in cell size over the same time as compared with apoptotic HL-60 cells while low levels of mechanical stress exacerbated this. Also, mechanical stress increased the circularity of apoptotic endothelium. Both the rapid reduction in cell size and increased circularity of endothelial apoptotic particles are consistent with the idea that endothelial cells are adapted to minimize micro-embolic potential during apoptosis. In the course of performing this work, additional previously unpublished ultrastructural features of endothelial apoptosis and canalicular fragmentation were observed.

In Chapter 5, the overall experimental findings of this thesis are discussed as are the directions that future work may take. To facilitate reading and help clarify the findings of work completed, the experimental Chapters 2 to 4 are written in a brief, "manuscript style". The Appendix, however, outlines work performed to establish experimental systems, description of which would interrupt the flow of reading in

earlier experimental chapters. The Appendix also contains descriptions of work not completed due to the limited time available for this thesis.

CHAPTER ONE

Blood Coagulation, Endothelium and Apoptosis

1.1. Endothelial Cells and Blood Coagulation

1.1.a. The Structure and Biological Role of Endothelium

1.1.a.i. The Location of Endothelial Cells

Most multicellular organisms are provided with a system of vascular channels serving the distribution of needed metabolites to cells and the removal of catabolites from the tissues of the body. The vessel wall forms the barrier between the circulating blood and the underlying tissues. The endothelial cells form a continuous luminal monolayer on all the blood vessels and heart chambers and while vascular endothelium appears structurally simple, it is functionally complex and its integrity is essential for normal vascular function (Cliff, 1976).

1.1.a.ii. The Role of Endothelium in Metabolic Exchange

Once regarded as an inert lining, the vascular endothelium is in fact a multifunctional 'organ', which can directly influence circulating blood components as well as other cells within the vessel wall. It synthesizes and releases biologically active substances that control aspects of the integrity and metabolism of the vessel wall. Endothelial cells play many roles in modulating physiological and pathophysiological processes including haemostasis, thrombosis, inflammation and immune responses (Wu *et al.*, 1988). Endothelial cell functions include the transport of nutrients and solutes across the endothelium, maintenance of vascular tone, maintenance of a thromboresistant surface, and activation and inactivation of various vasoactive hormones (Wu and Thiagarajan, 1996). Endothelial functional specialisations vary from one anatomical site to another so that endothelium can contribute significantly to the differentiated

functions of organs. Endothelial cells are metabolically active, physically responsive and susceptible to injury.

1.1.a.iii. Structural Features of Endothelial Cells

Endothelial cells can vary in thickness from several micrometers at their widest points, which is generally in the region of nucleus, to peripheral extensions of the cell so thin that they would not incorporate two adjacent plasmalemmal vesicles (Pittilo, 1988). Endothelial cells have a polygonal outline, particularly at branches, which may be related to flow disturbances in this area (Silkworth and Stehbens, 1975; Roach, 1977). The orientation of endothelial cell nuclei has been related to the pattern of blood flow (Flaherty *et al.*, 1972). The vascular endothelium constitutes approximately 1% of the human body mass with a surface area of approximately 5,000 m^2 (Jaffe, 1984). The normal lifespan of human endothelial cells is estimated to be approximately 30 years (Vanhoutte, 1996).

Endothelial cells possess the full complement of organellar structures seen by transmission electron microscopy (TEM) in mammalian cells, but additionally contain complex multilaminar Weibel-Palade bodies seemingly unique to endothelium (Weibel and Palade, 1964). This seems to be a storage site for von Willebrand Factor (VWF) as well as for the leukocyte adhesion molecule P-Selectin which achieves surface expression when these granule like structures fuse with the plasma membrane upon stimulation of endothelium with thrombin and other agonists (Warhol and Sweet, 1984; Bonfanti *et al.*, 1989; McEver *et al.*, 1989; Vischer and Wagner, 1993; Carlos and Harlan, 1994).

1.1.a.iv. The Biological Need for Anti-Coagulant Activity in Endothelium

Circulating blood contains myriad cells, proteins and other biologically active molecules while platelets and plasma proteins, especially the coagulation factors, are needed to form blood clots. As a pre-requisite for survival, the circulating blood must stay fluid even when the blood vessel is severely damaged so that blood normally does not clot inside its endothelial 'container' and maintenance of normal blood fluidity is an important function of endothelium. In the absence of significant insult, steady flow and negative cell-surface charge helps maintain blood fluidity by maintaining separation of endothelium and potentially aggregatory platelets. Substances expressed upon the outer endothelial surface such as thrombomodulin (TM), heparins and ADPase, or released by endothelial cells such as prostaglandin I_2 (PGI_2), nitric oxide (NO) and plasminogen activators (PA) serve to inhibit platelet activation or clotting and also lyse thrombi in the event of fibrin formation (Wu, 1992; Wu and Thiagarajan, 1996; Pearson, 1999; Toborek and Kaiser, 1999).

1.1.a.v. Diseases with Reduced Anti-Coagulant Endothelial Activity

Although endothelium serves a predominantly anticoagulant function (Gimbrone *et al.*, 1990), endothelium also appears capable of being procoagulant when it is perturbed by some physiological and chemical factors (Muller and Griesmacher, 2000). The balance between the anticoagulant and procoagulant roles has implications for a variety of disease processes and the most obvious are disorders involving thrombosis and thrombolysis. Other conditions where this seems important are restenosis after angioplasty, atherosclerosis, and early and late coronary artery bypass graft closure (Harrison, 1992).

1.1.b. Mechanisms of Blood Clotting

1.1.b.i. An Overview of Blood Clotting

Blood clotting is initiated by vessel damage in order to prevent exsanguination after trauma. The coagulation process can also be triggered by mediators of the inflammatory response and fibrin deposition has been noted in a variety of pathological states (Hoffbrand and Pettit, 1993). Upon damage to the vessel wall, platelets adhere to the exposed sub-endothelial matrix and become activated, releasing serotonin, thromboxane A_2 (TXA_2) and ADP which stimulate further platelet activation and aggregation. During this, there is vasoconstriction due to both neurogenic mechanisms and the activity of serotonin and thromboxane A_2. The initial haemostatic platelet plug is stabilized by the formation of a fibrin meshwork generated by the coagulation system, resulting in a stable blood clot. Coagulation may be initiated by tissue factor in the so called "extrinsic" coagulation pathway, as well as by contact of blood with the collagen and other extracellular matrix components in the "intrinsic pathway". Blood coagulation involves an enzymatic amplification system in which there is sequential proteolytic activation of circulating precursor pro-enzymes to culminate in the generation of active thrombin. Thrombin then converts soluble plasma fibrinogen into fibrin, enmeshing platelet aggregates and converting the unstable primary platelet plug to a firm, definitive and stable haemostatic plug. Platelets support this enzymatic process, in part by supplying phospholipid which acts as a co-factor for some clotting factors and also by providing specific clotting factors and clotting factor binding sites. A broad outline of blood clotting is illustrated in Fig. 1.1. It may be important to note the distinction drawn in this thesis between clotting, by which is meant the response of whole blood including

platelets, and the coagulation system, which is focussed upon the proteolytic coagulation system of blood clotting.

1.1.b.ii. The Intrinsic and Extrinsic Coagulation Cascade Systems Operate in Concert

The coagulation cascade is classically viewed as composed of separate extrinsic and intrinsic pathways (Figure 1.2). The extrinsic system is initiated by interaction of the phospholipid tissue factor with factor VII to activate factor X. Activated factor X is also generated by the intrinsic system which is initiated by contact activation of factor XII, also known as Hageman factor, by the negatively charged surfaces underlying endothelium (Hoffbrand and Pettit, 1993; Ferguson *et al.*, 1998). Factor XI is cleaved by activated Hageman factor and then activates factor IX which is subsequently capable of cleaving factor X. In the final common pathway of both the intrinsic and extrinsic systems, activated factor X converts prothrombin to thrombin. Thrombin then cleaves fibrinogen to fibrin which self-aggregates into a fibrous mesh. This is then stabilized by factor XIII which is activated by thrombin cleavage and cross-links adjacent fibrin molecules. The final common pathway is greatly accelerated by factor V, which stimulates prothrombin cleavage by factor X and like factor XIII is also activated by thrombin.

Factor VIII is a further protein co-factor activated by thrombin cleavage and stimulates cleavage of factor X by factor IX in the intrinsic pathway. Thrombin may also activate factor XI within the intrinsic pathway, providing a further mechanism for amplification of clotting. Calcium and phospholipid are additional important co-factors necessary for activity at numerous steps throughout the coagulation cascade and their role as well as the overall structure of the coagulation cascade is shown in

Figure 1.2 (Roberts and Lozier, 1992; Hoffbrand and Pettit, 1993; Ferguson *et al.*, 1998).

The intrinsic and extrinsic pathways are highly interdependent and a major interaction between the two sequences is mediated by the tissue factor/factor VIIa complex (Roberts and Lozier, 1992) which not only activates Factor X directly in the extrinsic pathway, but also activates factor IX in the intrinsic pathway (Roberts and Lozier, 1992; Hoffbrand and Pettit, 1993; Ferguson *et al.*, 1998).

A more integrated view of blood clotting from that implied by the separate intrinsic and extrinsic pathways is described by Roberts and Lozier (1992). In this three stage model, clotting is divided into phases of initiation, amplification and propagation (Roberts and Lozier, 1992). Clotting is initiated by tissue factor/factor VIIa surface complexes, which activate factor X and factor IX, generating a small amount of thrombin. Apart from causing the formation of fibrin, the thrombin thus generated also activates platelets as well as factors V, VIII and XI while the activated factors V and VIII consequently bind to specific platelet surface receptors. Since activation of factors V and VIII greatly accelerate production of thrombin and factor Xa respectively, while the activated factor XI generated by thrombin also activates more factor X via factor IX which also binds to specific platelet receptors, this sequence of events is defined as the amplification stage. Importantly, by binding factors V, VIII and IX, platelets essentially focus blood clotting to their surface and promote stabilization of the clot. Also, there is surface expression of phospholipids such as phosphatidyl serine normally confined to the inner platelet plama membrane leaflet, and these support activation of factor X and thrombin. Once initiated in this way,

there is explosive propagation of thrombin formation with cleavage of fibrinogen and fibrin polymerization, comprising the propagative stage of clotting (Roberts and Lozier, 1992; Ferguson *et al.*, 1998).

Hageman Factor (factor XII) is named after a patient who was deficient in this factor but appeared not to suffer any consequent bleeding disorder. From the above discussion, it seems clear that the major role of the proteins "beneath" factor XII in the intrinsic clotting cascade is to amplify thrombin formation. Factor XII's biological role appears to be primarily to link clotting to the kinin and fibrinolytic cascades (Ratnoff, 1993; Cotran *et al.*, 1999). Also, factor XII is activated by binding to strongly negatively charged surfaces and this may also play a minor role in coagulation.

1.1.b.iii. Platelets

1.1.b.iii.1. Platelet Morphology

Blood platelets are anucleate disc-shaped cells generated from megakaryocytes in the bone marrow. Resting human platelets measure 1 x 3.1 μm^2 in dimension and have a volume of from 4 to 7.6 μm^3 (Iyengar *et al.*, 1979; Frojmovic and Milton, 1982). Platelets normally circulate for up to 9-10 days before removal from the circulation.

Platelets display a complex internal morphology and as indicated in Figure 1.3, four major ultrastructural regions are recognized: the peripheral zone, the sol-gel zone, the organelle zone and membrane system (Nichols *et al.*, 1981; White *et al.*, 1981; 1983).

The peripheral zone consists of the membranes and closely associated structures forming the surface of the platelet and walls of the tortuous channels making up the surface-connected open canalicular system (OCS). The peripheral zone is viewed as composed of three structural domains, the exterior coat, the unit membrane and the submembrane region. Rich in glycoproteins, the exterior coat or glycocalyx, provides the receptors for stimuli triggering platelet activation and substrates for the adhesion-aggregation reaction. The unit membrane, the middle layer of the peripheral zone, is rich in asymmetrically distributed phospholipids that provide an essential surface for interaction with coagulant. The submembrane region is closely linked to the unit membrane and translates signals received on the outside surface into chemical messages and physical alterations required for platelet activation.

The sol-gel zone is the matrix of the platelet cytoplasm. It contains several fibre systems, including microfilaments, microtubules and the submembrane filaments, in various states of polymerisation. These support the discoid shape of unaltered platelets and provide a contractile system involved in shape change, pseudopod extension, internal contraction, and secretion (Mackie and Neal, 1988).

The organelle zone, comprising granules, electron-dense bodies, peroxisomes, lysosmes, and mitochondria randomly dispersed in the cytoplasm, serves in metabolic processes and for the storage of enzymes, nonmetabolic adenine nucleotides, serotonin, a variety of protein constituents and calcium destined for secretion. Three main types of granule have been described: lysosomes, dense bodies and alpha granules. Platelet lysosomes contain a large range of enzymes active at acid pH, such as beta glucuronidase, beta galactosidase, acid phosphatase, cathepsins and nuclease

(Mackie and Neal, 1988). The function of these enzymes is thought to be disposal of cell debris during wound healing. Alpha granules are approximately 0.2 μm in diameter and contain a range of proteins, many of which have a haemostatic function. Dense bodies are much smaller than alpha granules and store adenine nucleotides, serotonin, calcium and pyrophosphate, which are involved in platelet aggregation and vessel wall tone (Mackie and Neal, 1988). Both dense bodies and alpha granules can fuse with the surface connecting system and release their contents to the platelet exterior after contraction (Mackie and Neal, 1988; Wu, 1996).

The membrane system has a special place in platelet anatomy. Two membrane systems can be distinguished in the platelet - the OCS connecting to the surface, and the dense tubular system (DTS). The OCS is derived from the megakaryocyte plasma membrane (Mackie and Neal, 1988) which invaginates into the platelet interior to form an open membrane canalicular network in contact with the exterior via surface pores. In this way the OCS greatly increases the surface area of the platelet exposed to plasma and provides a communication route bringing stimuli into the deeper parts of platelets and also an exit route for platelet products (Mackie and Neal, 1988). The extensive cytoskeletal elements appear important for the significant shape changes which occur upon platelet activation while the OCS is involved in the release mechanism in which alpha granules and dense body contents are secreted in to the extracellular medium (White, 1970a; 1970b; White and Estensen, 1972). During stimulus-induced shape change, the canaliculi of resting normal human platelets disappear and seem to contribute to the cell surface processes formed (White *et al.*, 1990). The OCS seems also to play a role in phagocytosis by platelets (Mackie and Neal, 1988). The channels of the DTS are found randomly throughout the cytoplasm

and interdigitate with the OCS. The DTS originates from the smooth endoplasmic reticulum of the parent megakaryocyte and seems to represent this in the platelet (Mackie and Neal, 1988). Calcium, magnesium and ADPase are localised in the channels of DTS (Statland *et al.*, 1969; Mackie and Neal, 1988). This membrane system is also considered to be the site of prostaglandin synthesis, since it is rich in the enzymes of both cyclooxygenase and lipoxygenase pathways (Gerrard *et al.*, 1978; Mackie and Neal, 1988).

1.1.b.iii.2. Platelet Adhesion

One of the earliest events following vascular damage is the adhesion of platelets to areas denuded of endothelial cells and this seems an important first step in haemostasis and thrombosis (Baumgartner, 1977; Sixma, 1981). Platelet adhesion occurs under flow conditions, and requires a unique cell adhesion mechanism involving specific structural components of the subendothelium, plasma proteins and receptors on the platelet membrane. Platelet vessel wall interactions and subsequent platelet-platelet interactions are mediated by glycoproteins on the platelet outer membrane and by adhesive proteins, such as VWF, fibronectin, fibrinogen and thrombospondin, present in plasma, platelet alpha granules and the vessel wall (Packham and Mustard, 1984; van Zanten *et al.*, 1997).

VWF, also known as Factor VIII associated antigen, is a large multimeric glycoprotein that plays an important role in platelet adhesion, thrombus formation and as a carrier protein for factor VIII. It is synthesised by endothelial cells and megakaryocytes and released into the plasma and deposited to the extracellular matrix by endothelial cells (Rand *et al.*, 1980; Wagner and Marder, 1983). The size of VWF

molecules varies from a dimer of 500 kDa to high multimeric forms of up to 20,000 kDa (Wagner, 1990). VWF mediates platelet adhesion by forming a bridge between collagen or other components in the damaged vessel wall and specific platelet receptors. Two distinct platelet receptors seem most important for VWF binding to platelets, the glycoprotein Ib and the glycoprotein IIb/IIIa receptors. Platelets must first be activated before glycoprotein IIb/IIIa can bind its ligands. Glycoprotein Ib is already active on nonactivated platelets, but can only bind surface associated ligand (van Zanten *et al.*, 1997). Interaction of platelets with VWF via glycoprotein Ib causes activation of glycoprotein IIb/IIIa leading to platelet spreading and firm attachment (Savage *et al.*, 1992). The interaction of glycoprotein IIb/IIIa with VWF is also involved in platelet spreading and thrombus formation on the subendothelium (Sakariassen *et al.*, 1986; Weiss *et al.*, 1989; 1991).

1.1.b.iii.3. Platelet Activation

Platelet activation usually follows adhesion and can be initiated by several mechanical and chemical stimuli. Collagen and other components of the subendothelial matrix and thrombin are among the strongest stimulators of platelet activation (Lefkovits *et al.*, 1995). Platelet activation leads to a very fast change in platelet shape from the normal resting disc-shaped cell to a spiny sphere with multiple processes. This metamorphosis into Filipodia covered structures greatly enhances the ability of platelets to cling to the vessel wall, each other and fibrin fibrils, thus facilitating their task in haemostasis (Holmsen, 1986). Besides shape change, the activation of platelets is also associated with stimulation of several metabolic pathways, activation of the glycoprotein IIb/IIIa receptor, and induction of platelet coagulant activity (Coller, 1991). TXA_2, thrombin, norepinephrine, collagen, and ADP are all

substances that stimulate these intracellular pathways through various receptors and secondary messengers to ultimately increase mobilization of calcium and mediate degranulation (Lefkovits *et al.*, 1995). ADP, serotonin, and TXA_2 are released by activated platelets leading to recruitment and activation of further surrounding platelets (Fuster and Jang, 1994).

1.1.b.iii.4. Platelet Shape Change

There is some variability in the shapes adopted by stimulated platelets. Depending on the stimulus, these changes may include development of a spherical shape, extension of filopodia, or surface flattening and spreading to cover an exposed connective tissue matrix. The cytoskeleton provides scaffolding that dictates the membrane's contours and is primarily responsible for regulating the platelet's shape (Fox, 2001). In the early stages of platelet activation, shape change is reversible, but strong stimuli cause the centralisation of organelles, degranulation and release accompanied by irreversible shape change and aggregation (Mackie and Neal, 1988).

1.1.b.iii.5. The Release Reaction

A significant event following platelet activation is contraction of microtubules accompanied by release of endogenous granules and their contents. Activated platelets degranulate and secrete chemotaxins, clotting factors and vasoconstrictors, thereby promoting thrombin generation, vasospasm and additional platelet accumulation (Ferguson *et al.*, 1998). Alpha granular contents are released at lower concentrations of agonists than are the contents of dense bodies, whilst lysosomes seem only released at high levels of agonist (Mackie and Neal, 1988).

Alpha granules are the most abundant intraplatelet organelles. They contain a myriad of active compounds including growth factors, adhesive molecules and coagulant factors. Amongst the contents of alpha granules are fibrinogen (Broekman *et al.*, 1975), albumin (Sixma *et al.*, 1984), factor V (Chesney *et al.*, 1981), VWF (Slot *et al.*, 1978), fibronectin (Zucker *et al.*, 1979), high molecular weight kininogen (Schmaier *et al.*, 1983), platelet-derived growth factor (Heldin *et al.*, 1979) and transforming growth factor-β (TGF-β) (Assoian *et al.*, 1983). These factors play an important role in promoting vascular smooth muscle cell migration and proliferation, platelet-platelet and platelet-neutrophil interactions and fibrin formation (Wu, 1996). Alpha granules also contain beta thromboglobulin and platelet factor 4, which are useful specific markers for detecting *in vivo* platelet activation (Wu, 1996). Alpha granules appear to be released by one of two postulated mechanisms. Either they are centralized to first coalesce into a larger structure before release via the OCS, or individual alpha granules fuse with the OCS to mediate release (Wu, 1996; Furie *et al.*, 2001).

The dense granules appear to fuse first with the plasma membrane where they release their granular contents such as ADP, serotonin, and calcium into the extracellular milieu. ADP is an important secondary platelet activator and aggregator and the release of internally stored ADP and thromboxane amplifies the process of platelet activation by secondary feedback loops to recruit further platelets (Wu, 1996; Ferguson *et al.*, 1998). Large amounts of serotonin are also stored in dense bodies and this both acts synergistically with other inducers of aggregation and also causes vasoconstriction to limit blood loss (Mackie and Neal, 1988). Platelets may also

provide high local concentrations of calcium ions, thus potentiating the calcium dependent components of the coagulation system (Mackie and Neal, 1988).

1.1.b.iii.6. Platelet Aggregation

During platelet aggregation, platelets adhere to one another and this process requires energy, intracellular messengers and initiators. Irrespective of the agonist, aggregation is the final common pathway leading to formation of the platelet plug (Ruf *et al.*, 1997).

In terms of the molecular mechanisms mediating platelet aggregation, the platelet membrane glycoprotein IIb/IIIa complex is essential (Kieffer and Phillips, 1990; Nurden, 1994). The glycoproteins IIb and IIIa are members of the integrin family of proteins, forming a heterodimeric complex in the platelet membrane (Hynes, 1987; Ruoslahti, 1991). The complex constitutes a prosmiscuous receptor for several ligands including fibrinogen, VWF, fibronectin, vitronectin and possibly other cytoadhesive proteins (Haverstick *et al.*, 1985; Plow *et al.*, 1985; Lefkovits *et al.*, 1995). Binding of fibrinogen to glycoprotein IIb/IIIa receptors seems to be the principal mechanism for platelet aggregation (Gogstad *et al.*, 1982), although the other adhesive glycoproteins, including VWF, fibronectin, and vitronectin, also bind to these receptors and play a greater part in platelet adhesion to subendothelial structures (Plow *et al.*, 1985; Phillips *et al.*, 1988). In addition, VWF appears particularly important for glycoprotein IIb/IIIa mediated platelet aggregation and thrombus formation under conditions of high shear, with fibrinogen being more important at low shear rates (Weiss *et al.*, 1989).

1.1.b.iii.7. Platelet Cytoskeleton

The platelet membrane is supported by a scaffolding of cytoskeletal proteins. The platelet cytoskelen consist of a submembranous microtubule coil, a submembranous membrane skeleton and a network of long interconnecting actin filaments that fill the cytoplasm (Spangenberg, 1990; Fox, 1993; 2001). One function of the platelet cytoskeleton is to control the membrane contours of both unstimulated and activated platelets (Fox, 2001). Actin filaments throughout the cytoplasm are thought to connect with the membrane skeleton lining the plasma membrane. In this way, the entire cytoskeleton functions together to support the plasma membrane and direct its contours (Fox, 2001). Only 30 - 40 % of the actin is polymerized into filaments in unstimulated platelets whereas there is a rapid increase in actin polymerization upon activation. In this way, new filaments fill the extending filopodia and form a network at the periphery of the cells. A further event in activation is that myosin binds to cytoplasmic actin filaments, causing them to move towards the center of the platelet (Fox, 1993). Additional cytoskeletal reorganizations also occur during aggregation where glycoprotein IIb/IIIa ligand binding results in the association of glycoprotein IIb/IIIa, membrane skeleton proteins and signaling molecules with cytoplasmic actin (Fox, 1993). Another function of the cytoskeleton is to bind other cellular components and form new structures (Fox, 2001). One property of the cytoskeleton that allows it to regulate activities within the cell is its ability to bind numerous cellular proteins, including membrane receptors, membrane lipids and signaling molecules (Fox, 2001). In this way, the platelet cytoskeleton regulates both spatial organization and integration of cellular activities (Fox, 2001).

1.1.b.iii.8. Platelet Surface Proteins and Receptors

As indicated above (1.1.b.iii.2. and 1.1.b.iii.6.), glycoproteins IIb/IIIa are important glycoproteins on the platelet surface with approximately 40,000 to 80,000 receptors per platelet (Ferguson *et al.*, 1998). The glycoprotein IIb/IIIa receptor is a member of the integrin family and is particularly important in platelet aggregation and spreading. It consists of noncovalently linked alpha (alpha $_{IIb}$) and beta (beta$_3$) subunits (Phillips *et al.*, 1988; Madan *et al.*, 1998). There are two binding sites on glycoprotein IIb/IIIa, one that recognises the amino acid sequence Arg-Gly-Asp (RGD) and another that recognises the Lys-Gln-Ala-Gly-Asp-Val sequence (Pytela *et al.*, 1986). Fibrinogen is the principal ligand for this receptor and other RGD-containing ligands include fibronectin, vitronectin and VWF (Madan *et al.*, 1998).

The binding of VWF by the platelet membrane glycoprotein Ib-IX-V complex has been recognised as initiating a cascade of events leading to both thrombosis and haemostasis. The glycoprotein Ib-IX-V complex consist of four transmembrane subunits coded by separate genes present on different chromosomes, each of which is a member of the leucine-rich repeat protein superfamily (Clemetson and Polgar, 1997; Berndt *et al.*, 2001). The major physiological role of the glycoprotein Ib-IX-V complex is to mediate initial adhesion of circulating platelets to VWF in the subendothelial matrix at high shear (Berndt *et al.*, 2001). However, it has been suggested that the glycoprotein Ib-IX-V complex may play a much broader role through binding ligands such as P-selectin and the leukocyte adhesion receptor, Mac-1 (Berndt *et al.*, 2001). Platelets can utilize the glycoprotein Ib-IX-V complex to roll on activated endothelium via surface expressed endothelial P-selectin while binding to Mac-1 mediates adhesion to leukocytes. Also the glycoprotein Ib-IX-V complex may

initiate thrombosis by mediating platelet binding to the subendothelial matrix via VWF (Berndt *et al.*, 2001). In addition, the glycoprotein Ib-IX-V complex may lead to platelet activation by binding to VWF resulting in up-regulated glycoprotein IIb/IIIa binding affinity. Glycoprotein Ib-IX-V also seems to bind thrombin focussing fibrin formation to the cell surface (Berndt *et al.*, 2001).

Glycoprotein Ib-IX-V seems to play an early role in platelet adhesion to the subendothelial matrix (Clemetson and Polgar, 1997; Rao, 1998). The multiple attachments formed stop the platelet, hold it on the surface and start the activation process involving other receptors. The collagen receptor, glycoprotein Ia/IIa, is an important secondary receptor for platelet adhesion and activation, contributing to platelet spreading across the surface. Other secondary receptors, such as fibronectin, laminin and vitronectin receptors, also help to strengthen the links to the surface and act as a reserve in pathological situations (Clemetson and Polgar, 1997). When platelets are activated, previously inactive glycoprotein IIb/IIIa receptors on the platelet membrane surface undergo structural modification and become available to bind fibrinogen and VWF (Ferguson *et al.*, 1998). Additional glycoprotein receptors such as for thrombospondin probably strengthen and stabilise the interaction between platelets (Clemetson and Polgar, 1997). With continuing platelet activation, additional glycoprotein IIb/IIIa receptors stored within platelets are expressed at the surface to become available for ligand binding. With progressive platelet aggregation, large amounts of activated platelet membrane accumulate, supporting thrombus formation. Finally, the platelet has a further group of receptors involved in binding other cells contributing to the repair process such as neutrophils and monocytes. PECAM for example, is expressed on resting platelets while P-selectin is stored in

alpha granules and is only expressed on activated platelets that have undergone release (Clemetson and Polgar, 1997).

1.1.b.iii.9. Arachidonate Metabolism in Platelets

The major product of arachidonic acid metabolism in platelets is the cyclooxygenase product TXA_2 (Halushka *et al.*, 1997). Eicosanoids are not stored in cells, but are synthesized de novo in response to cell-specific proteolytic or hormonal stimuli. Since most arachidonic acid in resting cells is esterified to specific phospholipids, eicosanoid synthesis must occur in two stages. First, arachidonic acid is liberated from membrane phospholipids while second, there is metabolism of free arachidonic acid by specific enzymes (Halushka *et al.*, 1997). In human platelets, arachidonic acid is the most prevalent fatty acid, particularly in the sn-2 position of phosphatidylcholine, phosptidylethanolamine and phosphatidylinositol (Halushka *et al.*, 1997). The amount of free arachidonic acid and the rate of eicosanoid synthesis are very low in unactivated platelets while upon stimulation, the concentration of unesterified arachidonic acid rapidly increases as does the rate of eicosanoid synthesis (Habenicht *et al.*, 1990). Similarly, the addition of free arachidonic acid to resting cells results in a rapid increase in eicosanoid synthesis. These findings suggest that arachidonic acid cleavage from membrane phospholipids is a major trigger for eicosanoids synthesis (Bettazzoli *et al.*, 1990). Several mechanisms have been proposed for agonist-stimulated arachidonic acid mobilization. Activation of phospholipase A_2, which selectively cleaves fatty acids from the sn-2 position of membrane phospholipids, has been considered the prime enzymatic route (Bartoli *et al.*, 1994). In platelets, TXA_2 causes shape change, aggregation, secretion and exposure of fibrinogen binding sites (Arita *et al.*, 1989). It also acts as an

amplification system for weaker agonists as it lowers the activation threshold to other agents and affects calcium ion flux, liberating calcium from intracellular stores and possibly promoting the influx of extracellular calcium (Mackie and Neal, 1988). The free arachidonic acid, liberated by the hydrolysis of phospholipids (predominantly phosphatidylcholine) by phospholipase A_2, is converted by cyclooxygenase to prostaglandins G_2 and H_2 and subsequently by thromboxane synthetase to TXA_2 (Rao, 1998)

1.1.b.iii.10. Platelet Calcium

Calcium is an agent that plays a key role in triggering many platelet functions. Complete activation by all of the platelet agonists involves elevation of cytosolic calcium levels (Body, 1996; Authi, 1997). The low cytosolic calcium concentrations necessary to keep platelets in the resting state and to reestablish the resting state after low levels of activation are achieved by a combination of $Ca^{2+} + Mg^{2+}$ ATPase and a Na^+ / Ca^+ exchanger (Authi, 1997). When platelets are activated by agonists, cytosolic calcium elevation occurs by release of calcium from intracellular stores, particularly the dense tubular system (Berridge, 1993). This is mediated in large part by increasing levels of inositol 1,4,5-trisphosphate which functions as a messenger for calcium mobilization. Also, there is influx of calcium from the outside medium (Rink and Sage, 1990; Authi, 1997). The consequences of intracellular calcium mobilization include phosphorylation of myosin light chain by a specific calcium-dependent kinase and this contributes to shape change and secretion (Rao, 1998). Arachidonic acid release from phospholipids is also dependent upon calcium as phospholipase A_2 is activated by this ion and this is the rate-limiting step in TXA_2 synthesis.

1.1.b.iii.11. Platelet Coagulant Activity

Platelet activation is closely linked to coagulation and the phospholipid membrane of activated platelets acts as a cofactor for coagulation (Schafer, 1994). Predominantly negatively charged membranes rich in phosphatidylserine and phosphatidylinositol provide the surface on which enzymes of the coagulation cascade function with maximum efficiency. Activated platelets alter their cell membranes to conform to exactly these requirements. Thus activated platelet membranes are the major source of phospholipid surface on which the coagulation cascade proceeds (Ferguson *et al.*, 1998). The plasma membranes of activated platelets facilitate the conversion of factor X to Xa, so called "tenase activity" and prothrombin to thrombin (Tracy, 1988; Mann *et al.*, 1990). Catalysis of X to Xa requires assembly of a coagulation factor complex on the activated platelet surface. Also, binding of IXa is promoted by factor VIIIa on activated platelets, while binding of factor VIIIa to its specific receptors occurs on activated platelet membranes (Nesheim *et al.*, 1993). Following binding of VIIIa to activated platelet membrane, factor IXa, factor X and calcium form a complex with VIIIa. This facilitates catalytic conversion of factor X to Xa by factor IXa (Wu, 1996). Similarly, it has been suggested that factor Va binds to a specific receptor on activated platelets. Factor Xa and calcium form a complex with Va. This complex, called prothrombinase, catalyses the conversion of prothrombin to thrombin (Wu, 1996).

1.1.b.iii.12. Factors Stimulating Platelet Activation and Aggregation

The fundamental stimulus for platelet activation is binding of an agonist, such as thrombin, ADP, collagen, VWF or TXA_2, to a cell membrane-bound receptor on the platelet and this stimulation can occur by contact activation with the subendothelial

surface or in the plasma phase (Body, 1996). The agonists of particular relevance to the work in this thesis are thrombin, ADP and collagen.

Thrombin is a potent platelet activator and aggregator as mediated by specific receptors on the platelet surface. The cloned human platelet thrombin receptor belongs to the family of the seven-transmembrane domain G-protein-coupled receptors (Vu *et al.*, 1991). Several studies demonstrate that the platelet thrombin receptor is necessary as well as sufficient for thrombin-induced platelet activation (Siess, 1997). Thrombin is important not only in initiating but also in recruiting and amplifying platelet aggregate formation (Wu, 1996). Its dual roles in the coagulation cascade and platelet activation coordinate and amplify the concurrent formation of platelet and fibrin masses in haemostatic and thrombotic plugs (Wu, 1996).

The discovery of the first thrombin receptor (protease-activated receptor-1, or PAR 1) and the recognition that this receptor was activated by the proteolytic cleavage of its extracellular domain provided a new paradigm for receptor activation (Vu *et al.*, 1991). Proteolytic cleavage releases a small peptide and unmasks a new amino-terminal domain that can activate the receptor through intramolecular interactions. Proof of such a mechanism is demonstrated by the fact that synthetic peptides analogous to these unmasked sequences (the tethered ligands) can activate these receptors with an efficiency equivalent to that of the proteases themselves (Patterson *et al.*, 2001). Presently, four members of the protease-activated receptor family have been identified as PAR 1, PAR 2, PAR 3 and PAR 4 (Vu *et al.*, 1991; Nystedt *et al.*, 1994; Ishihara *et al.*, 1997; Kahn *et al.*, 1998; Xu *et al.*, 1998). Of these, PAR 1, PAR 3 and PAR 4 can be cleaved and activated by thrombin (Vu *et al.*, 1991; Ishihara *et*

al., 1997; Kahn *et al.*, 1998), although thrombin-mediated cleavage of PAR 4 occurs at higher concentrations of thrombin than are required for activation of PAR 1 and PAR 3, raising the possibility that activation of this receptor may occur in an atypical fashion (Patterson *et al.*, 2001). In contrast, PAR 2 is not activated directly by thrombin but can be activated under experimental conditions by trypsin and factor VIIa (Nystedt *et al.*, 1994; Kahn *et al.*, 1998). The existence of multiple thrombin receptors indicates that cellular responses to thrombin may be determined at least in part by the presence of different combinations of thrombin receptors on different cell types (Patterson *et al.*, 2001).

ADP was the first low-molecular-weight platelet aggregating agent to be identified and plays a key role in the development and extension of arterial thrombosis (Gachet and Cazenave, 1997). Contained at very high concentrations in platelet dense granules (Reimers, 1985), ADP is released by exocytosis when platelets are stimulated by other aggregating agents, such as thrombin or collagen, and thus contributes to and reinforces platelet aggregation (Gachet and Cazenave, 1997). Therefore, ADP is an important secondary platelet activator and aggregator that plays a major role in recruiting naive platelets to sites of vascular damage (Wu, 1996). In addition, low concentrations of ADP potentiate or amplify the effects of all other agents, even weak agonists such as adrenaline or serotonin (MacFarlane, 1987; Lanza *et al.*, 1988).

Collagen comprises structural macromolecules of the extracellular matrix and plays a major role in thrombogenesis at sites of vascular injury (Baumgartner, 1977). Collagen is considered the most thrombogenic matrix component and, together with

thrombin is the physiologically strongest platelet agonist (Brass, 1991). At high shear rates the rapid and reversible interaction between glycoprotein Ib-IX-V and VWF adherent on collagen types I, III and VI (Pareti *et al.*, 1987; Rand *et al.*, 1991; 1997), is crucial for slowing the platelets, allowing them to undergo stable binding with other elements of the damaged vessel wall (Moroi *et al.*, 1997; Savage *et al.*, 1998). Collagen not only offers a substrate for adhesion but also induces platelet activation (Alberio and Dale, 1998). At present the major direct platelet collagen receptors are thought to be glycoprotein Ia/IIa and glycoprotein Ib-IX-V complex (Clemetson and Clemetson, 2001). The further platelet receptors glycoprotein IIIb, glycoprotein IV (CD 36) and glycoprotein VI, may also bind collagen (Clemetson and Polgar, 1997; Clemetson and Clemetson, 2001).

High shear stress can also trigger platelet activation, cause release of granules and induce platelet aggregation (Moake *et al.*, 1986; Peterson *et al.*, 1987). High shear stress occurs at arteries partly occluded by atherosclerosis and around artificial valves or circulation-assistant devices, so that shear-stress-induced aggregation may encourage thrombi in these sites (Wu, 1996).

It has been reported that endothelial cells undergoing apoptosis become proadhesive for nonactivated platelets and highly procoagulant (Bombeli *et al.*, 1997; 1999). Also, *in vivo* studies have revealed that nonactivated platelets roll on the endothelium when it is activated with proinflammatory stimuli (Frenette *et al.*, 1995; 1998).

1.1.c. Anti-Coagulant Mechanisms

1.1.c.i. The Central Role of Endothelial cells in the Anti-Coagulant System

Thrombosis rarely occurs in healthy or undamaged vessels because of the thromboresistant activity of endothelial cells, acting upon coagulation, fibrinolysis and platelet aggregation (Hoak *et al.*, 1982; Hubbard *et al.*, 1984; Hawiger, 1987; Radomski *et al.*, 1987a; Dittman and Majerus, 1990; Pearson, 1994b; Schiffrin, 1994; Smith, 1996; Zoellner *et al.*, 1998). Furthermore, the negative surface charge of endothelium repulses the likewise negatively charged surface of platelets while endothelial cells also appear to express an anti-adhesive factor (Sawyer and Srinivasan, 1972; Gimbrone *et al.*, 1990; Wu, 1992). Also, tissue factor pathway inhibitor (TFPI) is released by endothelial cells and blocks the factor VIIa-TF-factor Xa complex (Broze, 1992).

The TM / protein C pathway contributes greatly to the anti-coagulant activity in endothelium (Engelberg, 1989). TM is expressed at the surface of endothelial cells and binds thrombin in a highly specific manner. Thrombin thus bound alters its substrate specificity such that it activates the pro-enzyme protein C. Activated protein C subsequently inactivates the clotting factors Va and VIIIa that have been previously activated by thrombin, as described above in 1.1.b.ii. In addition, activated protein C promotes fibrinolysis by complexing with the fibrinolytic inhibitor plasminogen activator inhibitor-1 (PAI-1) (Sakata *et al.*, 1986; Vervloet *et al.*, 1998). The activity of protein C is greatly enhanced by Protein S, a cofactor synthesised by endothelium (Nawroth *et al.*, 1987). A further anti-coagulant factor released by endothelial cells is heparin sulfate protroglycan, which activates antithrombin III to rapidly inactivate

thrombin or prevent its formation (Engelberg, 1989). The potent anti-platelet aggregatory factors NO and PGI_2 are also released by endothelial cells, so that both the coagulation cascade as well as platelets are inhibited by endothelium (Hoak *et al.*, 1982; Hubbard *et al.*, 1984; Hawiger, 1987; Radomski *et al.*, 1987a; Dittman and Majerus, 1990; Schiffrin, 1994). ADPase / CD39 may also be expressed on the surface of endothelium, providing a mechanism for removal of this potent platelet activator (Cote *et al.*, 1991; Marcus *et al.*, 1991; Yagi *et al.*, 1991). Finally, should the anti-coagulant mechanisms of endothelial cells fail and fibrin form within a vessel, endothelial cells are capable of producing plasminogen activators (PA) to generate active plasmin and lyse the offending clot. Balancing this fibrinolytic potential, however, are plasminogen activator inhibitors (PAI) also synthesised by endothelium (Loskutoff, 1991; Wu, 1992; Wu and Thiagarajan, 1996). The anti-coagulant mechanisms available for endothelial cells are illustrated in Figure 1.4.

1.1.c.ii. The Fibrinolytic System

The fibrinolytic system generates plasmin which proteolytically degrades fibrin in blood clots and thrombi (Wu *et al.*, 1988; Engelberg, 1989; Booth, 1999). Plasminogen is converted to the active fibrinolytic enzyme - plasmin, by the proteolytic action of PA while endothelial cells produce both tissue-type PA (t-PA) and urokinase PA (u-PA) (Levin and Loskutoff, 1982; Philips *et al.*, 1984; Zoellner *et al.*, 1998). u-PA is capable of activating plasminogen in the fluid phase while t-PA is most efficient when attached to fibrin (Booth, 1999). PAI occurs in two forms, designated PAI-1 and PAI-2 and these are capable of inhibiting both t-PA and u-PA (Loskutoff and Curriden, 1990; Wojta *et al.*, 1991; 1993; Zoellner *et al.*, 1993; Kooistra *et al.*, 1994). u-PA can bind a specific cell surface receptor (u-PAR), thus

focusing fibrinolytic activity to the cell surface (Barnathan *et al.*, 1990). u-PAR binds both u-PA and its precursor single-chain form (scu-PA), also known as pro-urokinase, and provides an environment for the conversion of scu-PA to u-PA (Ellis, 1996). Also, a receptor for t-PA has been described (Hajjar *et al.*, 1994). The fibrinolytic system is outlined in Figure 1.5. For this thesis, it will be important to recall that endothelial cells synthesize all of these forms of PA, PAI and PA receptor. Because plasminogen is always present in excess, the rate limiting factor for fibrinolysis is the amount of active PA available. From this, endothelial cells have the capacity to both stimulate and inhibit fibrinolysis, depending upon the balance between PA and PAI released (Hajjar *et al.*, 1987; Hajjar and Nachman, 1988) (Figure 1.5).

Because t-PA activity is maximal when bound to fibin, t-PA appears to play the most important anti-thrombotic role (Wu, 1992). Normal endothelial cells continuously produce and release t-PA to maintain a steady-state blood level of t-PA while this activity is checked by PAI-1. Therefore, maintaining a normal fibrinolytic ability seems to depend upon the balance between endothelial t-PA and PAI-1 production and release (Wu, 1992). However, transgenic and knockout mouse studies indicate that t-PA and u-PA are able to compensate for one another *in vivo* (Carmeliet and Collen, 1995).

1.1.c.iii. Thrombomodulin

TM is a 75 KD integral membrane protein expressed exclusively by endothelial cells and it has been estimated that there are from 30,000 to 100,000 TM molecules on the surface of each endothelial cell (Wu and Thiagarajan, 1996; Sagripanti and Carpi, 2000). TM is a potent activator of protein C which converts the protease thrombin

from a procoagulant to an anticoagulant protein. Apart from modifying the activity of thrombin, TM also acts as a thrombin receptor, thus removing any free thrombin from the circulation (Sagripanti and Carpi, 2000). Since the amount of TM in the capillaries is more than 1000-fold higher than in the major vessels, any thrombin circulating freely in larger vessels is thought to be extracted by TM in smaller vessels (Wu and Thiagarajan, 1996; Sagripanti and Carpi, 2000). Importantly, high levels of TM in the microcirculation allow rapid protein C activation (Esmon, 2001).

1.1.c.iv. Protein C and Protein S

Protein C is synthesised in the liver while the thrombin-TM complex at the endothelial cell surface catalyses the generation of activated protein C (APC) (Wu, 1992; Wu and Thiagarajan, 1996; Sagripanti and Carpi, 2000). APC is a potent anti-coagulant enzyme capable of inactivating factors V and VIII as well as PAI-1 (Pearson, 1994a; Sagripanti and Carpi, 2000). Protein S is synthesised by endothelium, the liver and megakaryocytes and is a vitamin K-dependent glycoprotein. Serving as a cofactor, it enhances the anticoagulant activity of APC to proteolytically inactivate clotting factors V and VIII (Walker, 1980; 1987; Sagripanti and Carpi, 2000). The main features of the TM / protein C anti-coagulant system are outlined in Figure 1.6. In plasma, 30-40% of protein S is free and functionally active. Protein S has high affinity for negatively charged phospholipids, which is important for the ability of protein S to interact with APC on cell membranes (Dahlback, 1994). Clinical studies indicate that the protein C anti-coagulant pathway is a major mechanism in controlling microvascular thrombosis (Dejana, 1987; Stern *et al.*, 1988; Esmon *et al.*, 1997; Esmon, 2000; Grinnell and Joyce, 2001).

1.1.c.v. Endothelial Surface Heparin Sulfate

Endothelial cell surface associated heparin molecules appear to play an important role in maintaining blood fluidity by enhancing thrombin neutralization (Wu, 1992). This is largely via heparin binding to antithrombin III, which is a major plasma serine protease inhibitor while another plasma protease inhibitor activated by heparin has been defined as heparin cofactor II (Wu and Thiagarajan, 1996). Both of these molecules require heparin or its analogues as a cofactor to achieve maximal thrombin binding and inhibition (Wu, 1992; Wu and Thiagarajan, 1996; Sagripanti and Carpi, 2000). It may be important to note that most of the heparin sulfate is in the basal membrane and subendothelial space, while only a relatively small amount of heparin is normally expressed on the luminal endothelial surface helping to maintain a non-thrombogenic surface (Marcum *et al.*, 1984; Gensini *et al.*, 1984; Marcum and Rosenberg, 1985; Kojima *et al.*, 1992a; 1992b).

1.1.c.vi. Inhibition of Platelet Aggregation

Under normal circumstances platelets do not interact with endothelial cells. Thus, the surface of endothelial cells does not promote platelet attachment, although platelets readily adhere to the subendothelial surface exposed when endothelial cells are removed (Gordon, 1986). The negative surface charge of endothelium repulses the likewise negatively charged surface of platelets so that surface charge seems an important anti-adhesive factor (Sawyer and Srinivasan, 1972). Furthermore, endothelial cells actively regulate their membrane properties, release platelet inhibiting factors and inactivate platelet stimuli such as thrombin, so preventing platelet activation and thrombus formation (Heller and Bevers, 1997). The most important endothelial products that influence platelet function are PGI_2 and NO

(Moncada *et al.*, 1991; Luscher, 1993). Both substances are inhibitors of platelet adhesion (Weiss and Turitto, 1979; de Graaf JC *et al.*, 1992) and aggregation, while they are also potent vasodilators. In addition, ecto-ADPase / CD 39 expressed at the luminal endothelial surface is thought to provide a further mechanism inhibiting platelet reactivity (Pearson and Gordon, 1985; Marcus and Safier, 1993). This enzyme hydrolyzes ADP released by activated platelets to AMP, which is further converted to adenosine by 5'-nucleotidase (Luthje, 1989; Zimmermann, 1992). Thus, the expression of ecto-ADPase / CD 39 may be critical for the inhibition of platelet aggregation (Zimmermann, 1992).

1.1.c.vi.1. Prostacyclin

Prostacyclin was discovered in 1976 and is a prostaglandin synthesized mainly by vascular endothelium but also by subendothelial smooth muscle cells (Moncada *et al.*, 1976; Kerins *et al.*, 1991; Vane and Botting, 1995). An alternative name for prostacyclin is PGI_2, while this extremely labile and fast-acting metabolite of arachidonic acid inhibits platelet activation, secretion and aggregation and induces vascular smooth muscle cell relaxation (Moncada and Vane, 1978). Its platelet-inhibitory activities are mediated by binding receptors at the platelet surface, which in turn activate the intracellular enzyme adenylate cyclase, leading to an increase in cyclic adenosine monophosphate (cAMP) levels (Gorman *et al.*, 1977; Tateson *et al.*, 1977). PGI_2 is highly unstable and rapidly degrades to 6-keto-prostaglandin $F_{1\alpha}$ while *in vivo*, enzymatic degradation also occurs to produce 6,15-diketo-13,14-dihydroprostaglandin $F_{1\alpha}$. The metabolic half-life of PGI_2 in the bloodstream is less than one circulation time (Vane *et al.*, 1990). Because of the extreme lability of PGI_2, it is necessary to measure synthesis of this compound through detection of its

degradation products (FitzGerald *et al.*, 1981; Fischer *et al.*, 1982; Koos and Clark, 1982; Watson *et al.*, 1983; Wu, 1992).

PGI_2 is not stored by endothelial cells, but is synthesised de-novo (Piper and Vane, 1971; Bhagyalakshmi and Frangos, 1989; Wu, 1992). Its synthesis is catalyzed by a series of enzymes, the first of which is phospholipase A_2. Phospholipase A_2 is activated by diverse mechanical and chemical stimuli including pulsatile pressure, bradykinin, thrombin, and stimuli released from activated platelets, such as serotonin, platelet-derived growth factor, interleukin-1, and adenine nucleotides (Forsberg *et al.*, 1987; Wu and Thiagarajan, 1996). The arachidonic acid liberated by activated phospholipase A_2 serves as a substrate for prostaglandin H synthase (PGHS), which is also known as cyclooxygenase. PGHS is a bifunctional enzyme with two distinct enzymatic activities: cyclooxygenase catalyzes the oxygenation of arachidonic acid to prostaglandin G_2 (PGG_2) and peroxidase catalyzes the reduction of PGG_2 into prostaglandin H_2 (PGH_2) (Vane *et al.*, 1990; Wu and Thiagarajan, 1996). PGH_2 is the common precursor for synthesis of prostaglandins, prostacyclin and thromboxane and in endothelial cells, PGH_2 is converted primarily to PGI_2 by prostacyclin synthase (Vane *et al.*, 1990; Wu and Thiagarajan, 1996). PGI_2 is the predominant form of the eicosanoids formed during endothelial cell activation *in vitro* (Wu, 1992). The main steps in the synthesis and degradation of PGI_2 are illustrated in Figure 1.7.

Normal endothelium probably produces a constant basal level of PGI_2, as evidenced by urinary excretion its metabolite, 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ (FitzGerald *et al.*, 1983). PGI_2 inhibits platelet aggregation at much lower concentrations than those required to inhibit platelet adhesion to collagen (Higgs *et al.*, 1978). Thus PGI_2 may permit platelets to bind to damaged vascular tissue and facilitate repair of the vessel

wall while at the same time preventing or limiting thrombus formation (Vane and Botting, 1995).

1.1.c.vi.2. Nitric Oxide

NO is a potent intra- and intercellular messenger that has been implicated in a number of diverse physiological processes including regulation of vascular tone, inhibition of platelet aggregation, neurotransmission and host defence (Radomski *et al.*, 1987a; Garthwaite *et al.*, 1988; Hibbs *et al.*, 1988; Vallance *et al.*, 1989). In 1980, Furchgott & Zawadzki discovered that vasodilator action of acetylcholine required the presence of an intact endothelium (Furchgott and Zawadzki, 1980). They showed that binding of acetylcholine to muscarinic receptors on endothelial cells triggers the release of a potent vasodilator that was termed endothelium relaxing factor (Furchgott and Zawadzki, 1980). This was later identified as NO, a surprising finding at the time as it was considered unlikely that such a simple molecule could display such highly specific signaling behaviour (Palmer *et al.*, 1987).

NO is produced from the guanidino nitrogen of L-arginine by the action of enzyme NO synthase (NOS), which is constitutively expressed in endothelial cells and regulated by intracellular calcium levels (Moncada and Higgs, 1993). NO diffuses to nearby cells including vascular smooth muscle and platelets, where it stimulates the intracellular enzyme guanylate cyclase that acts to increase cytoplasmic levels of cyclic guanosine monophosphate (cGMP). The rise in cGMP concentration mediates the relaxation of vascular smooth muscle and inhibition of platelet adhesion, activation, and aggregation (Radomski *et al*, 1987b). *In vivo*, NO appears important in inhibiting vasoconstriction caused by the release of TXA_2 by platelets (Yao *et al.*,

1992). Platelet products including serotonin and ADP/ATP also stimulate endothelial NO release, thus indirectly opposing vasoconstriction and inhibiting platelet function. NO is also released in small amounts by platelets themselves, although it is difficult to assess the relative importance of this autocrine activity (Radomski *et al.*, 1990). NO and prostacyclin act synergistically not only to inhibit platelet adhesion and aggregation, but also to reverse platelet aggregation and this is thought to be important *in vivo* (Radomski *et al.*, 1987d; Wu and Thiagarajan, 1996).

Three isoforms of NOS have been identified and vascular endothelium possesses a constitutive NOS (NOS-III), which shares about 50-60% amino acid sequence identity with neuronal constitutive NOS (NOS-I) and inducible NOS (NOS-II) (Wu, 1995). There are two principal pathways for the formation of NO in endothelial cells. The inducible isoform of NOS (NOS-II) in endothelial cells is expressed only in the presence of bacterial lipopolysaccharids and / or cytokines and does not seem to be subjected to any cellular control mechanisms other than the availability of L-arginine and / or other necessary cofactors (Moncada *et al.*, 1991). The constitutive isoform in endothelium (NOS-III) appears to be activated by a number of different stimuli. In principle, two types of constitutive release of NO from the vascular endothelium can be defined: basal and stimulated release (Busse *et al.*, 1993). The continuous basal release of NO, which keeps the vasculature in a dilated state, represents a sizable portion of total NO releasing capacity of native endothelial cells and failure of the continuous release of NO could contribute to hypertension (Vane *et al.*, 1990; Busse *et al.*, 1993). A stimulus may affect endothelial NO synthase (NOS-III) expression and NO synthesis can be further enchanced by both receptor-dependent agonists in response to receptor dependent factors including acetylcholine, ATP, and bradykinin,

and receptor-independent agonists such as calcium ionophores, polycation or calcium-ATPase inhibitors (Newby and Henderson, 1990; Hecker *et al.*, 1992). Physiological stimuli for NO release above the basal level include platelet products, such as ADP, serotonin, thrombin, shear stress, and pulsatile stretching of the vessel wall (Tada *et al.*, 1975; Pohl *et al.*, 1986; Hutcheson and Griffith, 1991). Figure 1.8 illustrates the main features of NO synthesis and activity. Shear stress in particular, seems to be a major factor regulating NO release. It has been argued that this response to shear stress is important in the regulating vascular tone controling nutritive blood flow to active organs while the agonist-mediated release of NO can be considered as a reserve to satisfy a transient and local requirement for high concentrations of NO, such as in counteracting the formation of microthrombi (Busse *et al.*, 1993). Defective endothelial NO production may contribute to the pathogenesis of essential hypertension (Gryglewski *et al.*, 1988), while diminished NO production also appears to accelerate atherogenesis by allowing unopposed platelet-endothelial interactions and loss of NO-mediated inhibition of vascular smooth muscle proliferation (Dinerman *et al.*, 1993).

1.1.c.vi.3. Ecto-ADPase / CD 39

Besides releasing factors that directly inhibit platelet activation, endothelial cells may control platelet reactivity by degrading or inactivating the platelet agonist ADP. Endothelial cells possesses an enzyme activity that functions on the cell surface to convert ADP released from erythrocytes and activated platelets to adenosine AMP and orthophosphate, thereby limiting platelet recruitment and the extent of platelet aggregation (Marcus *et al.*, 1991). This enzyme is named ecto-ADPase although it also has ATPase activity that dephosphorylates ATP and other nucleotide di-and tri-

phosphates, so that it may be classified as an ATP-diphosphohydrolase or apyrase (Cote *et al.*, 1991; Marcus *et al.*, 1991; Yagi *et al.*, 1991). With reference to the CD classification system for cell surface molecules, ADPase is identical to CD 39 (Kaczmarek *et al.*, 1996; Wang and Guidotti, 1996; Marcus *et al.*, 1997). In CD 39 gene knockout mice, fibrin deposition is increased in multiple organs whereas the bleeding times are also prolonged (Enjyoji *et al.*, 1999). This was shown to be a consequence of desensitization of platelet ADP receptors *in vivo*, which could be reversed by addition of exogenous apyrase to degrade extracellular ADP, confirming the importance of CD 39 in controling platelet activation (Pearson, 1999).

1.2. Endothelial Cell Apoptosis

1.2.a. The General features of Apoptosis

1.2.a.i. The Biological Role of Apoptosis

Apoptosis plays a critical role in the normal development of all multicellular organisms and is important for controlling cell population size in the adult organism. It occurs throughout mammalian development, from the formation of the inner and outer cell mass in the blastocyst to the ongoing cell death in adulthood (Coucouvanis and Martin, 1995; Brison and Schultz, 1997). Larval organ regression during metamorphosis, deletion of interdigital areas, retina development and palatal fusion are good examples of cell population deletion by apoptosis during development (Farbman, 1968; Hammar and Mottet, 1971; Kerr *et al.*, 1974; Penfold and Provis, 1986). Apoptosis has also been described in the immune system where it appears to

play an important role in deletion of lymphocyte clones (Shi *et al.*, 1989). It has been observed in regression of the lactating mammary gland, ovarian follicle atresia, the neonatal adrenal cortex, megakaryocytes, and leukocytes (Wyllie *et al.*, 1973; O'Shea *et al.*, 1978; Radley and Haller, 1983; Savill *et al.*, 1989; Walker *et al.*, 1989). Lymphocytes, such as T cells, K cells and NK cells, can induce apoptosis in target cells (Don *et al.*, 1977; Sanderson and Thomas, 1977; Bishop and Whiting, 1983). Deregulated apoptosis has been implicated as a fundamental pathogenic mechanism in a variety of human diseases. Excessive apoptotic cell death may cause organ atrophy and organ failure, as suggested for neurodegenerative diseases and viral infections. On the other hand, inefficient elimination of malignant, autoreactive, infected or redundant cells may lead to the development of neoplasia, autoimmunity, viral persistence and congenital malformation (Haunstetter and Izumo, 1998). Excessive endothelial and smooth muscle cell apoptosis may also play a role in a variety of cardiovascular diseases including myocardial infraction, heart failure and atherosclerosis (Haunstetter and Izumo, 1998).

1.2.a.ii. The Structural Features of Apoptosis

Apoptosis was first defined strictly on the basis of morphological and ultrastructural features by Kerr *et al.* (1972; 1994). The onset of apoptosis is charatcterised by shrinkage of the cell and nucleus due to loss water (Fadeel *et al.*, 1999). An early event is condensation of nuclear chromatin into sharply delineated masses that become marginated against the nuclear membrane. Later, the nucleus progressively condenses and fragments into small rounded particles, so that fragments of condensed nuclear material may be found in the cellular particles generated during apoptosis (Saraste and Pulkki, 2000). Apoptotic cells detach from their supportive matrix and

become highly convoluted through the formation of blebs and complex cell extensions. These surface blebs eventually detach to form multiple apoptotic particles. Apoptotic bodies are crowded with tightly packed cellular organelles and fragments of nuclear material. Organellar structures are well preserved, indicating maintained homeostatic activity in apoptotic particles (Kerr *et al.*, 1972; 1994; Saraste and Pulkki, 2000).

In vivo, apoptotic bodies are specifically recognised and rapidly phagocytised by neighbouring cells including macrophages and parenchymal cells. Apoptotic shrinkage, disassembly into apoptotic bodies and engulfment of individual cellular fragments characteristically occur without associated inflammation. This differs from necrosis in which release of intracellular contents into the tissues elicits an inflammatory response (Saraste and Pulkki, 2000).

Necrosis occurs when cells are unable to maintain homeostasis due to some critical cellular injury, such as hypoxia, hyperthermia, exposure to exogenous toxins, or attack by complement (Israels and Israels, 1999). Necrotic cell death can be distinguished from apoptosis on the basis of morphological and ultrastructural changes (Searle *et al.*, 1982; Compton, 1992; Israels and Israels, 1999). Necrosis is characterized by early mitochondrial swelling and rupture, detachment of ribosomes from the rough endoplasmic reticulum (RER), loss of plasma membrane integrity, cell swelling and eventual cellular disintegration. Necrotic cells do not fragment into small apoptotic bodies but instead may rupture with spillage of cytoplasmic contents and inflammation *in vivo* (Israels and Israels, 1999; Mostafapour *et al.*, 1999).

When isolated cultured cells undergo apoptosis, the resulting fragments are so numerous that the surviving cells are unable to remove them by phagocytosis. Despite the maintained haemostasis of apoptotic bodies, eventually the homeostatic capacity of apoptotic particles is overwhelmed by the progressive biochemical degradation and the apoptotic bodies become necrotic. This necrotic change in apoptotic bodies is a common culture artifact and has been termed secondary necrosis (Kerr *et al.*, 1972; Savill *et al.*, 1993; Kerr *et al.*, 1994; Shah *et al.*, 1997). It is important to appreciate the artifactual nature of secondary necrosis as well as to recognize the difference between this and true necrosis which may occur both *in vitro* and *in vivo*.

1.2.a.iii. Non Morphological Methods for Detecting Apoptosis

A widely accepted biochemical marker of apoptosis is internucleosomal cleavage of DNA to produce a 180 to 200 base pair ladder in DNA gel electrophoresis (Kerr *et al.*, 1972; Wyllie, 1980; 1984; Arends *et al.*, 1990; Vaux and Strasser, 1996; Poirier J., 1997; Martins and Earnshaw, 1997). This differs significantly from events in necrosis, in which DNA breakdown is random and seen as a smear after electrophoresis. The internucleosomal DNA fragmentation is almost always detected when the morphological changes of apoptosis are recognised (Wyllie, 1980; Arends *et al.*, 1990). DNA cleavage can also be detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL), in which dUMP is attached to the 3' end of genomic DNA by TdT. Positive cells are visualised by fluorescent dyes conjugated to dUTP or using enzyme mediated chromogenic systems (Gavrieli *et al.*, 1992). An alternative technique, is in situ end-labeling (ISEL), in which the Klenow fragment of *Escherichia coli* DNA polymerase I is used to

recognize only 3' recessed ends, and this differs from the activity of TdT which recognises all types of free 3' DNA ends although a higher sensitivity is reported for TUNEL as compared with ISEL (Wijsman *et al.*, 1993; Mundle *et al.*, 1995). However, it is important to be cautious in interpreting TUNEL or ISEL results. This is because exposure of 3' DNA ends occurs not only in apoptosis but also during necrosis so that these methods are only meaningful in context of clearly apoptotic morphology (Eastman and Barry, 1992; Gold *et al.*, 1994). Sensitivity for the detection of DNA fragmentation in individual cultured cells may be increased as compared with TUNEL and ISEL by use of the "comet assay" (Fairbairn *et al.*, 1995; Krown *et al.*, 1996). In this assay, cells suspended and lysed in agarose are subjected to gel electrophoresis. Fragmented DNA migrates out of the nucleus and forms a characteristic comet tail. Although this technique displays a high sensitivity for fragmented DNA, it does not provide definite proof of the internucleosomal DNA cleavage characteristic of apoptosis. This is because the migration of Okazaki fragments from cells in S-Phase as well as of fragmented material from necrotic cells produces false positive comet results for apoptosis (Lazebnik *et al.*, 1994). A further effective method for detecting fragmentation of DNA in populations of cells is fluorescence activated cell scanning (FACS) analysis of cells in which DNA of has been labelled with a fluorescent DNA marker such as propidium iodide or ethydium bromide. In this method, small cellular particles with reduced DNA content can be detected and displayed in the same way as a cell cycle histogram to reveal a sub-diploid "apoptotic" peak (Darzynkiewicz *et al.*, 1992).

Phosphatidylserine is normally only found on the inner plasma membrane surface. During apoptosis, however, this phospholipid is transferred to the outer plasma

membrane leaflet. Because of this, surface binding by the phosphatidylserine specific ligand, annexin V may be used to detect apoptosis and has been suggested as a marker for the early phases of apoptosis (Savill, 1998). In addition, specific cleavage of cellular target proteins, such as poly (ADP)-ribosylating protein, has been used to detect apoptosis (Lazebnik *et al.*, 1994; Nicholson and Thornberry, 1997; Porter *et al.*, 1997). Detection of protein fragments by Western blotting has been used to verify apoptotic cell death in cell culture models of apoptosis (Haunstetter and Izumo, 1998). Caspases are proteinases thought to be important in apoptosis and detection of these active enzymes has also been used to verify apoptosis (Durrieu *et al.*, 1998; Poot and Pierce, 1999; Bedner *et al.*, 2000; van Heerde *et al.*, 2000).

1.2.a.iv. Mechanisms Mediating Apoptosis

Apoptosis can be initiated by a wide variety of stimuli, including developmental signals, cellular stress and disruption of the cell cycle. In contrast, execution of apoptosis is remarkably uniform involving characteristic morphological and biochemical changes. Genetic studies on the nematode *Caenorhabditis elegans* led to identification of three genes essential for control and execution of apoptosis in developing worms (Hengartner and Horvitz, 1994). Subsequently, mammalian homologs of all these genes with seemingly similar functions were identified. Three main pathways of apoptotic signaling have been identified. The first is initiated by the withdrawal of growth factors and seems regulated by the Bcl-2 family of proteins. This pathway results in cytochorme c release from mitochondria, activation of apoptotic protease activating factor-1 (Apaf-1) and triggering of the caspase cascade. The second well-established apoptotic pathway involves signaling by cell surface death receptors such as tumour necrosis factor receptor or Fas, which through adapter

molecules can recruit and activate caspases. The third and least well characterized pathway is initiated by DNA damage. Although in part regulated by proteins such as p53 and ATM, it is not clear how this pathway results in caspase activation. In all three of these cell death pathways, caspases and members of the Bcl-2 protein family play a key role in the regulation and execution of apoptosis (Dragovich *et al.*, 1998).

Bcl-2 family proteins are important in regulating apoptosis, as members of the mammalian Bcl-2 protein family are shown to mediate both pro-apoptotic and anti-apoptotic events (Vaux *et al.*, 1988; Inohara *et al.*, 1997). Sixteen members of this family have been recognized: some, including Bcl-2 and Bcl-X_L, are apoptosis-inhibitory proteins, while others, such as Bax, Bad and Bid, are promoters of apoptosis (Israels and Israels, 1999). Homologous domains in these proteins permit the formation of homodimers or heterodimers to tip the cell either towards or against initiation of apoptosis. High expression of the Bax group promotes apoptosis while high expression of members of the Bcl-2 group inhibit apoptosis. An excess of Bax promotes cell death, but co-expression of Bcl-X_L or Bcl-2 can neutralized this effect and p53 probably functions by regulating the ratios of the Bax/Bax, Bax/Bcl-2 and Bcl-2/Bcl-2 groups (Israels and Israels, 1999). Most Bcl-2 family proteins have a conserved C-terminal hydrophobic domain that is necessary for insertion into outer mitochondrial, endoplasmic reticular and outer nuclear membranes, while the function of Bcl-2 is strongly related to the regulation of protein translocation from the mitochondral intermembrane space into the cytosolic compartment (Haunstetter and Izumo, 1998). The Bax proteins reside in the cytosol; upon receipt of apoptotic signals, Bax proteins migrate and bind to mitochondrial membrane "permeability transition pores," inducing loss of selective ion permeability. As a result of

membrane changes, there is release into the cytosol of the contents of the intermembrane space, including cytochrome c and apoptosis-inducing factor (AIF) (Yang *et al.*, 1995; Vander *et al.*, 1997; Israels and Israels, 1999). Then AIF moves directly to the nucleus, where it is associated with chromatin condensation and nuclear fragmentation, while cytosolic cytochrome c sets in motion the terminal events of apoptosis. Cytochrome c is the only known activator of cytoplasmic protein Apaf 1. Binding of cytochrome c to Apaf-1 is necessary for the subsequent activation of procaspase-9. Caspase-9 activates downstream caspases, including procaspase-3, responsible for the cytological changes characteristic of apoptosis (Hu *et al.*, 1998; Israels and Israels, 1999). In addition, caspase may act on mitochondrial membranes with further release of cytochrome c and of some intramitochondrial procaspases. Thus localization of members of the Bcl-2 family to the mitochondria as well as the role of cytochrome c in apoptosis have pointed toward a central role for mitochondria in apoptosis. Overexpression of either Bcl-2 or Bcl-X_L not only prevents cell apoptosis via neutralization of Bax but also inhibits cytochrome c release from mitochondria (Yang *et al.*, 1995; Vander *et al.*, 1997; Dragovich *et al.*, 1998; Reed, 1998).

The caspases are cysteine proteases that cleave their substrate proteins specifically at aspartate residues. They are formed constitutively and are normally present as inactive proenzymes. For the induction of full enzymatic activity, they require cleavage at specific internal aspartate residues, which separate large and small subunits from each other (Thornberry and Lazebnik, 1998). Studies on substrate specificity, prodomain structure and biological function indicate that caspases are activated during apoptosis in a self-amplifying cascade (Martin and Green, 1995;

Srinivasula *et al.*, 1996; Thornberry *et al.*, 1997; Hirata *et al.*, 1998; Thornberry and Lazebnik, 1998; Slee *et al.*, 1999). Activation of the upstream caspases, such as caspases 2, 8, 9 and 10, by pro-apoptotic signals leads to proteolytic activation of downstream or effector caspases 3, 6 and 7. However, proteolysis by caspases is restricted and limited in producing disassembly of the protein without general proteolysis. Caspase activity results in cleavage of cytoskeletal proteins, disruption of the nuclear membrane, disruption of cell-cell contact and the freeing of DNA nuclease, caspase-activated deoxyribonuclease from its associated protein inhibitor to allow DNA fragmentation. The restricted proteolysis does not result in cellular lysis but rather in the formation of membrane-bound sealed apoptotic bodies. These irreversible proteolytic events are responsible for the morphological changes characteristic of apoptotic cells and the DNA agarose gel pattern that is the signature of apoptosis (Israels and Israels, 1999; Saraste and Pulkki, 2000). Caspase activation appears to take place either within death receptor complexes of the cytoplasmic membrane or by a mitochondrion-dependent mechanism within the cytosol. The death receptor pathway for the initiation of apoptosis involves the binding of extracellular death signal proteins to their cognate cell surface receptors, such as Fas and TNF receptors. The death receptors contain a distinct cytoplasmic domain that is critical for their proapoptotic function (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). Given its importance in the transmission of proapoptotic signals, this domain was designated the "death domain". Caspase 8 is the most apical caspase in this pathway (Srinivasula *et al.*, 1996; Hirata *et al.*, 1998). The mitochondrial pathway integrates apoptotic signals caused by various cytotoxic agents, aberrant oncogene expression and p53 (Fearnhead *et al.*, 1998; Soengas *et al.*, 1999). The crucial step for this mechanism of caspase activation is the release of cytochrome c and other caspase

activators, such as AIF, into the cytosol from the miochondrial transmembrane space (Liu *et al.*, 1996; Kluck *et al.*, 1997; Kroemer *et al.*, 1997; Green and Reed, 1998).

A family of caspase inhibitors, inhibitors of apoptosis (IAPs), selectively inhibit effector caspases, thus blocking the apoptosis process and these are over-expressed in many malignant cells. The IAP family also inhibits apoptosis through non-caspase dependent mechanisms by modulation of transcription factors and by involvement in cell-cycle control (Israels and Israels, 1999).

1.2.b. Apoptosis in Endothelial Cells

Endothelial apoptosis is clearly important for microvascular remodelling and has been observed during tissue remodeling in a variety of biological settings including the involuting breast, pressure atrophy of the parotid gland, wound healing, the cycling ovary and the regressing uterus (Walker and Gobe, 1987; Walker *et al.*, 1989; Augustin *et al.*, 1995; Meeson *et al.*, 1996; Sgonc *et al.*, 1996; Darby *et al.*, 1997; Desmouliere *et al.*, 1997; Tatarczuch *et al.*, 1997). Apoptosis of endothelial cells has also been implicated in several human diseases such as atherosclerosis, hypretensive microvasculopathy, diabetes and development of metastasis (Mizutani *et al.*, 1996; Gobe *et al.*, 1997; Dimmeler *et al.*, 1998; Kebers *et al.*, 1998).

1.2.b.i. Factors Stimulating Endothelial Apoptosis

A number of stimuli can induce endothelial apoptosis by: loss of survival factors; modulation of survival pathways; or induction of pro-apoptotic pathways. Examples of pro-apoptotic factors which inhibit survival pathways in endothelium are tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). These cytokines suppress

endothelial cell $\alpha_v\beta_3$ integrin activity leading to a decreased $\alpha_v\beta_3$ -dependent endothelial cell adhesion and survival (Ruegg *et al.*, 1998). TNF-α also markedly increases endothelial cell apoptosis via activation of caspase 3 and this is abrogated by caspase inhibitors or by shear stress (Dimmeler *et al.*, 1996). Transforming growth factor β1 (TGF-β1), bacterial lipopolysaccharide (LPS), ionizing radiation and oxidized low density lipoprotein may also initiate endothelial cell apoptosis (Tsukada *et al.*, 1995; Lizard *et al.*, 1996; Dimmeler *et al.*, 1997; Escargueil-Blanc *et al.*, 1997; Lindner *et al.*, 1997).

1.2.b.ii. Factors Inhibiting Endothelial Apoptosis

Cell-to-cell and cell-to-matrix contacts are necessary for maintenance of endothelial cell survival (Meredith *et al.*, 1993; Brooks *et al.*, 1994; Re *et al.*, 1994; Zoellner *et al.*, 1996b; Levkau *et al.*, 1998; Scatena *et al.*, 1998). Loss of cell-to-cell contact leads to the activation of a default death programme in a variety of cell types. Growth factors, such as vascular endothelial growth factor, fibroblast growth factor-2 and angiopoietin-1, also have anti-apoptotic activity through inhibition of stimulated apoptosis or alternatively, by acting as survival signals for endothelium (Alon *et al.*, 1995; Haimovitz-Friedman *et al.*, 1997; Karsan *et al.*, 1997; Fujikawa *et al.*, 1999; Hayes *et al.*, 1999; Kwak *et al.*, 1999). Variations in blood flow play an important role in vessel growth and regression. Shear stress abrogates endothelial cell apoptosis induced by different stimuli and shear stress-mediated inhibition of endothelial cell apoptosis might contribute significantly to the functional integrity of the endothelial cell monolayer (Dimmeler *et al.*, 1996). However, since endothelial cells can be cultured in static conditions, the role of shear stress in inhibiting endothelial apoptosis appears to be secondary to other more potent factors. It seems that chemical plasma

factors have a predominant role over shear stress in regulating endothelial apoptosis (Araki *et al.,* 1990a; Zoellner *et al.,* 1996b; 1999). Withdrawal of serum results in rapid endothelial apoptosis, so it is clear that plasma contains some anti-apoptotic factors and serum albumin appears to be one of the serum factors protecting endothelial cells in this way (Zoellner *et al.,* 1996b; 1999). It has been argued that in the microcirculation, redundant vessels are removed when plasma flow ceases, depriving the endothelial cells of a supply of anti-apoptotic serum factors (Zoellner *et al.,* 1996b; 1999) while *in vivo* observations indicating degeneration of poorly perfused vessels supports this suggestion (Sandison, 1928; Lang and Bishop, 1993; Lang *et al.*, 1994; Meeson *et al.*, 1996; 1999).

If cultured endothelial cells are deprived of adhesion, they rapidly become apoptotic and this has been argued as having biological relevance with regard to cells detached into the circulation by trauma. Importantly, serum factors including albumin seem unable to rescue endothelium from apoptosis in the absence of adhesion, suggesting that endothelial survival is dependent upon the dual signals of successful cell adhesion and the presence of plasma factors (Meredith *et al.*, 1993; Zoellner *et al.*, 1996b).

1.2.b.iii. The Role of Endothelial Apoptosis in Vascular Remodelling

Vascular remodelling is characterized by a reorganization of blood vessel geometry in response to physiological alterations in blood flow or to pathophysiological stimuli. Because of its strategic location, the endothelium should serve as the primary mediator of flow-mediated mechanotransdction to initiate vascular remodelling (Dimmeler and Zeiher, 2000). Using implanted transparent chambers in rabbit ears to observe vascular changes in the formation of reparative granulation tissue and

subsequent vascular regression during maturation to scar tissue, Sandison in 1928 recorded the appearance of new blood vessels and the subsequent degeneration of seemingly excess vessels in numerous elegant camera-lucida diagrams (Sandison, 1928). In these experiments, he recognized that endothelial cells in apparently excess vessels were removed by a degenerative process and this was clearly associated with low levels of blood flow in vessels destined to degenerate (Sandison, 1928). This was later confirmed by others in their *in vivo* works (Lang and Bishop, 1993; Lang *et al.*, 1994; Meeson *et al.*, 1996; 1999). It now seems clear that endothelial cells in poorly perfused vessels are deprived of critical anti-apoptotic signals.

Physiological remodelling of blood vessels before and after birth has been shown to be the result of a balance between apoptosis and cell proliferation. Developmentally programmed capillary regression in the developing eye is initiated by macrophage-mediated endothelial cell apoptosis (Lang and Bishop, 1993). The dying cells project into the capillary lumen causing temporary or permanent blockage to blood flow. Following cessation of flow, secondary synchronous apoptosis of vascular endothelial cells occurs, leading to capillary regression (Meeson *et al.*, 1996). It can be hypothesised that during development, the selection of capillary beds is under the control of blood flow-dependent endothelial cell survival and apoptosis (Mallat and Tedgui, 2000).

1.2.c. The Possible Micro-Thrombotic and Micro-Embolic Activity of Apoptotic Endothelium

1.2.c.i. Detachment of Endothelial Cells During Trauma and Apoptosis

One of the features of endothelial apoptosis is rapid detachment (Araki *et al.,* 1990a; Zoellner *et al.,* 1996b; 1999), so that apoptotic endothelial particles seem likely to enter the circulation during microvascular remodelling. Traumatic vascular injury may also result in detachment of endothelial cells from their adhesive surface (Lai *et al.*, 1996) while it is known that endothelial cells become apoptotic if deprived of matrix adhesion (Meredith *et al.*, 1993; Zoellner *et al.*, 1996b; Solovey *et al.*, 1997). Supporting this idea are several studies which report elevated levels of circulating endothelial cells in the blood after injury (Lai *et al.*, 1996). From this, it seems that there are at least two mechanisms whereby apoptotic endothelium may enter the circulation, either from microvascular remodelling or alternatively, following traumatic detachment of previously non-apoptotic cells.

1.2.c.ii. The Possible Accumulation of Apoptotic Endothelium in the Microvasculature of the Lungs and Elsewhere

Endothelial integrity is maintained in order to preserve efficient thromboresistance and macromolecular barrier functions of the vessels. Detachment of apoptotic endothelial cells has the potential to expose the subendothelial matrix, which may initiate platelet attachment and blood coagulation (Section 1.1.b.i). It seems likely that endothelial cell apoptosis would result in local micro-thrombosis *in vivo* which would activate further apoptosis of surviving endothelial cells throughout the affected vessel. Furthermore, as indicated in 1.1.c, endothelial cells have potent anti-

thrombotic activities and loss of these activities during apoptosis in microvascular remodelling or after traumatic detachment would be expected to encourage the formation of microthrombi about apoptotic endothelial particles. Such microthrombi would exacerbate the difficulty posed by the relatively large size of endothelial cells in passing through the comparatively small capillaries of tissues, with microembolism a likely consequence. The organ most likely to suffer from such micro-embolic events is the lung, as this single organ effectively filters the blood from the entire remainder of the body on each circulatory passage while it is from these peripheral sites that traumatic endothelial detachment during movement or frank traumatic injury is most likely.

The potential of microemboli to cause biologically meaningful damage is illustrated by the disease of sickle cell anaemia, in which microvascular infarction by aggregated red blood cells can produce clinically significant ischaemia while in disseminated intravascular coagulation and adult respiratory distress syndrome, micro-infarction is by microthrombi (Cotran *et al.*, 1999). With respect to this, it is interesting to note that acute respiratory distress syndrome may be the consequence of massive trauma and it is tempting to speculate that endothelial particles may contribute to this.

1.2.c.iii. Canalicular Fragmentation: A Unique Ultrastructural Feature of Apoptosis in Endothelium

The formation of large numbers of tunnel like canaliculi by infolding of the plasma membrane is an apparently unique ultrastructural feature of apoptosis in endothelial cells and this has been termed canalicular fragmentation (Zoellner *et al.*, 1996a; 1999). This has been observed for cultured endothelium from both macrovascular and

microvascular sources as well as in both human and animal apoptotic microvascular endothelial cells in tissues (Zoellner *et al.,* 1996a; 1999). The honeycomb structures produced appear to make apoptotic cells so fragile that they readily fragment into smaller particles. Although this mechanical effect has not been experimentally established, it has been speculated that canalicular fragmentation facilitates rapid mechanical disintegration of apoptotic endothelial cells and that this is an adaptation to minimize the micro-embolic potential of such particles (Zoellner *et al.,* 1996a).

1.2.c.iv. Maintained Fibrinolytic Proteins in Apoptotic Endothelium

Cultured human macrovascular and microvascular endothelial cells maintain u-PA levels during apoptosis, despite massive proteolysis, and this is not due to compensatory synthesis of new u-PA during apoptosis or binding to u-PAR (Zoellner *et al.*, 1998). It appears that u-PA is in some way protected from degradation during endothelial apoptosis and it has been argued that this may represent a specific adaptation in endothelium to minimise the development of micro-thrombi around apoptotic endothelial cells both at sites of apoptosis as well as in circulating apoptotic endothelial particles (Zoellner *et al.*, 1998). This observation is consistent with the induction of u-PA by TNF-α, which also causes endothelial apoptosis (Robaye *et al.*, 1991; Wojta *et al.*, 1993). This interpretation is also consistent with the suggested role of canalicular fragmentation in minimising the micro-embolic potential of apoptotic endothelial particles (Zoellner *et al.,* 1996a).

1.2.c.v. Reports of Platelet Binding and Pro-coagulant Changes in Apoptotic Endothelium

Although endothelial cells normally maintain an anti-coagulant surface, these cells are also capable of stimulating coagulation in some circumstances (Muller and Griesmacher, 2000). The stimuli inducing procoagulant activities of vascular endothelium may also be able to cause endothelial cell death. During the work described in this thesis, two papers were published by Bombeli *et al.* (Bombeli *et al.*, 1997; 1999) which described the interactions between endothelial cells and blood clotting systems. These reports both concluded that apoptotic endothelial cells posses pro-coagulant activities. In particular, it was found that during endothelial apoptosis there was redistribution of phosphatidylserine to the cell surface to support increased tenase activity while there was also reduced surface heparin expression. Also, these workers demonstrated reduced thrombomodulin activity, lower levels of TFPI and elevated thrombin generation. In addition, tissue factor activity appeared to increase in apoptotic endothelium, but only when cells were stimulated with LPS (Bombeli *et al.*, 1997). Further work demonstrated platelet binding to apoptotic endothelium, as well as a low level of platelet aggregation as evidenced by the limited release of β-thromboglobulin and platelet factor 4 (Bombeli *et al.*, 1999). Binding of apoptotic endothelium was mediated by β_1-integrins expressed by the platelets, although the endothelial cell ligand(s) remains unidentified (Bombeli *et al.*, 1999). In addition to these papers by Bombeli *et al.*, papers were also published describing increased tissue factor activity in apoptotic endothelium (Casciola-Rosen *et al.*, 1996; Greeno *et al.*, 1996), supporting the idea that apoptotic endothelium is essentially pro-coagulant, although the biological significance of these findings has not as yet been established.

1.3. Questions Arising from the Literature and Addressed in This Thesis

Although the micro-embolic and micro-thrombotic potential of apoptotic endothelium may be biologically important, it is still not clear from the literature if apoptotic endothelium maintains a primarily pro or anti-thrombotic potential. On the one hand, increased tissue factor, tenase activity and platelet binding in apoptotic endothelium implies increased micro-thrombotic potential (Casciola-Rosen *et al.*, 1996; Greeno *et al.*, 1996; Bombeli *et al.*, 1997; 1999) as does reduced heparin and TFPI (Bombeli *et al.*, 1997), while on the other hand, maintained u-PA expression suggests the ability to remove any fibrin which may form around apoptotic endothelium (Zoellner *et al.*, 1998).

Because platelets play a central role in blood clotting, this thesis investigated the effect of apoptotic endothelium upon the aggregatiory activity of platelets. Importantly, even when during this experimental work a manuscript was published by others describing the binding of platelets to apoptotic endothelium, no data were presented relating to the effect of this upon the critical biological activity of platelet aggregation. Interestingly, low levels of platelet activation were seen, but no comment was made regarding the failure of platelet activation to continue despite having been initiated (Bombeli *et al.*, 1999). This fundamental question is addressed in the first two experimental chapters, Chapters 2 and 3, of this thesis.

Separate to this issue, but nonetheless related to the question of microembolism, is the biological significance of canalicular fragmentation in apoptotic endothelium. Since the role of canalicular fragmentation in mechanically reducing the size of apoptotic

endothelial particles was only assumed in earlier work (Zoellner *et al.,* 1996a), it was considered important to study the size and shape of endothelial cells during apoptosis and this is the question addressed in the third experimental chapter of this thesis, Chapter 4.

By pursuit of these two separate but related questions, this thesis further investigates the micro-thrombotic and micro-embolic potential of apoptotic endothelium.

Figure 1.1. Diagram outlining blood clotting. Injury damages vessels, exposing the subendothelial matrix. Platelets bind collagen and other matrix components and subsequently become activated. During platelet activation, serotonin and thromboxane A_2 (TXA_2) are released and stimulate smooth muscle cell contraction with the effect that there is reduced blood flow. This reduces stress upon the primary haemostatic plug formed by aggregating platelets. TXA_2 also stimulates platelet aggregation, as does ADP also released by activated platelets. Tissue factor is released by injured cells and this initiates the blood coagulation cascade. Blood coagulation is supported by platelet phospholipid so that the local concentration of thrombin close to the platelet plug is greatly elevated. Thrombin cleaves fibrinogen which then auto-polymerises to form fibrin. Fibrin stabilizes the primary haemostatic platelet plug to form the final stable haemostatic plug. It should be noted that coagulation refers to the proteolytic coagulation cascade initiated primarily by tissue factor while clotting describes the combined effect of platelet activation and coagulation.

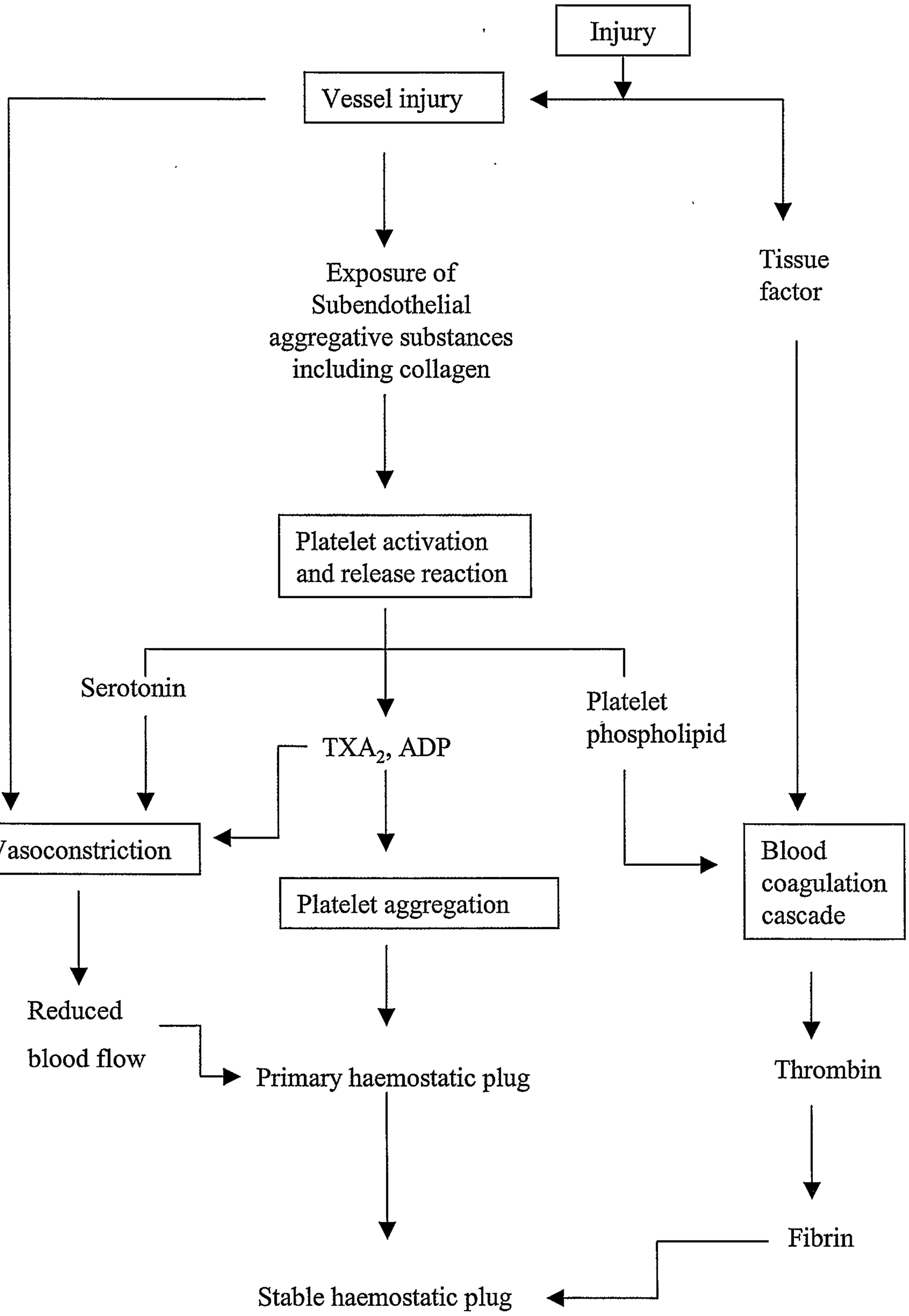

Injury
Vessel injury
Exposure of
Subendothelial
aggregative substances
including collagen
Platelet activation
and release reaction
Tissue
factor
Serotonin
TXA_2, ADP
Platelet
phospholipid
Vasoconstriction
Platelet aggregation
Blood
coagulation
cascade
Reduced
blood flow
Primary haemostatic plug
Thrombin
Fibrin
Stable haemostatic plug

Figure 1.2. Diagram outlining the main features of the blood coagulation system.

The extrinsic system is initiated by tissue factor from injured cells. This converts factor VII to VIIa which then cleaves factor IX to produce IXa. IXa cleaves factor X to form Xa, and both of these cleavages are facilitated by the presence of Ca^{2+} and phospholipid primarily from activated platelets (PL). Xa cleaves prothrombin to produce active thrombin which subsequently generates fibrin monomer from fibrinogen. Polymerisation of fibrin is followed by cross-linking by XIIIa, which is produced by thrombin cleavage of factor XIII. Amplification of this process is mediated through several mechanisms and these are indicated with dotted lines in the diagram. Thrombin plays an important role in amplification through activation of factors V, VIII and XI which each accelerate defined stages of the cascade as indicated in the diagram. Also, factor Xa amplifies the cascade through further activation of factor VII. Factor XII is activated by surface contact, and initiates the intrinsic clotting cascade. However, it appears that this is not physiologically critical, as patients lacking this clotting factor seem largely unaffected. Instead, factor XII plays a more important role linking the coagulation cascade with the kinin cascade through interaction with prekallikrein (PK) and high molecular weight kininogen (HK).

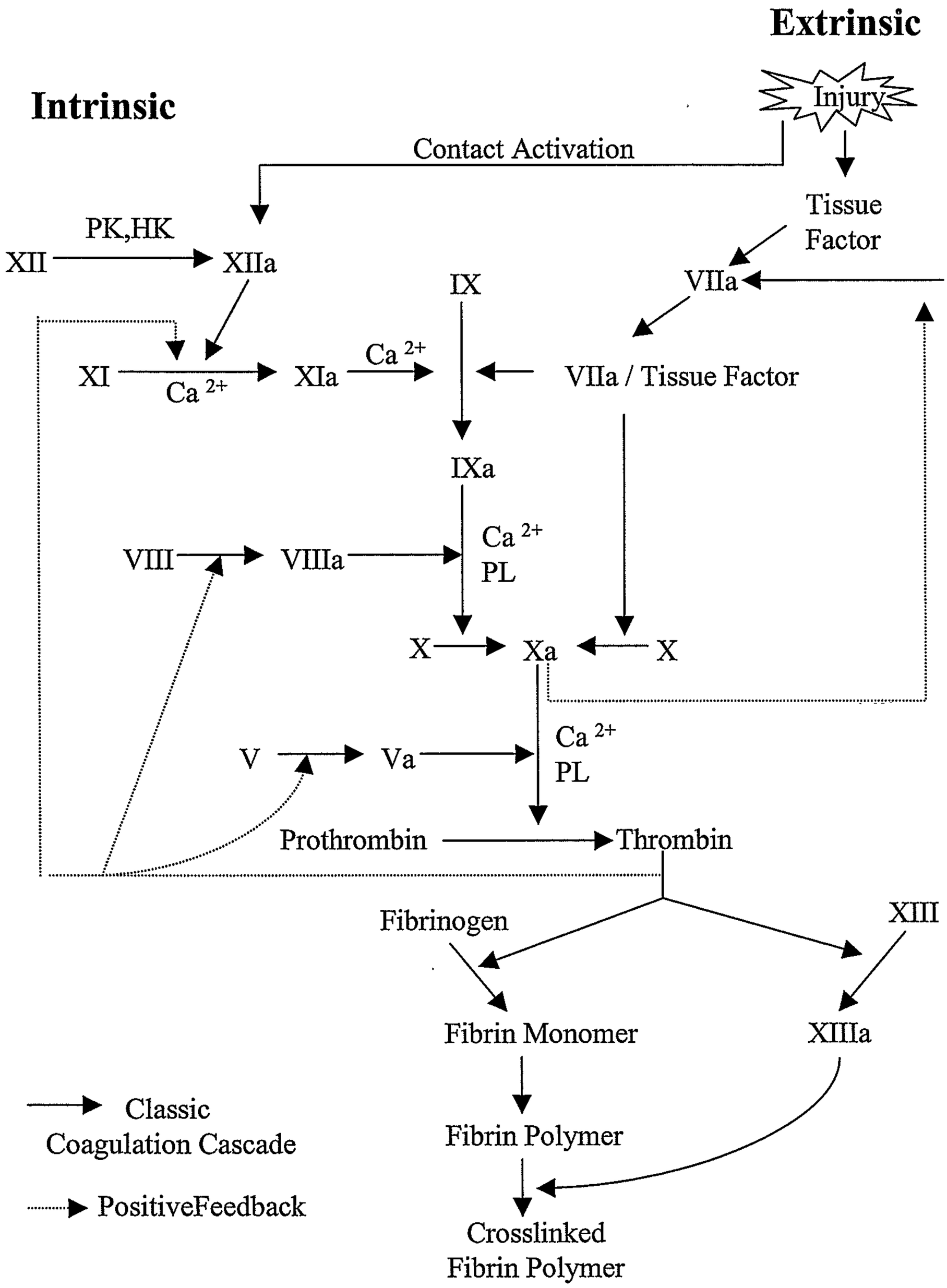
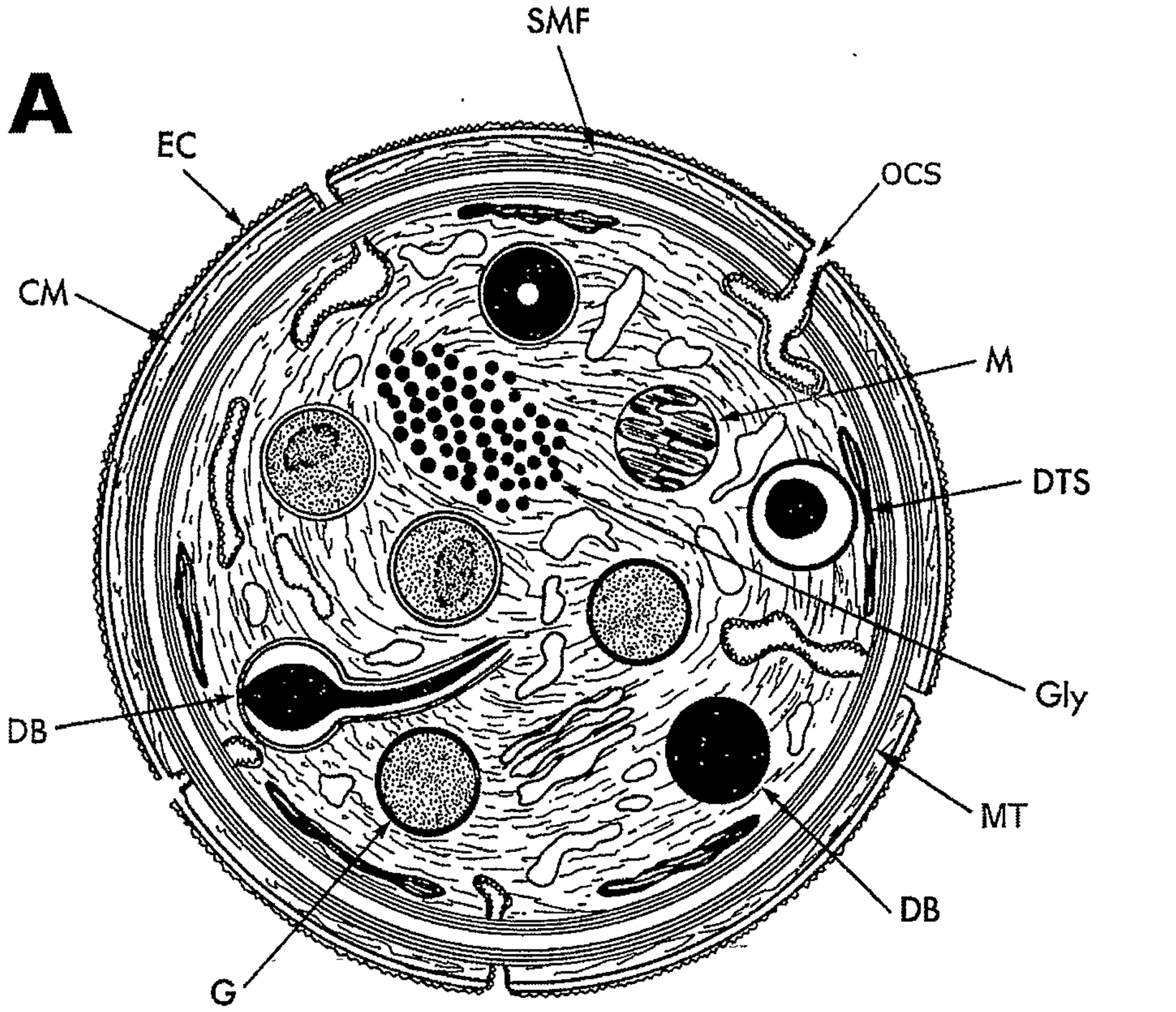

Extrinsic
Intrinsic
Injury
Contact Activation
Tissue Factor
PK,HK
XII
XIIa
VIIa
VII
IX
XI
Ca 2+
XIa
Ca 2+
VIIa / Tissue Factor
IXa
VIII
VIIIa
Ca 2+
PL
X
Xa
X
V
Va
Ca 2+
PL
Prothrombin
Thrombin
Fibrinogen
XIII
Fibrin Monomer
XIIIa
Classic
Coagulation Cascade
PositiveFeedback
Fibrin Polymer
Crosslinked
Fibrin Polymer

Figure 1.3. Diagrammatic representation of the ultrastructure of platelets in an equatorial plane (A), and in cross section (B). Components of the peripheral zone include the exterior coat (EC), trilaminar unit membrane (CM), and submembrane area containing specialised filaments (SMF) that form the platelet wall and line channels of the surface-connected open canalicualr system (OCS). The matrix of platelet cytoplasm is defined as the sol-gel zone and contains active microfilaments, structured filaments, the circumferential band of microtubules (MT) and glycogen (Gly). Organelles are embedded in the sol-gel zone and include mitochondria (M), granules (G) and electron-dense bodies (DB) while some alpha-granules appear to contain tubular structures (GT). The membrane systems include the OCS and the dense tubular system (DTS). Occasionally, Golgi apparatus (GZ) is found in some platelets. (Redrawn from White JG: Am J Clin Pathol 71: 363, 1979).


A
SMF
EC
OCS
CM
M
DTS
Gly
DB
MT
DB
G

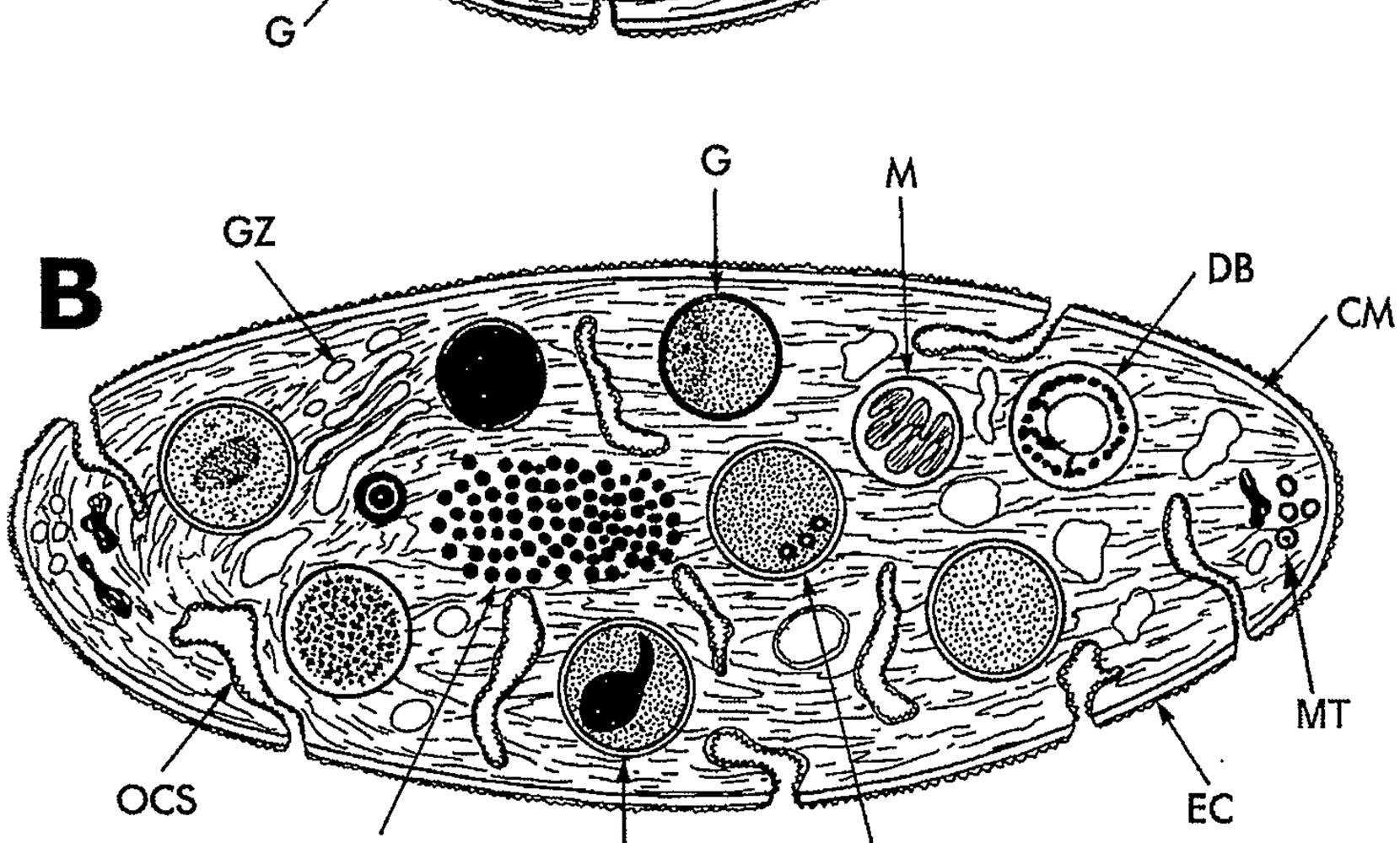

B
G
M
GZ
DB
CM
MT
EC
OCS
Gly
G + DB
GT

Figure 1.4. Diagram outlining the anti-thrombotic activities of endothelial cells. Endothelial cells have several specific cell products which actively inhibit thrombosis. Firstly, endothelial cells contain both forms of plasminogen activator (A) (t-PA and u-PA), which are both able to cleave plasminogen to generate active plasmin which in turn degrades any fibrin which may form at the cell surface. Also, should thrombin be formed at the cell surface, binding to thrombomodulin (B) activates protein C with the effect that factors Va and VIIIa are degraded while PAI-1 is inactivated. This is accelerated by the release of protein S, also produced by endothelium (C). Heparin (D) bound to the cell surface activates anti-thrombin III (AT-III) to inhibit thrombin activity. In addition, platelet activation is inhibited by the release of PGI_2 (E) and NO (F) while the platelet derived platelet agonist ADP is inactivated by ADPase (CD39) (G) to produce AMP, depriving platelets of an important autocrine agonist. In addition, the tissue factor pathway is inhibited by TFPI (H).

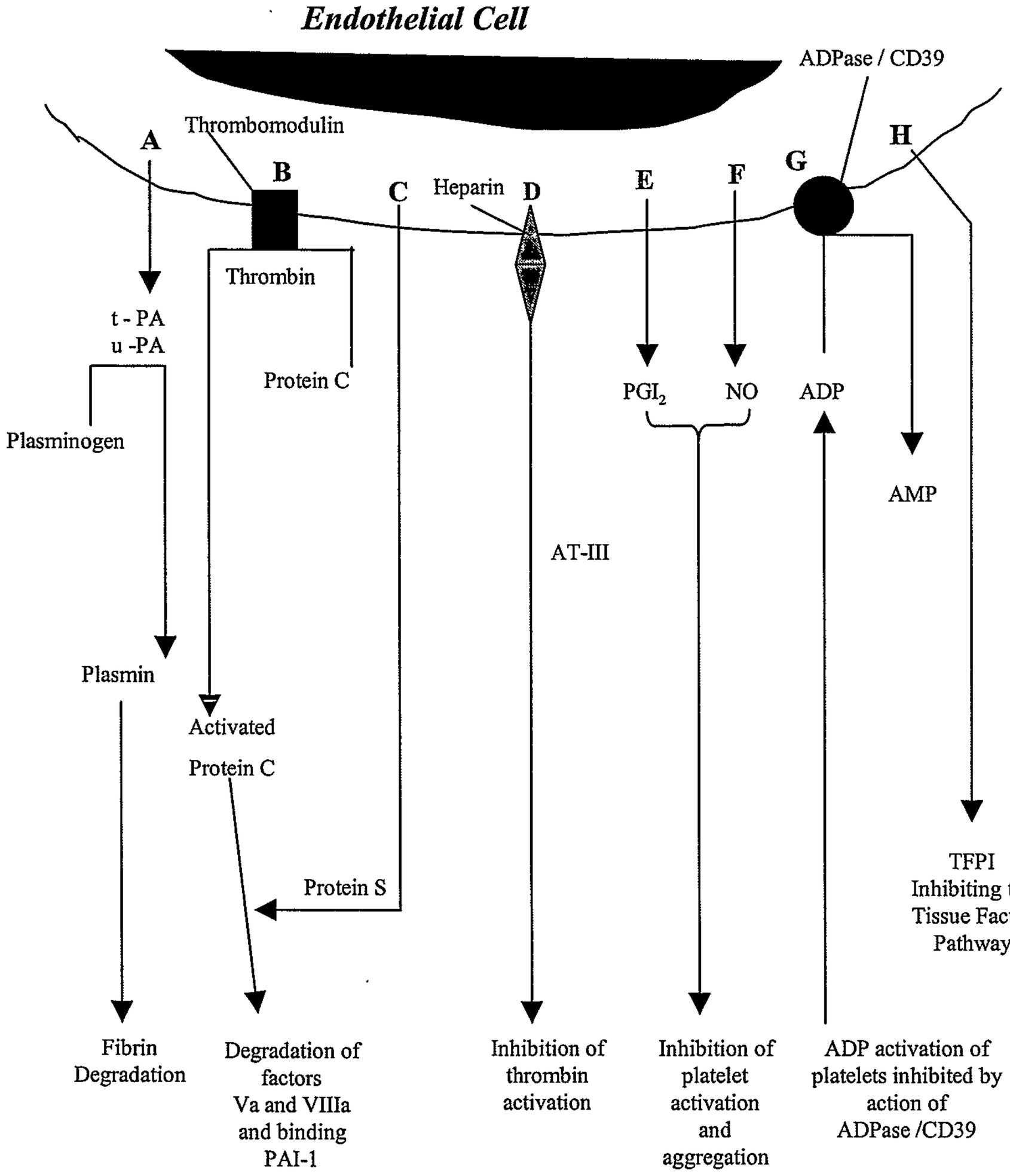

Endothelial Cell
ADPase / CD39
Thrombomodulin
A
B
C
Heparin
D
E
F
G
H
Thrombin
t - PA
u -PA
Protein C
Plasminogen
PGI2
NO
ADP
AMP
AT-III
Plasmin
Activated
Protein C
Protein S
TFPI
Inhibiting the
Tissue Factor
Pathway
Fibrin
Degradation
Degradation of
factors
Va and VIIIa
and binding
PAI-1
Inhibition of
thrombin
activation
Inhibition of
platelet
activation
and
aggregation
ADP activation of
platelets inhibited by
action of
ADPase /CD39

Figure 1.5. Diagram outlining the main features of the fibrinolytic system. Plasminogen is cleaved by either t-PA or u-PA to produce plasmin which is capable of fragmenting polymerised fibrin. t-PA is activated by binding fibrin. u-PA occurs as a pro-enzyme, single chain u-PA (scu-PA) which is activated by plasmin to generate active u-PA. PAI is able to inactivate both t-PA and u-PA through stochimetric binding. Active plasmin may be neutralised though binding α_2-plasmin inhibitor (α_2-PI). There are two forms of PAI (PAI-1 and PAI-2), which are not indicated separately in this diagram, while also not indicated is the u-PA receptor. It is important to note that endothelium is able to produce all forms of PA and PAI, while these cells also bear the u-PA receptor.

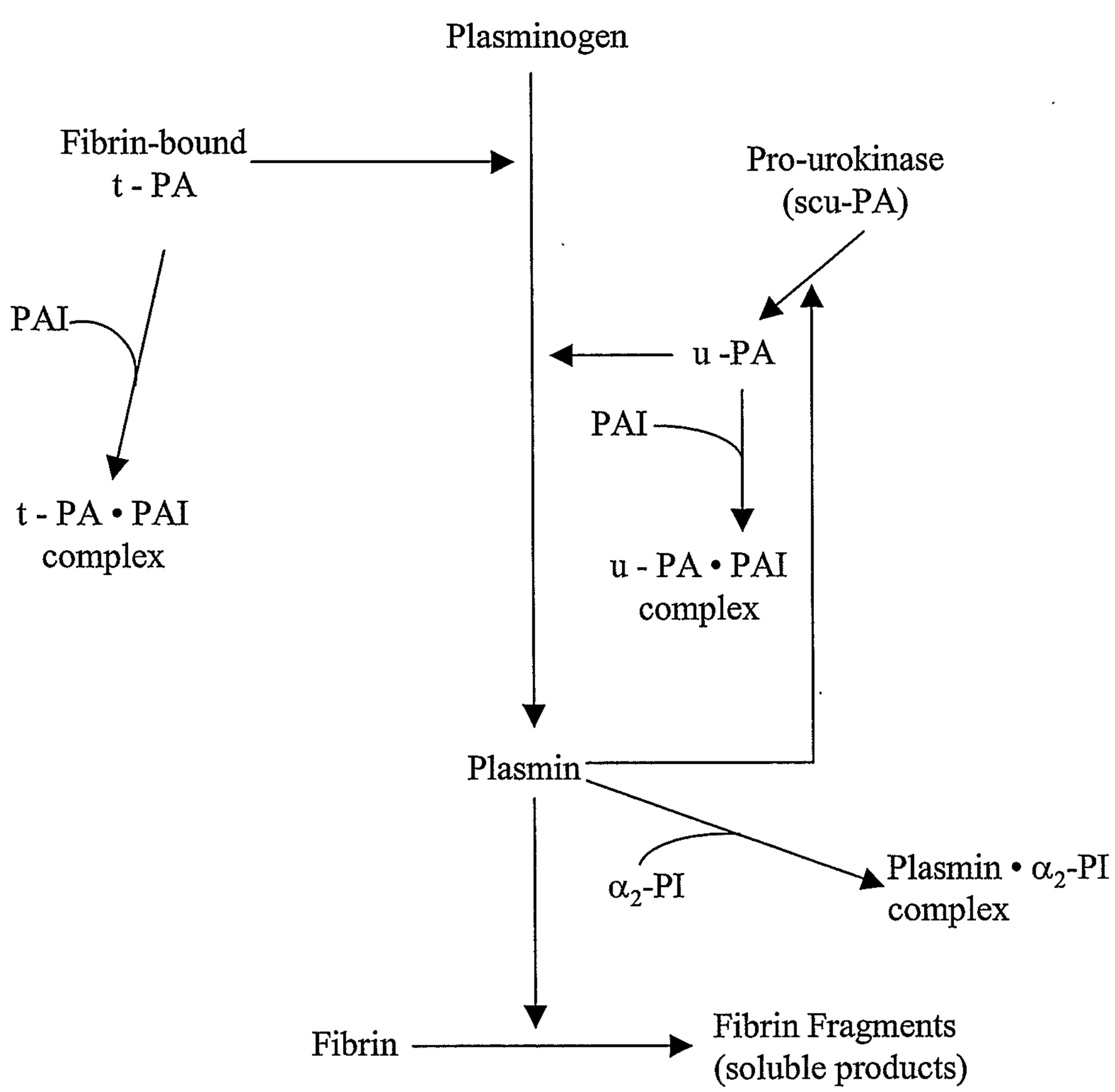

Plasminogen
Fibrin-bound
t - PA
Pro-urokinase
(scu-PA)
PAI
u -PA
PAI
t - PA • PAI
complex
u - PA • PAI
complex
Plasmin
α_2-PI
Plasmin • α_2-PI
complex
Fibrin
Fibrin Fragments
(soluble products)

Figure 1.6. Diagram of the thrombomodulin / protein C anti-coagulant system. Thrombomodulin (TM) on the endothelial surface is able to bind active thrombin (T) with the effect that the specificity of thrombin is altered so that it now cleaves inactive protein C. Activated protein C is then capable of inactivating clotting factors Va and VIIIa, inhibiting blood coagulation. This is greatly accelerated by protein S, also released by endothelium. A further action of activated protein C, is the inactivation of PAI-1, effectively facilitating fibrinolysis.

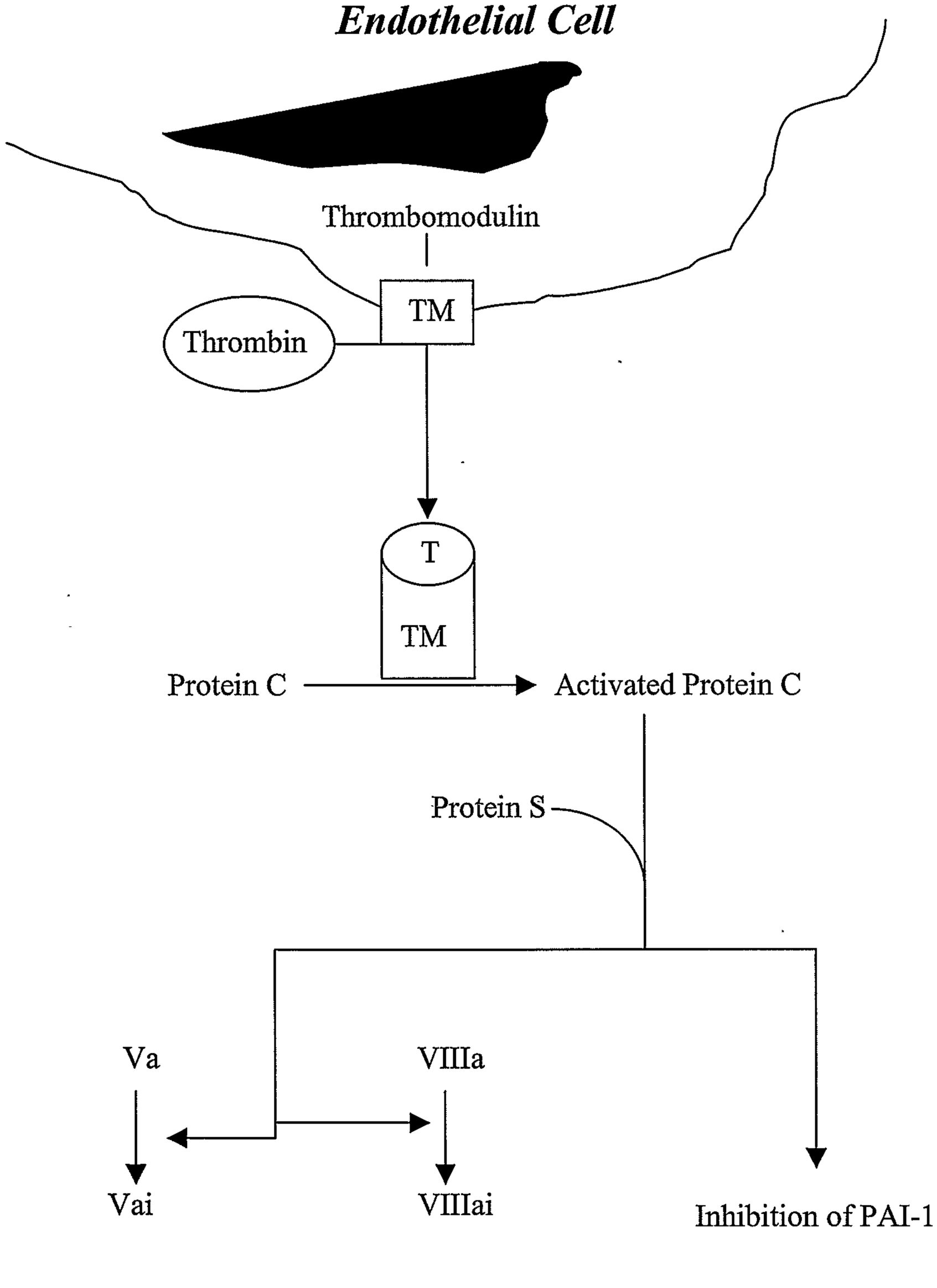

Endothelial Cell
Thrombomodulin
TM
Thrombin
T
TM
Protein C
Activated Protein C
Protein S
Va
VIIIa
Vai
VIIIai
Inhibition of PAI-1

Figure 1.7. Diagram outlining the production of PGI_2 by endothelium. Endothelial cells produce PGI_2 both constitutively and in response to specific agonists including thrombin, histamine and IL-1 which each bind defined receptors. When thrombin or histamine bind their receptors, arachidonic acid is released through the action of phospholipase A_2. This is then metabolised to produce PGG_2 which exerts an inhibitory effect on it's own production. PGG_2 is in turn converted to PGH_2 which is the precursor for PGI_2, also known as prostacyclin. Both PGG_2 and PGH_2 are products of the same enzyme, PGH synthase, also known as cyclooxygenase. Because PGG_2 and PGH_2 both readily cross the plasma membrane barrier, these arachidonic acid products may be obtained indirectly by endothelium from platelets at the cell surface so that platelets may indirectly assist endothelium to produce anti-aggregatory PGI_2, in part counterbalancing the pro-aggregatory platelet derived TXA_2. The increased production of PGI_2 stimulated by IL-1 is similar to that seen in response to thrombin and histamine, with the difference that PGH synthase levels are increased through production of new enzyme, resulting in increased long term PGI_2 production. PGI_2 is highly unstable, and readily undergoes degradation in aqueous solutions to produce 6-keto-$PGF_{1\alpha}$. This is also unstable, and undergoes β oxidation to form the stable but essentially biologically inert end product, 2,3 Dinor-6-keto-$PGF_{1\alpha}$.

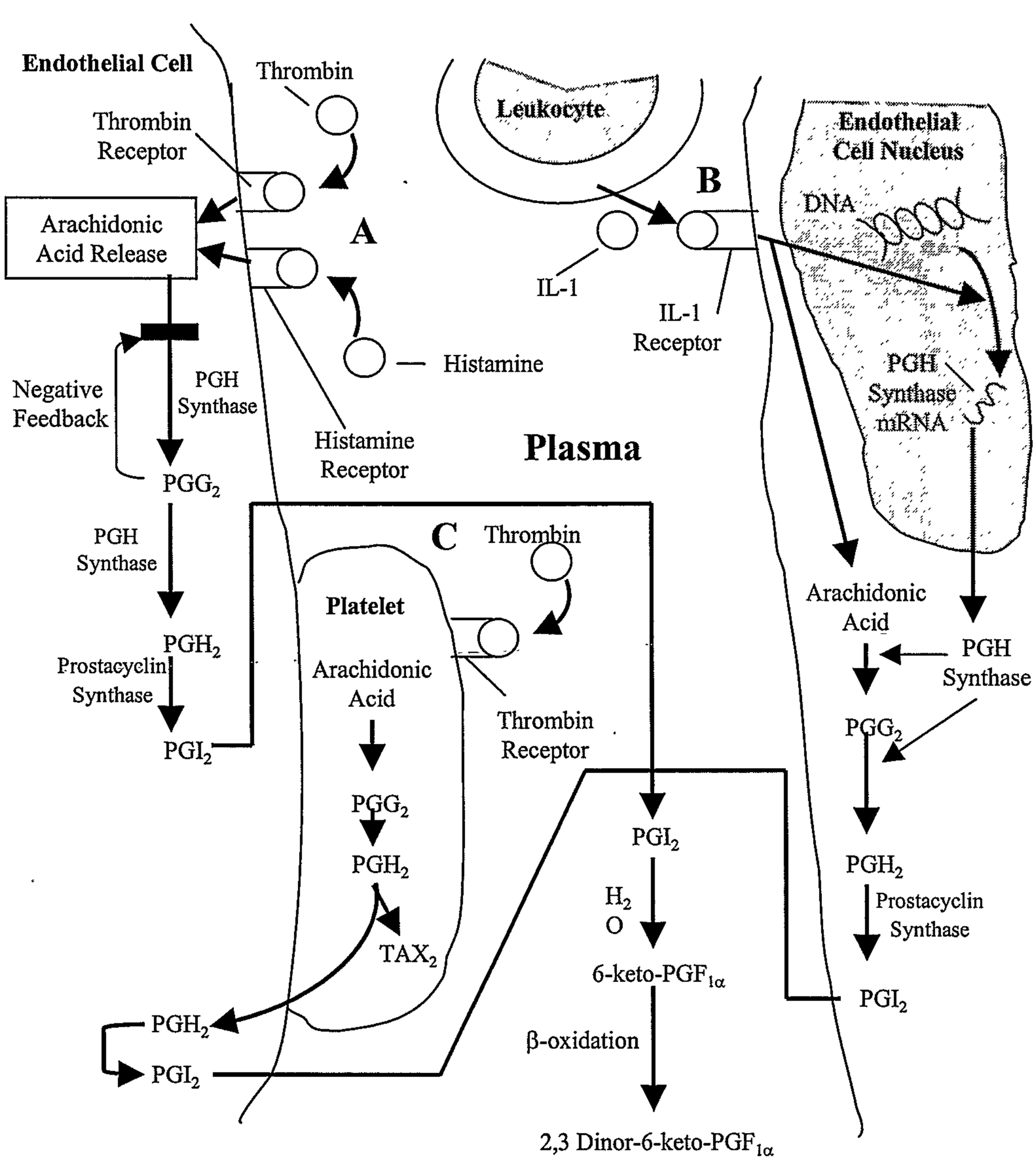
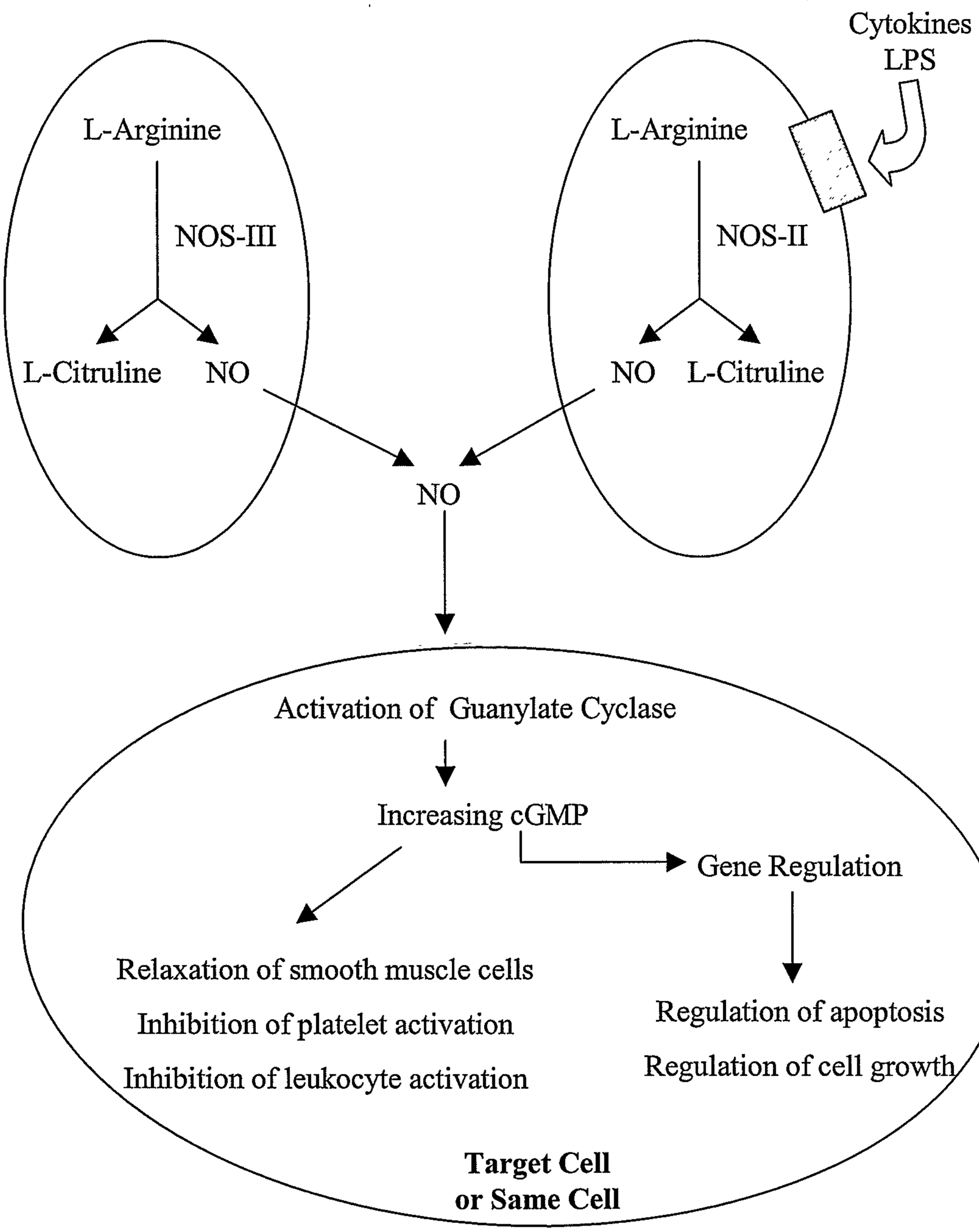

Endothelial Cell
Thrombin
Thrombin Receptor
Arachidonic Acid Release
A
Histamine
Histamine Receptor
Negative Feedback
PGH Synthase
PGG_2
PGH Synthase
PGH_2
Prostacyclin Synthase
PGI_2
Leukocyte
B
IL-1
IL-1 Receptor
Endothelial Cell Nucleus
DNA
PGH Synthase mRNA
Plasma
C
Thrombin
Platelet
Arachidonic Acid
Thrombin Receptor
PGG_2
PGH_2
TAX_2
PGH_2
PGI_2
PGI_2
H_2O
6-keto-$PGF_{1\alpha}$
β-oxidation
2,3 Dinor-6-keto-$PGF_{1\alpha}$
Arachidonic Acid
PGH Synthase
PGG_2
PGH_2
Prostacyclin Synthase
PGI_2

Figure 1.8. Diagram outlining the production of NO by endothelium and the activation of target cells by NO. NO and the by-product L-citruline are produced by the oxidation of the guanidino nitrogen moiety of L-arginine by NO synthase (NOS). Endothelium possesses both constitutive NOS (NOS-III) and inducible NOS (NOS-II), which is expressed by cells stimulated with cytokines or bacterial endotoxin (LPS). NO readily passes across the cell membrane to enter other near-by cells where it stimulates guanylate cyclase to increase cGMP levels. This relaxes smooth muscle cells and also inhibits platelet activation. NO reduces leukocyte activation while in some cells NO controls apoptosis and regulates cell growth.

Unstimulated Endothelial Cells

Stimulated Endothelial Cells



Target Cell
or Same Cell

CHAPTER TWO

The Effect of Apoptotic Endothelial Cells Upon Platelet Aggregation

2.1. Introduction

2.1.a. Do Apoptotic Endothelial Cells Affect Platelet Aggregation?

As discussed in 1.1.c, endothelial cells have a potent anti-aggregatory activity for platelets as mediated by NO, PGI_2 and CD39. Malignant tumour cells gaining access to the vasculature during metastasis often express pro-aggregatory activity for platelets and this has been argued as facilitating metastasis to distant tissues (Heinmoller *et al.*, 1996). Pro-coagulant activity can be modified by a variety of agents including cytokines and bacterial products (Colucci *et al.*, 1983; Bevilacqua *et al.*, 1984; 1985; Nawroth and Stern, 1986; Moore *et al.*, 1987) so that it seems reasonable to suggest that apoptosis may also affect similar pro-coagulant activity.

Also, as outlined in 1.2.c, there seem to be at least two ways in which apoptotic endothelial cells may gain access to the circulation. Firstly, the early detachment of apoptotic endothelium during microvascular remodelling constitutes a physiological shedding of apoptotic endothelial particles into the circulation (Araki *et al.*, 1990b; Zoellner *et al.*, 1996b; 1999). Secondly, and perhaps of greater biological importance, endothelium detached by trauma and thus deprived of adhesion to an underlying matrix becomes apoptotic (Meredith *et al.*, 1993; Zoellner *et al.*, 1996b; Solovey *et al.*, 1999). The possibility that apoptotic endothelial particles may act as sites for microthrombosis, resulting in microembolism is discussed in 1.2.c. Also discussed in 1.2.c, is the apparent contradiction between reports suggesting pro-thromboembolic and anti-thromboembolic properties of apoptotic endothelium. On the one hand, apoptotic endothelial cells pro-coagulant changes and increased platelet binding (Casciola-Rosen *et al.*, 1996; Greeno *et al.*, 1996; Bombeli *et al.*, 1997; 1999), while

on the other, there is maintained u-PA expression and canalicular fragmentation (Zoellner *et al.*, 1996a; 1998; 1999). At the time that the work in this thesis was commenced, there were no reports of interactions between platelets and apoptotic endothelium. However, when a single report of platelets binding to apoptotic endothelium was made available during the course of this work, no data on platelet function was discussed (Bombeli *et al.*, 1999). This chapter thus presents data from experiments investigating the effect of apoptotic endothelium upon platelet aggregation.

2.1.b. Isolated Cultured Endothelial Cells as a Source of Apoptotic Endothelium

2.1.b.i. Human Umbilical Vein Endothelial Cells as an Endothelial Cell Culture Model

Isolated micro- and macro-vascular endothelial cells from a wide range of both animal and human vessels including umbilical vein have been used to study endothelial function. Human umbilical vein endothelial cells (HUVEC) isolated from umbilical cords are a highly convenient and widely accepted model for study of endothelial function *in vitro* (Hughes, 1996). Despite the convenience afforded by HUVEC in cell culture studies, it is nonetheless necessary to appreciate the limitations of this model system. Perhaps significantly, HUVEC are macrovascular vein cells derived from a foetal tissue destined for mechanical shedding after delivery and this seems a reasonable objection to the use of HUVEC for study of physiological endothelial function in adult microvascular or arterial vascular beds. Differences are noted in the behavior of endothelium from umbilical veins as compared with endothelium from microvascular, arterial and even adult venous source (Kumar *et al.*, 1987; Bicknell,

1993; Jackson and Nguyen, 1997; Bachetti and Morbidelli, 2000; Lang *et al.*, 2001; Otto *et al.*, 2001). However, it is also noteworthy that there are further differences in cultured microvascular endothelium from separate tissues and also between species (Del Vecchio *et al.*, 1992; Bachetti and Morbidelli, 2000; Murugesan *et al.*, 2000). Importantly for this study, HUVEC can be obtained in very large numbers from individual donors while only comparatively small numbers of microvascular cells can be obtained for such a study (Jackson *et al.*, 1990; Hewett and Murray, 1993). Because the experiments described in this chapter required very large numbers of endothelial cells, in order to repeat experiments sufficiently often to obtain statistically meaningful findings, it was necessary to use HUVEC as the endothelial cell culture model.

Isolated cells can be identified as being endothelial in origin by morphological, biochemical and immunological criteria. The typical polygonal shape of endothelial cells nestled within in a cobblestone arrangement is characteristic of HUVEC and readily observed during cell culture (Jaffe *et al.*, 1973; 1980; Bachetti and Morbidelli, 2000). In addition to this, the presence of VWF (previously termed Factor VIII related or associated antigen) (Jaffe *et al.*, 1973; 1980) and the binding of *Ulex europeaus* lectin type 1 (UEA-1) (Holthofer *et al.*, 1982) as detected by immunohistochemistry can be used as highly specific and sensitive markers for the endothelial origin of HUVEC and other endothelial cells.

2.1.b.ii. Different Methods for Preparation of Apoptotic HUVEC as Models for Apoptotic Endothelium from Different Biological Settings

When microvascular segments are deprived of flow, they are removed by endothelial cell apoptosis (Sandison, 1928; Meeson *et al.*, 1996). It is believed that chemical plasma factors are important in maintaining survival of endothelium in functional vessels, while deprivation of these plasma factors causes endothelial apoptosis in poorly perfused vessels (Sandison, 1928; Araki *et al.*, 1990b; Zoellner *et al.*, 1996b; Meeson *et al.*, 1996; Zoellner *et al.*, 1999). Also, detachment is an early event during apoptosis in serum deprived endothelium both in isolated cell culture and in tissues (Araki *et al.*, 1990a; Zoellner *et al.,* 1996b; 1999). This suggests that detached-floating apoptotic cells induced by serum deprivation represent a reasonable experimental model to study apoptotic endothelial cells generated during vascular remodelling. In the current study, apoptotic endothelial cells obtained in this way were used and designated serum deprived detached apoptotic endothelial cells (SDDA-EC).

Also in the current study, the adherent cells remaining after serum deprivation constituted a convenient non-apoptotic control population for comparison with the serum deprived apoptotic cells, and these were designated serum deprived non apoptotic endothelial cells (SDnA-EC).

Detachment of endothelial cells by trauma has been reported *in vivo* and loss of matrix adhesion by endothelium appears to cause apoptosis (Meredith *et al.*, 1993; Zoellner *et al.*, 1996b; Lai *et al.*, 1996; Solovey *et al.*, 1999). In the current study, matrix deprivation was used to obtain apoptotic endothelial cells believed to be

similar to those generated by traumatic detachment *in vivo*. These cells were designated as matrix deprived apoptotic endothelial cells (MDA-EC).

To permit comparison of MDA-EC with non-apoptotic endothelial cells also exposed to serum, untreated non-apoptotic endothelial cells (UnA-EC) were used as a control population.

Since one of the differences between SDDA-EC and MDA-EC was the absence of serum in SDDA-EC, it seemed important to have a further control apoptotic endothelial population which was deprived of both matrix adhesion and serum and this apoptotic cell type was designated as matrix and serum deprived apoptotic endothelial cells (M&SDA-EC).

The identity and culture conditions of these five populations of cells are summarized in Table 2.1.

Apoptotic cells derived by deprivation of either serum or matrix have identical structural and DNA fragmentation features suggesting that study of these different populations may not be entirely necessary (Meredith *et al.*, 1993; Zoellner *et al.*, 1996b). Nonetheless, it was felt that the two biological settings outlined in which endothelium become apoptotic are fundamentally different, and that it was important to assess this in this study. Also, by comparing results of SDDA-EC and MDA-EC with those from M&SDA-EC, it was considered possible to detect any synergy in platelet regulatory activity between these two mechanisms for stimulating endothelial

apoptosis. It should be noted that M&SDA-EC were used by Bombeli et al. in studying apoptotic endothelium (Bombeli *et al.*, 1997; 1999).

2.1.c. The Choice of Platelet Agonists for Study of the Effect of Apoptotic Endothelium Upon Platelet Aggregation

In this study, it was important to use agonists which were both highly potent and physiologically relevant. Because of the central role of thrombin in blood clotting as outlined in 1.1.b. as well as the very intense aggregatory response of platelets to thrombin, this agonist was chosen as an effective and physiologically relevant factor for study (Harker *et al.*, 1995; Wu, 1996). Unlike thrombin, ADP is derived from activated platelets but is also an intense platelet agonist so that this was also selected as a biologically important agonist for investigation (Gachet and Cazenave, 1997; Puri and Colman, 1997). Both ADP and thrombin can be viewed as "intravascular" agonist signals, which may be generated in the absence of platelet contact with subendothelial matrix. To investigate the possible effect of an "extravascular" or matrix associated agonist, collagen was used as this is widely accepted as a potent platelet stimulant and is also readily available in commercial preparations (Brass, 1991; Alberio and Dale, 1998).

Table 2.1. Populations of non-apoptotic and apoptotic HUVEC used in experiments

Apoptotic Status	Non Apoptotic HUVEC		Apoptotic HUVEC		
Full Designation of Cell Population	Untreated Non Apoptotic Endothelial Cells	Serum Deprived Non Apoptotic Endothelial Cells	Serum Deprived Detached Apoptotic Endothelial Cells	Matrix Deprived Apoptotic Endothelial Cells	Matrix and Serum Deprived Apoptotic Endothelial Cells
Abbreviation	UnA-EC	SDnA-EC	SDDA-EC	MDA-EC	M&SDA-EC
Culture Conditions Used to Obtain Cell Population	Adherent cells in complete medium. Effectively a starting population.	Adherent cells surviving 24 hr in serum free conditions	Detached cells from cultures deprived of serum for 24 hr	Cells deprived of matrix adhesion by culture in bacterial culture plates but in the presence of serum for 24 hr	Cells deprived of both serum and matrix adhesion by culture in bacterial culture plates for 24 hr

HUVEC were cultured in the presence or absence of serum or matrix adhesion in order to obtain populations of apoptotic endothelial cells. UnA-EC constituted a convenient control for MDA-EC obtained in the presence of serum, while SDnA-EC provided a control population for SDDA-EC and M&SDA-EC were studied to determine any interaction between serum and matrix deprivation.

2.2. Materials and Methods

2.2.a Materials

2.2.a.i. Cell Culture Materials

Tissue culture flasks (25, 75 and 225 cm^2) were obtained from Costar (Cambridge, MA, USA) while bacterial culture plates (60 cm^2) were purchased from Techno-Plas (South Australia, Australia). Disposable plastic centrifuge tubes were purchased from Iwaki, Scitech Division (Chiba, Japan) and disposable membrane filters were supplied by Sartorius, Minisart (Gottingen, Germany). Type 1A collagenase, Hank's Balanced Salt Solution (HBSS), Dimethylsulphoxide (DMSO) and gelatin were obtained from Sigma (St. Louis, USA) whereas Medium 199 (M199), iron fortified fetal bovine calf serum (FCS) and trypsin/EDTA were supplied by JRH Biosciences (Lenexa, USA). Heparin was purchased from Pharmacia and Upjohn (Perth, WA, Australia). Endothelial cell growth supplement (ECGS) was prepared from bovine hypothalamus as described elsewhere (Maciag *et al.*, 1984). Penicillin/streptomycin solution used in all cell cultures was obtained from CSL Biosciences (Parkville, Vic, Australia) while amphotericin B was supplied by ICN Biomedicals (Ohio, USA).

2.2.a.ii. Materials for Histology and Immunological Reagents

Selby's Biotechnology (Sydney, Australia) supplied absolute methanol and Paraplast paraffin was purchased from Oxford Lanware (St. Louis, USA). Normal goat serum (NGS) was obtained from Hunter Antisera (Jesmond, NSW, Australia) while hydrogen peroxide was purchased from Laboratory Supply (Milperra, NSW, Australia). UEA-1, haematoxylin, diamino-benzo-tetrahydrochloride (DAB) and

bovine serum albumin (BSA) were provided by Sigma (St. Louis, USA) and histolene was obtained from Fronine laboratory Products (Riverstone, NSW, Australia). Dako (Carpinteria, California, USA) supplied rabbit anti VWF and peroxidase labelled goat anti-rabbit antibody while streptavidin peroxidase conjugate was obtained from BioSource International (Camberwell, Vic, Australia). Triton X-100 and aquamount mounting medium were purchased from BDH Laboratory Supplies (Poole, England). Mediglass microscopy slides were obtained from Lomb Scientific (Sydney, Australia).

2.2.a.iii. Materials for Platelet Aggregometry

ADP was obtained from Sigma (St. Louis, USA) while Ortho Diagnostic Systems Inc. (USA) supplied thrombin (human) fibrindex. Collagen was purchased from Nycomed Horm (Germany). Standard buffer sodium citrate was obtained from BD Vacutained System (Becton, Dickinson and Company, UK). Latex Beads (diameter 6.4 μm and 25.7 μm) were supplied by Sigma (St. Louis, USA).

2.2.a.iv. Materials for Transmission and Scanning Electron Microscopy

Cacodylate buffer and uranyl acetate were supplied by Fluka (Buchs, Switzerland) while the Spurr's low viscosity resin prepared by Taab Laboratories Equipment Inc (Aldermaston, Berkshire, UK). Reynold's lead citrate was prepared by stoichiometric reaction of lead nitrate provided by Fluka (Buchs, Switzerland) and trisodium citrate obtained from Ajax Chemicals (Auburn, NSW, Australia). Glutaraldehyde, osmium tetroxide, slot grids and 300 mesh copper grids were purchased from ProSciTech (Thuringowa, QLD, Australia). Absolute ethanol and acetone were obtained from BDH Laboratory Supplies (Poole, England) while methylene blue was purchased

from Medos Co. Pty. Ltd. (Lidcombe, NSW, Australia). Nuclepore filters were provided by Holgate Scientific Pty. Ltd. (Terrigal, NSW, Australia). Kodak electron microscope film, type 4489, was used to record results.

2.2.a.v. Reagents for DNA Gel Electrophoresis

RNAse A and Proteinase K were obtained from Roche Molecular Biochemicals (Nutley, New Jersey, USA) while glycerol was purchased from BDH Laboratory Supplies (Poole, England). Sigma (St. Louis, USA) supplied Tris, Tris-HCL, Borate, EDTA, sodium acetate, ethidium bromide and bromophenol blue. Ajax Chemicals (Auburn, NSW, Australia) supplied phenol, absolute ethanol, sodium chloride and potassium chloride. Molecular biology grade agarose, electrophoresis units, Minisub DNA Cell and DNA Sub Cell, as well as a Model 200/2.0 Power Supply power pack were obtained from Bio-Rad (Hercules, California, USA).

2.2.b. Isolation, Culture and Identification of Human Umbilical Vein Endothelial Cells (HUVEC)

2.2.b.i. Isolation of HUVEC from Umbilical Cords

HUVEC were isolated from human umbilical cords obtained from normal vaginal deliveries by using the collagenase digestion method (Jaffe *et al.*, 1973). Isolation was performed in a laminar flow hood. Umbilical cords were collected from the maternity ward of Westmead Hospital and stripped of as much blood as possible. Then umbilical veins were canulated at one end and washed 3 times with 20 ml volumes of warmed HBSS to remove blood clots from veins. 10 to 20 ml of sterile

crude collagenase solution (1 mg/ml) in HBSS were perfused into veins and the distal ends of cords clamped. Afterwards, cords were left in HBSS for 30 minutes at room temperature and massaged gently to enhance the release of endothelial cells. Cord and veins were cut and the contents containing endothelial cells were collected into 50 ml centrifuge tubes containing 5 ml of FCS. Remaining loosely adherent endothelial cells were collected by further washing twice with 20 ml of warmed HBSS and draining to the same tube. Cells were concentrated by centrifugation at 1,000 rpm for 5 minutes and the supernatant was discarded. Cell pellets were resuspended in 5 ml of HUVEC culture medium comprising M199 with FCS (20%), heparin (30 U/ml) and ECGS (50 µg/ml). Penicillin (100 U/ml), streptomycin (100 µg/ml) and fungizone B (2.5 µg/ml) were used as antibiotics throughout cell culture. The resuspended cells were then seeded into 25 cm^2 culture flasks that had been pre-coated with gelatin (0.1%) in PBS. Flasks were incubated at 37 °C in 100% relative humidity under CO_2 (5%). Medium was changed the following day and cells were fed every 3 to 4 days.

2.2.b.ii. Culture of HUVEC

Primary cultures of HUVEC were in 25 cm^2 culture flasks and once confluent, cells were passaged with Trypsin-EDTA into either 75 cm^2 or 225 cm^2 flasks at a split ratio of 1 to 3. Briefly, culture medium was first removed from flasks and HUVEC monolayers washed twice with M199. Cells were then treated with 5 to 15 ml of Trypsin (0.25%), EDTA (0.02%) in HBSS. Cells were then observed by phase contrast microscopy and once detached were collected and added to 5 ml of FCS to neutralise Trypsin activity. After centrifugation at 1,000 rpm for 5 minutes, cell pellets were resuspended in appropriate volumes of complete culture medium, being

15 ml for 75 cm^2 and 45 ml for 225 cm^2 flasks, before seeding in flesh gelatin coated flasks for further culture. HUVEC were cultured to a maximum of fifth passage and fed with fresh complete culture medium at least twice a week. Experiments were performed in this study with cells from fourth to fifth passages. Cells were stored frozen in liquid nitrogen with DMSO (10%) and FCS (20%) in M199 for subsequent thawing and plating at confluence.

2.2.b.iii. Identification of HUVEC in Culture

HUVEC were identified as endothelial cells by their cobblestone morphology, staining for VWF (Jaffe *et al.*, 1973; 1980) and the binding of UEA-1 (Holthofer *et al.*, 1982) detected by immunophosphatase histochemistry.

2.2.b.iii.1. Preparation of Paraffin Sections of Umbilical Cords and Monolayers for Immuno and Lectin Histochemistry

Umbilical cord sections were dehydrated through a series of graded ethanols and cleared in histolene before being infiltrated and blocked in paraffin. Sections were cut using a Leitz Microtome 1516 supplied by Leica Microsystems Pty. Ltd. (Gladesville, NSW, Australia) and mounted on microscope slides. Prior to staining, the slides were baked at 60 °C for 30 minutes before de-paraffinising with histolene and re-hydrating with a series of graded ethanols. The slides were then washed in water and stained as appropriate. HUVEC were grown on tissue culture coverslips pre-coated with gelatin and fixed with formalin (10%) in PBS for 10 minutes before extensive washing with PBS.

2.2.b.iii.2. Immuno and Lectin Histochemistry

Before application of labelling reagents, endogenous peroxidase activity was inhibited by incubation at room temperature for 10 minutes in H_2O_2 (3%) in distilled water followed by washing with PBS. Then, paraffin sections and tissue culture coverslips were pre-incubated with a solution of PBS with FCS (1%), NGS (3%) and Triton X-100 (0.3%) for 20 minutes to reduce non-specific binding. The same solution was used to dilute all labelling reagents and antibodies. Primary label, consisting of either biotin labelled UEA-1 at a final concentration of 0.02 mg/ml or rabbit antiserum against VWF at 1 in 100 dilution, was then applied to specimens in a humidified chamber for 60 minutes at room temperature. Specimens were washed three times with PBS for 15 minutes and treated with either streptavidin peroxidase (0.01 mg/ml) or peroxidase labelled goat anti rabbit immunoglobulin (1 in 100 dilution) dependent upon the primary label. After 30 minutes incubation, the specimens were again washed three times with PBS for 15 minutes. The peroxidase label was detected by incubation with the chromogenic agent DAB (2.2 mg/ml) in PBS with H_2O_2 (0.03%) for 5 to 10 minutes at room temperature. Specimens were then rinsed with water for 2 to 3 minutes before counter staining with haematoxylin and further washing with water. Sections were dehydrated with serial ethanols followed by histolene before coverslipping whereas tissue culture coverslips were air dried prior to mounting on slides with Aquamount. Using this procedure, it was possible to identify HUVEC as both UEA-1 and VWF positive cells.

2.2.c. The Preparation of Apoptotic Endothelial Populations

MDA-EC were prepared by collecting confluent, washed HUVEC from a single 225cm^2 culture flask using Trypsin-EDTA. The enzyme was inhibited with 5 ml of FCS and the HUVEC collected by centrifugation prior to resuspension in 10 ml of M199 containing serum (20%) and antibiotics but not ECGS. Cells were then transferred to bacterial culture flasks, where they were unable to establish normal matrix adhesion and thus became apoptotic over the following 24 hr of culture (Meredith *et al.*, 1993; Zoellner *et al.*, 1996b). Apoptotic cells were then harvested by washing and centrifugation for use in experiments. Since apoptotic endothelial cells undergo fragmentation, it was necessary to determine MDA-EC number before actual apoptosis occurred and this was done by haemocytometer counts of the cells at the time they were plated onto bacterial culture dishes. To determine the effect of combined matrix and serum deprivation, M&SDA-EC were prepared in the same way as MDA-EC, with the difference that no serum was present during incubation on non-adherent culture surfaces.

Since endothelial cells rapidly detach during apoptosis, it was possible to separate SDDA-EC from the remaining SDnA-EC by washing HUVEC cultures deprived of serum for 24 hr (Araki *et al.*, 1990a; 1990b; Zoellner *et al.*, 1996b; 1998; 1999). Briefly, HUVEC were grown to confluence in 225cm^2 flasks before being washed with M199 and then cultured with M199 containing antibiotics but no serum or ECGS. After 24 hr the SDDA-EC were collected by washing and centrifugation while the remaining SDnA-EC were harvested with Trypsin-EDTA and transferred to centrifuge tubes containing 5 ml volumes of FCS and pelleted by centrifugation. The number of SDnA-EC was determined by cell counts using a haemocytometer while

the number of SDDA-EC was determined by subtracting the number of SDnA-EC from the number of MDA-EC determined at the time of serum deprivation. This method for obtaining SDDA-EC and SDnA-EC is identical to that used in earlier studies and has been accepted as a reasonable approach to quantitation of apoptotic endothelial particles (Zoellner *et al.*, 1998).

UnA-EC were also collected from 225 cm^2 culture flasks using Trypsin-EDTA and centrifugation in the presence of FCS immediately prior to resuspension. Cell number was determined using a haemocytometer immediately prior to use in experiments.

Fourth to fifth passage HUVEC were used for aggregation experiments, with all five HUVEC populations (UnA-EC, SDnA-EC, SDDA-EC, MDA-EC and M&SDA-EC) being obtained from the same donor within each experiment. Cells were resuspended in HBSS to a final concentration of 10^7 cells per ml immediately prior to use in platelet aggregation experiments.

2.2.d. Verification of Endothelial Apoptosis

2.2.d.i. DNA Gel Electrophoresis

SDDA-EC, MDA-EC and M&SDA-EC as described in 2.2.c. were collected by washing while UnA-EC and SDnA-EC as described in 2.2.c. were harvested with Trypsin-EDTA into a small amount of serum. The five populations were immediately concentrated by centrifugation at 2,000 rpm for 10 minutes at 4 °C, and transferred to Eppendorf tubes in 1 ml of Tris buffered saline with EDTA (10 mM pH 7.2). Following centrifugation, cell pellets were lysed in 50 µl of a Tris (50 mM pH 8.0)

buffer with EDTA (10 mM) and SDS (0.5%). The lysed cells were frozen at -80 °C for 30 minutes before addition of RNAase A to a final concentration of 0.5 mg/ml. Preparations were then incubated at 37 °C for 1 hour before addition of proteinase K to a final concentration of 0.5 mg/ml and incubation at 50 °C for a further three hours. Samples were then taken up to a final volume of 500 µl with Tris (10 mM), EDTA (1 mM, pH 7.5) and extracted twice with 500 µl volumes of phenol before isopropanol precipitation of DNA by addition of 50 µl of sodium acetate (3 M) and 1 ml of ice cold absolute ethanol. Samples were mixed by inversion before being stored at -80 °C overnight. Prior to electrophoresis, solutions were centrifuged at 13,000 rpm for 20 minutes to pellet precipitated DNA. DNA pellets were further washed with ice cold ethanol (70%) and recentrifuged before removal of the supernatant and allowing the pellets to dry at room temperature. The dried pellets were resuspended in 15 µl of TE buffer (Tris 10 mM, EDTA 1 mM pH 7.5) before adding 5 µl of loading buffer, comprising bromophenol blue (0.25%) and glycerol (30%) in water. DNA samples were subjected to electrophoresis in agarose gels (2%) with ethydium bromide (0.5%) at 70 V for 30 to 45 minutes and electrophoretic motility assessed by visualisation with UV light. Results were recorded photographically using a DS34 Polaroid Direct Screen Instant Camera and Polaroid 667 Black and White film.

2.2.d.ii. Transmission Electron Microscopy

All five populations of endothelial cells (SDDA-EC, MDA-EC and M&SDA-EC as well as UnA-EC and SDnA-EC) were harvested by either washing or Trypsin-EDTA as described in 2.2.d.i. TEM was performed upon cells pre-fixed with gluteraldehyde (0.25%) in PBS for 15 minutes at 4 °C before pelleting by gentle centrifugation. The pellets were then further processed by fixation with gluteraldehyde (2.5%) in PBS for

1 hour at 4 °C. Fixed pellets were then washed three times with PBS before further fixation with osmium tetroxide (2%) in cacodylate buffer (0.1 M, pH 7.4) for 3 hour at 4 °C. Specimens were dehydrated with serial 10 minutes immersions in graded alcohols followed by two final 10 minutes treatments with acetone (100%). Acetone was replaced with an acetone / resin mixture (1:1) for one hour after which samples were treated 3 times with resin (100%) for 10 minutes each at 70 °C. Specimens were embedded in fresh resin, which was polymerised at 70 °C for 10 hours. Semi-thin sections were prepared using a Reichert Ultracut E microtome (Vienna, Austria) and stained with methylene blue (1%) for 2 minutes. Ultrathin sections were collected on copper grids before staining with uranyl acetate (2%) for 15 minutes followed by a wash with ultrapure water for at least 15 seconds. Grids were then further stained with Reynold's lead citrate for 4 minutes followed by washing with ultrapure water. Grids were examined using a Philips CM 120 BioTwin, transmission electron microscope (Eindhoven, The Netherlands) at 100 kV.

2.2.e. Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to further investigate the possibility that endothelial cells bind to collagen fibres. Platelets in the presence and absence of collagen and or endothelial cells were pre-fixed with glutaraldehyde (0.25%) in PBS for 10 minutes at 4 °C and placed dropwise onto nuclepore filters with a 3.0 μm pore size. Filters were then fixed with 2.5 % glutaraldehyde in PBS at 4 °C for 1 hour followed by washing three times using PBS. Samples were dehydrated using graded ethanols before critical point drying using a semi-automatic critical point drying apparatus (Balzers Union, Leichtenstein). All specimens were sputter coated with gold using a Balzers Union sputter coater (Balzers Union, Leichtenstein) and

examined using a JSM 840 scanning electron microscope from JEDL SEM (Tokyo, Japan). The images were captured by using Agfapan AP x 100 Professional 120 photographic film.

2.2.f. Platelet Aggregometry

2.2.f.i. Platelet Preparation

Blood was obtained by venipuncture into standard buffered sodium citrate (1 volume of 0.105 M citrate to 9 volumes of blood) from healthy adult volunteers. Platelet-Rich-Plasma (PRP) was prepared by centrifuging whole blood at 200g for 10 minutes at room temperature and carefully transferring the PRP fraction into fresh plastic tubes. Platelet-Poor-Plasma (PPP) was prepared by centrifuging the residual blood at 1200g for 20 minutes at room temperature, and carefully transferring the PPP into fresh plastic tubes, taking care not to disturb the PPP/cell pellet interface.

2.2.f.ii. Platelet Aggregometry in the Presence and Absence of Endothelial Populations

PRP was diluted with PPP to achieve a final concentration of 200 x 10^9 platelets per liter for use in aggregometry experiments. Platelet aggregation in the presence and absence of the five endothelial populations was monitored using the light transmission method with an aggregometer (Payton Scientific, Lumiaggregation Module 100; Ontario, Canada) using a PPP blank. Zero transmission was defined as light transmission in the presence of PRP while 100% transmission was defined as transmission with PPP. Changes in optical transmission in response to aggregating agents were monitored using a chart recorder (Bausch and Lamb, Houston

Instruments, Austin, Texas, Model EB5216) at a rate such that 2.5 cm was equivalent to 1 minute.

In aggregometry experiments, a small magnetic stirring bar was used to stir 400 µl of platelet suspension (200 x 10^9 platelets per liter) at a standard 1000 revolutions / minute. After 3 minutes of stirring, 50 µl of either HBSS or HUVEC cell preparation at a concentration of 10^7 HUVEC per ml were added to the platelets to achieve a final concentration of 10^6 UnA-EC, SDnA-EC, SDDA-EC, MDA-EC or M&SDA-EC per ml. The addition of these cells or HBSS had a negligible effect upon light transmission and did not initiate platelet aggregation. After one minute, 50 µl of agonist was added to the platelet suspension and changes in light transmission recorded. In most experiments at least three recordings were made for each agonist and cell type. Platelet agonists used were ADP (2.5 µM final concentration) and thrombin (0.25 Units / ml final concentration), while some experiments were also performed using collagen (0.5 µg / ml final concentration) (Wilson *et al.*, 1992; Rasko *et al.*, 1995). The slope of primary aggregation per 2.5 cm of record paper was used to calculate the aggregation rate. Lag phase and maximum percentage aggregation were also measured in the study (Wilson *et al.*, 1992; Rasko *et al.*, 1995).

2.2.f.iii. Experiments Using Latex Beads in Place of HUVEC

To exclude the possibility that changes in platelet aggregation reflected mechanical effects of HUVEC particles, experiments were performed using latex beads of two sizes. The smallest beads measured 6.4 µm in diameter, a size similar to that of apoptotic HUVEC particles. The larger beads measured 23 µm in diameter, a range similar to non-apoptotic HUVEC. Briefly, beads were washed twice with HBSS and

then adjusted to a final concentration of 10^7 beads / ml with HBSS. These were then used directly in platelet aggregation experiments in a similar way to HUVEC populations. Aggregation was monitored by using the above described light transmission method (Born and Cross, 1963).

2.2.g. Statistical Procedures

In order to overcome some inherent variability in platelet sensitivities and HUVEC activities between experiments, data was expressed as a ratio between aggregation rates in the presence or absence of either endothelial cells or latex beads. In addition, measurements within experiments were made relative to the light transmission of PPP and PRP, further standardising result from experiment to experiment. This allowed meaningful comparison of data from experiments performed on different days as well as permitting statistical analysis. Experiments examining the effect of endothelial cells upon platelet aggregation were performed from six to ten times for each cell type studied, using a total of twelve separate donors of HUVEC. Statistical analysis was performed using the two tailed Wilcoxon's Matched-Pairs-Ranks Test and p values of less than 0.05 were considered statistically significant.

2.3. Results

2.3.a. Cells Isolated from Umbilical Cords were HUVEC

HUVEC isolated from umbilical veins using the collagenase digestion method (Section 2.2.b.i.) and cultured in medium as described in Section 2.2.b.ii formed small clusters of adherent cells and usually became confluent within one week of isolation. Monolayers displayed the characteristic cobblestone morphology of HUVEC and no significant changes in cellular morphology were found in subsequent passages. Importantly, immuno-hisochemistry for VWF and lectin histochemistry for UEA-1 binding reveled strong labelling of endothelial cells in both paraffin sections of umbilical veins and isolated cultured endothelium. No labelling was seen for either VWF or binding of UEA-1 when the primary antibody or lectin was excluded from the labelling protocol (Figures 2.1 and 2.2). These data confirmed that isolated cultured cells used in the study were HUVEC.

2.3.b. Endothelial Apoptosis Resulted from Matrix and Serum Deprivation

DNA gel electrophoresis revealed internucleosomal DNA fragmentation typical of apoptosis in: SDDA-EC, MDA-EC and M&SDA-EC, while UnA-EC and SDnA-EC showed no sign of DNA fragmentation (Figure 2.3). TEM confirmed the cellular fragmentation, nuclear condensation, canalicular fragmentation and maintained organellar integrity expected in apoptotic endothelial cells, while non-apoptotic cells had large vesicular nuclei and did not show any sign of cellular or organellar fragmentation (Figure 2.4). The three apoptotic populations had an identical appearance to each other, as did the two non-apoptotic populations studied.

2.3.c. Apoptotic Endothelial Cells Maintained an Anti-Aggregatory Activity for Platelets Stimulated with Thrombin or ADP, but Failed to Inhibit Platelet Aggregation in Response to Collagen

Representative traces for platelets in the presence of UnA-EC or M&SDA-EC stimulated with thrombin or ADP are shown (Figure 2.5). In multiple experiments with cells obtained from separate donors, all five populations of HUVEC reduced the aggregation rate of platelets in response to both thrombin and ADP relative to aggregation in the absence of endothelial cells ($p < 0.05$) (Figure 2.6). No consistent difference in ability to inhibit platelet aggregation was seen between any of the apoptotic and non-apoptotic populations studied.

The maximum percentage aggregation (Figure 2.7) and lag phase (Figure 2.8) were also analysed in these experiments, and with the exception of occasional experiments, the results for maximum percentage aggregation were similar to those for aggregation rate ($p < 0.05$). A reduction in lag phase shown in both ADP and thrombin stimulated platelets seemed most prominent in the presence of non-apoptotic cells ($p < 0.05$). However, this tendency was not statistically significant in apoptotic endothelial cells (Figure 2.8).

Experiments were also performed using collagen as a platelet agonist. It was found that although non-apoptotic endothelial cells (UnA-EC and SDnA-EC) as well as MDA-EC reduced platelet aggregation ($p < 0.05$), SDDA-EC and M&SDA-EC did not reproducibly express this activity (Figure 2.9). The results for maximum percentage aggregation were broadly similar while when examining results for lag-phase, non-apoptotic endothelial cells reduced lag phase ($p < 0.05$) while MDA-EC

appeared to produce a prolonged lag phase ($p < 0.05$) and no clear effect was observed for SDDA-EC and M&SDA-EC (Figure 2.9).

2.3.d. The Anti-Aggregatory Activity of Endothelial Cells for Thrombin and ADP Stimulated Platelets was Not due to a Passive Mechanical Effect of Cells

It was considered possible that reduced platelet aggregation in the presence of endothelial cells may be artifactual, reflecting mechanical interference of platelet to platelet contact by endothelial particles. To examine this possibility, experiments were performed with latex beads of similar dimension to apoptotic and non-apoptotic endothelial cells. When latex beads were substituted for HUVEC no reproducible effect upon thrombin or ADP stimulated platelet aggregation was seen, excluding a significant passive mechanical effect of endothelial cells upon platelet aggregation in response to these two agonists. However, when collagen was used as an agonist, the small beads reduced the aggregation rate in most experiments while the large beads always reduced the aggregation rate (Figure 2.10).

2.3.e. Collagen Fibres were Mopped-up by Endothelial Cells and Latex Beads

The above described observations with latex beads indicated that inhibition by apoptotic and non-apoptotic cells of thrombin and ADP induced platelet aggregation was not likely due to mechanical interference and most likely reflected a biological response. However, latex beads did significantly reduce platelet aggregation in response to collagen as an agonist. One possible explanation for this was that latex beads mechanically interfered with platelet interactions during collagen induced aggregation. However, in view of the absence of any effect of beads upon thrombin or ADP stimulated aggregation, the other possibility that collagen binding by latex

beads deprived platelets of agonist, thus reducing the aggregation rate, was considered more likely (Figure 2.10). This also raised the further possibility that endothelial cells similarly bound collagen fibres, and that this may have accounted for the reduced aggregation rate in response to collagen (Figure 2.9).

To investigate this possibility, SEM of platelet aggregates in the presence and absence of collagen and endothelial cells was performed. Aggregation of platelets was seen in the presence of collagen both with and without apoptotic endothelium and importantly, collagen fibres were seen in association with both clumps of platelets and endothelial particles (Figure 2.11). Also, it was noted that platelets displayed significant shape change when activated by collagen alone (Figures 2.11 and 2.12A) but that when platelets were exposed to apoptotic endothelial cells alone, no shape change occured (Figure 2.12B). At higher magnification, activated platelets were seen to bind to the surface of apoptotic endothelial cells via the membrane processes generated during platelet activation (Figure 2.13). These data suggested that at least some of the anti-aggregatory effect seen in collagen treated platelets was due to the mechanical mopping up of collagen fibres by endothelium.

In examining apoptotic cells by SEM, numerous cells were found with small surface pores, membrane rests and extensive irregular fragmented regions (Figure 2.13). These appeared consistent with surface morphology expected for cells undergoing canalicular fragmentation, initially through invagination of the plasma membrane via surface pores and latter through gross mechanical disruption in highly honeycombed areas.

Additional experiments were performed with collagen solutions pre-treated with the five endothelial cell populations and latex beads before removal of the cells or beads by gentle centrifugation. It was found that the non-apoptotic endothelial cells (UnA-EC and SDnA-EC), MDA-EC and latex beads inactivated collagen solutions as compared with parallel solutions of collagen, centrifuged but not exposed to cells or beads (Table 2.2). This was interpreted as reflecting the binding and removal of collagen fibres by cells and beads.

Figure 2.1. Photomicrographs of paraffin sections of human umbilical vein labeled by immuno-histochemistry for VWF (A) and lectin histochemistry with UEA-1 (B). Endothelial cells (arrows) were visible as a monolayer separating the vessel lumen (L) from the underlying smooth muscle cells (SMC). Endothelium labeled for both VWF (*A*) and UEA-1 lectin binding (*B*) while underlying SMC failed to label with either of these markers. No significant labeling was seen in control sections in which the primary antibody or lectin was omitted (*C*). (Immuno or lectin histochemistry and Haematoxylin, Bars = 250 μm)

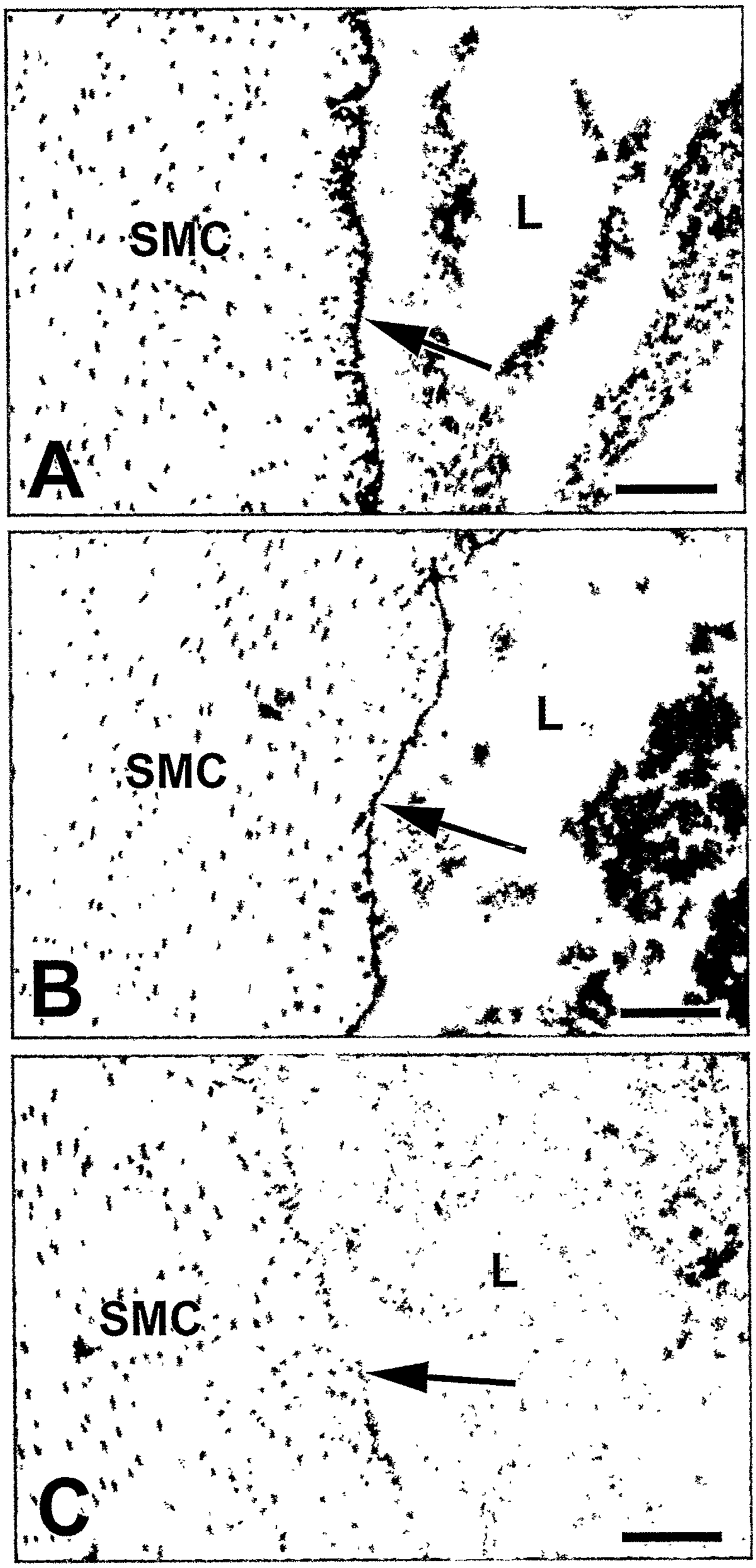
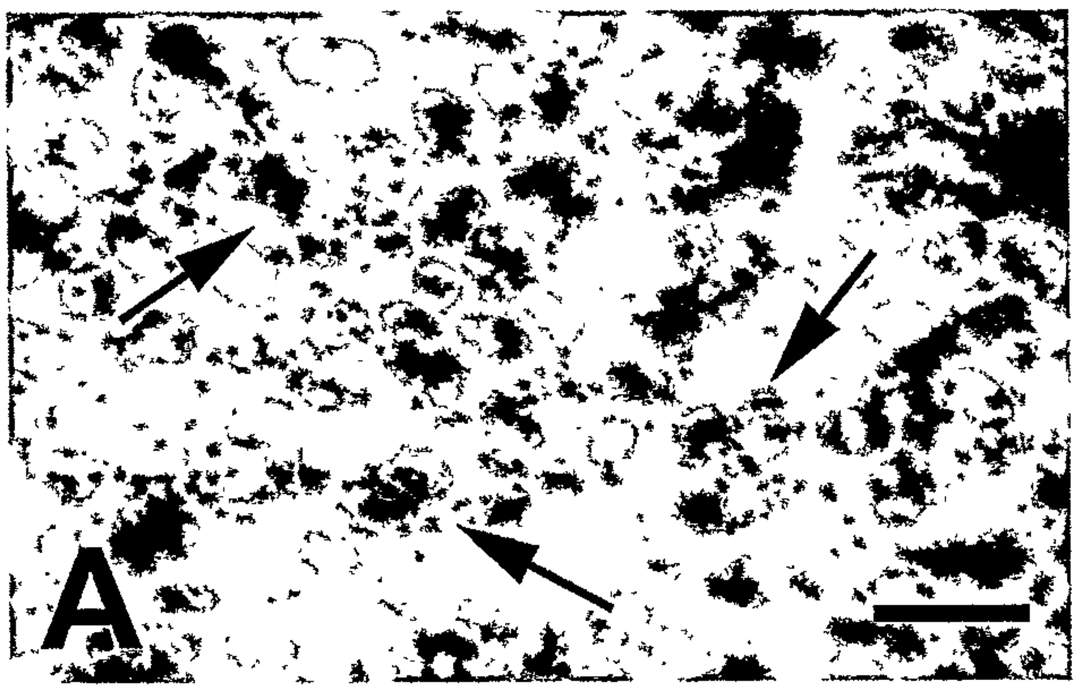

SMC
L
A
SMC
L
B
SMC
L
C

Figure 2.2. Photomicrographs of isolated cultured cells from umbilical vein labeled by immuno-histochemistry for VWF (A) and lectin histochemistry with UEA-1 (B). Cells cultured from umbilical veins displayed punctate labelling VWF (arrows) (*A*) and a more diffuse surface labelling for UEA-1 binding (arrows) (*B*), typical of endothelium and confirming the identity of these cells as HUVEC. No labeling was seen for cells (arrows) in control specimens where either of the primary antibody or lectin was omitted (*C*). (Immuno or lectin histochemistry and Haematoxylin, Bars = 80 μm)


A

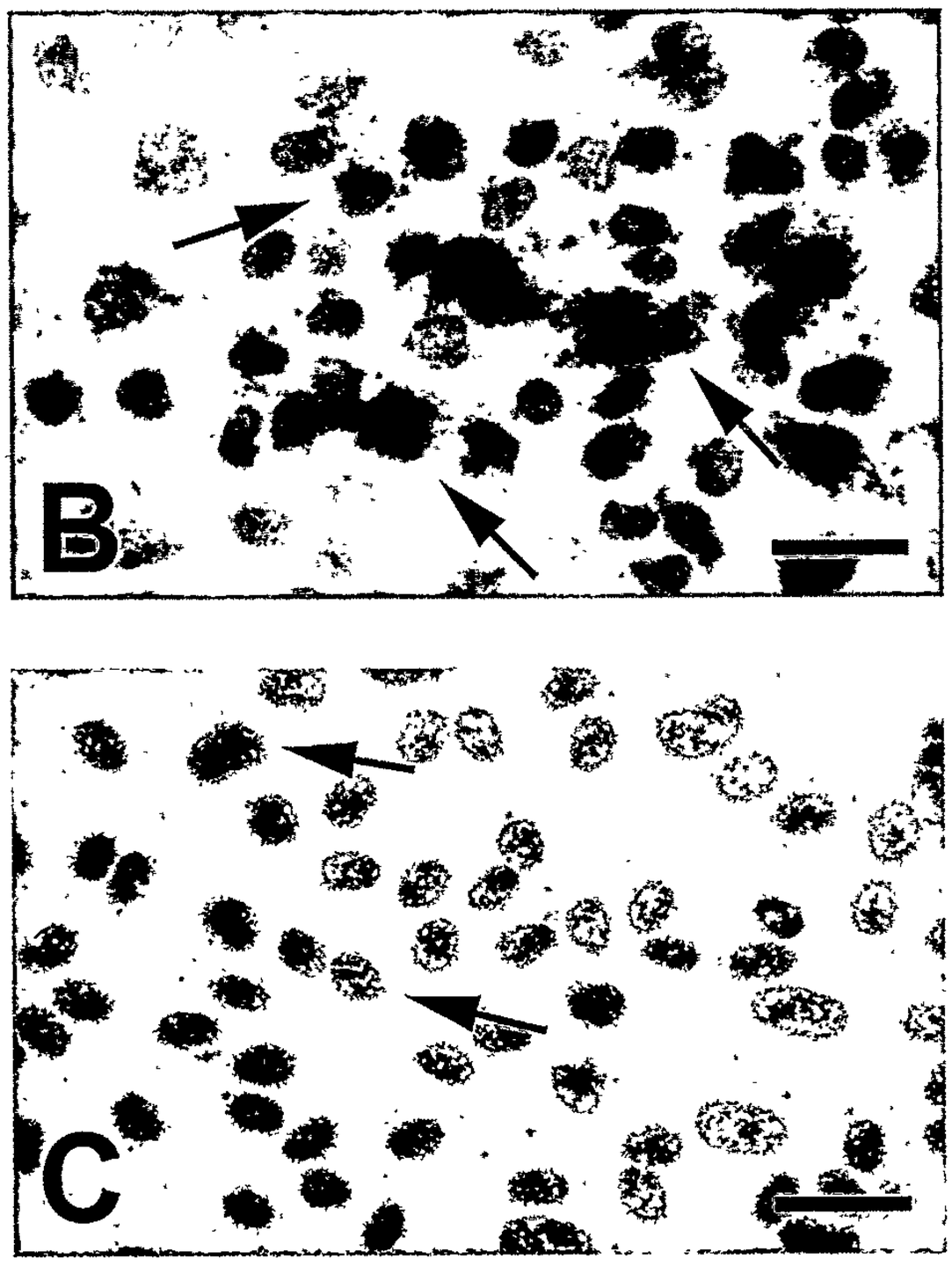

B
C

Figure 2.3. Agarose gel electrophoresis with ethydium bromide staining of DNA from the apoptotic and non-apoptotic HUVEC populations used in this study. DNA from UnA-EC and SDnA-EC was not fragmented while internucleosomal fragmentation to produce a 180 base pair ladder was seen in DNA from SDDA-EAC, MDA-EC as well as M&SDA-EC. Molecular size markers are indicated.

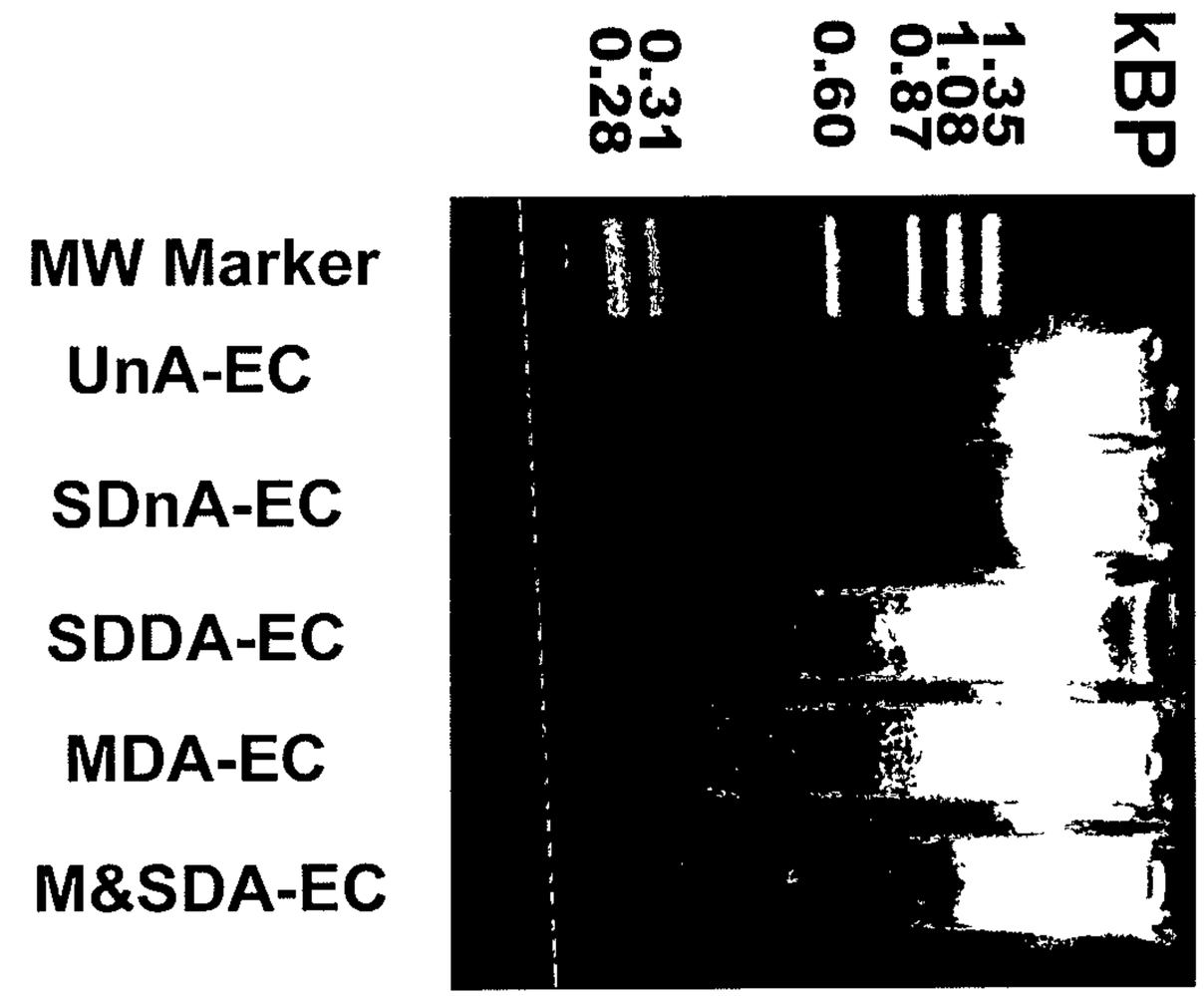

kBP
1.35
1.08
0.87
0.60
0.31
0.28
MW Marker
UnA-EC
SDnA-EC
SDDA-EC
MDA-EC
M&SDA-EC

Figure 2.4. Transmission electron micrographs of SDnA-EC (A) and SDDA-EC (B). (*A*) Non-apoptotic HUVEC had large vesicular nuclei (vN) with loose chromatin, intact mitochondria (m) and no sign of cellular or organellar fragmentation. (*B*) This contrasted with apoptotic HUVEC where changes typical of endothelial apoptosis were seen including: the formation of apoptotic particles, nuclear fragmentation and condensation (N), canalicular fragmentation (c) and maintained integrity of mitochondria (m) and RER (arrows). (Bar for A = 1 μm, Bar for B = 2 μm)

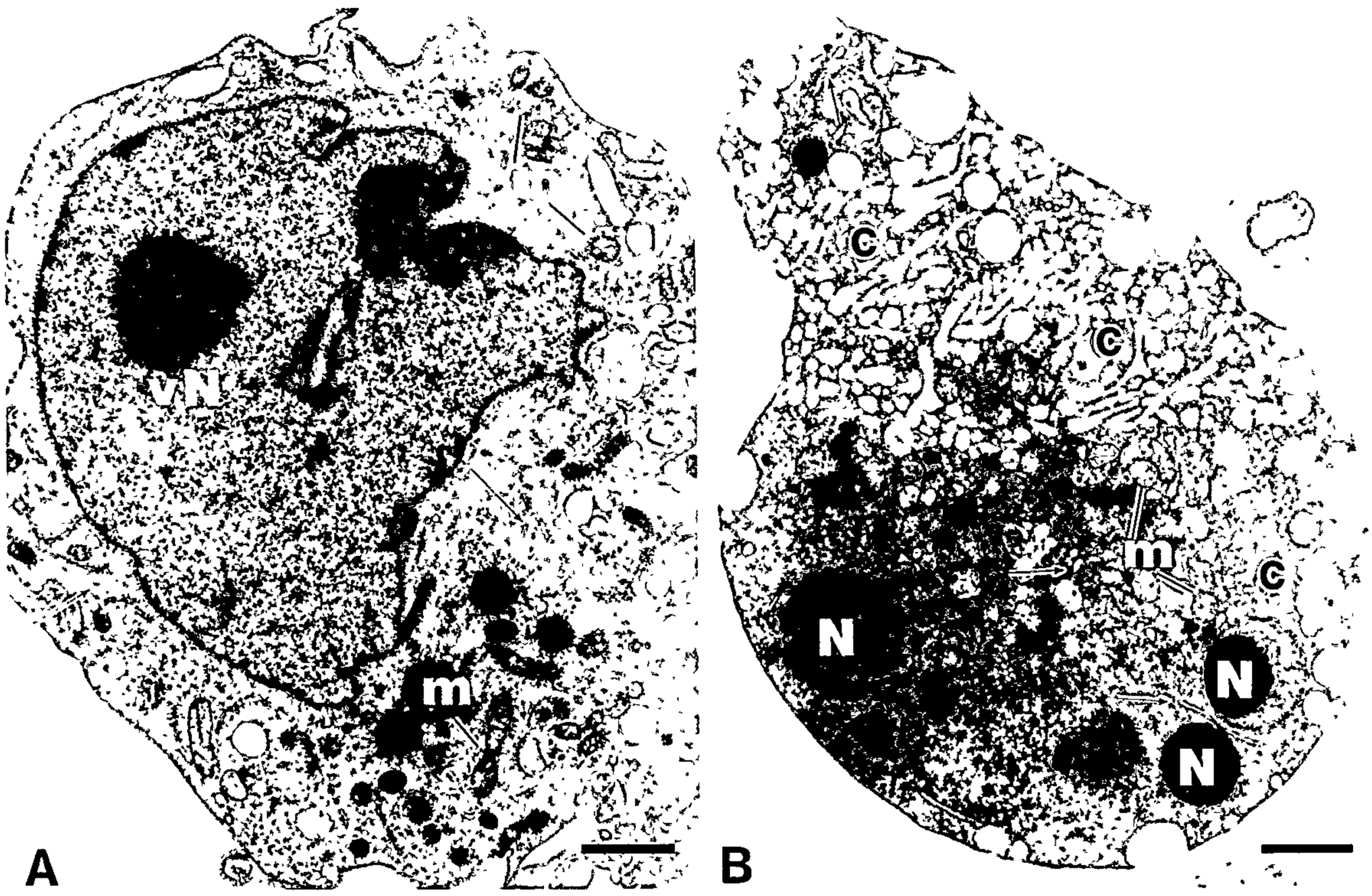

VN
m
A
C
C
C
m
C
N
N
N
B

Figure 2.5. Representative aggregometry traces for platelets stimulated with thrombin (0.25 U/ml) or ADP (2.5 μM) in the presence of added apoptotic endothelial cells (M&SDA-EC), non-apoptotic endothelial cells (UnA-EC) or buffer (HBSS). Both non-apoptotic and apoptotic populations of endothelial cells reduced the aggregation rate, which is determined from the slope of the aggregometry trace curve. The maximum percentage aggregation revealed the size of platelet aggregates and indicated by the height achieved by the aggregometry trace curve. This was lower in the presence of either apoptotic or non-apoptotic endothelium as compared with buffer alone. Lag phase was defined as the time between addition of the platelet agonist and the onset of aggregation. Shape change with the formation of platelet membrane surface processes is an early event during platelet aggregation and was revealed by a modest reduction in light transmission soon after addition of the agonist. Using traces such as these, it was possible to obtain reproducible and interpretable data for aggregation rate, maximum percentage aggregation and lag phase, allowing comparison of the response of platelets to agonists in the presence of endothelial cells from numerous separate donors. Although endothelium did reduce platelet aggregation, no consistent difference was found between any of the endothelial populations studied.

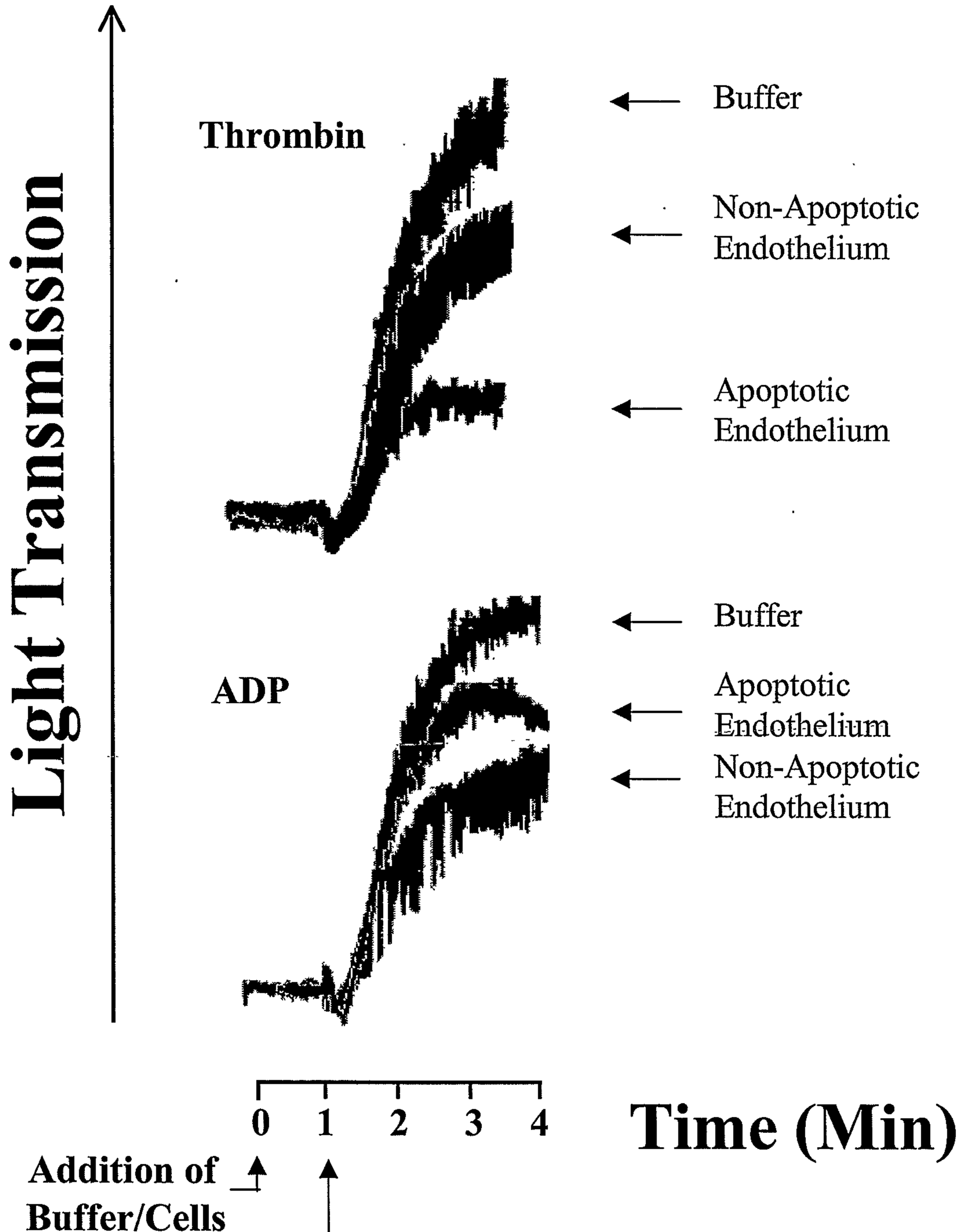
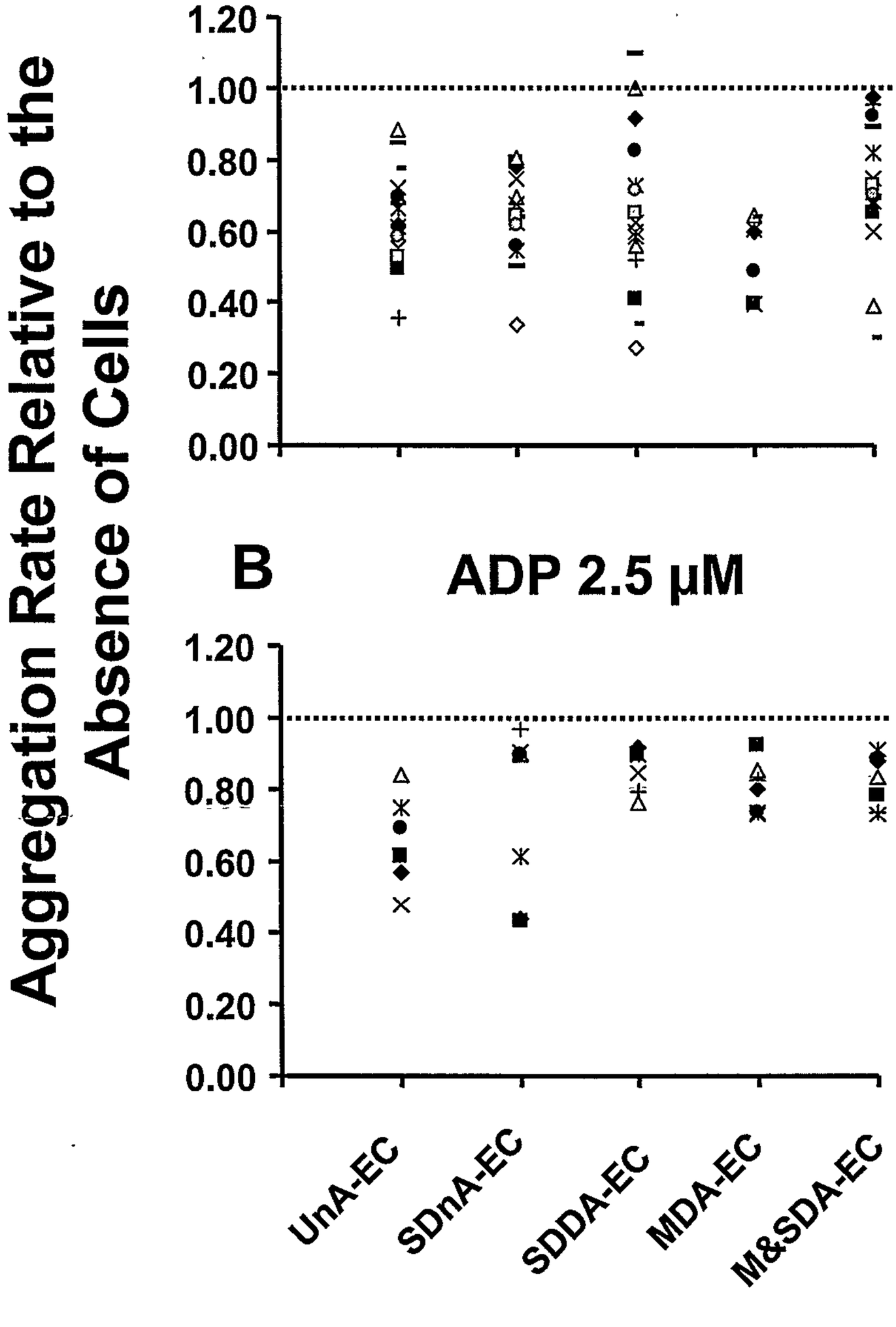

Light Transmission
Thrombin
Buffer
Non-Apoptotic Endothelium
Apoptotic Endothelium
ADP
Buffer
Apoptotic Endothelium
Non-Apoptotic Endothelium
0
1
2
3
4
Time (Min)
Addition of Buffer/Cells
Addition of Agonist

Figure 2.6. The relative aggregation rate of thrombin (0.25 U/ml) (A) and ADP (2.5 μM) (B) stimulated platelets in the presence of non-apoptotic (UnA-EC, SDnA-EC) and apoptotic (SDDA-EC, MDA-EC, M&SDA-EC) endothelium as compared with aggregation in the absence of endothelial cells (Value of 1 as indicated by the horizontal line). All five endothelial populations had anti-aggregatory activity for platelets activated with thrombin or ADP ($p < 0.05$) as evidenced by the preponderance of data points below the horizontal line with a value of 1.



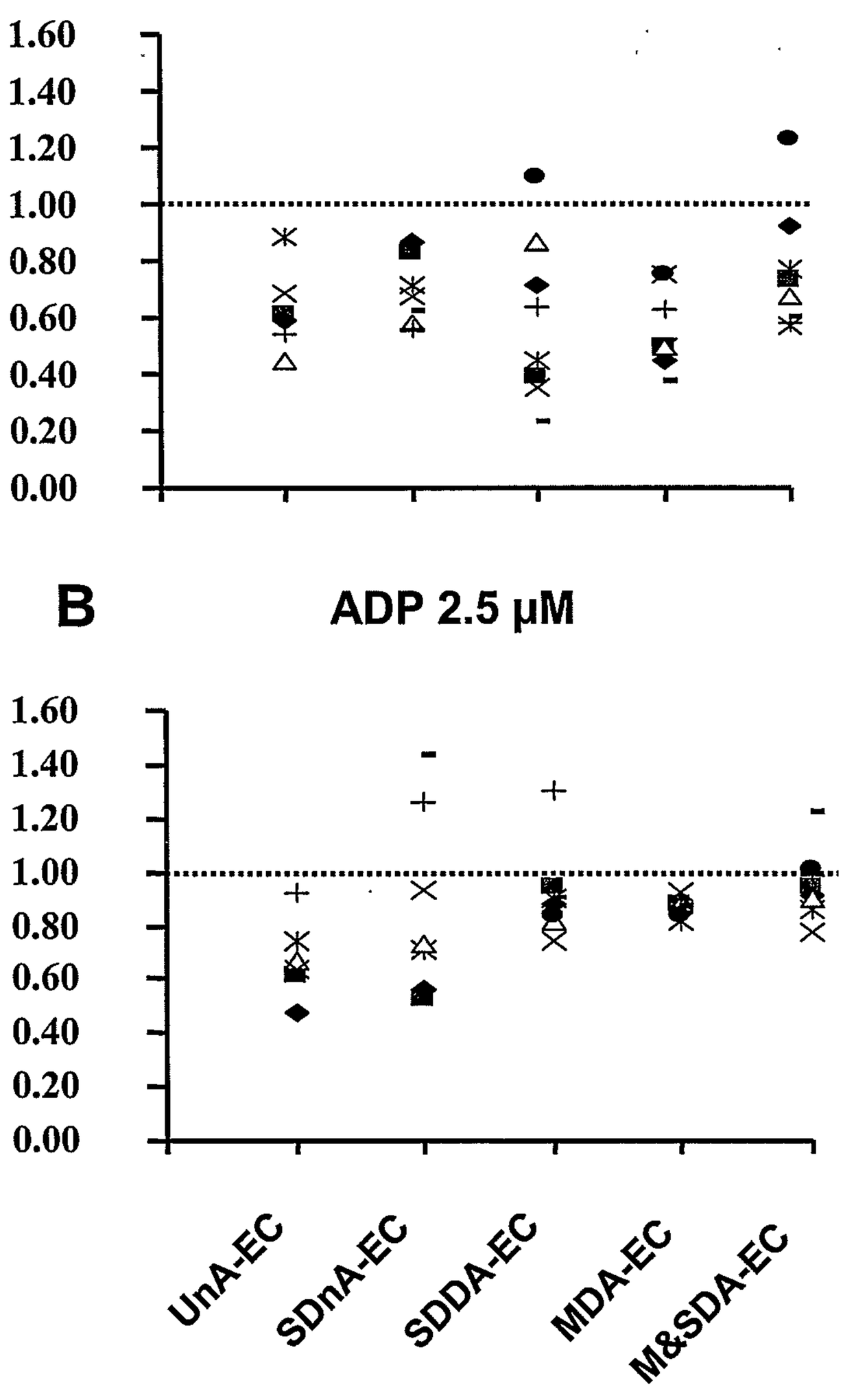
Cell Type Included with Platelets

Figure 2.7. The relative maximum percentage aggregation seen in thrombin (0.25 U/ml) (A) and ADP (2.5 μM) (B) stimulated platelets in the presence of non-apoptotic (UnA-EC, SDnA-EC) and apoptotic (SDDA-EC, MDA-EC, M&SDA-EC) endothelium as compared with aggregation in the absence of endothelial cells (Value of 1 as indicated by the horizontal line). In almost all experiments, the maximum percentage aggregation was lowered by both apoptotic and non-apoptotic populations of HUVEC, indicating that endothelial cells not only reduced the rate of platelet aggregation but also reduced the size of any aggregates which formed ($p < 0.05$).

A Thrombin 0.25 U/ml

B ADP 2.5 µM

Maximum Percentage Aggregation Relative to the Absence of Cells



Cell Type Included with Platelets

Figure 2.8. The relative lag phase of thrombin (0.25 U/ml) (A) and ADP (2.5 μM) (B) stimulated platelets in the presence of non-apoptotic (UnA-EC, SDnA-EC) and apoptotic (SDDA-EC, MDA-EC, M&SDA-EC) endothelium as compared with lag phase in the absence of endothelial cells (Value of 1 as indicated by the horizontal line). Reduced lag phase for both thrombin and ADP stimulated platelets in the presence of non-apoptotic endothelium was seen ($p < 0.05$), while this trend was not seen in the presence of apoptotic endothelium ($p > 0.05$). This suggested that the onset of platelet activation was longer in the presence of apoptotic endothelium as compared with that with non-apoptotic endothelium.

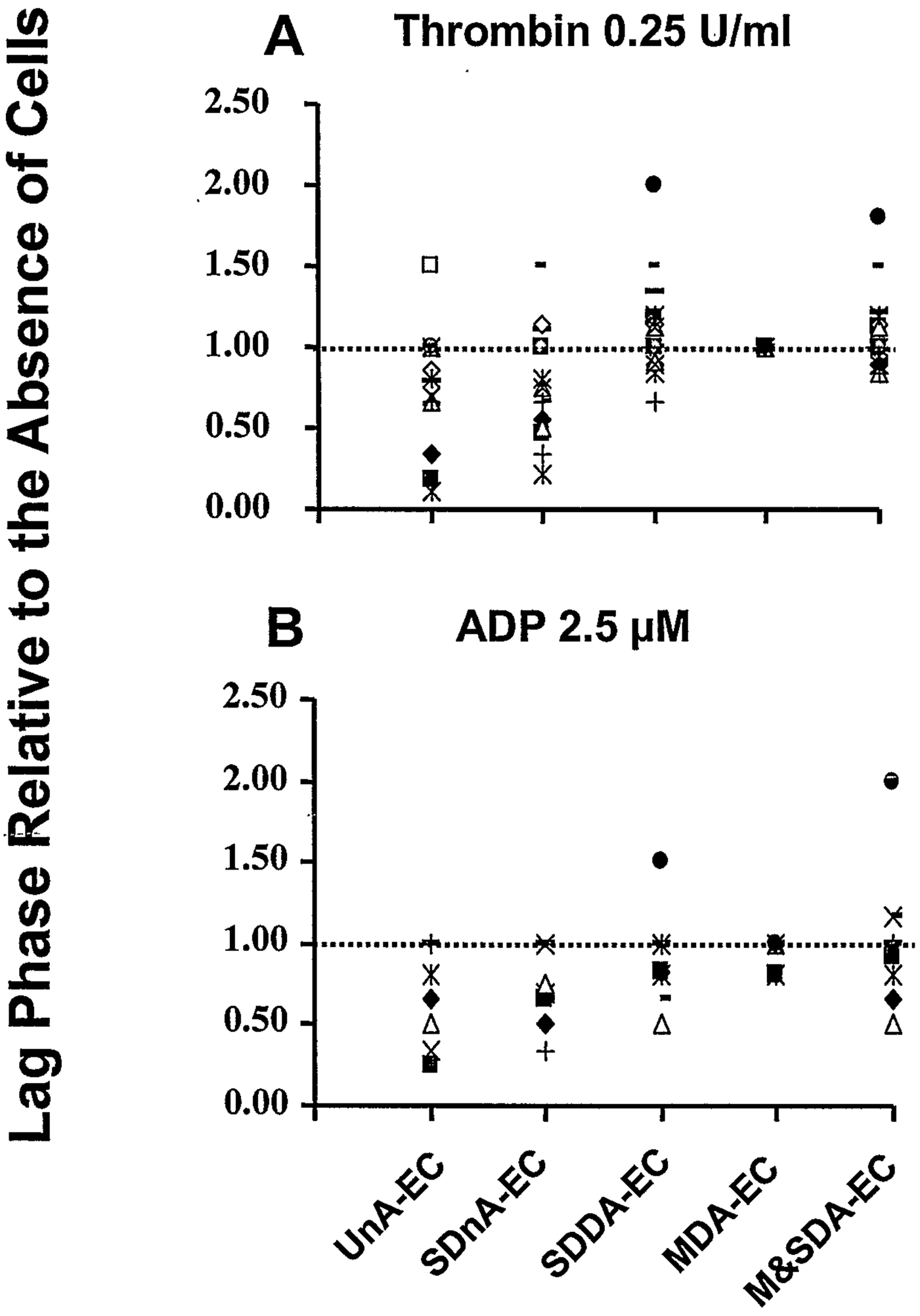

A
Thrombin 0.25 U/ml
2.50
2.00
1.50
1.00
0.50
0.00
B
ADP 2.5 µM
2.50
2.00
1.50
1.00
0.50
0.00
UnA-EC
SDnA-EC
SDDA-EC
MDA-EC
M&SDA-EC
Lag Phase Relative to the Absence of Cells
Cell Type Included with Platelets

Figure 2.9. The relative lag phase, aggregation rate and maximum percentage aggregation of collagen stimulated platelets in the presence of non-apoptotic (UnA-EC, SDnA-EC) and apoptotic (SDDA-EC, MDA-EC, M&SDA-EC) endothelium as compared with responses in the absence of endothelial cells (Value of 1 as indicated by the horizontal line). The aggregation rate was markedly reduced by the presence of both populations of non-apoptotic endothelium as well as by MDA-EC ($p < 0.05$), however, SDDA-EC and M&SDA-EC did not affect platelet aggregation. Lag phase was also reduced by the presence of non-apoptotic cells ($p < 0.05$), but not for apoptotic cells, where a rise in lag phase was seen for MDA-EC ($p < 0.05$). The maximum percentage aggregation appeared unaffected in all of the endothelial populations studied, with the exception again of MDA-EC, where a reduction occurred in most experiments ($p < 0.05$). Although these data suggested an interesting role for anti-aggregatory activities of endothelium for collagen induced platelet aggregation, further experiments described in this chapter revealed that these data were complicated by collagen binding or other mechanical effects.

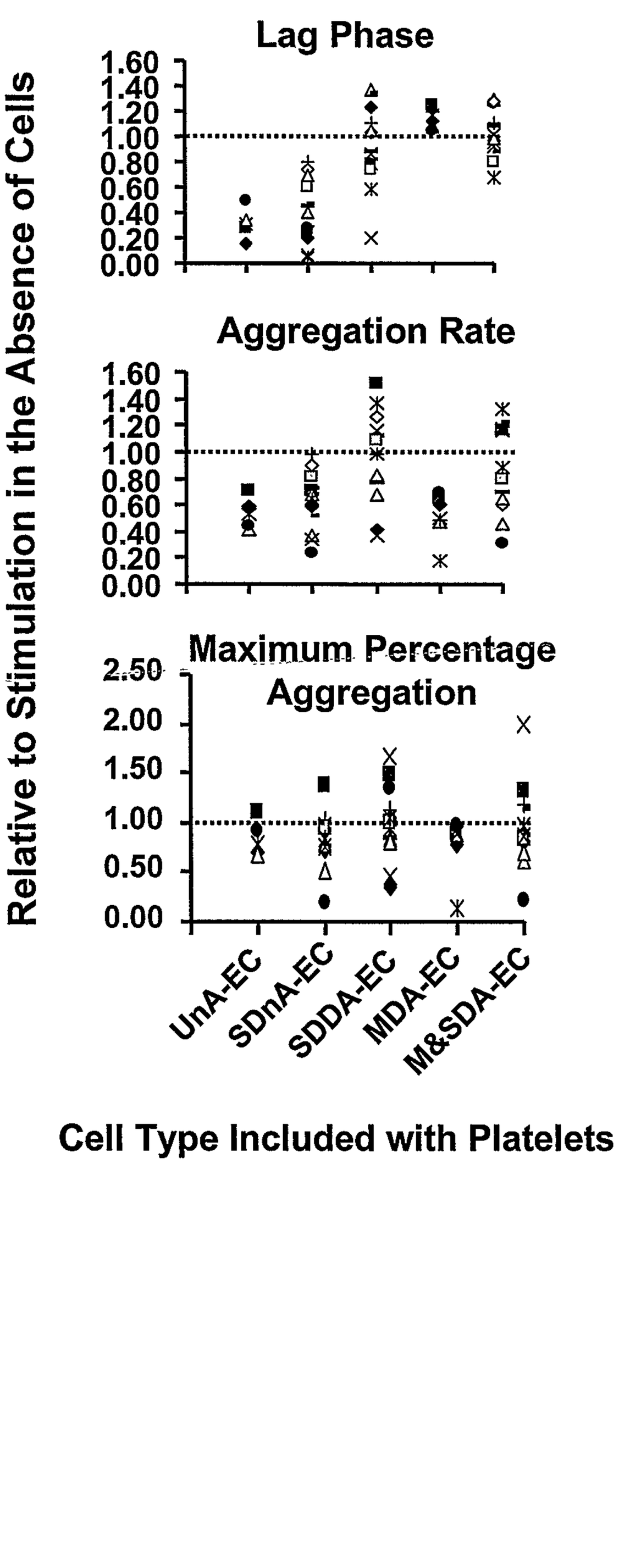

Relative to Stimulation in the Absence of Cells
Lag Phase
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Aggregation Rate
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Maximum Percentage Aggregation
2.50
2.00
1.50
1.00
0.50
0.00
UnA-EC
SDnA-EC
SDDA-EC
MDA-EC
M&SDA-EC
Cell Type Included with Platelets

Figure 2.10. The relative aggregation rate of thrombin (A), ADP (B) and collagen (C) stimulated platelets in the presence of latex beads with diameters of either 6.4 μm or 23 μm as compared with aggregation in the absence of beads (Value of 1 as indicated by the horizontal line). Beads of both sizes had no effect upon aggregation rate in response to either thrombin or ADP. These data indicate that the effect of apoptotic and non-apoptotic cells upon aggregation rate stimulated by thrombin and ADP were not due to a passive mechanical effect. However, when collagen was used as an agonist, the small beads reduced the aggregation rate in most experiments while the large beads always reduced the aggregation rate. This suggested that endothelial cells may mechanically interfere with platelet aggregation in response to collagen, either by interspersing between platelets and preventing platelet adhesion or alternatively, perhaps by binding collagen.

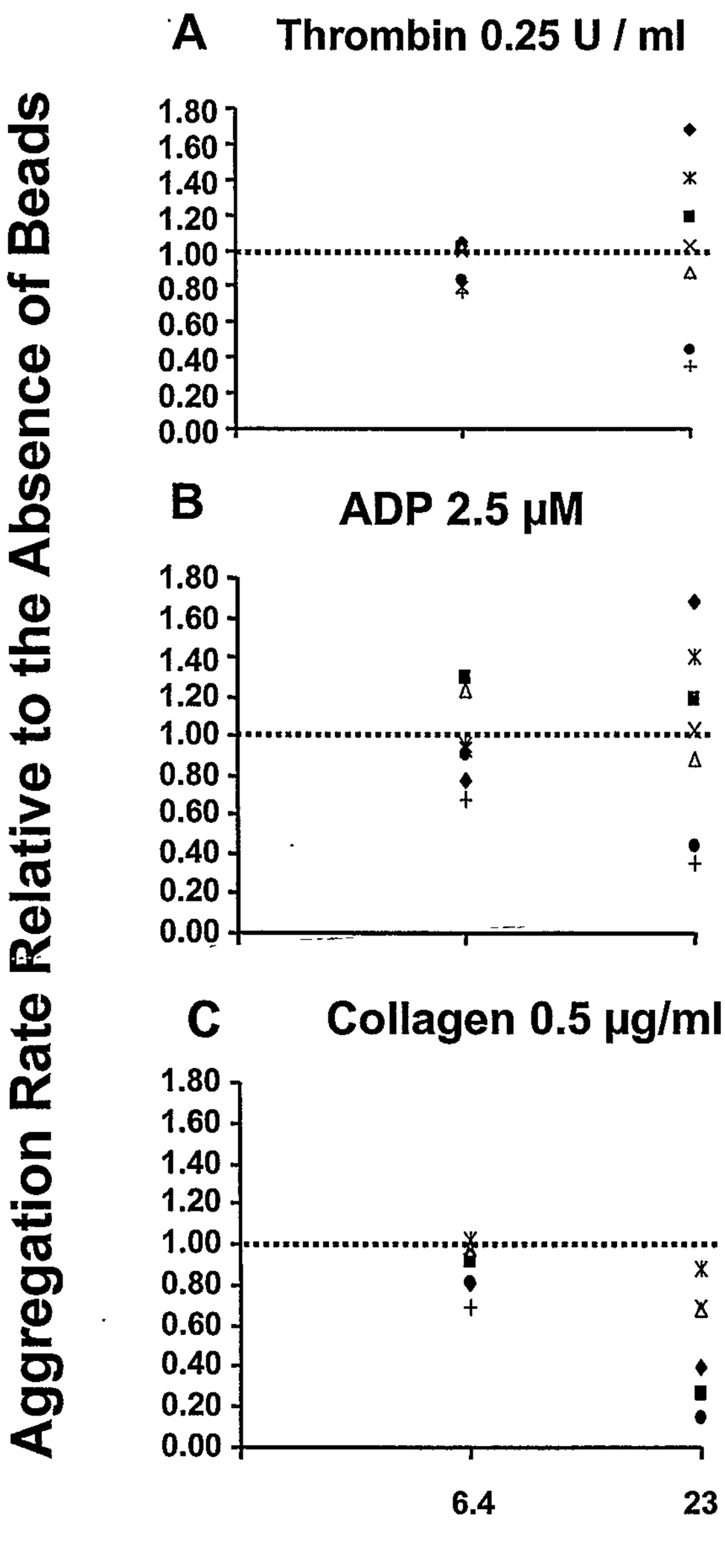

A Thrombin 0.25 U / ml
B ADP 2.5 µM
C Collagen 0.5 µg/ml
Aggregation Rate Relative to the Absence of Beads
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
6.4
23
Size of Beads (µm)

Figure 2.11. Scanning electron micrographs of platelet aggregates with apoptotic endothelial cells and collagen at low (A) and high magnification (B). Collagen fibres (CF) were seen as linear structures subtended in a string-like fashion between cellular structures or across the supporting membrane surface. At higher magnification, a slight banding pattern was seen in collagen fibres, expected for this molecule. Platelet aggregates were seen (large arrow heads). In addition individual platelets were seen, often with many extended cell processes characteristic of platelet aggregation (small arrow heads). Amongst these platelet aggregates, occasional endothelial cells were seen as large, often roughly spherical structures (arrows). These were often loosely associated with platelet aggregates while occasionally, collagen fibres were seen bound to the endothelial surface (*B*). Very occasional contaminant erythrocytes (E) were identified in micrographs, although it is not felt likely that these contributed significantly to the data obtained. (Bars = 10 μm)

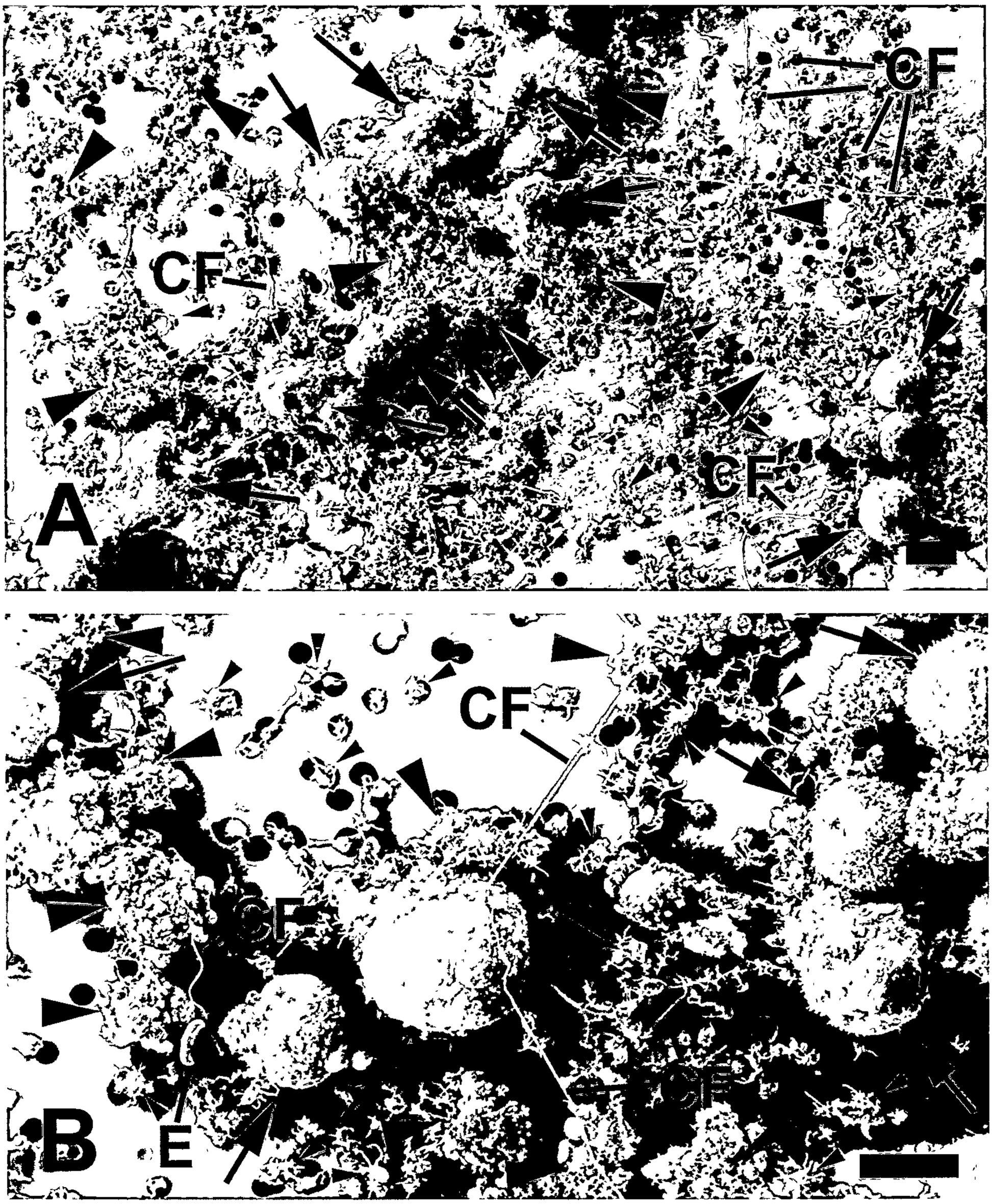
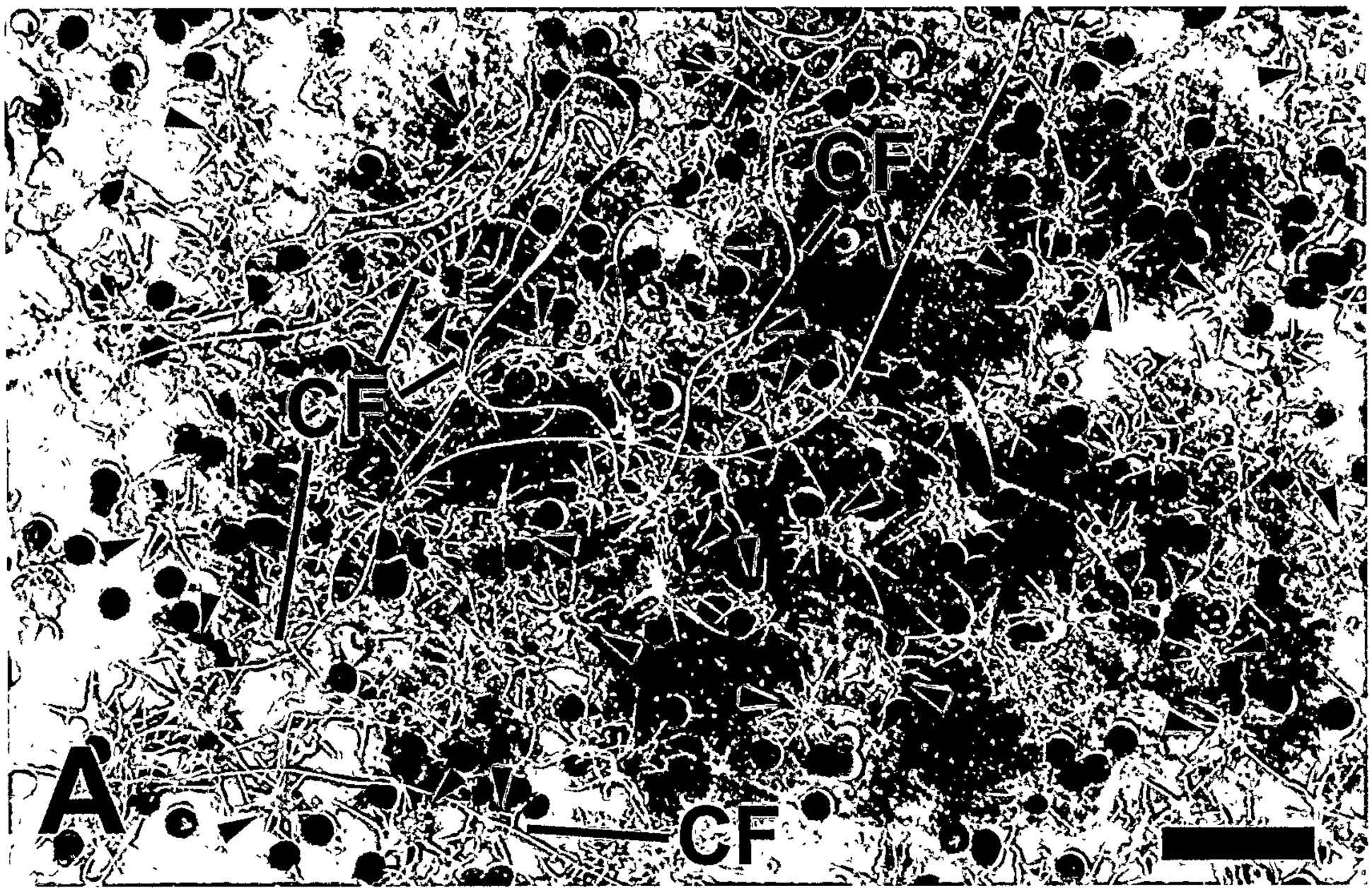

CF
CF
CF
A
CF
CF
B
E
CF

Figure 2.12. Scanning electron micrographs of platelets exposed to collagen alone (A) or apoptotic endothelial cells alone (B). (*A*) In this micrograph, collagen fibres (CF) were seen spread across the surface of the membrane support while activated platelets with highly fimbriated surfaces were present (small arrow heads), consistent with activation of platelets by collagen fibres. (*B*) In the absence of collagen, platelets appeared much less activated, with only occasional surface processes present (small arrow heads) and seemed to fall into the holes of the supporting membrane (large arrow heads). Apoptotic endothelial cells (arrows) did, however, appear to bind occasional platelets with the formation of small aggregates in the absence of collagen, suggestive of low levels of platelet aggregation frustrated by anti-aggregatory activity from endothelial cells. (Bars = 10 μm)


CF
CF
CF
A

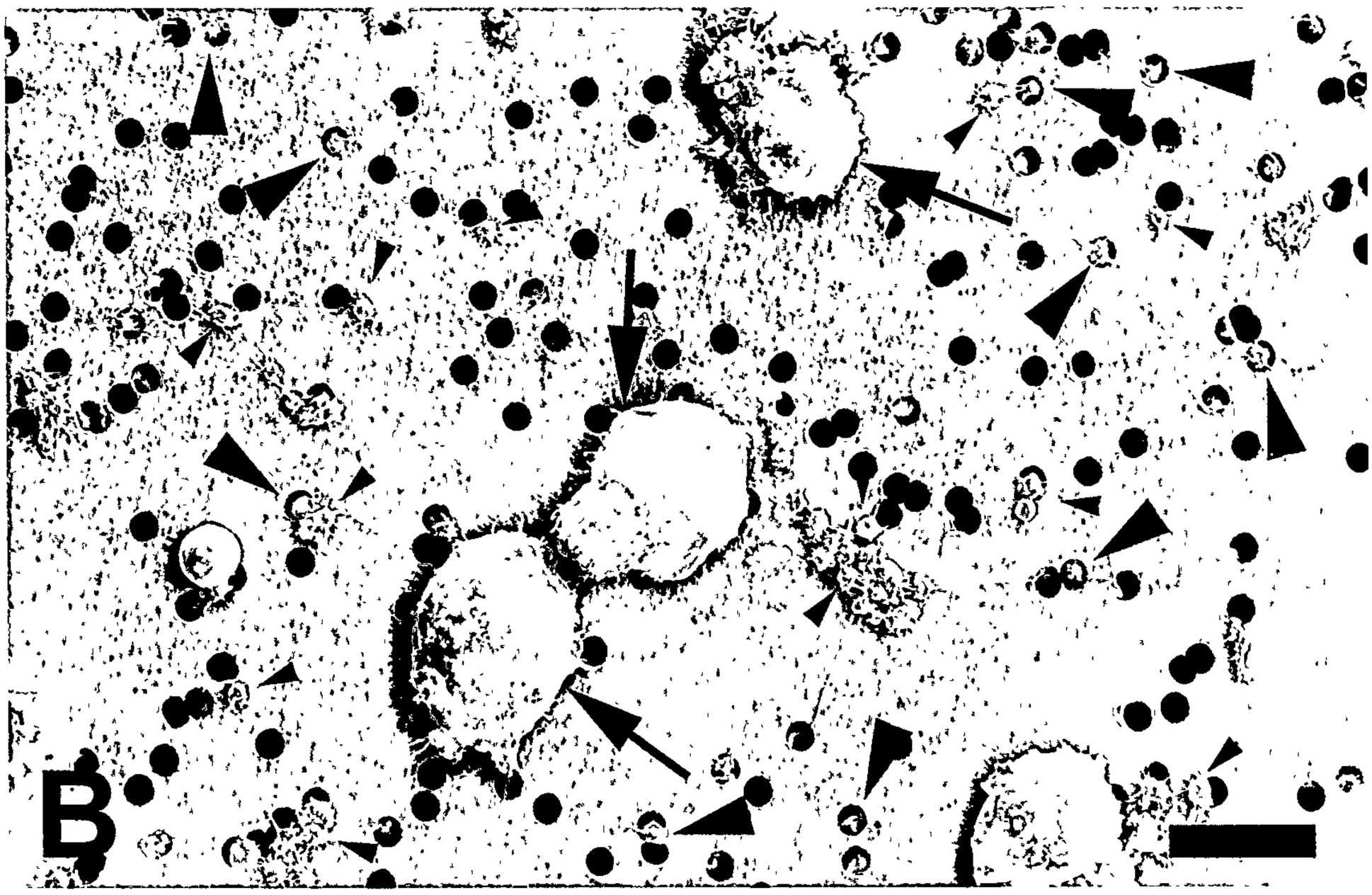

B

Figure 2.13. Scanning electron micrograph of an apoptotic endothelial cell in the presence of collagen stimulated platelets. Many apoptotic endothelial cells had surface features consistent with those expected from TEM of cells undergoing canalicular fragmentation. In this micrograph, an apoptotic endothelial cell was seen with a smooth surface frequently marked by surface pores (P), consistent with the surface pores through which canaliculi appear to form. Rising from the surface of this cell was a single membrane-like sheet (m), similar to the membranous rests also reported in transmission electron micrographs of apoptotic endothelium. A highly irregular surface suggestive of fragmentation with exposure of underlying canalicular membranous structures and cell contents was seen on one surface of the cell (F), suggestive of the fragmentation that canaliculi formation has been proposed to result in. In addition, numerous activated platelets with extensive plasma membrane process formation were seen (arrow heads). Some of these appeared to have an association with the endothelial cell surface via their cell processes (arrows). (Bar = 3 μm).

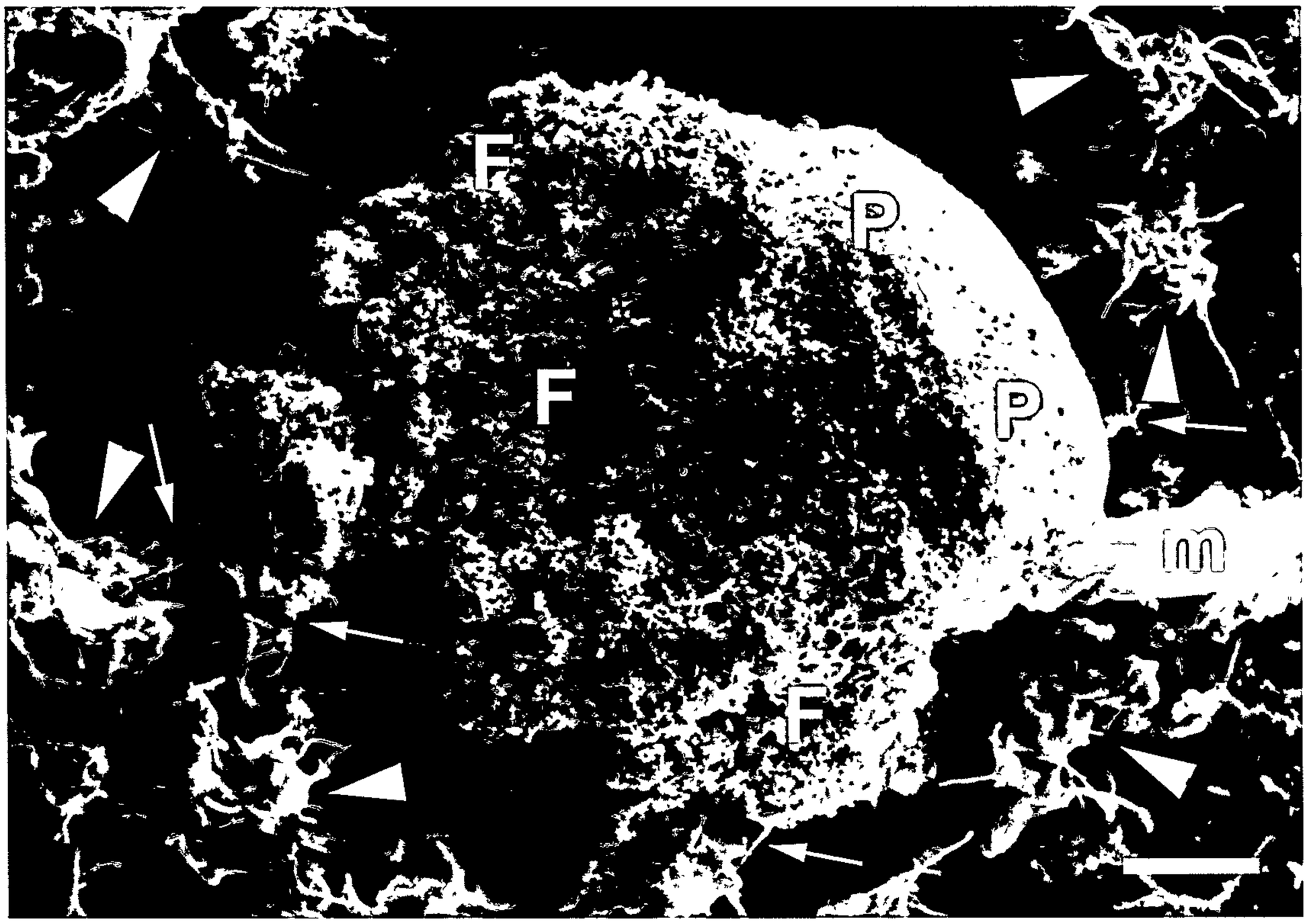

F
P
F
P
m
F

Table 2.2 The effect of pre-treatment of collagen solutions with endothelium or latex beads upon the agonist activity of collagen for platelets

	Aggregation Rate	Lag Phase	Maximum Percentage Aggregation
Buffer	2.25 ± 0.55	71.2 ± 20.1	45.91% ± 0.16%
UnA-EC	0.56 ± 0.46	34.4 ± 23.4	9.74% ± 0.07%
SDnA-EC	0.55 ± 0.55	26.4 ± 26.4	9.69% ± 0.10%
SDDA-EC	0.92 ± 0.47	34.0 ± 20.1	15.15% ± 0.12%
MDA-EC	0.52 ± 0.32	51.6 ± 27.6	9.06% ± 0.06%
M&SDA-EC	2.11 ± 0.11	56.4 ± 20.8	30.21% ± 0.09%
Beads (*D=6.4* μ)	--	--	--
Beads (*D=23* μ)	--	--	--

The aggregation rate was reduced by pre-treatment with either endothelium or beads, as was the maximum percentage aggregation. Lag phase was also affected in most experiments. Where latex beads were used, all of the agonist activity was lost upon centrifugation, so that no platelet aggregation was obtained. These data indicate binding and / or inactivation of collagen by the particulate material removed by centrifugation prior to platelet activation.

2.4. Discussion

The cells used in these studies had the morphological, immunohistochemical and lectin binding properties expected for HUVEC, so that there can be reasonable confidence that the results obtained in experiments reflect endothelial behavior. Reduction in platelet aggregation with HUVEC has been reported by others (Alheid *et al.*, 1987; Broekman *et al.*, 1991; Kato *et al.*, 1992; Durante *et al.*, 1992; Ohno *et al.*, 1994), so that reduced aggregation in the presence of UnA-EC further supported the identity of cells used as HUVEC.

The ultrastructural and DNA degradative features typical of endothelial apoptosis verified SDDA-EC, MDA-EC and M&SDA-EC as apoptotic while UnA-EC and SDnA-EC displayed all the features expected of non-apoptotic HUVEC (Kerr *et al.*, 1972; Zoellner *et al.*, 1996a; 1996b; 1998; 1999). Further supportive of the endothelial origin of cells used was observation of canalicular fragmentation, an ultrastructural feature seen only during apoptosis in endothelium (Zoellner *et al.*, 1996a; 1999).

As discussed in 2.1.b.ii, SDDA-EC are suggested as a reasonable experimental model for study of apoptotic endothelial cells generated during vascular remodelling. SDnA-EC provided a convenient control population for comparison with SDDA-EC, and this experimental model has been used in earlier studies comparing the fibrinolytic properties of SDnA-EC with SDDA-EC (Zoellner *et al.*, 1998). Also discussed in 2.1.b.ii, MDA-EC seem a reasonable model for apoptotic cells generated through traumatic detachment of endothelial cells. As expected from earlier studies

(Meredith *et al.*, 1993; Zoellner *et al.*, 1996b), these MDA-EC had identical apoptotic features to SDDA-EC. In this way, by comparing SDDA-EC with MDA-EC, it was possible to compare the behaviour of apoptotic endothelial cells obtained by mechanisms similar to the two main causes of endothelial cell apoptosis *in vivo*, vascular remodelling and trauma. Also, study of M&SDA-EC allowed investigation of any possible synergy with regard to platelet activation between these two mechanisms of stimulating endothelial cell apoptosis. Also M&SDA-EC from HUVEC have been used by others to study coagulation related events in apoptotic endothelium (Bombeli *et al.*, 1997; 1999). UnA-EC provided a further control population of non-apoptotic endothelial cells exposed to serum.

The fragmentation of individual cells into an unpredictable number of smaller particles, accelerated in endothelial cells by canalicular fragmentation (Zoellner *et al.*, 1996a), makes the precise quantitation of apoptotic cell number after apoptosis impossible. To overcome this difficulty, SDDA-EC number was calculated by subtracting the number of surviving adherent cells (SDnA-EC) from the number of cells in the starting population before 24 hr of serum deprivation. The preparation of MDA-EC and M&SDA-EC by 24 hr of matrix deprivation provided a convenient opportunity to determine the starting cell number at the beginning of both matrix deprivation for MDA-EC, M&SDA-EC and serum deprivation of SDnA-EC and SDDA-EC. It is known that there is no significant HUVEC proliferation in the conditions used to obtain SDDA-EC and SDnA-EC and electron microscopy reveals all cells detached during serum deprivation to be apoptotic (Zoellner *et al.*, 1996a; 1996b; 1999). Because of this, it is reasonable to assume that SDDA-EC number obtained by the above described calculation provides a good measure of apoptotic cell

number. This approach has been successfully used to quantitate apoptotic cell number for normalization of antigen levels between apoptotic and non-apoptotic HUVEC (Zoellner *et al.*, 1998). Similarly, since electron microscopic examination revealed all MDA-EC to be apoptotic, the cell count at the time of seeding into bacterial culture plates provides a reasonable measure of apoptotic cell number after 24 hr of matrix deprivation.

One unavoidable difficulty in this approach is the loss of apoptotic cells by secondary necrosis, which occurs when apoptotic particles are no longer able to maintain homeostasis (Cejna *et al.*, 1994; Hebert *et al.*, 1996; Papassotiropoulos *et al.*, 1996; Shah *et al.*, 1997) (please see section 1.2.a.ii). The number of these necrotic-apoptotic particles is difficult to assess as they rapidly disintegrate or are phagocytosed by adjacent cells. It is, however, important to note that relatively few of the particles observed by TEM exhibited signs of secondary necrosis. Also, it must be stressed that because secondary necrosis can only reduce and never increase apoptotic cell number, the effects of apoptotic cells described in this study represent a conservative assessment of the activity of apoptotic endothelial cells. For these reasons, it is argued that the methods used to quantitative apoptotic endothelial cell number in this study are reasonable and enforce a conservative interpretation of the data.

HUVEC from different donors were used in the current study and the quantitative differences observed between experiments may reflect the variability seen in other studies of HUVEC behaviour (Zoellner *et al.*, 1998). Expression of data as a ratio between the platelet response in the presence and absence of endothelial cells or beads largely overcame these problems and permited meaningful analysis of data using non-

parametric statistical methods. Using this approach it was possible to demonstrate consistent responses in the aggregation rate despite variability in the data, while the consistency of aggregation rate data provide confidence in the values obtained for maximum percentage aggregation and lag phase from the same experiments.

Thrombin and ADP are both physiologically important activators of platelet aggregation in blood (Harker *et al.*, 1995; Puri and Colman, 1997), while collagen is thought to only contribute to aggregation when platelets escape the circulation through a damaged vessel wall (Bankowski *et al.*, 1967; Trelstad, 1978; Madri *et al.*, 1980). The markedly reduced aggregation rate seen for all populations of both apoptotic and non-apoptotic endothelial cells studied indicate that anti-aggregatory activity is maintained for thrombin and ADP stimulated platelets during endothelial apoptosis. The maximum percentage aggregation provides a measure of the relative size of platelet aggregates attained during aggregation and the generally reduced magnitude of this measure in the presence of endothelial cells is consistent with the anti-aggregatory activity observed.

Reduction in lag phase is interpreted as revealing a more rapid response of platelets to the agonist. Since platelets bind apoptotic endothelium (Bombeli *et al.*, 1999), it seems reasonable to suggest that the trend for reduced lag phase in the presence of apoptotic endothelium reflects low level activation of bound platelets (Bombeli *et al.*, 1999). It seems possible that this low level of activation is largely counterbalanced by anti-aggregatory activity of the endothelium. The complexity of these interactions may be increased by surface binding of agonist, perhaps explaining the variability in data obtained from different experiments.

The possibility that endothelial cells mediated their anti-aggregatory effect for thrombin or ADP stimulated platelets by mechanically interfering with platelet to platelet contact was excluded in experiments demonstrating the absence of an anti-aggregatory activity by latex beads of a similar size to the endothelial cells used.

However, these experiments also suggested that collagen could be readily adsorbed by particulate matter and that this inhibits the activity of this agonist. SEM confirmed binding of collagen by apoptotic endothelium as well as the shape changes expected in activated platelets. In scanning electron micrographs, it was interesting to note the absence of significant shape change in platelets exposed to apoptotic endothelium in the absence of collagen agonist, despite some degree of platelet binding and the formation of small aggregates at the endothelial surface. This was consistent both with the earlier report of platelet adhesion to apoptotic endothelium (Bombeli *et al.*, 1999) and the suggestion made above, that low levels of platelet aggregation stimulated by adhesion to apoptotic endothelial cells are inhibited by anti-aggregatory activities released by the endothelium.

Additional experiments investigating the ability of cells and latex beads to deplete agonist activity from collagen suspensions confirmed adsorption of collagen agonist activity by both cells and latex beads. From this, it was concluded that at least some of the anti-aggregatory activity expressed by endothelium for collagen stimulated platelets was due to removal of available collagen agonist by adsorption to particulate matter.

The absence of a clear reduction in aggregation rate for collagen stimulated platelets in the presence of SDDA-EC and M&SDA-EC may reflect reduced ability to bind collagen in these cells as compared with non-apoptotic endothelium. It is tempting to speculate that serum may be able to in some way compensate for this in MDA-EC, where a clear reduction in collagen induced platelet aggregation was seen. If it is agreed that such binding removes agonist activity, the slight increase in lag phase seen in MDA-EC would seem consistent with this interpretation.

SEM also revealed surface features of apoptotic endothelial cells expected from earlier transmission electron microscopic reports in which canalicular fragmentation produces surface pores, membrane rests and fragmented regions of membranous and cellular debris. These preliminary observations of the surface features of apoptotic endothelium were extended in further experiments described in Chapter 4.

In conclusion, these data support the suggestion that endothelial cells have maintained anti-aggregatory activity during apoptosis, consistent with the absence of reported microthrombi around apoptotic endothelial cells. Earlier work described canalicular fragmentation and maintained urokinase plasminogen activator expression during endothelial cell apoptosis (Zoellner *et al.*, 1996a; 1998), and it was suggested that these activities are specific endothelial cell adaptations to minimise the micro-embolic and micro-thrombotic potential of these particles. The current study is consistent with this idea, in that maintained anti-aggregatory activity for platelets could be viewed as a further anti-microthrombotic adaptation in apoptotic endothelium.

Platelets are reported to bind to the surface of apoptotic endothelial cells (Bombeli *et al.*, 1999). It might be expected that this would increase platelet aggregation. However, it is also possible that this focuses anti-aggregatory activities of endothelial cells upon potentially activated platelets, so that platelets binding to apoptotic endothelial cells may help inhibit aggregation. Increased levels of tissue factor, surface phosphatidyl serine and tenase activity by apoptotic endothelial cells may be pro-thrombotic in apoptotic endothelial cells (Casciola-Rosen *et al.*, 1996; Greeno *et al.*, 1996; Bombeli *et al.*, 1997). Similarly, reduced TFPI, heparin and thrombomodulin expression also appears to increase the micro-thrombotic potential of apoptotic endothelial particles (Bombeli *et al.*, 1997). However, since thrombosis is the outcome of altered balance between pro and anti coagulant, fibrinolytic and platelet aggregatory events, it is argued that all of these factors need to be considered together in assessment of the micro-thrombotic potential of apoptotic endothelial cells.

As discussed in 1.1.b.iii, platelet activation contributes greatly to development of thrombi and clots *in vivo*, so that deficient platelet function is associated with bleeding disorders (Rodgers, 1999). It seems possible, that the anti-aggregatory activity of apoptotic endothelium sufficiently overcomes the known apoptotic pro-thrombotic changes (Casciola-Rosen *et al.*, 1996; Greeno *et al.*, 1996; Bombeli *et al.*, 1997; 1999) to prevent the formation of biologically significant microthrombi. This idea is supported by the absence of progressive platelet activation despite initial adhesion and low levels of activation reported by Bombeli et al. (1999) and seen by SEM in the current study (Bombeli *et al.*, 1999). Also, it seems likely that any fibrin formed in

association with apoptotic endothelium would be degraded by the u-PA seen in these cells (Zoellner *et al.*, 1998).

No difference was noted in these experiments between apoptotic endothelial cells derived by either serum or matrix deprivation, while no synergy was seen between these two aoptotic stimuli with regard to platelet aggregation. This suggests that interactions between platelets and apoptotic endothelial cells are similar regardless of the biological setting from which the apoptotic cells are derived.

It appears that endothelial cells have maintained anti-aggregatory activity for ADP and thrombin stimulated platelets during apoptosis, however, how apoptotic endothelial cells mediate platelet functions is not established by the data shown in this chapter. Of the three anti-aggregatory mechanism known to be expressed by endothelium, release of PGI_2, NO synthesis and expression of CD39, it seems unlikely that CD 39 contributed to the effects seen. This is because experiments were performed in HBSS containing high levels of phosphate which inhibits this enzyme. Further experiments are described in Chapter 3 investigating the possible mechanisms responsible for mediating the anti-aggregatory activity of apoptotic endothelium.

CHAPTER THREE

Mechanisms of the Anti-Aggregatory Activity of Apoptotic Endothelial Cells for Platelets

3.1. Introduction

3.1.a. The Experimental Question: What is the Basis for the Anti-Aggregatory Activity in Apoptotic Endothelium?

The experiments performed in Chapter 2 indicated that endothelial cells maintain anti-aggregatory activity for platelets during apoptosis. However, the mechanisms responsible for this were not established. As outlined in 1.1.c.vi, unperturbed endothelial cells regulate their membrane properties, release platelet inhibiting factors and inactivate platelet stimuli, thereby preventing platelet activation and thrombus formation (Heller and Bevers, 1997). PGI_2 and NO are believed to be the most important endothelial products regulating platelet function (Moncada *et al.*, 1991; Luscher, 1993). Both substances are inhibitors of platelet adhesion and aggregation, while they are also potent vasodilators (Weiss and Turitto, 1979; de Graaf JC *et al.*, 1992). In addition, ecto-ADPase / CD 39 expressed at the luminal endothelial surface is thought to provide a further mechanism inhibiting platelet reactivity and may be critical for the inhibition of platelet aggregation (Pearson and Gordon, 1985; Zimmermann, 1992; Marcus and Safier, 1993). The experiments described in this chapter investigate the possible role of PGI_2, NO and ecto-ADPase / CD39 in mediating the anti-aggregatory activity of apoptotic endothelial cells.

3.1.b. Methods for Defining Anti-Aggregatory Activity for Platelets in Endothelium

Because PGI_2 is a product of the cyclooxygenase pathway of arachidonic acid metabolism, it is possible to regulate the synthesis of this product by applying inhibitors of cyclooxygenase (Carmignani *et al.*, 1980; Hadhazy *et al.*, 1983). Indomethacin is a widely accepted inhibitor of cyclooxygenase activity, so that altered

endothelial activity following exposure to indomethacin has in the past been accepted as indicating a role for the principal endothelial cyclooxygenase product, PGI_2 (Az-ma and Yuge, 1995; Gelety and Chaudhuri, 1995; Nosaka *et al.*, 1997; Pawlak *et al.*, 1998; Cha *et al.*, 2000). To further verify the potential role of PGI_2 in biological processes, it is desirable to demonstrate the presence of this metabolite in the biological system under study. However, PGI_2 is chemically unstable with a half-life of approximately three to four minutes in aqueous solution (Wu, 1992; Maclouf *et al.*, 1998). This is because it rapidly undergoes non-enzymatic hydrolysis to a chemically stable and biological inactive intermediate metabolite, 6-keto-$PGF_{1\alpha}$, which can be further converted *in vivo* by β-oxidation to 2,3-dinor-6-keto-$PGF_{1\alpha}$ (Wu, 1992; Maclouf *et al.*, 1998). Measurement of these metabolites is possible using immune-based assays and has been accepted as a method for estimating PGI_2 synthesis in biological systems (Vane *et al.*, 1990; Wu, 1992; Maclouf *et al.*, 1998). In the current study, both inhibition of cyclooxygenase activity by indomethacin and direct detection of 6-keto-$PGF_{1\alpha}$, by a competitive enzyme immune assay were used to determine the possible role of PGI_2 in regulation of platelet aggregation by apoptotic endothelium.

As outlined in 1.1.c.iv, NO, synthesized and released by endothelium, diffuses to nearby platelets where it strongly inhibits platelet activation and aggregation (Radomski *et al.*, 1987b; Farrell and Blake, 1996). L-N^G-Nitroarginine methyl ester (L-NAME) is a potent inhibitor for NOS expressed in endothelium, so that altered endothelial behaviour in response to L-NAME is accepted as evidence for a role of NO (Bilfinger *et al.*, 2002; Rikitake *et al.*, 2002). Like PGI_2, NO is very unstable, reacting with many biological compounds including thiols and hemoglobin (Loscalzo and Welch, 1995). Chemiluminscence, electron paramagnetic resonance and

spectrophotometric detection of methemoglobin have been used to detect NO (Loscalzo and Welch, 1995). Because nitrite (NO_2^-) and nitrate (NO_3^-) are stable oxidative end products of NO, detection of these products is also accepted as a convenient and reliable method for the indirect detection of NO both *in vivo* and *in vitro* (Misko *et al.*, 1993; Viinikka, 1996). Nitrate is the main stable bioreaction product of NO in whole blood, while in tissue culture media devoid of hemoglobin, nitrite is the main stable bioreaction product (Kirkeboen and Strand, 1999). In the current study the Griess reaction, a widely accepted colorimetric method for detecting NO_2 for indirect determination of NO levels, was exploited to determine levels of NO in cultured apoptotic endothelium (Green *et al.*, 1982; Nithipatikom *et al.*, 1996; Privat *et al.*, 1997; Guevara *et al.*, 1998; Jang *et al.*, 1999). Also, the effect of L-NAME upon platelet aggregation in the presence and absence of endothelium was established.

Endothelial surface ADPase / CD39 has the potential to degrade ADP released by activated platelets and so may have contributed to the anti-aggregatory activity under investigation (Kaczmarek *et al.*, 1996; Wang and Guidotti, 1996; Marcus *et al.*, 1997). However, HUVEC are not reported to express CD39 antigen (Shaw, 1995) while the experiments conducted in Chapter 2 were in the presence of phosphate buffer, a potent inhibitor of ADPase activity. For these reasons, it was considered highly unlikely that CD39 contributed to the anti-aggregatory activity studied. Nonetheless, it was decided that it would be wise to verify the absence of CD39 in HUVEC isolated in this laboratory, and to achieve this objective, fluorescence activated cell scanning (FACS) analysis was performed. This is a highly sensitive and reliable

method for detecting antigen on cell surfaces, and was exploited in the current study to determine levels of antigen expression in HUVEC.

3.2. Materials and Methods

3.2.a. Materials

Indomethacin and L-NAME were supplied by Sigma (St. Louis, USA). Griess Reagent R1 and R2, as well as 6-keto Prostaglandin F 1α EIA kit were purchased in kit form from Cayman Chemical (MI, USA). Mouse anti human CD 39 and F $(ab')_2$ rabbit anti mouse Ig G: FITC were obtained from Serotec Ltd. (Oxford, UK). Phenol red-free M199 was purchased from Invitrogen Life Technologies (Victoria, Australia). Dulbecco's PBS was obtained from Sigma (St. Louis, USA). Other reagents and materials used in the study were as described in 2.2.a.

3.2.b. Preparation of Apoptotic and Non Apoptotic Endothelial Cells for Experiments

3.2.b.i. Pre-incubation of Endothelial Cells with Indomethacin and L-NAME

HUVEC from fourth to fifth passage were cultured from several separate donors as indicated in 2.2.b. In experiments in which indomethacin or L-NAME was used as metabolic inhibitors to probe the interactions between endothelium and platelets, all five endothelial populations were studied and prepared in the same way as indicated in 2.2.c. The only difference, however, was that before cells were used in platelet aggregometry experiments, cells were either pre-incubated with indomethacin (100 μM), L-NAME (300 μM) or buffer for 100 minutes at room temperature. (Rees *et al.*, 1990; Laneuville *et al.*, 1994). Cells were then used directly in experiments in the same way as in 2.2.e.ii. In most experiments, thrombin was used as the agonist while ADP was used in some experiments to confirm that the endothelial response was

independent of the agonist used. In some additional experiments, the effect of pre-incubation of platelets (200 x 10^9 per liter) with indomethacin or L-NAME was determined without pre-incubation of the endothelium. This was to confirm that the effect of these inhibitors was upon the endothelium and not the platelet population.

3.2.b.ii. Culture Conditions to Detect PGI_2 and NO Synthesis by Apoptotic Endothelium

In preliminary experiments attempting to detect PGI_2 and NO in the 5 populations studied, it was found that levels of antigen and NO_2^- were insufficient for reliable detection from SDDA-EC supernatants while background levels of PGI_2 and NO_2^- at the starting times during experiments often made detection of additional material synthesized during the experiment difficult. Also, the expense of purchasing the kit based assays made performance of large-scale experiments impossible. In addition, it was noted that there was no clear difference in the performance of apoptotic endothelium regardless of the mechanism used to induce apoptosis (2.3.c). For these reasons, it was decided to determine PGI_2 and NO levels only in MDA-EC, M&SDA-EC and UnA-EC. Also, the need to collect a zero-hour time point as well as to avoid interference of M199 containing phenol red with the chromogenic immune assay compelled slight modification of the protocol for generating these populations.

Briefly, MDA-EC and M&SDA-EC were prepared as in earlier experiments by seeding into plastic bacterial culture dishes. After 4 hr of incubation to provide time for endothelial apoptosis to commence, cells were harvested by gentle washing with M199 and centrifugation before washing once with fresh M199. Cells were then resuspended in 10 ml volumes of phenol red-free M199 either with or without FCS

(20%) for MDA-EC or M&SDA-EC respectively. These were then partitioned into triplicate wells in 6 well tissue culture plates for 24 hr of further incubation. Medium from individual wells was collected at the time cells were seeded into 6 well tissue culture plates and gently centrifuged to remove cells before collecting supernatant and storing frozen for determination of "zero hour" levels of PGI_2 and NO. Medium harvested after 24 hr, and in some experiments for NO at 48 hr, was collected and handled in the same way and used to determine if additional PGI_2 and NO had been synthesised by the endothelial cells studied. UnA-EC cultured in 6 well tissue culture plates were used as control cells in these experiments. All incubations were performed under CO_2 (5%) and 100% humidity at 37 °C in the presence of antibiotics as used in 2.2.b.

3.2.c. PGI_2 Assay Detecting the Stable Metabolite 6-keto $PGF_{1\alpha}$

PGI_2 was determined in the cell culture medium as its stable metabolite, 6-keto $PGF_{1\alpha}$, using the 96 well enzyme immunoassay kit obtained from Cayman Chemical (MI, USA). This enzyme immunoassay is based on the competition between free 6-keto $PGF_{1\alpha}$ and a 6-keto $PGF_{1\alpha}$ tracer (6-keto $PGF_{1\alpha}$ linked to an acetylcholinesterase molecule) for a limited number of 6-keto $PGF_{1\alpha}$ - specific rabbit antiserum binding sites. The concentration of the 6-keto $PGF_{1\alpha}$ tracer is held constant while the concentration of free 6-keto $PGF_{1\alpha}$, either in the standard or sample, varies. Thus, the amount of 6-keto $PGF_{1\alpha}$ tracer that is able to bind to the rabbit antiserum is inversely proportional to the concentration of free 6-keto $PGF_{1\alpha}$ in the well. After washing with wash buffer five times, 200 µl of the chromogenic substrate (Ellman's Reagent) is added to wells to detect bound tracer. The samples were read at 415 nm in a Model 3350 Microplate Reader (Bio-Rad Laboratories, CA, USA) and the concentrations of

6-keto $PGF_{1\alpha}$ in samples were calculated by using the standards and the method provided by the manufacturer.

3.2.d. Nitric Oxide Assay Detecting the Stable Product NO_2^-

NO_2^-, a stable end product of NO, was measured in the culture medium by using the spectrophotometric Greiss reaction (Green *et al.*, 1982). Briefly, 180 µl aliquots of medium from each well were mixed with 40 µl volumes of Greiss reagent (20 µl of Greiss reagent R1 and 20 µl of Greiss reagent R2) obtained from Cayman Chemical (MI, USA). The mixture was incubated for 10 minutes at room temperature before spectrophotometric determination of absorbance at 550 nm using in a DU Series 600 Spectrophotometer (Beckman Instruments Inc, CA, USA). Concentrations were determined by comparison with a sodium nitrite standard.

3.2.e. Fluorescence Activated Cell Scanning Analysis for Ecto-ADPase / CD 39

Both apoptotic and non apoptotic endothelial cells were obtained as indicated in 2.2.c. Cells were pelleted by centrifugation at 2,000 rpm and 4 °C for 5 minutes before washing twice with 1 ml of cold buffer composed of Dulbecco's PBS and FCS (1%). Cells were then transferred to FACS tubes so that each tube contained 5 x 10^5 cells. After centrifugation and removing supernatant, cells were resuspended in 50 µl of cold buffer with 10 µl of anti-human CD 39 mAb (Ig G_1) at a concentration of 25 µg / ml and briefly vortexed before 30 minutes of incubation on ice in the dark. Cells were then washed twice and 10 µl the fluorescinated secondary antibody, rabbit anti-mouse Ig G: FITC at concentration of 10 µg / ml in cold buffer was added into the tubes. After gentle vortexing, cells were incubated for a further 30 minutes on ice in the dark. Finally, cells were resuspened in 1 ml of buffer and held on ice and protected

from light prior to analysis using a FACScan bench top model, applying Cell Quest version 3.2.1 f1 software (Becton Dickinson). Samples without primary and or secondary labels were used to control for non-specific binding.

3.2.f. Statistical Analysis

The two-tailed Wilcoxon's Matched-Pairs-Ranks Test was used to compare data obtained from multiple experiments while Student's t Test was performed within experiments. p values of less than 0.05 were considered as statistically significant.

3.3. Results

3.3.a. Inhibition of Prostacyclin and Nitric Oxide Synthesis Abrogated the Anti-Aggregatory Activity of Apoptotic Endothelial Cells

Pretreatment with indomethacin reduced the anti-aggregatory activity of all five HUVEC populations for thrombin stimulated platelets ($p < 0.05$) (Figure 3.1A). L-NAME also suppressed the anti-aggregatory activity for thrombin stimulated platelets of apoptotic populations as well as of SDnA-EC ($p < 0.05$) (Figure 3.1B). However, this inhibitor did not affect the aggregation rate in the presence of UnA-EC (Figure 3.1B). These data suggest a role for PGI_2 and NO in mediation of the anti-aggregatory activity of apoptotic endothelial cells. Additional experiments in which platelets but not HUVEC were pre-treated with either L-NAME or indomethacin failed to reveal an effect of these inhibitors upon platelet function (data not shown). With regard to the maximum percentage aggregation (Figure 3.2), only MDA-EC and to a lesser extent SDDA-EC appeared to be increased by indomethacin and L-NAME, with the increase in maximum percentage aggregation being consistent with the changes seen in aggregation rate. Lag phase was unaffected by either of the inhibitors studied (Figure 3.3). Similar results for aggregation rate, maximum percentage aggregation and lag phase were obtained when platelet aggregation was stimulated with ADP in a single experiment (data not shown).

3.3.b. Prostacyclin was Released by Apoptotic Endothelium

Because of unexpected difficulties with the stability of the 6-keto $PGF_{1\alpha}$ standard provided with the kit, it was difficult to achieve proper quantitation of 6-keto $PGF_{1\alpha}$ levels in all experiments performed. Nonetheless, very significant changes in optical

density were clearly seen, demonstrating clear increases in antigen levels over the 24 hr incubation period. Figure 3.4 illustrates the results of 4 separate experiments with HUVEC from separate donors in which supernatants from M&SDA-EC, MDA-EC and UnA-EC had greatly increased levels of antigen at the 24 hr time point as compared with the starting time. Figure 3.5 shows the result of a single experiment where quantitation of 6-keto $PGF_{1\alpha}$ was achieved, demonstrating again an increase in antigen levels with time.

3.3.c. Nitric Oxide was Produced by Apoptotic Endothelial Cells

Figure 3.6 summarises the optical density read-outs of 6 separate experiments demonstrating NO_2^- generation over the experimental period. Figure 3.7 shows the result of a single experiment in which there is a clear increase in NO_2^- levels over time in all three endothelial populations studied. These data indicate that the apoptotic and non-apoptotic endothelial cells investigated produce NO, consistent with the proposed role of this product in inhibiting platelet aggregation.

3.3.d. FACS Analysis Failed to Reveal Significant Levels of Ecto-ADPase / CD 39 in Cultured HUVEC

Although slight expression of CD39 by HUVEC was noted in one experiment, and no convincing antibody labelling was seen in any further experiments (n = 6) with either apoptotic or non-apoptotic cells (Figure 3.8).

Figure 3.1 The relative aggregation rate of thrombin stimulated platelets in the presence of non-apoptotic (UnA-EC, SDnA-EC) or apoptotic (SDDA-EC, MDA-EC, M&SDA-EC) endothelium pretreated with either indomethacin (A) or L-NAME (B) as compared with aggregation in the presence of endothelial cells not pretreated with inhibitors (Value of 1 indicated by the horizontal line). Pretreatment with indomethacin of all five endothelial cell types studied increased the aggregation rate ($p < 0.05$) (*A*) while pretreatment with L-NAME of SDnA-EC, SDDA-EC, MDA-EC and M&SDA-EC but not UnA-EC also increased the aggregation ($p < 0.05$) rate (*B*).

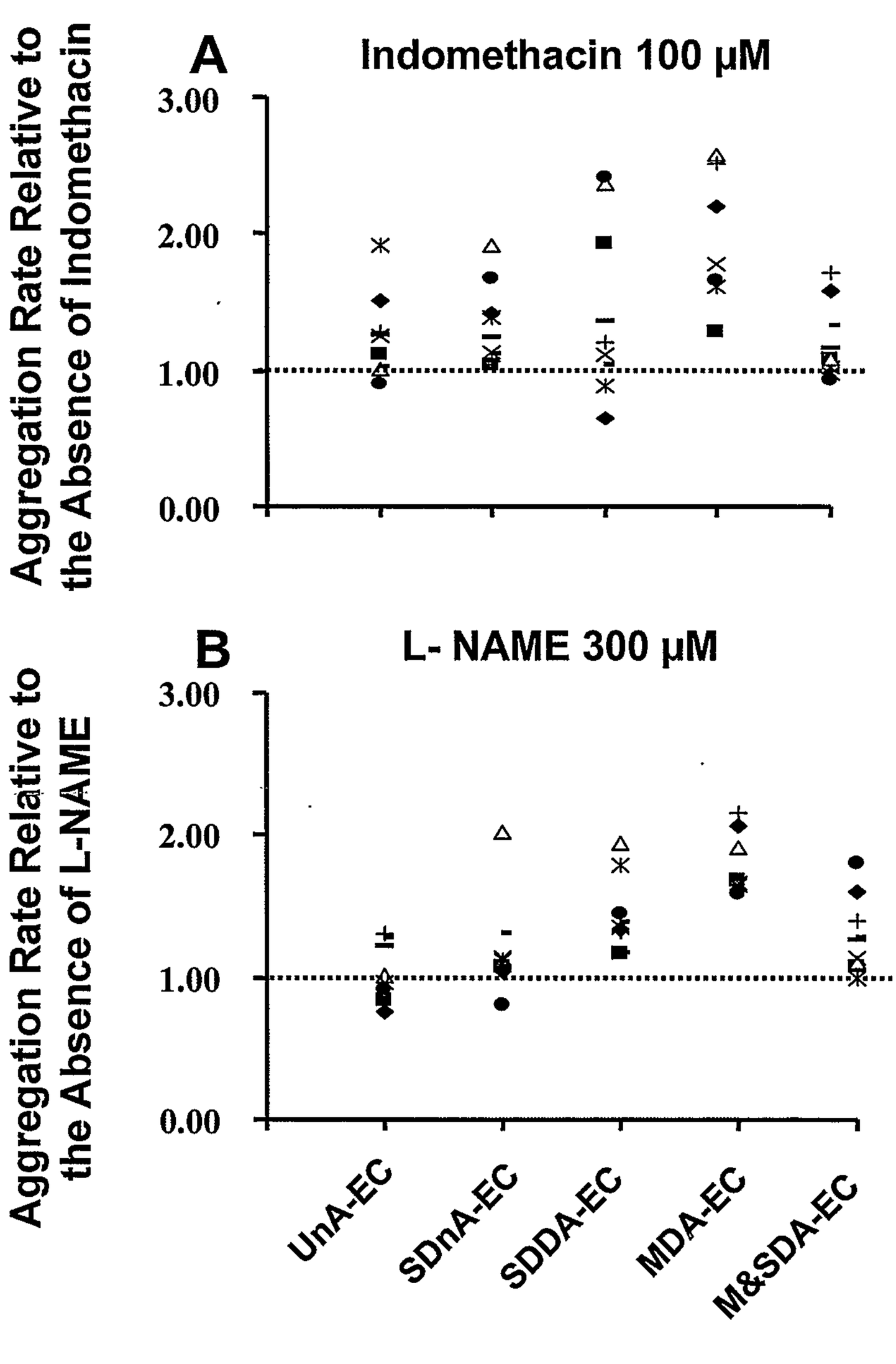

A
Indomethacin 100 µM
Aggregation Rate Relative to the Absence of Indomethacin
3.00
2.00
1.00
0.00
B
L- NAME 300 µM
Aggregation Rate Relative to the Absence of L-NAME
3.00
2.00
1.00
0.00
UnA-EC
SDnA-EC
SDDA-EC
MDA-EC
M&SDA-EC
Cell Type Included with Platelets

Figure 3.2 The relative maximum percentage aggregation of thrombin stimulated platelets in the presence of non-apoptotic (UnA-EC, SDnA-EC) or apoptotic (SDDA-EC, MDA-EC, M&SDA-EC) endothelium pretreated with either indomethacin (A) or L-NAME (B) as compared with that in the presence of endothelial cells not pretreated with inhibitors (Value of 1 indicated by the horizontal line). Only MDA-EC ($p < 0.01$) and to a lesser extent SDDA-EC appeared to be affected by indomethacin and L-NAME.

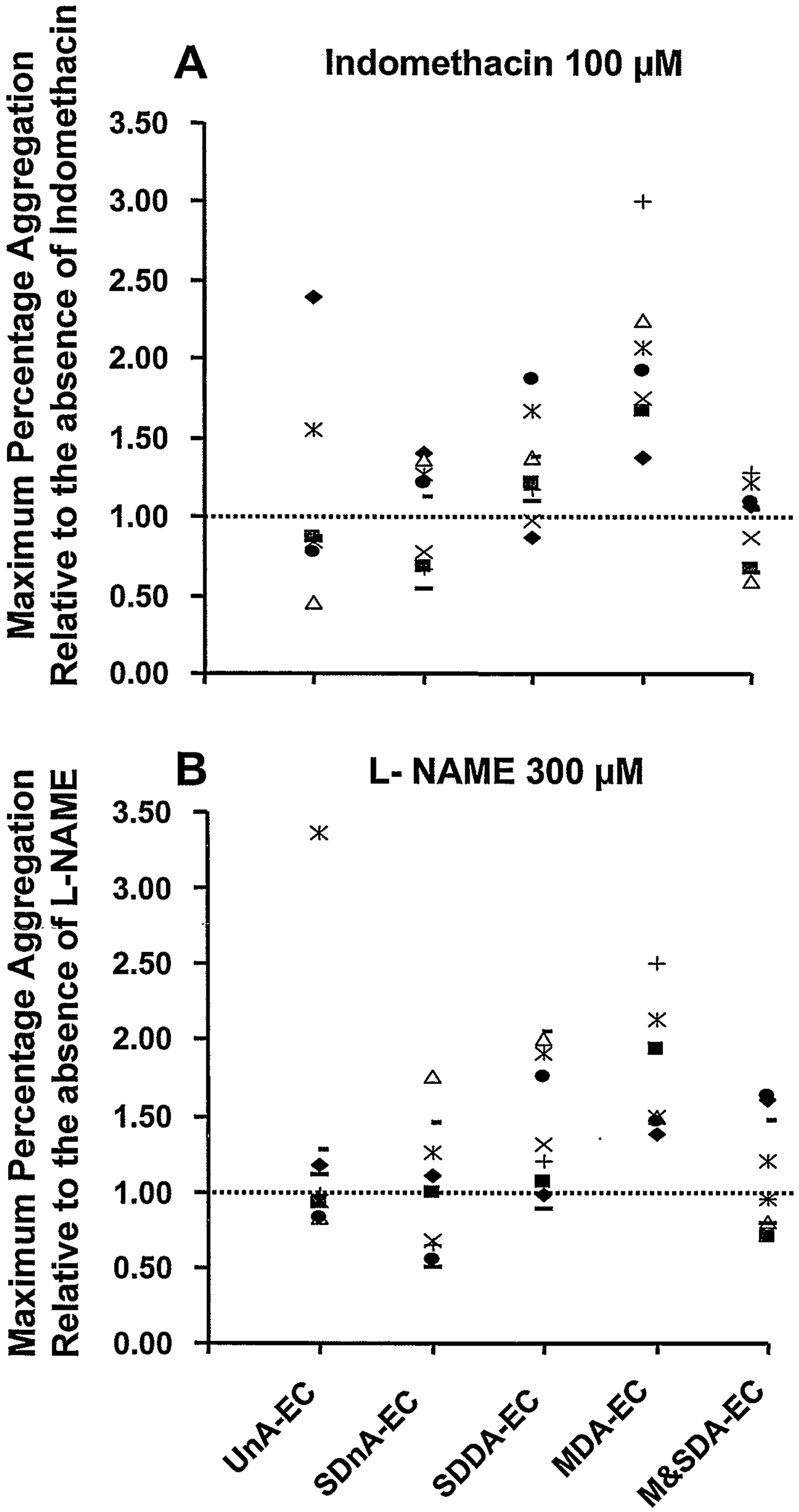

A
Indomethacin 100 µM
Maximum Percentage Aggregation Relative to the absence of Indomethacin
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
B
L- NAME 300 µM
Maximum Percentage Aggregation Relative to the absence of L-NAME
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
UnA-EC
SDnA-EC
SDDA-EC
MDA-EC
M&SDA-EC
Cell Type Included with Platelets

Figure 3.3 The relative lag phase of thrombin stimulated platelets in the presence of non-apoptotic (UnA-EC, SDnA-EC) or apoptotic (SDDA-EC, MDA-EC, M&SDA-EC) endothelium pretreated with either indomethacin (A) or L-NAME (B) as compared with that in the presence of endothelial cells not pretreated with inhibitors (Value of 1 indicated by the horizontal line). No clear effect upon lag phase was seen in any of the populations studied.

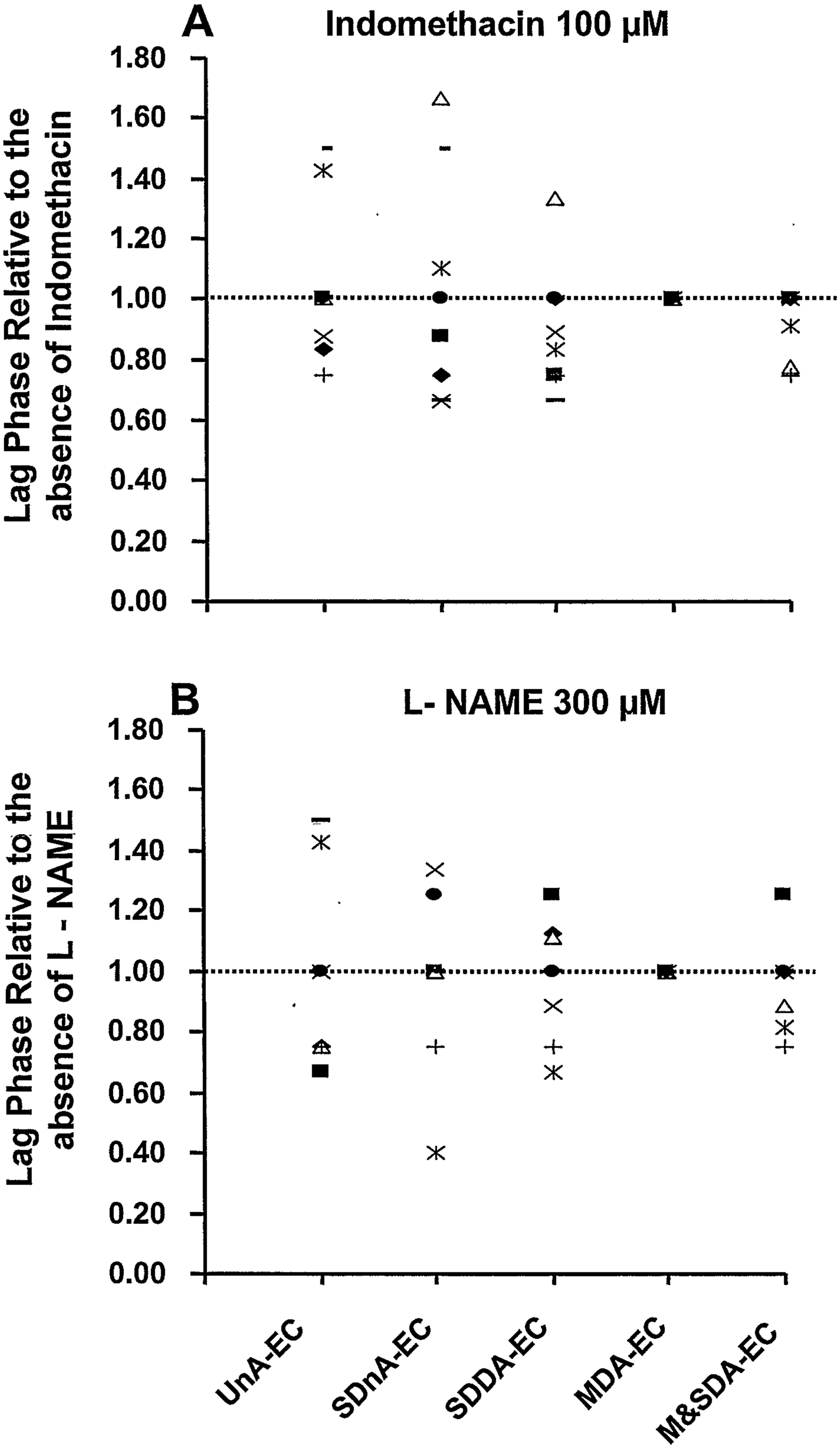
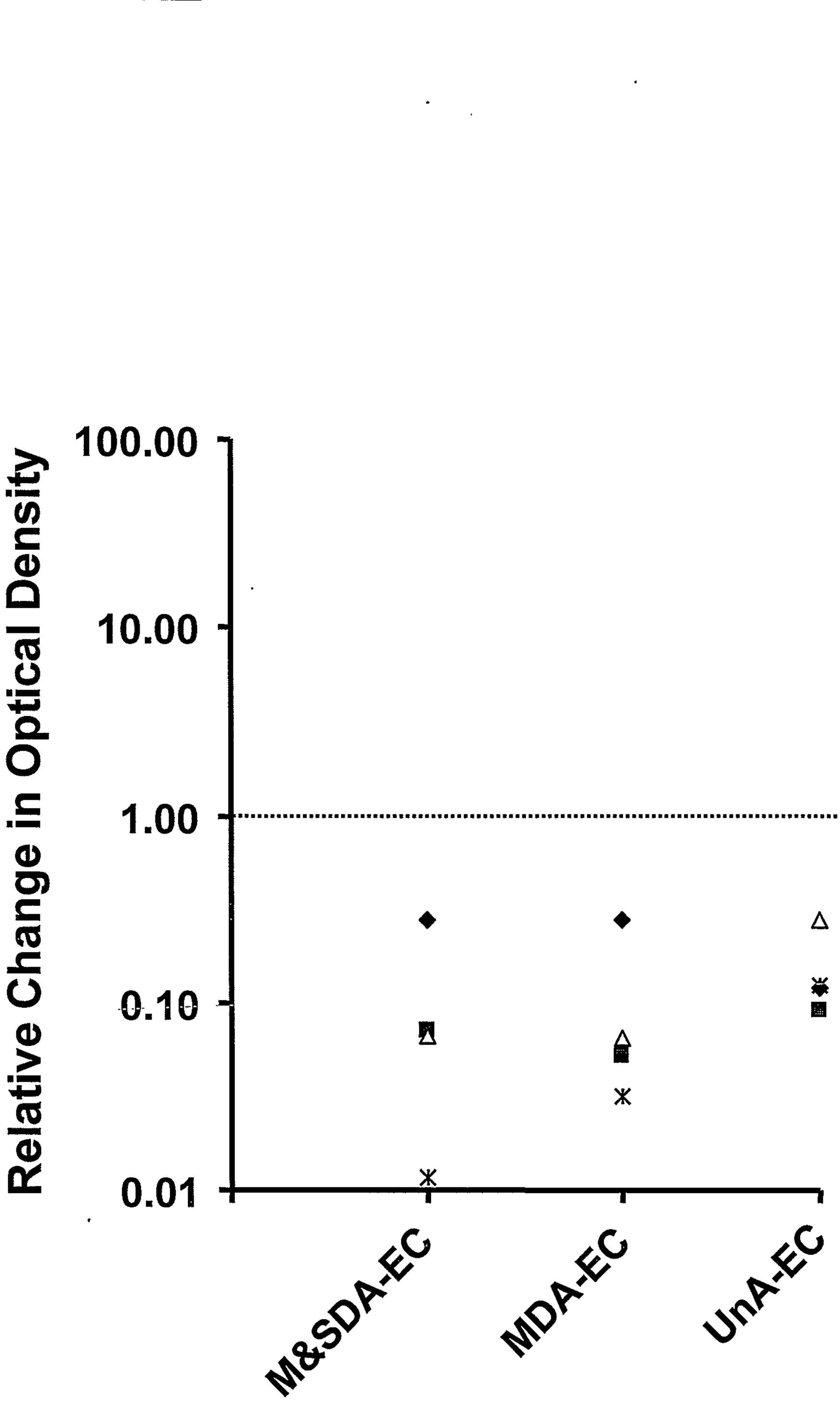

A
Indomethacin 100 µM
Lag Phase Relative to the absence of Indomethacin
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
B
L- NAME 300 µM
Lag Phase Relative to the absence of L - NAME
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
UnA-EC
SDnA-EC
SDDA-EC
MDA-EC
M&SDA-EC
Cell Type Included with Platelets

Figure 3.4 The relative change in optical density seen in competitive immune assays for 6-keto $PGF_{1\alpha}$ of supernatants collected over 24 hr from apoptotic (M&SDA-EC, MDA-EC) and non-apoptotic (UnA-EC) endothelium. A clear reduction in optical density within the 6-keto $PGF_{1\alpha}$ assay was seen in supernatant of all samples over the 24 hr period studied, relative to supernatant at the starting time (value of 1 indicated by horizontal line). These data reveal the production of PGI_2 by these endothelial populations.



Cell Populations Studied

Figure 3.5 The levels of 6-keto $PGF_{1\alpha}$ in culture supernatants from apoptotic (M&SDA-EC, MDA-EC) and non-apoptotic (UnA-EC) endothelial cells in a single experiment, 0 hr and 24 hr after incubation. Although some 6-keto $PGF_{1\alpha}$ was detected at the start of this experiment, possibly reflecting material carried over from the cell preparation phase, a clear increase in antigen was detected, revealing the production of PGI_2 by both apoptotic and non-apoptotic endothelium.

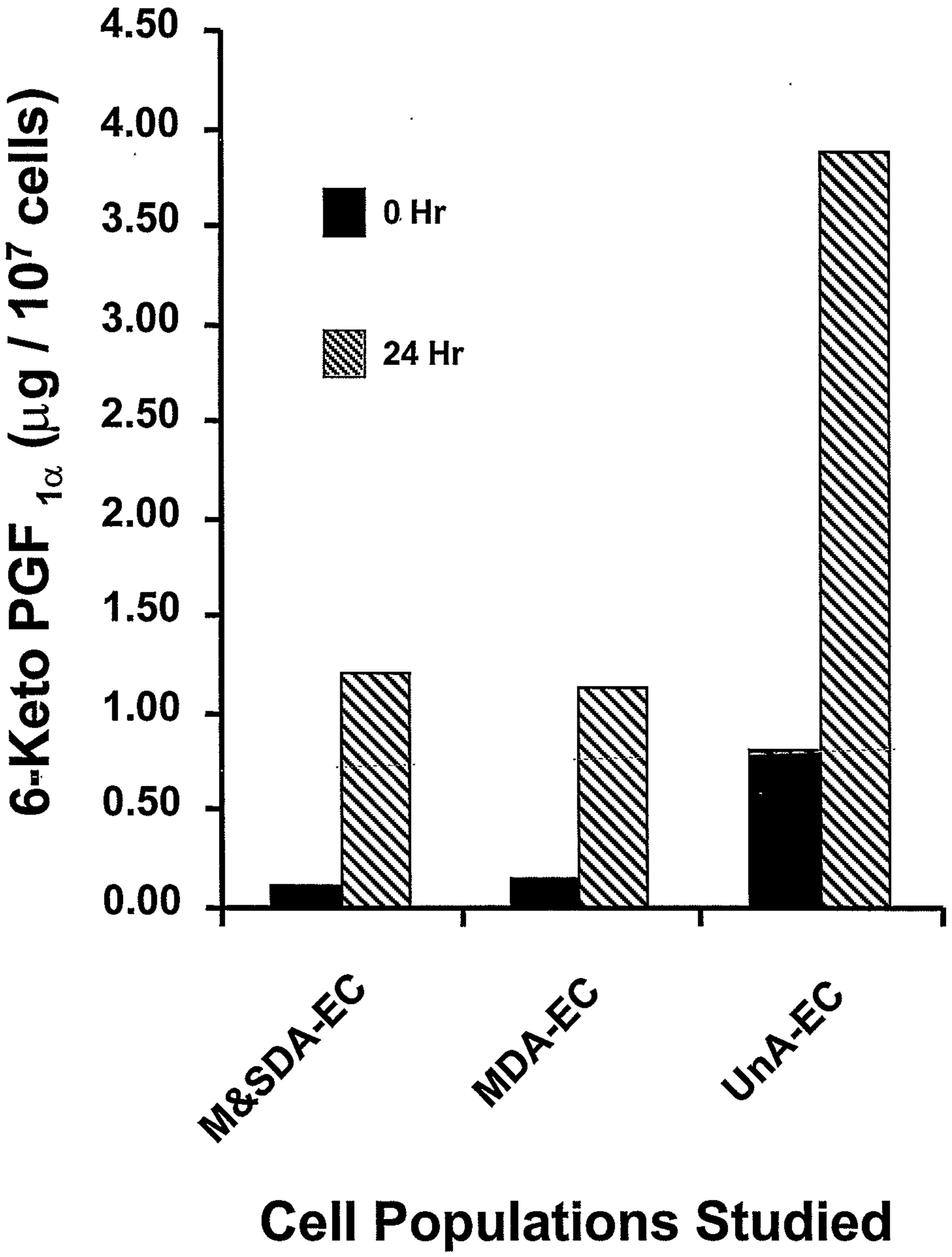

4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
6-Keto PGF 1α (µg / 10⁷ cells)
0 Hr
24 Hr
M&SDA-EC
MDA-EC
UnA-EC
Cell Populations Studied

Figure 3.6. The relative change in optical density seen in chromogenic assays for NO_2^- in supernatants collected over 24 hr from apoptotic (M&SDA-EC, MDA-EC) and non-apoptotic (UnA-EC) endothelium. A clear increase in optical density was seen for all cell types studied over the 24 hr period of study relative to the levels at the starting time (horizontal line at 1), indicating an increase in NO levels in all experiments performed.

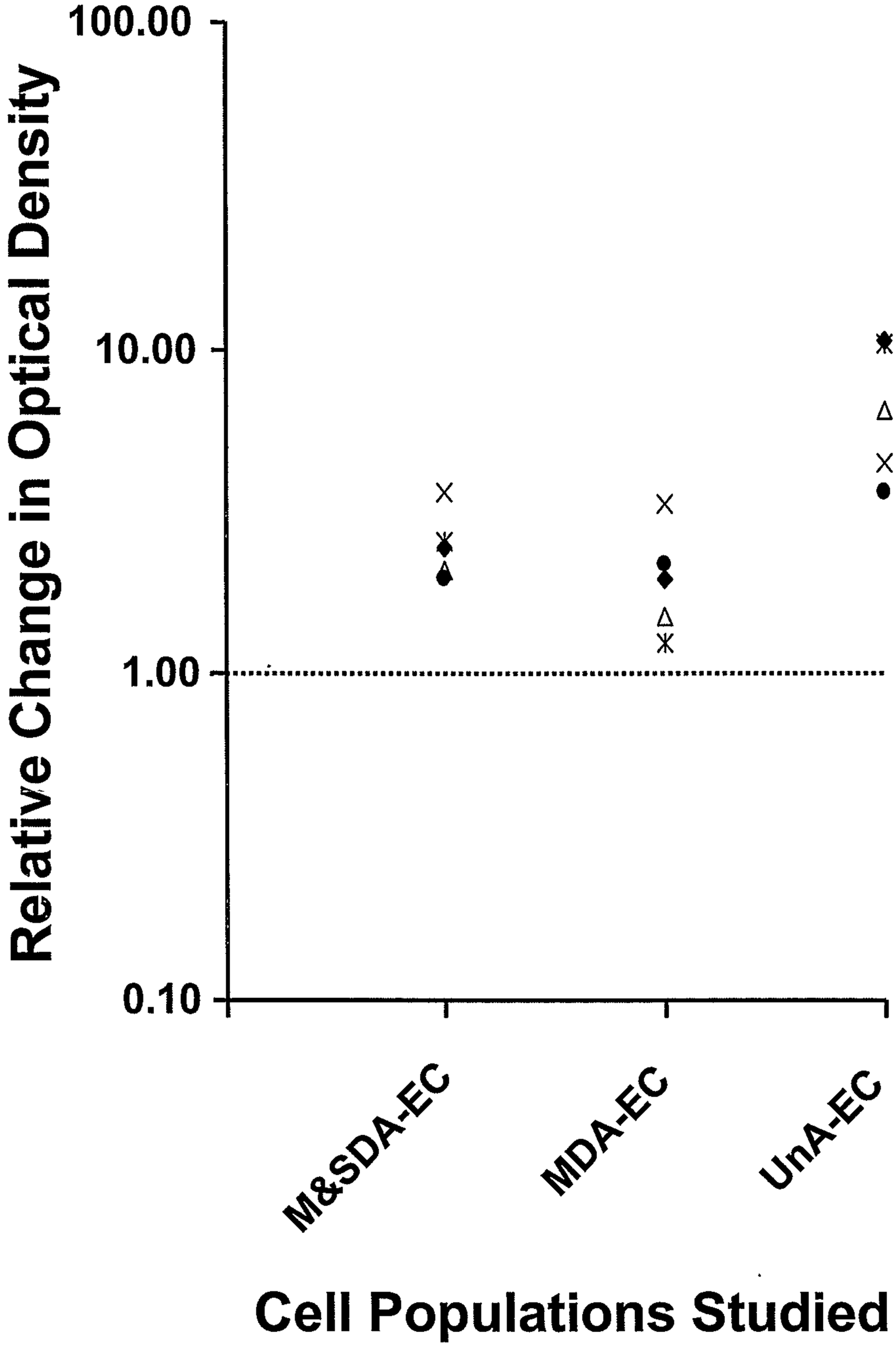
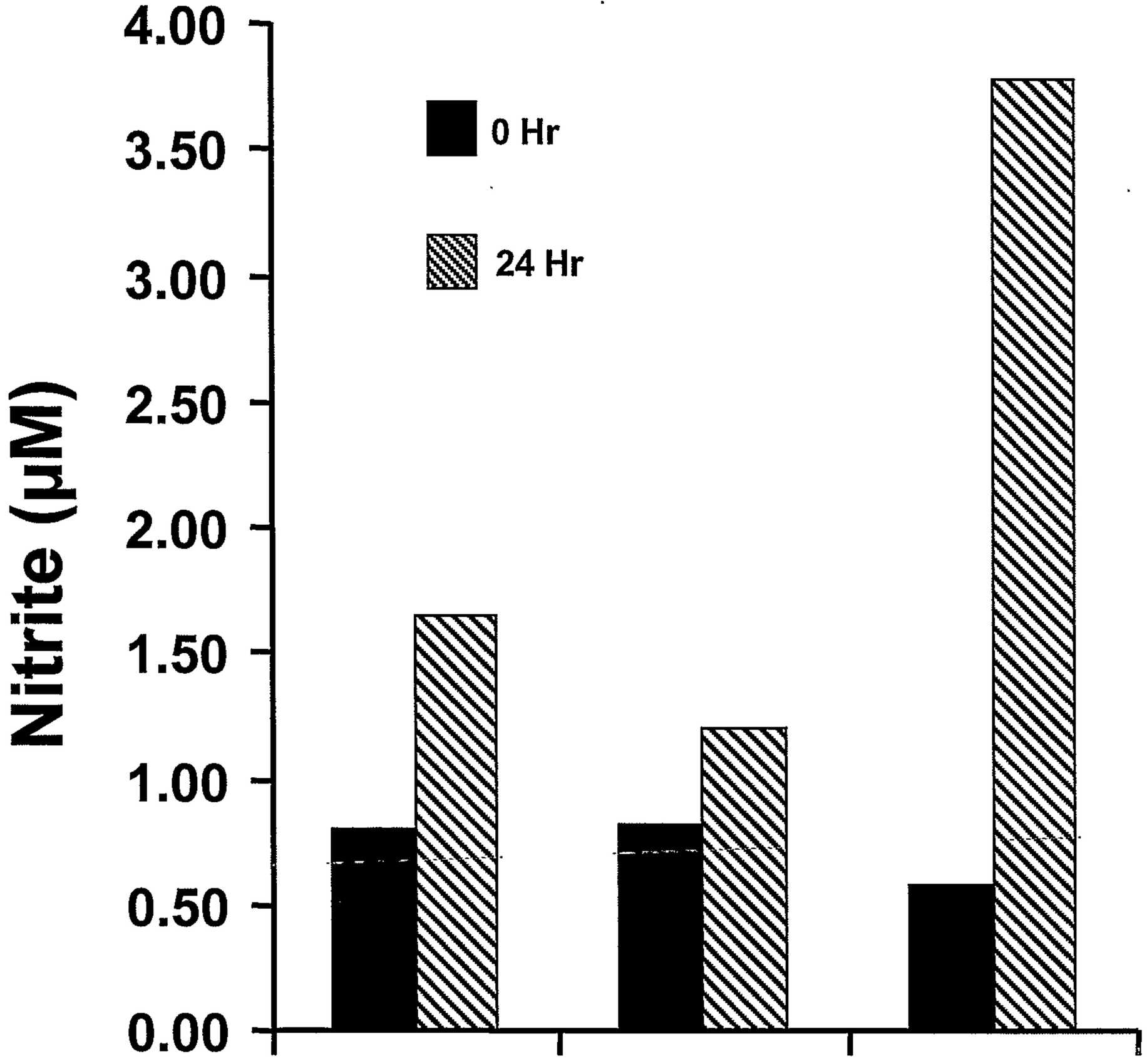

100.00
10.00
1.00
0.10
Relative Change in Optical Density
M&SDA-EC
MDA-EC
UnA-EC
Cell Populations Studied

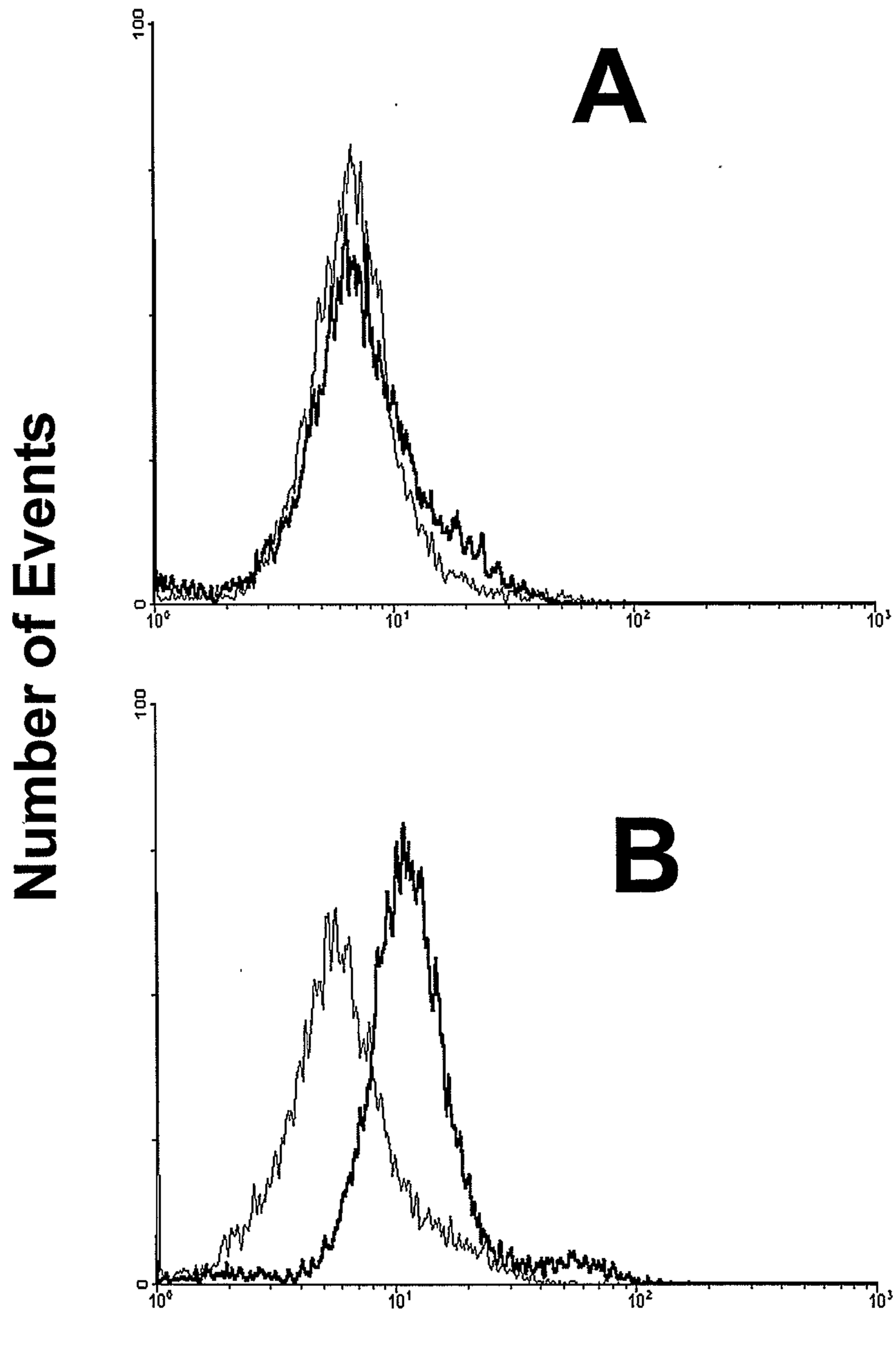
Figure 3.7. Levels of NO_2^- in culture supernatants from apoptotic (M&SDA-EC, MDA-EC) and non-apoptotic (UnA-EC) endothelial cells in a single experiment, 0 hr and 24 hr after incubation. Although some NO_2^- was present at the start of this experiment, possibly reflecting material carried over from the cell preparation phase, a clear increase in NO_2^- was detected, revealing the production of NO by both apoptotic and non-apoptotic endothelium.



Cell Populations Studied

Figure 3.8. Fluorescence activated cell scanning analysis for CD39 in HUVEC.

In most experiments, there was no significant expression of CD39 on the cell surface (bold line) as compared with cells exposed to isotype control antibodies (faint line) (*A*). However, in one experiment out of the seven performed a modest increase in CD 39 was detected in non-apoptotic endothelium (*B*). From this, it seems very unlikely that ecto-ADPase / CD39 activity contributed to the anti-aggregatory activity of apoptotic HUVEC.


A
B
100
0
10^0
10^1
10^2
10^3
Number of Events
Fluorescence

3.4. Discussion

The apoptotic and non-apoptotic cells used for these experiments were identical to those used and characterised by TEM and DNA gel electrophoresis in Chapter 2. Slight modifications in the ways these cells were used for experiments determining NO and PGI_2 levels are independent of the methods used to generate apoptosis, so these minor procedural differences are not thought to significantly affect the apoptotic endothelium. Once cells become apoptotic, they are unable to reverse the process. Thus, the MDA-EC and M&SDA-EC used in these experiments were unable to re-establish monolayers when seeded into 6 well tissue culture plates but continued in their apoptotic state. Seeding into culture wells was important to both generate the triplicate readings necessary for assays and achieve sufficient cell concentrations for antigen or NO_2^- detection. It was unfortunate that it was never possible to obtain sufficient numbers of SDDA-EC to reliably detect either NO or PGI_2 synthesis, although low levels of these products were seen in a few preliminary experiments. However, the absence of any clear difference between separate apoptotic endothelial populations with regard to their effect upon platelet aggregation as seen in Chapter 2, suggests that results for SDDA-EC would not be different to MDA-EC and M&SDA-EC.

Studies have shown that apoptosis can be divided into biochemically and morphologically distinct phases (Lazebnik *et al.*, 1993; Solary *et al.*, 1993). Firstly, pro-apoptotic stimuli trigger activation of the central molecular machinery of apoptosis, the initiation phase. In the second, committed or effector phase, the molecular execution machinery becomes fully activated as shown by the ability of

cytosolic extracts of committed cells to induce apoptotic changes in nuclei (Lazebnik *et al.*, 1993; Solary *et al.*, 1993). Only after this, in the third, or degradation phase, do the hallmarks of apoptosis, such as morphological changes and DNA fragmentation, become evident (Saraste and Pulkki, 2000). The asynchronous nature of apoptotic death in cell populations is mainly due to the highly variable duration of the initiation phase (Saraste and Pulkki, 2000). In cell culture video microscopy studies, the dynamic morphologic changes at the light microscopic level always take place in less than 2 hours (McCarthy *et al.*, 1997; Messam and Pittman, 1998). The point of no return occurs several hours before the appearance of morphologic features (Brunet *et al.*, 1998; Messam and Pittman, 1998). On this basis, as well as the fact that MDA-EC and M&SDA-EC failed to reattach after seeding into 6 well culture flasks, apoptotic cells used in detection of PGI_2 and NO are believed to have been committed to apoptosis after initial apoptosis induction for 4 hours. Low levels of PGI2 and NO detected at starting time points are suggested to have been released by cells during apoptosis and cell preparation.

As outlined above (3.2.b.ii), because of the difficulty in detecting PGI_2 and NO from SDDA-EC as well as the expense of working with kit products, it was necessary to compromise with regard to the number of cell populations studied, so that only two apoptotic and one non-apoptotic population were studied. Despite these difficulties, it was possible to make statistically meaningful observations of the populations studied while experiments with the metabolic inhibitors L-NAME and indomethacin provided important indirect evidence of a role for NO and PGI_2 in the anti-aggregatory activity of apoptotic and non-apoptotic cells and further reinforced the idea that there were no

significant differences between apoptotic endothelial cells obtained by different means.

PGI_2 and NO are both believed important in mediating anti-aggregatory activity for platelets by endothelial cells (Hoak *et al.*, 1982; Radomski *et al.*, 1987a; 1987c; Palmer *et al.*, 1988; Sneddon and Vane, 1988). This was confirmed in the current study, in which inhibitors for both of these factors reduced the anti-aggregatory activity of both apoptotic and non-apoptotic endothelial cells. However, inhibition NO synthesis did not affect anti-aggregatory activity in UnA-EC. This is difficult to explain, but may reflect activation of platelets by contaminating matrix components obtained during cell harvesting. This interpretation would be consistent with the lower lag phase seen in the presence of UnA-EC (Figure 2.8), although the absence of a difference between these cells and SDnA-EC makes this interpretation seem less likely.

Normal endothelium is thought to release a constant basal level of PGI_2 (FitzGerald *et al.*, 1983), and this was supported by observations in the current study where 6-keto $PGF_{1\alpha}$ was produced by UnA-EC over the 24 hr study period. Similar results were also found in the experiments performed with MDA-EC and M&SDA-EC. These data, together with the results of indomethacin treatment indicate that both non-apoptotic and apoptotic cells produce PGI_2 and that this is capable of inhibiting platelet aggregation.

Also as indicated in 1.1.c.vi, untreated endothelium produces basal levels of NO (Vane *et al.*, 1990; Busse *et al.*, 1993). This was confirmed in the current study by

detecting increased NO_2^- levels in the supernatants of UnA-EC over a 24 hr period. NO_2^- levels also rose in the supernatants of MDA-EC and M&SDA-EC, indicating NO production in these apoptotic populations.

No significant difference was found in the anti-aggregatory activity among the three apoptotic populations pretreated with inhibitors. Moreover, the results of detection of PGI_2 or NO from MDA-EC and M&SDA-EC were almost identical. These observations are consistent with the finding in chapter 2, which suggested that interactions between apoptotic endothelial cells are similar regardless of the biological setting from which the apoptotic cells are derived. Although SDDA-EC have not been included in the experiments of detection of PGI_2 and NO, it seems reasonable to assume that SDDA-EC also produce basal levels of PGI_2 and NO.

Although expression of ecto-ADPase / CD 39 is reported on some HUVEC cell lines (Kansas *et al.*, 1991; Marcus *et al.*, 1997) and very low levels were seen in one experiment, it was not possible to reproduce this observation over multiple experiments. Detailed expression data from the 5th CD workshop shows no expression of CD 39 on HUVEC, either resting or stimulated with TNFα (Shaw, 1995). From this, there seems to be some controversy regarding the expression of ecto-ADPase / CD 39 by HUVEC. However, as described in 2.4, the possibility that ecto-ADPase / CD 39 plays a role in the anti-aggregatory activity observed in this study seems remote, because of the high phosphate levels of the culture medium used.

In conclusion, endothelium was found to maintain anti-aggregatory activity for platelets during apoptosis as determined in the previous chapter. Experiments

performed in this chapter determined that the anti-aggregatory activity of apoptotic endothelial cells was abrogated by metabolic inhibitors of NO and PGI_2 production. Further investigation revealed that apoptotic endothelial cells released basal levels of NO and PGI_2. Unactivated platelets were reported to bind to apoptotic endothelial cells by others (Bombeli *et al.*, 1999) and this was also observed in Chapter 2. It is possible that the NO and PGI_2 produced by apoptotic endothelium inhibit activation of any platelets bound to apoptotic endothelial cells. Moreover, it is possible that the production of NO and PGI_2 increases when apoptotic endothelial cells interact with platelets stimulated by agonists, such as thrombin or ADP. Ecto-ADPase / CD 39 was not reliably detected in the cells studied and it is felt that this system did not contribute significantly to the anti-aggregatory effects seen.

CHAPTER FOUR

Cellular Fragmentation and Shape Change During Endothelial Apoptosis

4.1. Introduction

4.1.a. Canalicular Fragmentation During Endothelial Apoptosis

In canalicular fragmentation, large numbers of long branching tunnel-like structures form from invaginations of the plasma membranes of apoptotic endothelial cells. In earlier ultrastructural studies, highly canalicularised apoptotic endothelial cells appeared to be very fragile and liable to mechanical disruption (Zoellner *et al.*, 1996a; 1999). Since canalicular fragmentation has not been reported for apoptosis in any cell type other than endothelium, it seems important to propose some function for canalicular fragmentation specific to apoptosis in endothelial cells that would be irrelevant for other cell types. Also, because endothelial cells are uniquely stationed for accidental shedding into the circulation during trauma or microvascular remodelling while microembolism is known to cause clinically significant disease (Cotran *et al.*, 1999), it was suggested that canalicular fragmentation is a specific endothelial adaptation reducing micro-embolic potential (Zoellner *et al.*, 1996a).

Canalicular fragmentation occurs in HUVEC and cultured human microvascular endothelial cells during apoptosis, suggesting that this pattern of fragmentation is common to both macrovascular and microvascular human endothelial cells (Zoellner *et al.*, 1996a). Also, canalicular fragmentation is seen in apoptotic endothelium derived by either serum or matrix deprivation (Zoellner *et al.*, 1996a). Micrographs from one earlier study (Walker *et al.*, 1989) show detached apoptotic microvascular endothelial cells of the involuting rodent breast with what appear to be canalicular structures similar to those characterised in apoptotic cultured cells (Zoellner *et al.*, 1996a). Although the authors did not comment on the canalicular structures, the

published data indicated that canaliculi formation is not restricted to cultured human endothelial cells. Later studies of both human and rat tissue explants revealed canalicular fragmentation in microvascular endothelium in both rat and human tissue explants (Zoellner *et al.*, 1999), further confirming that this pattern of apoptosis occurs across species and in endothelial cells integrated into normal tissue architecture.

4.1.b. The Experimental Question: How Does the Size and Shape of Endothelial Cells Change During Apoptosis?

The reason for studying the anti-aggegatory activity of apoptotic endothelial cells for platelets in earlier chapters was to determine the likelihood or otherwise that apoptotic endothelium may act as centres for the formation of platelet micro-thrombi (1.2.c, 2.1.a). The data obtained indicated that endothelial cells actively inhibit platelet aggregation during apoptosis (2.3.c, 3.3.c). However, apart from consumption of platelets and associated clotting factors, the major impact of micro-thrombi is as micro-emboli and endothelium shed by trauma may be able to act as micro-emboli in the absence of microthrombosis. Since microembolism is a fundamentally mechanical event dependent upon cell size and also perhaps cell shape, it becomes important to know something of the size and shape of endothelial cells during apoptosis. Despite this, although there are numerous published micrographs indicating the size of individual endothelial cells during apoptosis (Walker *et al.*, 1989; Robaye *et al.*, 1991; Polunovsky *et al.*, 1994; Zoellner *et al.*, 1996a; Slowik *et al.*, 1997; Korff and Augustin, 1998; Zoellner *et al.*, 1998; 1999), there seem to be no published studies characterizing cell size and shape over time in whole populations of

apoptotic endothelial cells. Experiments described in this chapter address this unanswered question.

As discussed above (4.1.a), canalicular fragmentation has been suggested as a specific endothelial adaptation facilitating a rapid reduction in cell size during endothelial apoptosis to minimize the micro-embolic potential of the particles (Zoellner *et al.*, 1996a). It has been argued that the extensive canalicular structures make cells mechanically unstable so that even low levels of mechanical stress fracture cells into multiple small fragments. This suggestion arises from the electron microscopic appearance of cells with extensive canalicular structures and what appears to be evidence for the shedding of peripheral cytoplasmic fragments (Zoellner *et al.*, 1996a). With regard to this, it may be interesting to review Figures 2.4, 2.11 and 2.13 of this thesis showing apoptotic endothelium. In 2.4, the honeycombed appearance of an apoptotic endothelial cell is seen. A single contaminant erythrocyte in 2.11 provides a convenient blood cell for comparison with apoptotic endothelial particles. Knowing that erythrocytes must deform to squeeze through the microvessels of the lungs, it is easy to imagine that the much larger endothelial particles with which the erythrocyte in 2.11 are compared, would have difficulty negotiating the pulmonary microcirculation. In 2.13, the surface pores, irregular fragmented areas and membrane rests expected from transmission electron micrographs of apoptotic endothelium undergoing canalicular fragmentation (Zoellner *et al.*, 1996a) seem evident. However, the presumed rapid fragmentation of endothelial cells into smaller particles implied by these and earlier observations (Zoellner *et al.*, 1996a) has not been tested experimentally and there is no direct evidence indicating reduction in the size of apoptotic endothelial cells during mechanical stress. To address the absence of

any such data, experiments are described in this chapter investigating the effects of low levels of mechanical stress upon the size of endothelial cells during apoptosis.

Comparison between endothelial cells and the HL-60 macrophage cell line (Collins *et al.*, 1977) was made in the current study, in order to obtain some comparison between cells capable (endothelial cells) and incapable (HL-60 cells) of canalicular fragmentation. HL-60 cells were selected for this because the extensive membranous lysosomal structures of these cells superficially resemble the canalicular networks of apoptotic endothelial cells and so constitute a control for the presence of cytoplasmic membrane structures. It is important, however, to note that the lysosomal membranous structures in HL60 cells are neither confluent with the cell surface or interconnecting in the way that endothelial canaliculi are during apoptosis. Also important for this study, is that there are reports that HL60 cells become apoptotic in response to either serum deprivation (Barroso *et al.*, 1997; Durrieu *et al.*, 1999) or the calcium ionophore A23187 at a concentration of 10 μM (Ramachandra and Studzinski, 1995). As discussed below in 4.3.a, it was possible to reliably induce apoptosis in HL60 cells, although it was found necessary to modify culture conditions from those reported in the literature (Ramachandra and Studzinski, 1995).

4.2. Materials and Methods

4.2.a. Materials

RPMI 1640 medium was purchased from Sigma (St. Louis, USA). Other reagents and materials used in the study were as described in 2.2.a.

4.2.b. Preparation of Endothelial Cells

Experiments were performed with HUVEC from fourth to fifth passages cultured from seven separate donors as indicated in 2.2.b.

4.2.c. Culture of HL-60 Leukaemia Cells

HL-60 cells (Collins *et al.*, 1977) were obtained as a gift from the Department of Haemotology, Westmead Hospital. The cells are matrix independent and so were grown in suspension and cultured in RPMI 1640 medium supplemented with FCS (10%), glutamine (0.1mg/ml), penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were split at a ratio of 1 to 3 and fed every 2 to 3 days.

4.2.d. Culture Conditions for Study of Apoptotic Cell Suspensions Subjected to Low Levels of Mechanical Stress

M&SDA-EC were prepared as described in 2.2.c with the difference that cells were used immediately after release from the culture surface and placed into autoclaved eppendorf tubes instead of onto bacterial culture plates. The effect of this was that M&SDA-EC were not apoptotic at the very beginning of experiments, but developed apoptosis over time. M&SDA-EC were suspended in a medium consisting of M199 (80%) and HBSS (20%) for experiments. HL-60 cells were collected by washing and

concentrated by centrifugation. After washing twice with RPMI 1640 medium, HL-60 cells were resuspended in serum free RPMI 1640 medium with HBSS (20%) and calcium ionophore A23187 at concentrations ranging from 10 μM to 30 μM, with 30 μM being used in experiments where cell size and circularity was assessed. HL60 cells were aliquoted as 1 ml suspensions containing approximately 10^5 cells / ml into eppendorf tubes which were incubated at 37 °C either on a mechanical roller or in static tube racks. Please note that eppendorf tubes placed onto the mechanical roller were placed inside a further 50 ml centrifuge tube so as to facilitate regular circular motion of eppendorf tubes, while tubes rotated at a rate of 5 rotations per minute. Samples of these cell suspensions were collected at increasing time points for up to 18 hours.

4.2.e. Scanning Electron Microscopy

Briefly, cell suspensions of M&SDA-EC and HL-60 cells subjected to increasing time periods of either static or rotating culture were pre-fixed with glutaraldehyde (0.25 %) in PBS at 4 °C for 15 minutes before application dropwise onto nucleopore filters having a 3.0 μm pore size and fixed with glutaraldehyde (2.5 %) in PBS for 1 hour at 4 °C. Filters were then washed three times with PBS before dehydration with graded ethanols followed by critical point drying using a semi-automatic critical point drying apparatus (Balzers Union, Leichtenstein). All specimens were sputter coated with gold using a Balzer Union sputter coater (Balzers Union, Leichtenstein). A JSM 840 scanning electron microscope from JEDL SEM (Tokyo, Japan) was used while images were captured using Agfapan AP x 100 Professional 120 photographic film. A minimum of 7 separate sites was recorded in each filter studied.

4.2.f. Determination of Cell Size

Cell size was determined from scanning electron micrographs of M&SDA-EC and HL-60 cells, measuring a minimum of 100 cells per specimen to obtain a population distribution of cells according to size. The Scion Image analysis program (Scion Corporation, available at www.scioncorp.com) was used to determine cell size by manually outlining on screen individual cells and then calculating the surface area presented by the cell profile. This permitted objective measurement of the size of individual cells independent of their shape. Only objects which were clearly cellular were included in this study, while any cells obscured from view or seemingly degenerate in scanning electron micrographs were excluded from the analysis.

4.2.g. Analysis of Apoptotic Cell Shape

M&SDA-EC and apoptotic HL-60 cells were collected by washing and pelleted by centrifugation at 1,500 rpm for 5 minutes at 4 °C, before resuspension in M199 and RPMI 1640 medium, respectively. Cells were then transferred to eppendorf tubes before either processing for TEM (as described in 2.2.d.ii) to define the starting population, or alternatively incubation at 37 °C for 3 hr in either static or rolling culture as described in 4.2.d. Semi-thin sections were examined to determine the circularity of individual cells in cell populations using the Optimas 6.5 Image Analysis Program provided by Media Cybernetics L. P. (Silver Spring, MD, USA). This program determines the circularity of objects outlined manually on screen by the operator, by dividing the square of the circumference by the surface area, generating a unitless number, which reduces in magnitude with increasing circularity. In each experiment, at least 100 cells were measured from each specimen to obtain a population distribution of cell circularity.

4.2.h. Statistical Analysis

Statistical analysis was performed using the Mann-Whitney U test and p values of less than 0.05 were considered statistically significant.

4.3. Results

4.3.a. HL-60 Cells Became Apoptotic in Serum Free Condition with 30 μM A23187

TEM revealed only very occasional apoptotic cells in the starting population of HL-60 cells while there appeared to be little or no increase in apoptosis in response to serum deprivation (Figure 4.1A,B). Addition of the calcium ionophore A23187 at concentrations of either 10 μM or 30 μM also had little effect upon HL60 apoptosis (Figure 4.1C), however, when cells were simultaneously deprived of serum and exposed to A23187 at a concentration of 30 μM, all of the cells observed had the features of apoptosis. Some of these cells also displayed the hallmarks of secondary necrosis (Figure 4.1D). Importantly for this study, lysosomal membrane structures were prominent in both non-apoptotic and apoptotic HL-60 cells (Figure 4.1).

Figure 4.2 illustrates the ultrastructural apoptotic features of HL-60 cells including condensed and fragmented nuclear material, intact mitochondria and RER as well as lysosomal membrane structures seemingly "honeycombing" parts of the cells. Despite the presence of these occasionally extensive intracellular membranous structures, no canalicular structures were seen and none of the vacuoles appeared to communicate with the cell surface, clear differences from canalicular fragmentation seen in apoptotic endothelium.

DNA gel electrophoresis revealed internucleosomal fragmentation in the absence of serum and presence of A23187 (30 μM), further confirming the apoptotic status of these cells (Figure 4.3).

4.3.b. Scanning Electron Microscopy Revealed Intact HUVEC and HL-60 Cells

When non-apoptotic, starting populations of HUVEC and HL-60 cells were observed by SEM, both cell populations presented as irregular spherical structures, with HL-60 cells being considerably smaller than HUVEC. Some degree of seemingly artifactual flattening of cells was apparent in both populations, while the apparent rupture of occasional cells with loss of plasma membrane continuity indicated poor preservation of some cells (Figure 4.4).

Despite these artifacts, it was felt that cellular shape and integrity were sufficiently well preserved to initiate SEM studies comparing the size of HUVEC and HL-60 cells during apoptosis and low levels of mechanical stress.

4.3.c. Apoptotic HUVEC and HL-60 Cells Became Smaller and Degenerate with Time

Scanning electron micrographs of both HUVEC and HL-60 cells revealed reducing cell size and increasing degradation at increasing times after initiation of apoptosis (Figures 4.4 and 4.5). The artifactual flattening and loss of membrane preservation became more extreme at later time points, although many cells still maintained plasma membrane integrity and most cells were essentially rounded in shape. Degenerate cellular material was interpreted as representing debris from secondary necrosis. No clear difference in the appearance of cells was noted in conditions of low mechanical stress. Despite this, when the size of non-degenerate cells was quantitated and compared between static and rotating populations, significant changes were seen over time and in conditions of low mechanical stress. Figure 4.5 shows the result of a typical experiment in which a M&SDA-EC population reduced in size with

progressive apoptosis, and mechanical stress resulted in still further reduction in size at all time points studied ($p < 0.01$). Similar results were obtained in four separate experiments with HUVEC from four separate donors.

Although HL-60 cell size reduced with apoptosis, there was negligible difference between cells exposed to mechanical stress and those cultured in static conditions, with no difference seen in two separate experiments at all time points studied and a statistically significant difference being found only at the 18 hours time point in one further experiment only (Figure 4.6).

When data from all experiments were combined by plotting the means of the average size of cells from each time point as a percentage of the size of cells in the starting population, a slight difference between rotating and static populations in both HUVEC and HL60 cells was apparent. Also, a significant difference was seen between M&SDA-EC and HL-60 cells, in that M&SDA-EC clearly reduced in size at a much greater rate as compared with HL-60 cells ($p < 0.05$) (Fig. 4.7).

4.3.d. Intracellular Vesicles Appeared to Fuse with Canaliculi During Apoptosis in HUVEC

TEM revealed canalicular fragmentation with the formation of surface pores as expected from earlier studies (Figures 4.8 - 4.12). In many instances, canaliculi were closely packed to form honeycomb-like structure (Figures 4.8 - 4.12). On close examination of canaliculi, it was noted that canaliculi at the deepest parts of these honeycomb-like structures were often in close association with small vesicles while in some instances, these vesicles appeared to fuse with the canaliculi and this was the

case at all time points studied (Figures 4.8 - 4.12). Fusion did not seem to be limited between canaliculi and small vesicles, but in places adjacent canaliculi appeared to fuse together as well. In some cells, canaliculi appeared to contribute to larger vacuoles within apoptotic HUVEC, occasionally having an irregular border opening into a honeycomb of canaliculi (Figure 4.8), but more often being essentially spherical in appearance. This seemed to result in the formation of large round vacuoles (Figures 4.8 - 4.12). The vacuoles formed were often at the cell surface, seemingly about to "burst". By 18 hours canaliculi appeared to be much wider in diameter than at earlier time points and large round vacuoles were also often prominent (Figure 4.12).

These observations suggest that canaliculi form not only by invagination of the plasma membrane, but also by fusion with vesicles, and that there is further maturation of canaliculi to dilate and or form large vacuole like structures which can fuse with the cell surface.

4.3.e. Scanning Electron Microscopy of Apoptotic HUVEC Revealed Surface Features Consistent with Transmission Electron Microscopy

Blebbing of cells typical of apoptosis was seen in M&SDA-EC (Figure 4.13A). In addition, small pores were seen to open onto the surface of many cells, consistent with the pores opening into canaliculi seen by TEM (Figure 4.13B).

At later time points, many apoptotic HUVEC displayed large crater-like surface voids (Figure 4.13B, C), consistent with the large vacuoles seen by TEM. Also consistent with TEM, was the presence of wide channel-like structures opening at the cell

surface, and these are suggested as canaliculi opening "side on" to the plasma membrane (Figure 4.13B,C). One surprising observation, was the presence of elevated cords of plasma membrane, creating the appearance of "rope-like" structures snaking across the surface of some apoptotic cells (Figure 4.13D).

4.3.f. The Circularity of HUVEC Increased with Mild Mechanical Stress

Although rotating and static HUVEC had similar features by both TEM and SEM, cells subjected to low levels of mechanical stress appeared to be more round in shape as compared to static M&SDA-EC populations. To further study this, semi-thin sections of M&SDA-EC already clearly apoptotic after 12 hr of culture in static conditions were compared with similar sections of cells cultured for a further 3 hr in static conditions or alternatively in conditions of low mechanical stress.

Figure 4.14 shows the typical appearance of these cells in semi-thin sections. Discounting cells with the appearance of secondary necrosis, it was clear that static M&SDA-EC populations were not significantly more circular as compared with the starting population, while M&SDA-EC subjected to low levels of mechanical stress were very much more circular than either the starting or static populations ($p < 0.01$). This is illustrated in Figure 4.15 which shows the distribution of cells with different levels of circularity while similar results were obtained in four separate experiments with cells from four separate donors.

When HL-60 cells were studied, it was found that these cells were already highly circular at the beginning of experiment and that little or no change in circularity occurred in either static or rotating populations during experiments. The results of a

typical experiment are shown in Figure 4.16, with similar results being found in four separate experiments.

4.3.g. Cell Size Reduced in Semi-Thin Sections of Both Apoptotic Endothelium and HL-60 Cells in Response to Mild Mechanical Stress

Since scanning electron microscopic assessment of both apoptotic endothelium and HL-60 cells was complicated by cell flattening and degradative artifacts, cell size was further assessed in semi-thin sections of M&SDA-EC and HL-60 cells. Figure 4.17 shows the cell size distribution of both apoptotic HUVEC and HL-60 cells in semi-thin sections both at the beginning of a typical experiment, as well as after three hours of either static or rotating culture. Cell size was seen to reduce significantly for HUVEC with time ($p < 0.01$), while this was much more pronounced for HUVEC exposed to mechanical stress ($p < 0.01$). Similar results were observed in three out four experiments with M&SDA-EC from four separate donors. No difference in the size of HL-60 cells was seen with progressive apoptosis over the 3 hour time period studied (Figure 4.18). Similar results were obtained in four separate experiments.

These data were interpreted as indicating that M&SDA-EC had a greater tendency to reduction in cell size over time as compared with apoptotic HL-60 cells and were also more fragile than HL-60 cells, fragmenting more readily in response to low levels of mechanical stress.

These data are consistent with those observed by SEM, suggesting that despite the difficulties posed by the artifacts seen in scanning electron micrographs that the results obtained are meaningful.

Figure 4.1. Transmission electron micrographs of HL-60 cells cultured for 18 hr with complete culture medium (A), serum free medium (B), serum free medium and A23187 (10 μM) (C) and serum free medium and A23187 (30 μM) (D). HL60 cells often had prominent lysosomal vacuoles (arrows), reminiscent of canaliculi in apoptotic endothelium, but distinctly different in that the vacuoles in HL60 cells were not confluent with the surface or interconnecting. These extensive membranous structures were felt to provide a form of control for comparison with the membranous canaliculi of apoptotic endothelium. Apoptotic HL-60 cells (arrow heads) were readily recognized by the presence of condensed nuclear fragments (stars), increased cytoplasmic electron density and often an apparent reduction in physical size. The vacuolar structures present in non-apoptotic HL-60 cells (arrows) were often observed in apoptotic cells. Very few apoptotic cells were evident in growth culture conditions (*A*) while serum deprivation alone had little effect upon this (*B*). When cells were both deprived of serum and exposed to low levels of A23187 (10 μM), there was also little effect upon HL-60 apoptosis (*C*). Nonetheless, when serum deprived cells were subjected to higher concentrations of A23187 (30 μM), all of the HL-60 cells observed were apoptotic, or displayed features of secondary necrosis (SN) (*D*). (Bars = 5 μm)

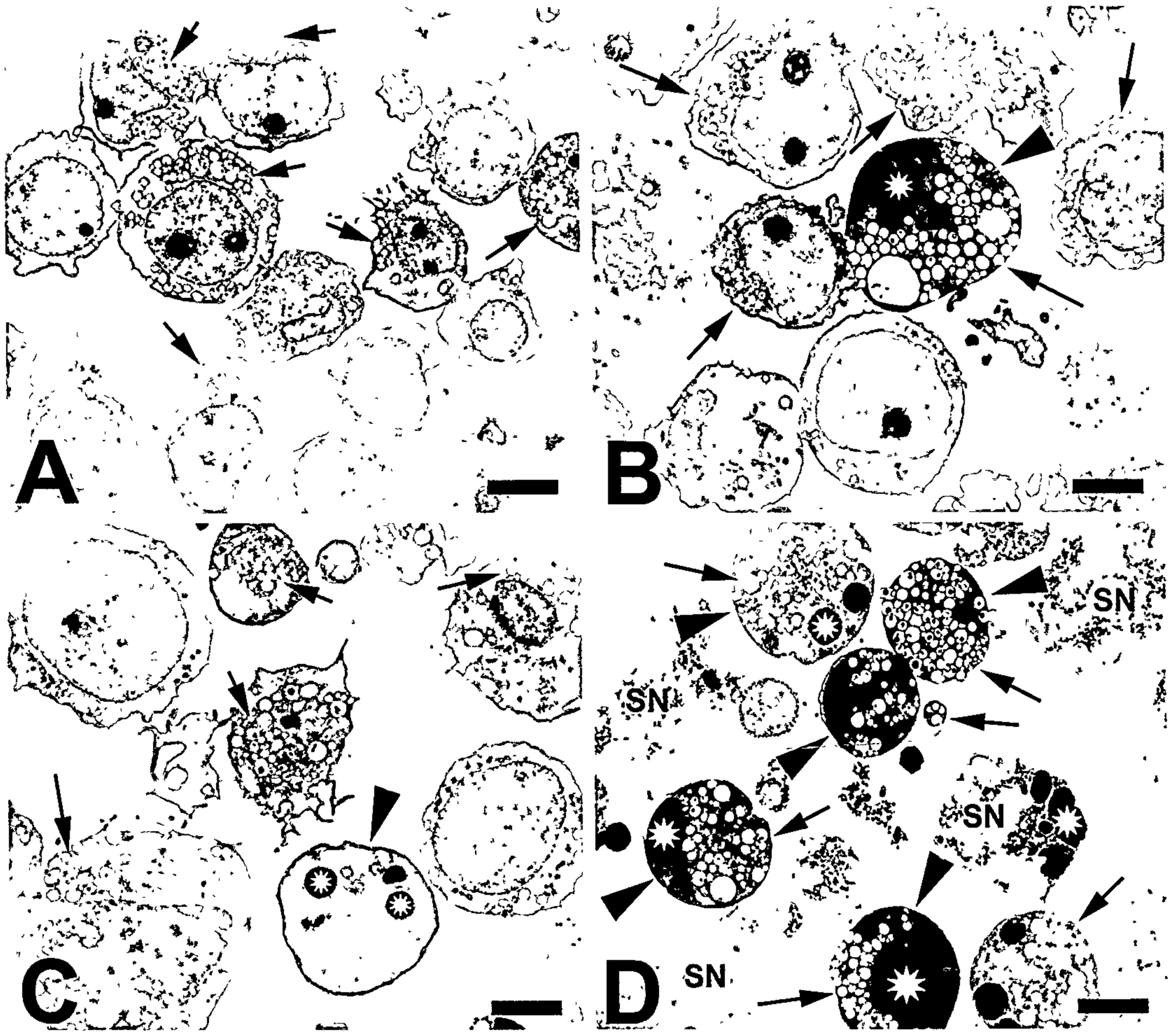
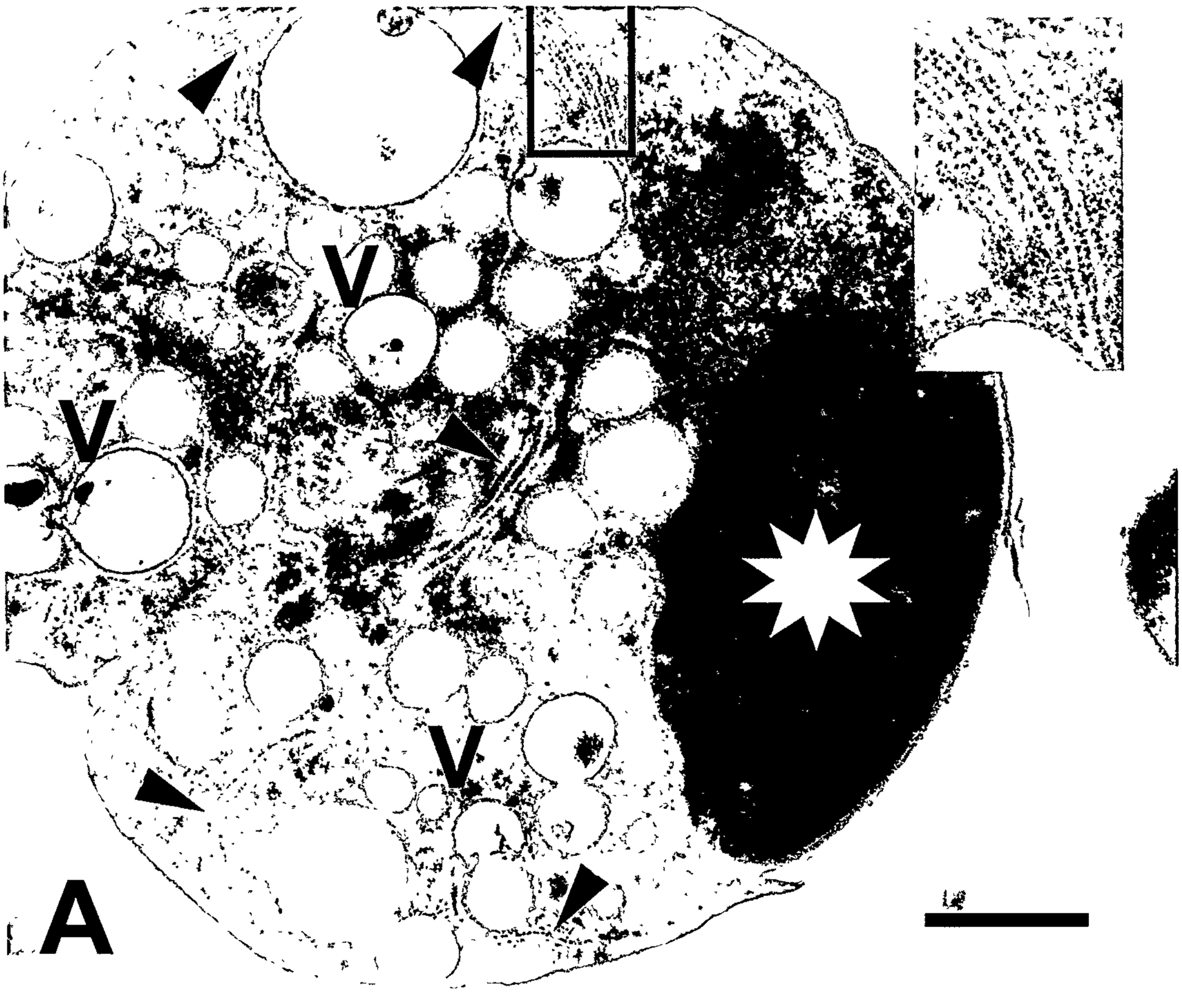

A
B
C
D
SN
SN
SN
SN

Figure 4.2. Transmission electron micrographs of HL-60 cells cultured for 18 hr in serum free medium with A23187 (30 μM). Condensed and fragmented nuclear material (stars) was present, typical of apoptosis. Organellar integrity was also maintained in these cells, with RER (arrow heads) (*A*) and mitochondria (insert) (*B*) being seen within the electron dense cytoplasm. The membrane bound vacuoles (V) seen in non-apoptotic HL-60 cells were also preserved during apoptosis. (Bars = 1 μm)


V
V
V
A

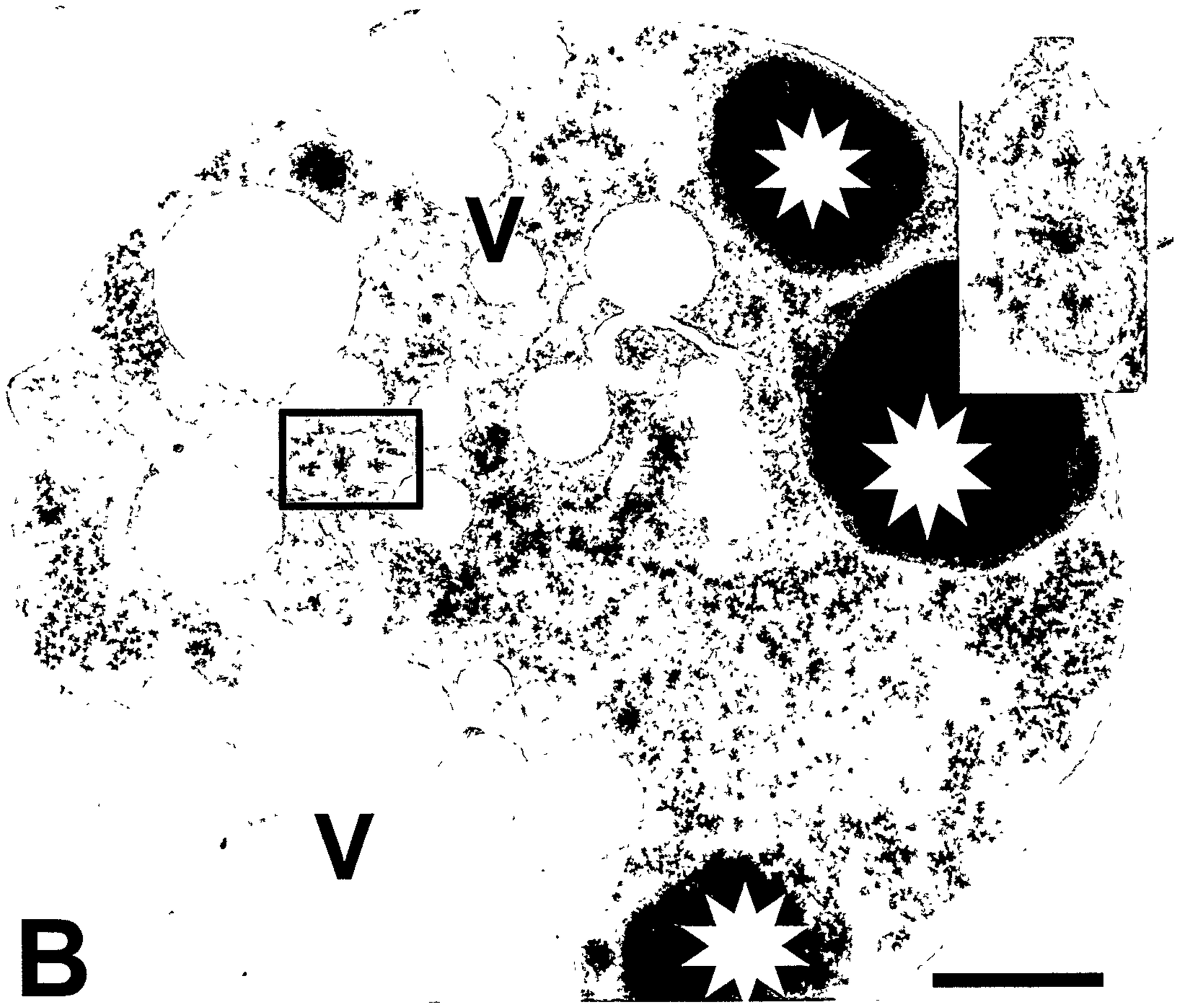

V
V
B

Figure 4.3. Agarose gel electrophoresis with ethydium bromide staining of DNA from HL-60 cells in complete culture medium as compared with that from cells deprived of serum in the presence of A23187 (30 μM). Almost all of the DNA from cells in complete culture medium occurred as high molecular mass material, penetrating only a small distance into the gel. This contrasted with that of DNA from cells deprived of serum with A23187 (30 μM), which was degraded into a 180 BP ladder typical of apoptosis. Molecular weight markers are indicated.

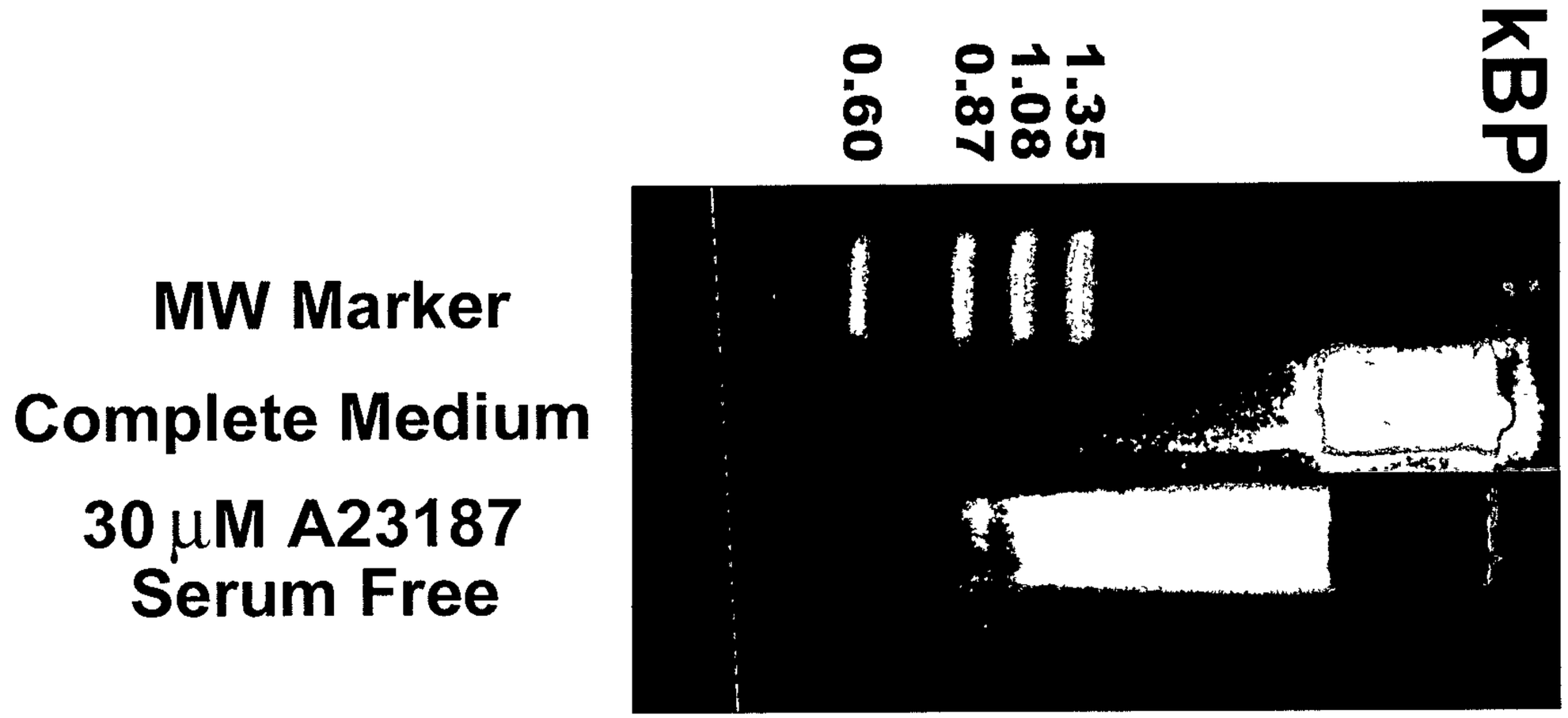
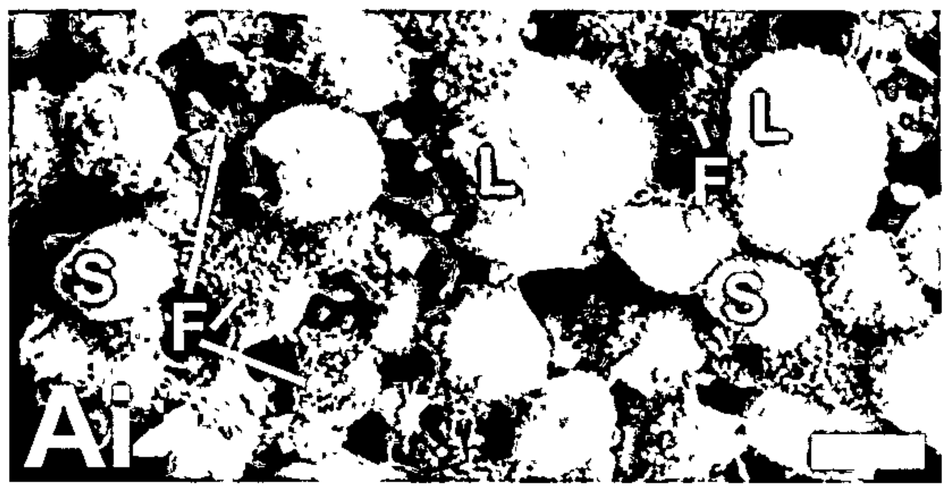
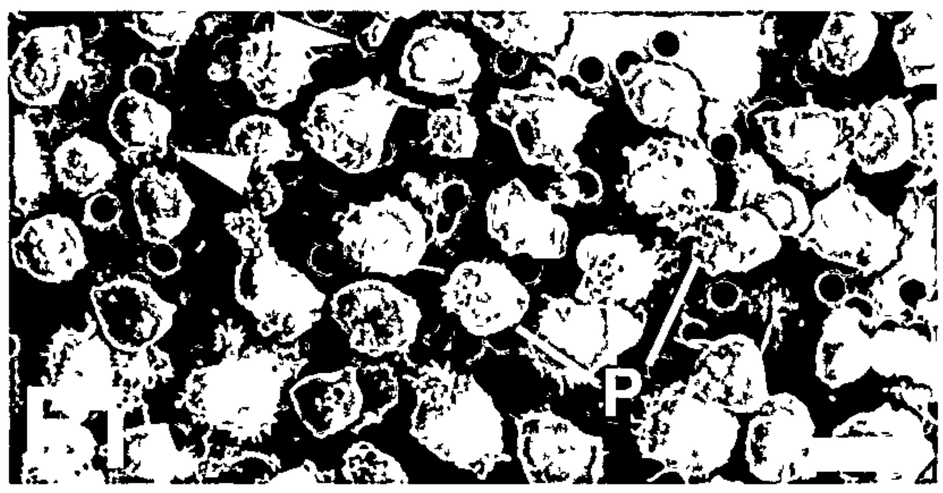
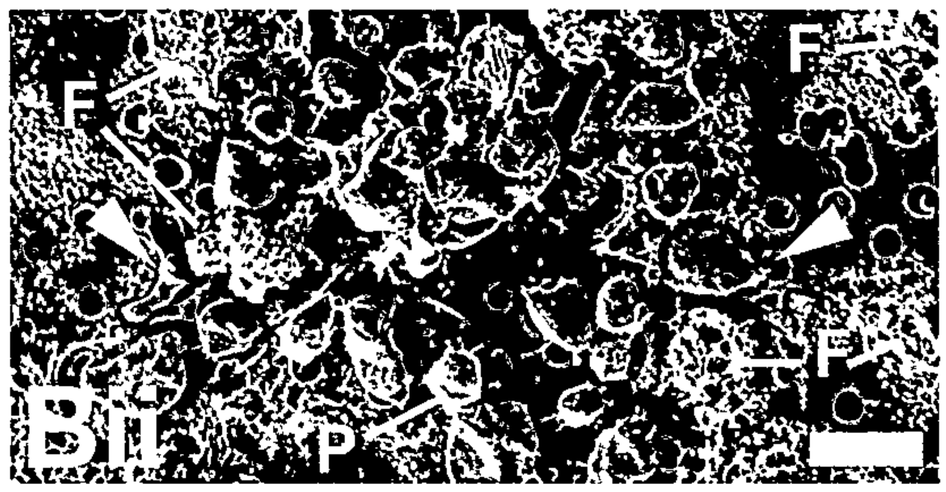

kBP
1.35
1.08
0.87
0.60
MW Marker
Complete Medium
30 μM A23187
Serum Free

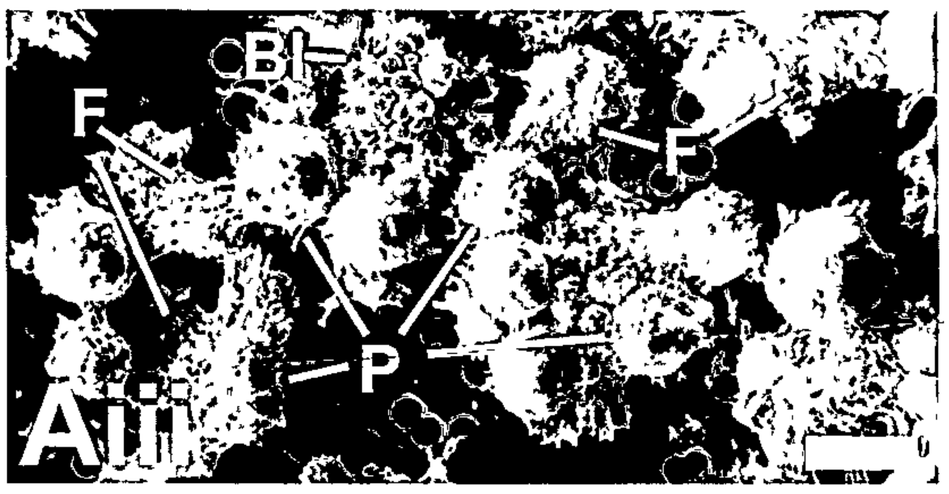
Figure 4.4. Scanning electron micrographs of HUVEC (A) and HL-60 cells (B), 0 hr (i), 6 hr (ii), 12 hr (iii) and 18 hr (iv) after induction of apoptosis by matrix and serum deprivation in the case of HUVEC and serum deprivation in the presence of A23187 (30 μM) in the case of HL-60 cells. Some clearly artifactual changes were noted including a degree of flattening amongst some cells. One prominent artifact was the apparent extension of some cell processes into pores of the underlying support membrane (arrow heads). This was known to not reflect active cellular behavior because all cells had been fixed prior to application to the membranes. Also, some fragmented and seemingly cellular debris was seen at early time points (F), suggestive of degradation during processing. These fragmented cellular masses may, however, have reflected secondary necrosis and this is supported by the prominence of this material at later time points (F) while fragmented material at early times may have been due to background apoptosis. Despite these difficulties, preservation of most cells seemed adequate for assessment of cell size and shape, even at the latest time points when cellular degradation was prominent (arrows). Large cells (L) were readily seen, particularly at the earliest time points amongst HUVEC and these contrasted with much smaller cells (S) in identical preparations. Surface pores (P) were noted in many M&SDA-EC as well as in occasional HL-60 cells. Apoptosis was also marked by the formation of cells with prominent surface blebs (Bl). Progressive degradation of apoptotic populations was evidenced by a reduction in size of the cells as well as by loss of membrane integrity with some cells at latter times seeming to consist only of cytoskeletal remnants (C). (Bars = 10 μm)

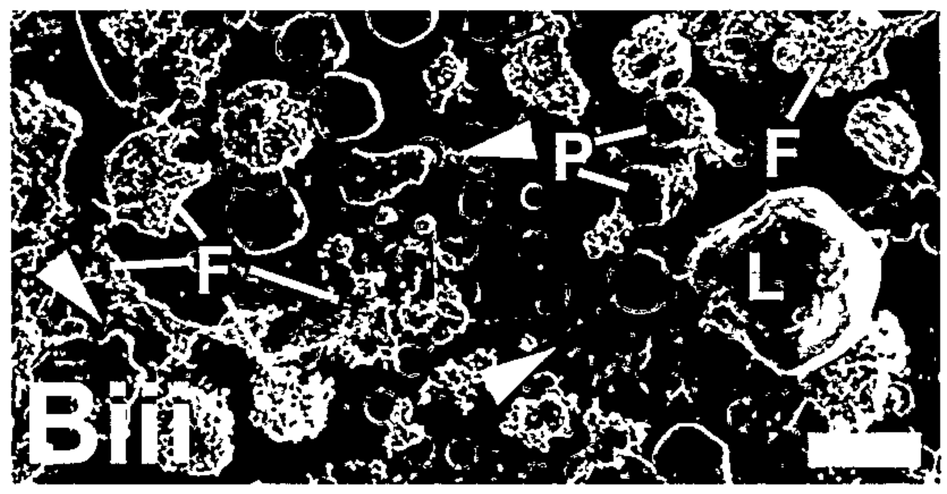

L
L
F
S
S
F
Ai

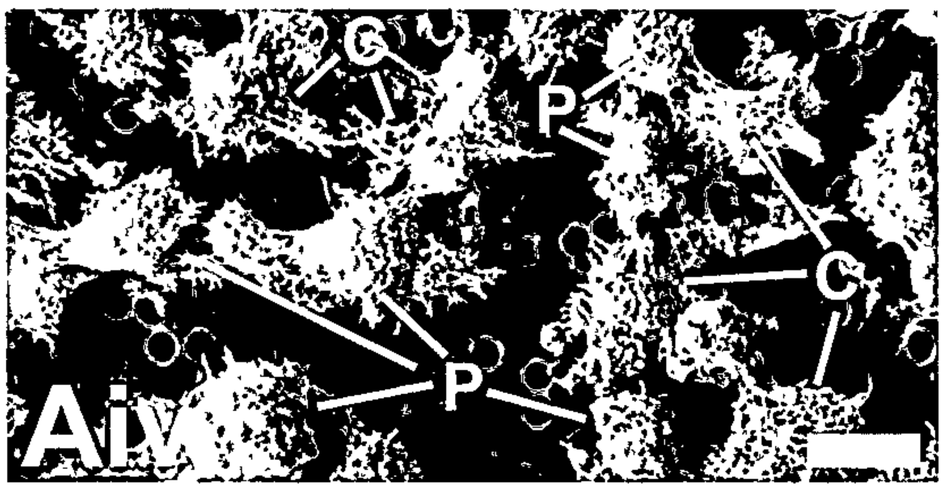

P
F


F
F
Bi
Bi
P
Aii


F
F
F
P
Bii


Bi
F
F
P
Aiii


P
F
F
L
Biii


C
P
C
P
Aiv

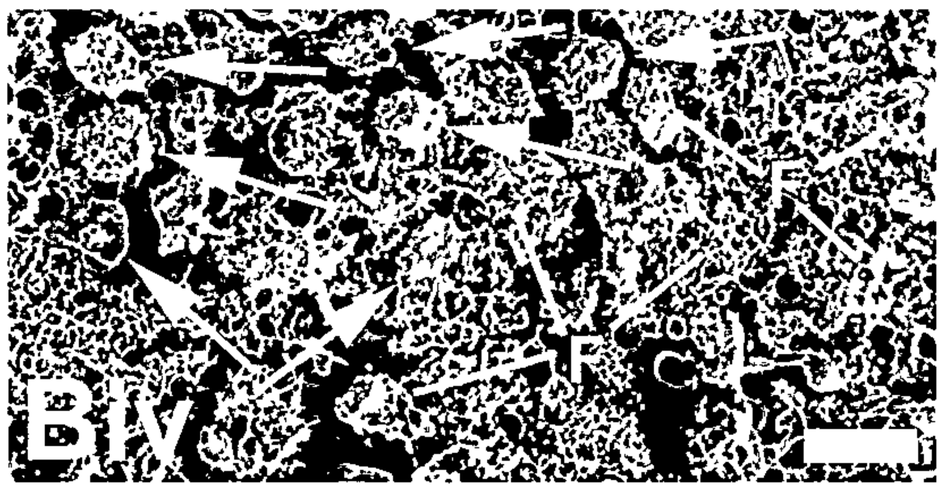

F
C
Biv

Figure 4.5. The distribution in size of M&SDA-EC over time in static culture (closed circles) as compared with cells exposed to low levels of mechanical stress (open circles). Non apoptotic cells in the starting population (0 hr) varied greatly in size with a skewed but almost normal distribution. After 6 hr of apoptosis, the size distribution of M&SDA-EC was narrower and shifted to the left in static culture conditions ($p < 0.01$) while with low levels of mechanical stress, this was even more pronounced ($p < 0.01$). This trend continued at later time points, with progressive reduction in size occurring over time ($p < 0.01$) and the mechanically stressed cells always being smaller than the cells in static culture ($p < 0.01$). Similar results were obtained in three further separate experiments with cells from different donors.

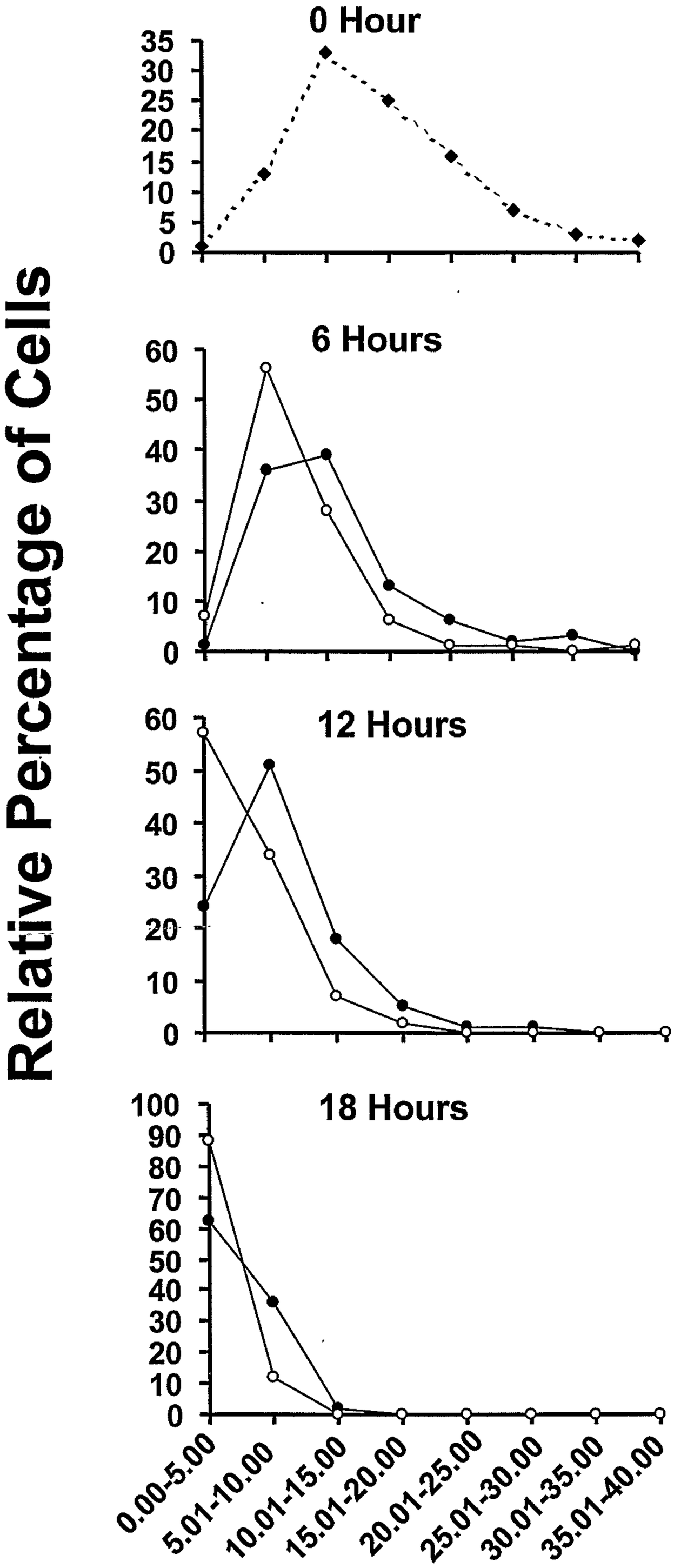

0 Hour
35
30
25
20
15
10
5
0
6 Hours
60
50
40
30
20
10
0
12 Hours
60
50
40
30
20
10
0
18 Hours
100
90
80
70
60
50
40
30
20
10
0
0.00-5.00
5.01-10.00
10.01-15.00
15.01-20.00
20.01-25.00
25.01-30.00
30.01-35.00
35.01-40.00
Relative Percentage of Cells
Visible Surface Area (μm²)

Figure 4.6. The distribution in size of apoptotic HL-60 cells over time in static culture (closed circles) as compared with cells exposed to low levels of mechanical stress (open circles). Non apoptotic cells in the starting population (0 hr) varied in size with a near normal distribution. After 6 hr of apoptosis by culture in serum free conditions with A23187 (30 μM) the size distribution of apoptotic HL-60 cells was shifted to the left ($p < 0.01$) while there was no clear difference in size between cells exposed to mechanical stress and those in static culture. These trends continued throughout all time points studied, with reducing cell size over time but no difference arising from the different culture conditions. Similar results were obtained in two further separate experiments.

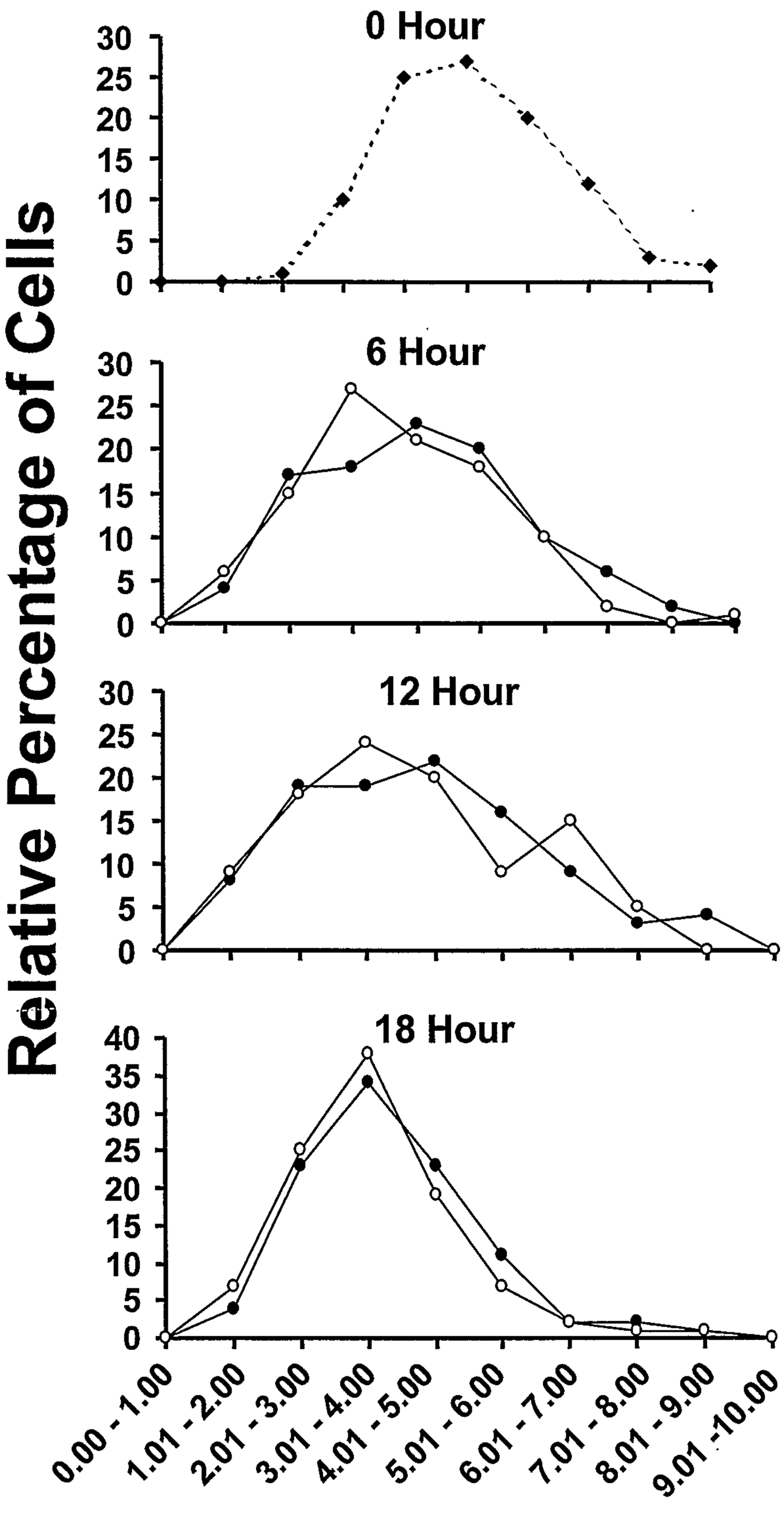

0 Hour
6 Hour
12 Hour
18 Hour
Relative Percentage of Cells
0
5
10
15
20
25
30
35
40
0.00 - 1.00
1.01 - 2.00
2.01 - 3.00
3.01 - 4.00
4.01 - 5.00
5.01 - 6.00
6.01 - 7.00
7.01 - 8.00
8.01 - 9.00
9.01 -10.00
Visible Surface Area (μm²)

Figure 4.7. Graph of the changes over time in the average cell size of HUVEC and HL-60 cells during apoptosis induced by serum and matrix deprivation in the case of HUVEC and serum deprivation with A23187 (30 μM) in the case of HL-60 cells. Data show the mean results from four separate experiments with HUVEC and three experiments with HL-60 cells. It was seen that apoptotic HUVEC reduced in size at a greater rate as compared with HL-60 cells ($p < 0.05$), while in both populations, low levels of mechanical stress had a further modest effect in reducing the relative size of cells.

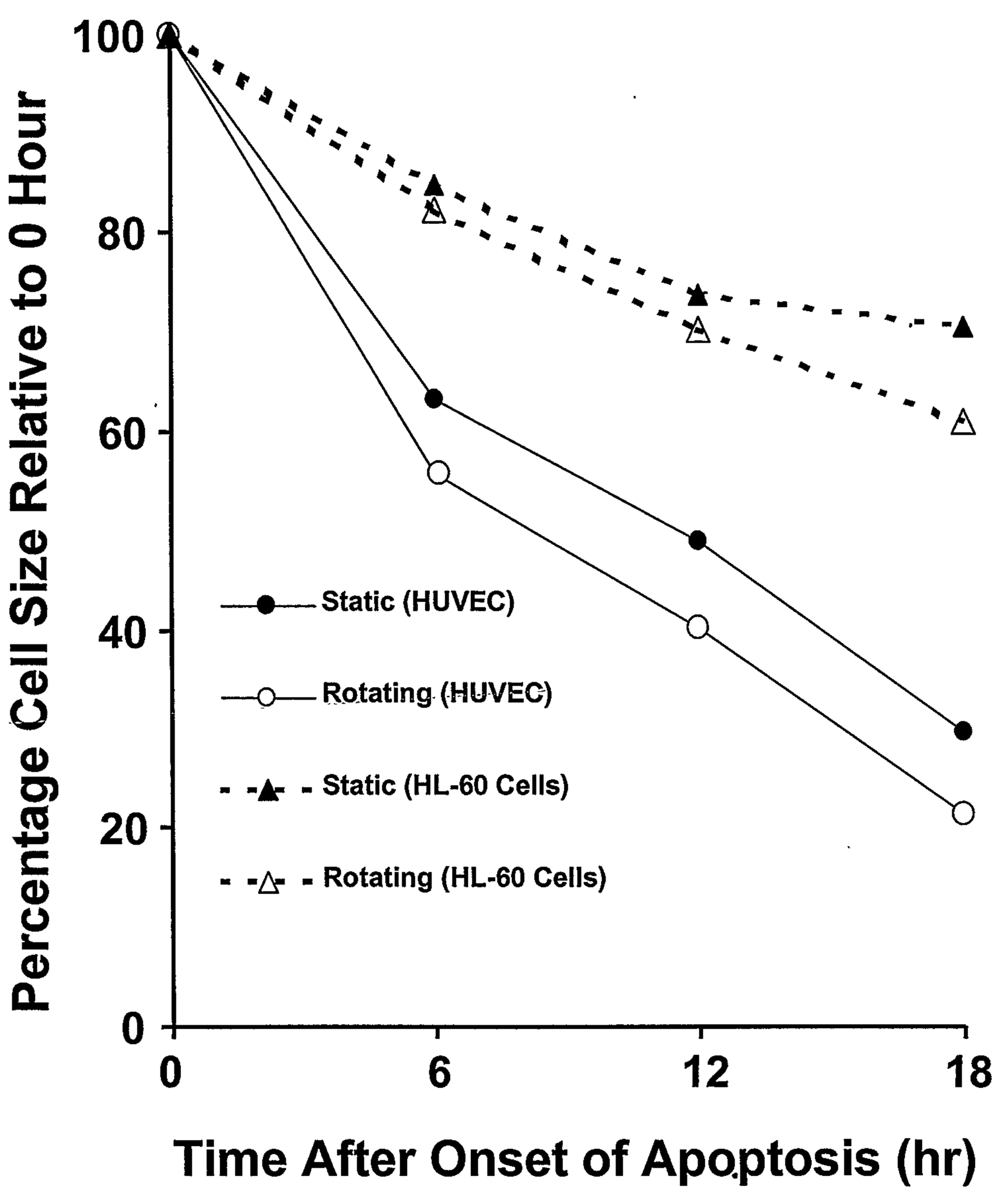

Percentage Cell Size Relative to 0 Hour
100
80
60
40
20
0
0
6
12
18
Static (HUVEC)
Rotating (HUVEC)
Static (HL-60 Cells)
Rotating (HL-60 Cells)
Time After Onset of Apoptosis (hr)

Figure 4.8. Transmission electron micrographs of a M&SDA-EC forming large vacuoles through the coalescence of canaliculi at low (A) and high (B) magnification. Features of canalicular fragmentation reported in earlier studies were seen including the formation of surface pores (P) and deep interconnecting canaliculi (C). Surface membrane rests (MR) were seen as were intact mitochondria (m) and RER (arrow heads). A previously unreported observation was the seeming coalescence of canliculi (arrows) to form larger irregular vacuoles (V). This was particularly seen at higher magnification (*B*), where multiple canaliculi appeared to open into a large irregular vacuole. Other vacuoles (V) appeared more regular and spherical. An autophagocytic vacuole was also seen in this cell (AV). (Bar for A = 2 μm, Bar for B = 1 μm)

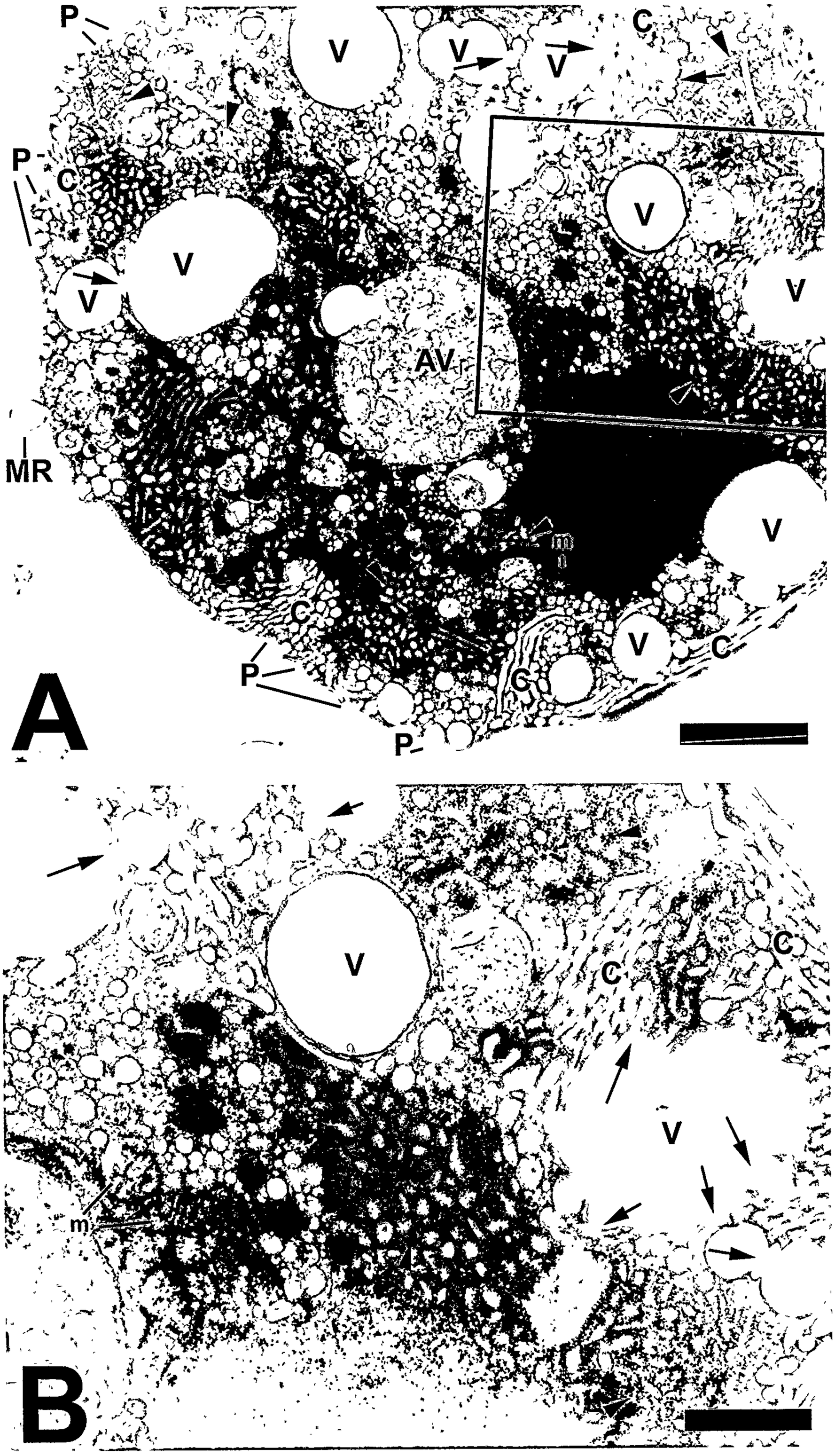
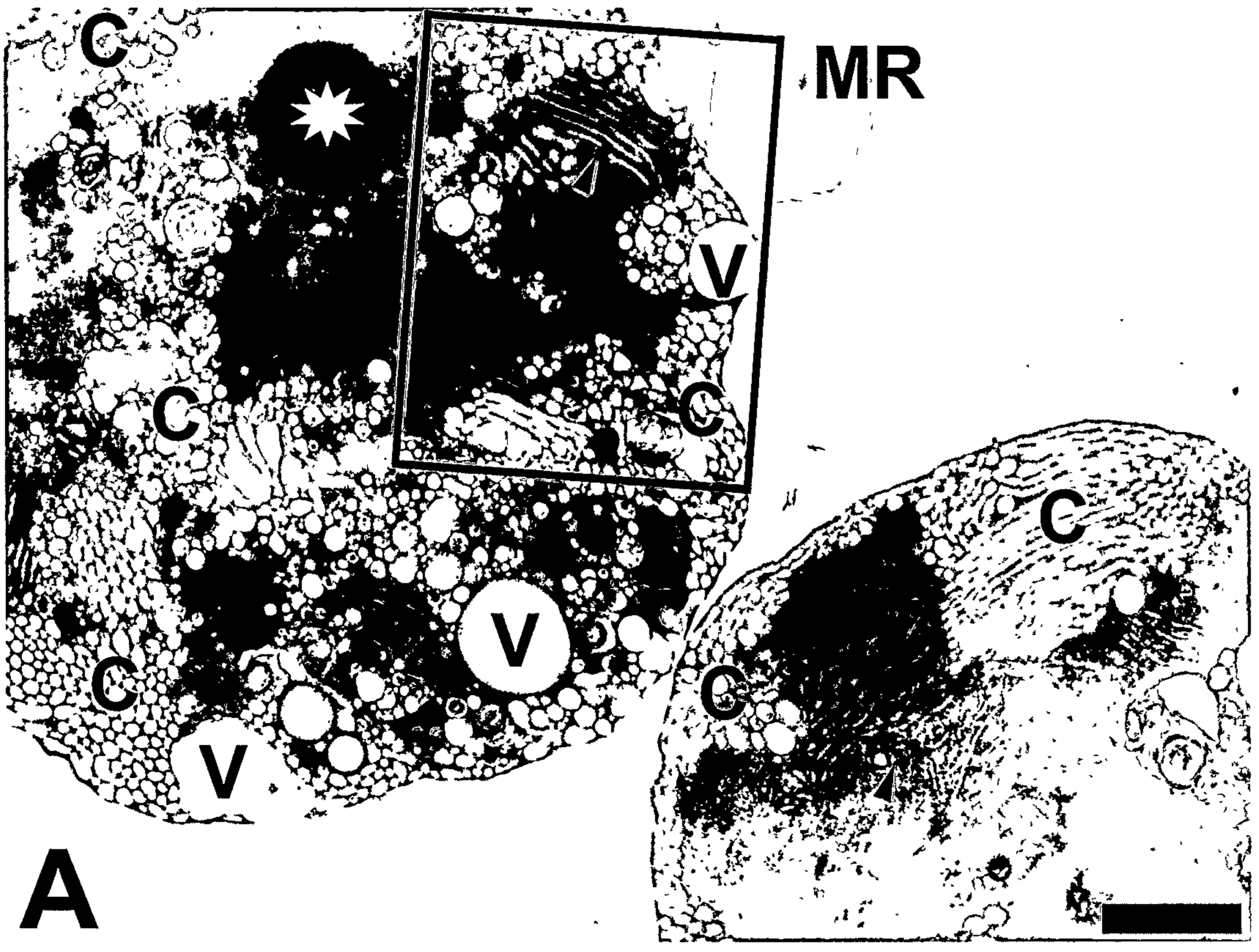

P
V
V
V
C
P
C
V
V
V
AV
MR
m
V
C
P
C
V
C
P
A
V
C
C
V
m
B

Figure 4.9. Transmission electron micrographs of M&SDA-EC at low (A) and high (B) magnification, in which canaliculi appear to be fusing with very small vesicles. Apoptotic endothelial cells with condensed nuclear fragments (stars), intact mitochondria (m), intact RER (arrow heads), surface membrane remnants (MR) and canalicular fragmentation (C) were seen. In addition, large vacuoles (V) were present, with some of these appearing to fuse with the cell surface. Canalicuili were seen to communicate with the surface via surface pores (P). An unexpected finding was the presence of multiple small vesicles seeming to fuse with canaliculi (arrows). In some instances, this appeared similar to the fusion of canaliculi with each other (arrows). These apparent fusion events were seen most clearly at higher magnification (*B*) while the insert illustrates this more clearly. (Bar for A = 2 μm, Bar for B = 1 μm)


C
MR
V
C
C
V
C
V
C
A

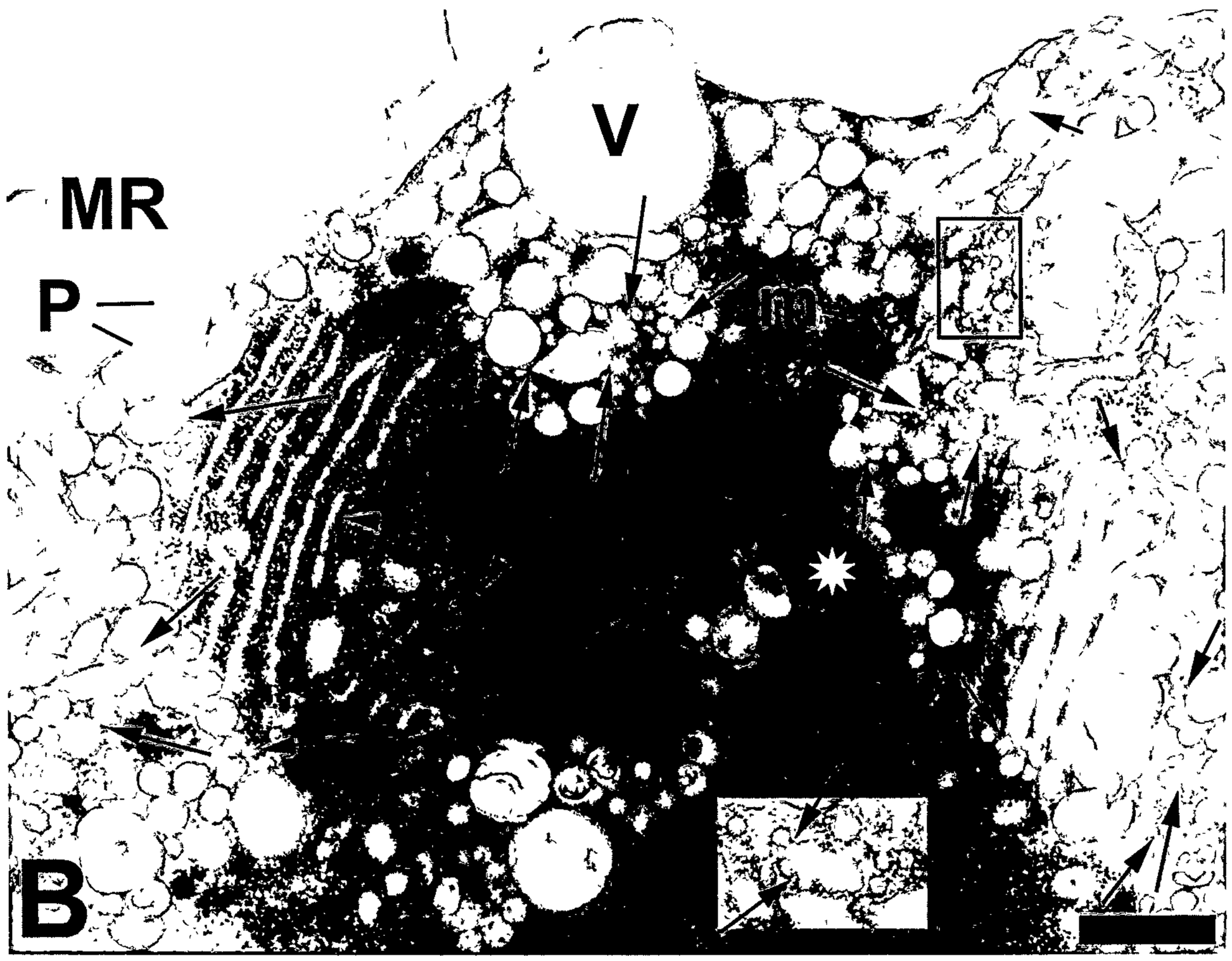
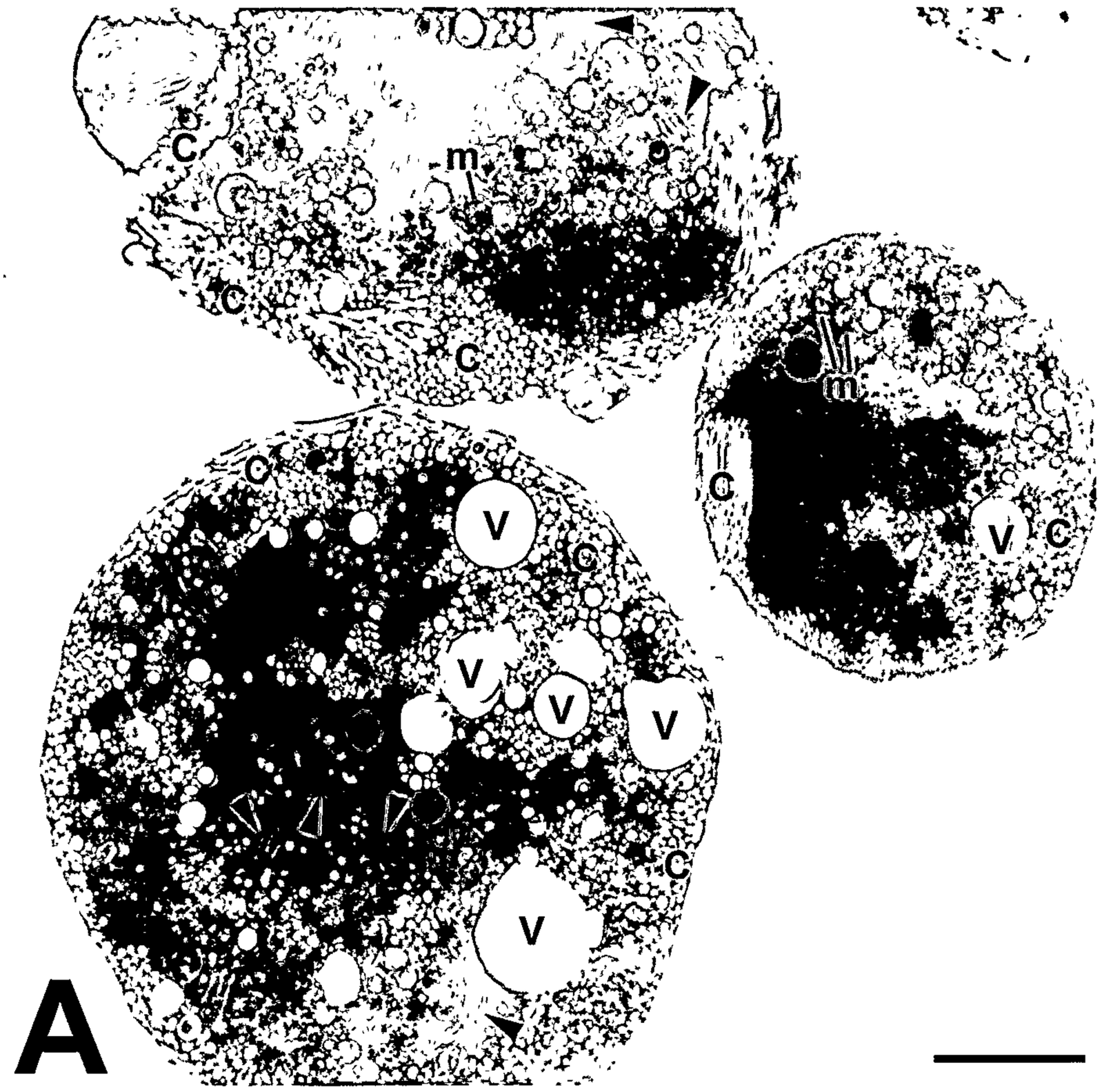

V
MR
P
B

Figure 4.10. Transmission electron micrographs of a M&SDA-EC at low (A) and high (B) magnification, in which canaliculi appear to be fusing with each other. Apoptotic endothelial cells with intact mitochondria (m), intact RER (arrow heads), surface membrane remnants (MR) and canalicular fragmentation (C) were seen. In addition, large vacuoles (V) were present. Canalicuili were seen to communicate with the surface via surface pores (P). At higher magnification, canaliculi appeared to fuse with one another (arrows). (Bar for A = 4 μm, Bar for B = 2 μm)


C
m
C
C
m
C
V
C
V
V
V
C
V
C
A

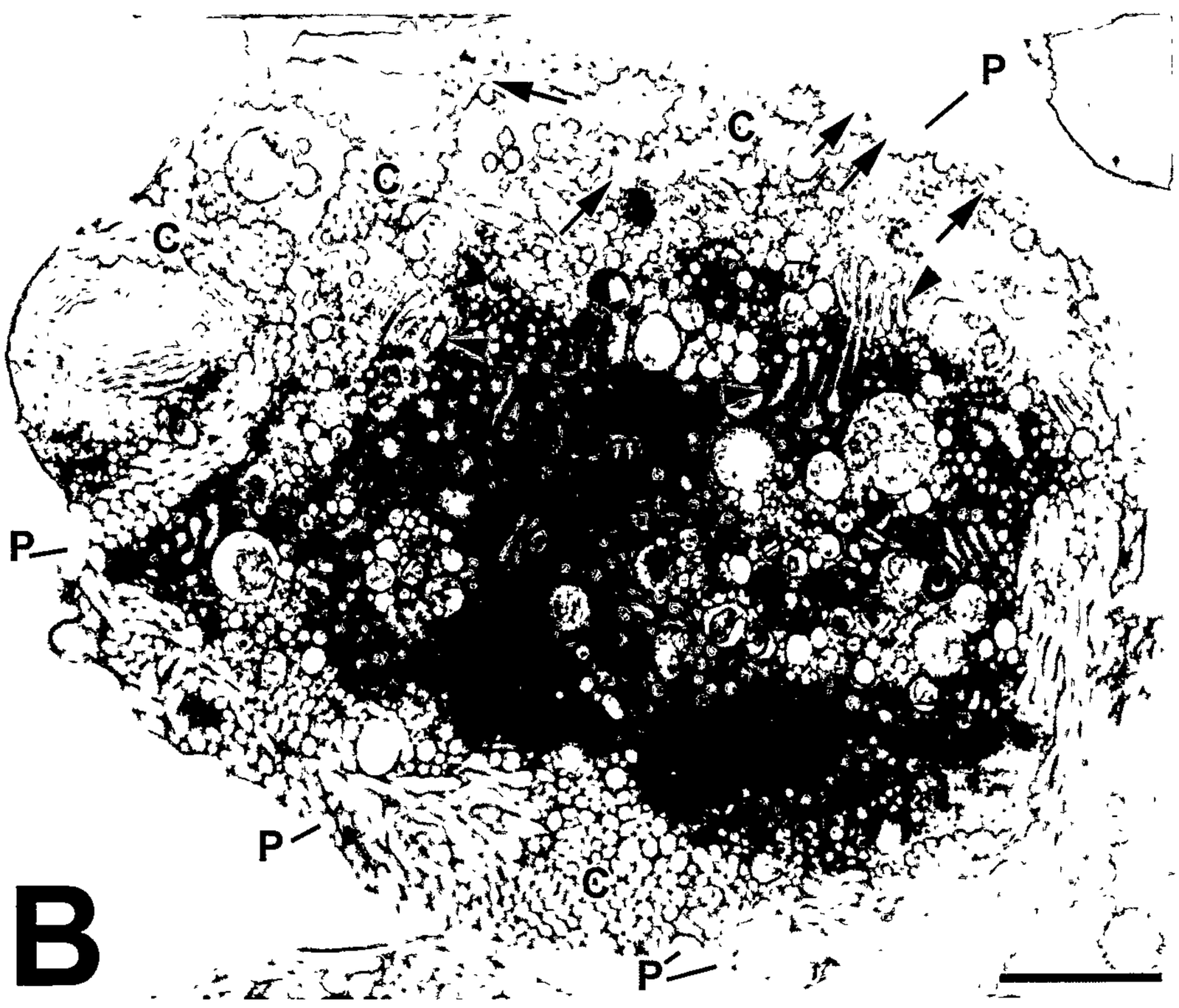

P
C
C
C
P
C
P
P
B

Figure 4.11. Transmission electron micrographs of a M&SDA-EC at low (A) and high (B) magnification, in which canaliculi appear to be fusing with one another as well as with very small vesicles. An apoptotic endothelial cell with condensed nuclear material (stars), intact mitochondria (m), intact RER (arrow heads), surface membrane remnants (MR) and canalicular fragmentation (C) was seen while a single large vacuole was present (V). Canalicuili were seen to communicate with the surface via surface pores (P). At higher magnification, canaliculi appeared to fuse to each other as well as to small vesicles (arrows). (Bar for A = 1 μm, Bar for B = 0.5 μm)

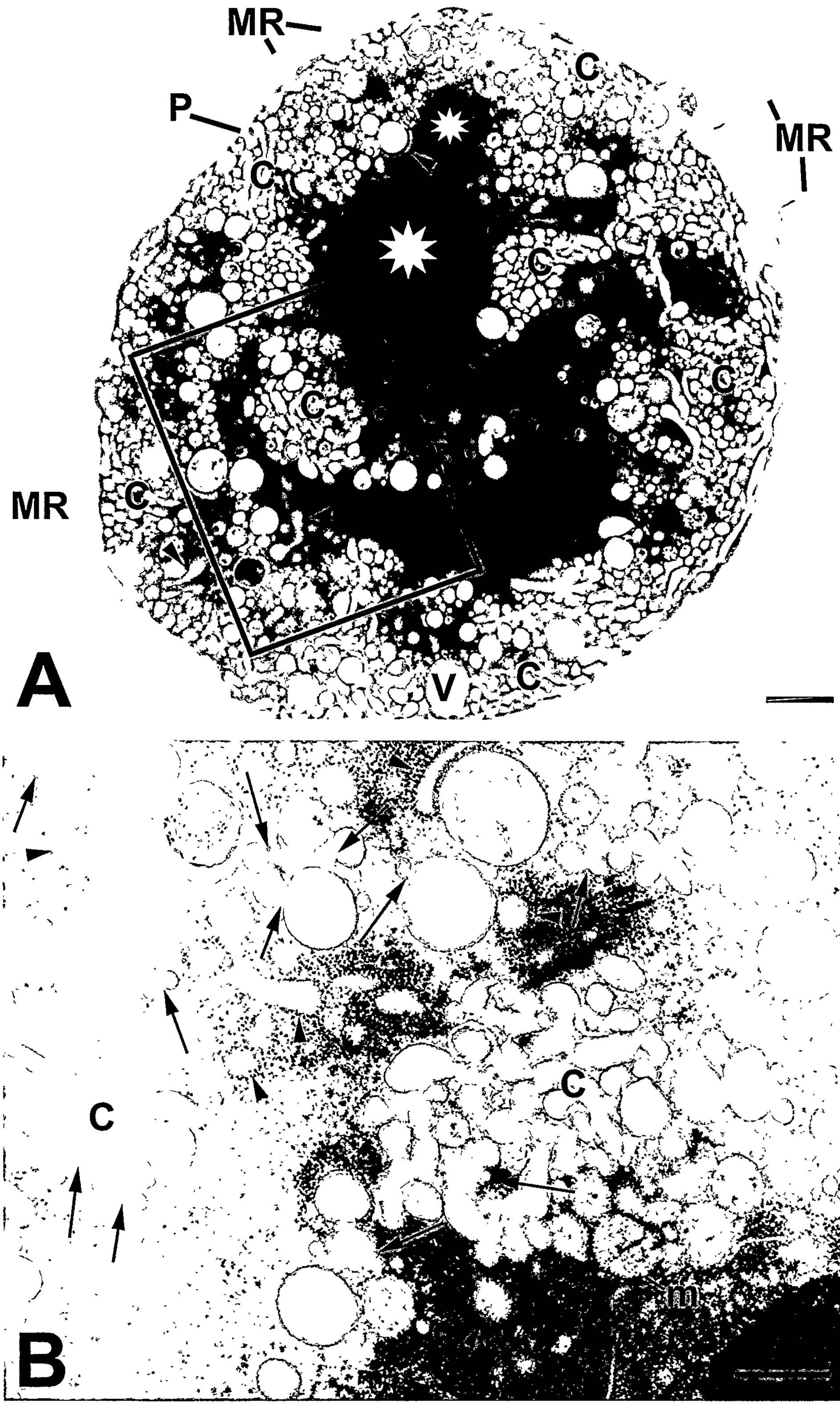
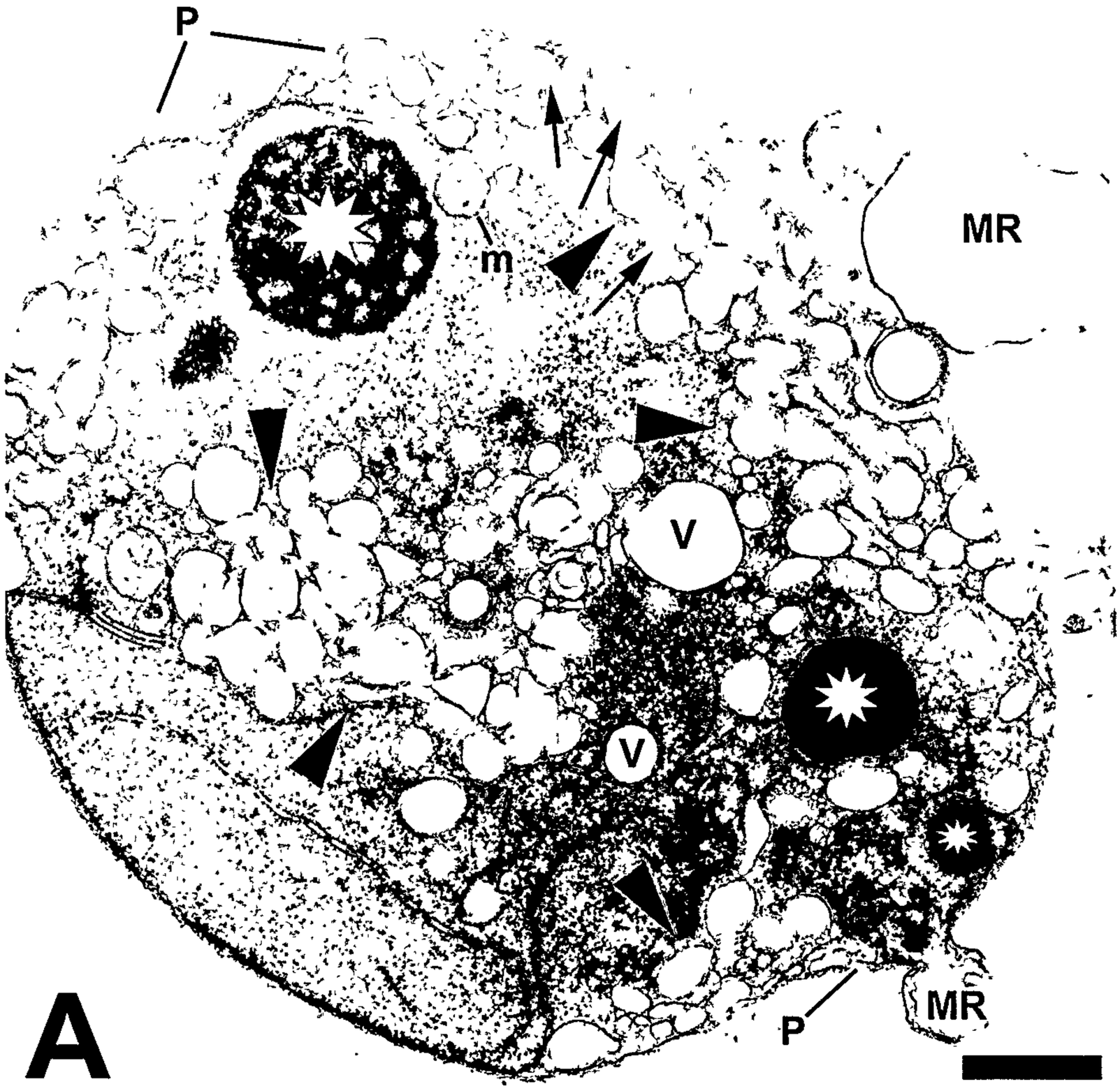
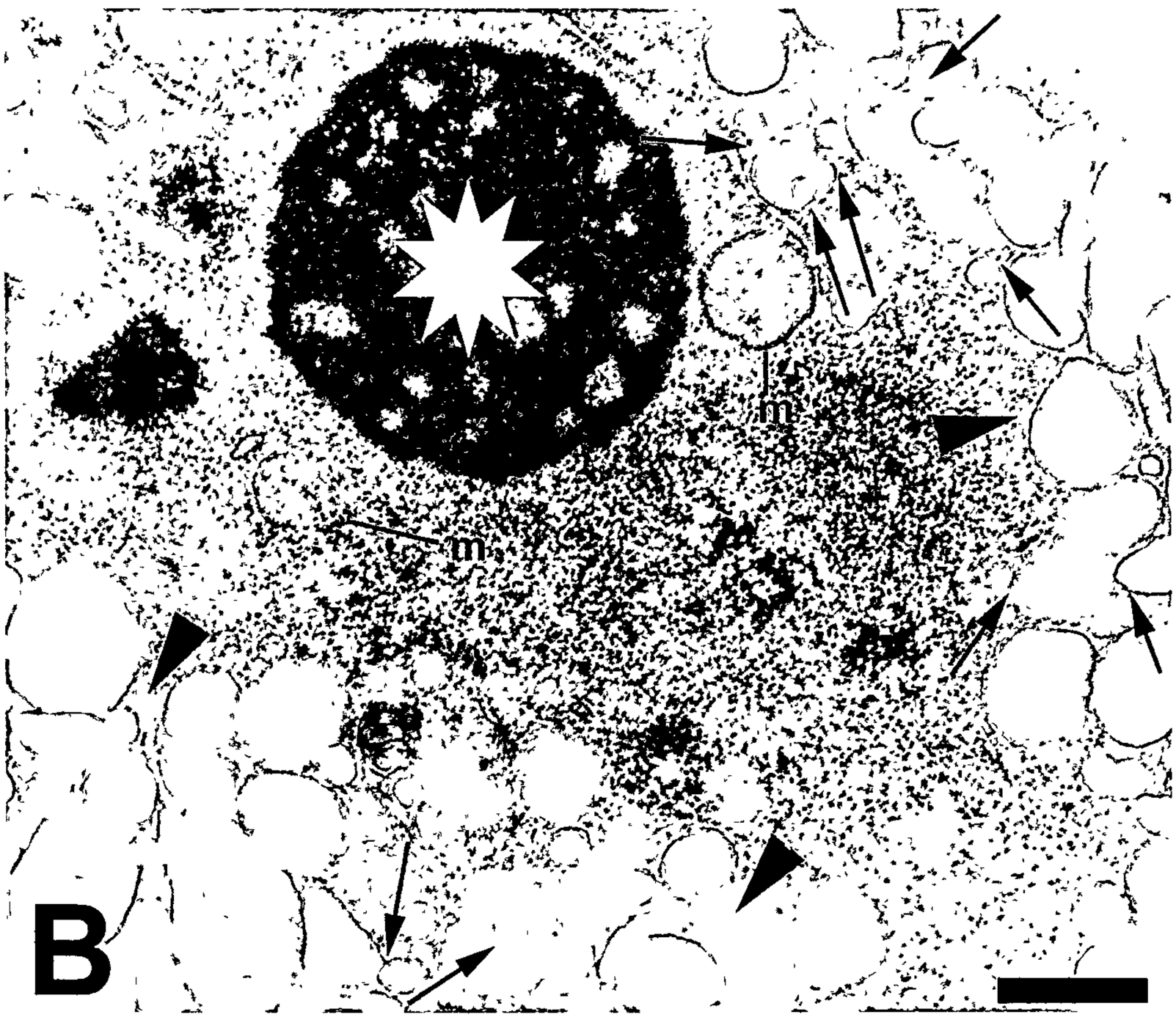

MR
P
C
C
C
C
C
C
MR
MR
C
V
C
A
C
C
m
B

Figure 4.12. Transmission electron micrographs of a M&SDA-EC at low (A) and high (B) magnification after 18 hr of apoptosis, in which canaliculi appear to be dilated. An apoptotic endothelial cell with condensed nuclear material (stars), intact mitochondria (m), surface membrane remnants (MR) and canalicular fragmentation (C) was seen while large vacuoles were present (V). Canalicuili were seen to communicate with the surface via surface pores (P) but also appeared to be dilated as compared with most cells seen (arrow heads). At higher magnification, canaliculi appeared to fuse to each other as well as to small vesicles (arrows). (Bar for A = 2 μm, Bar for B = 0.5 μm)



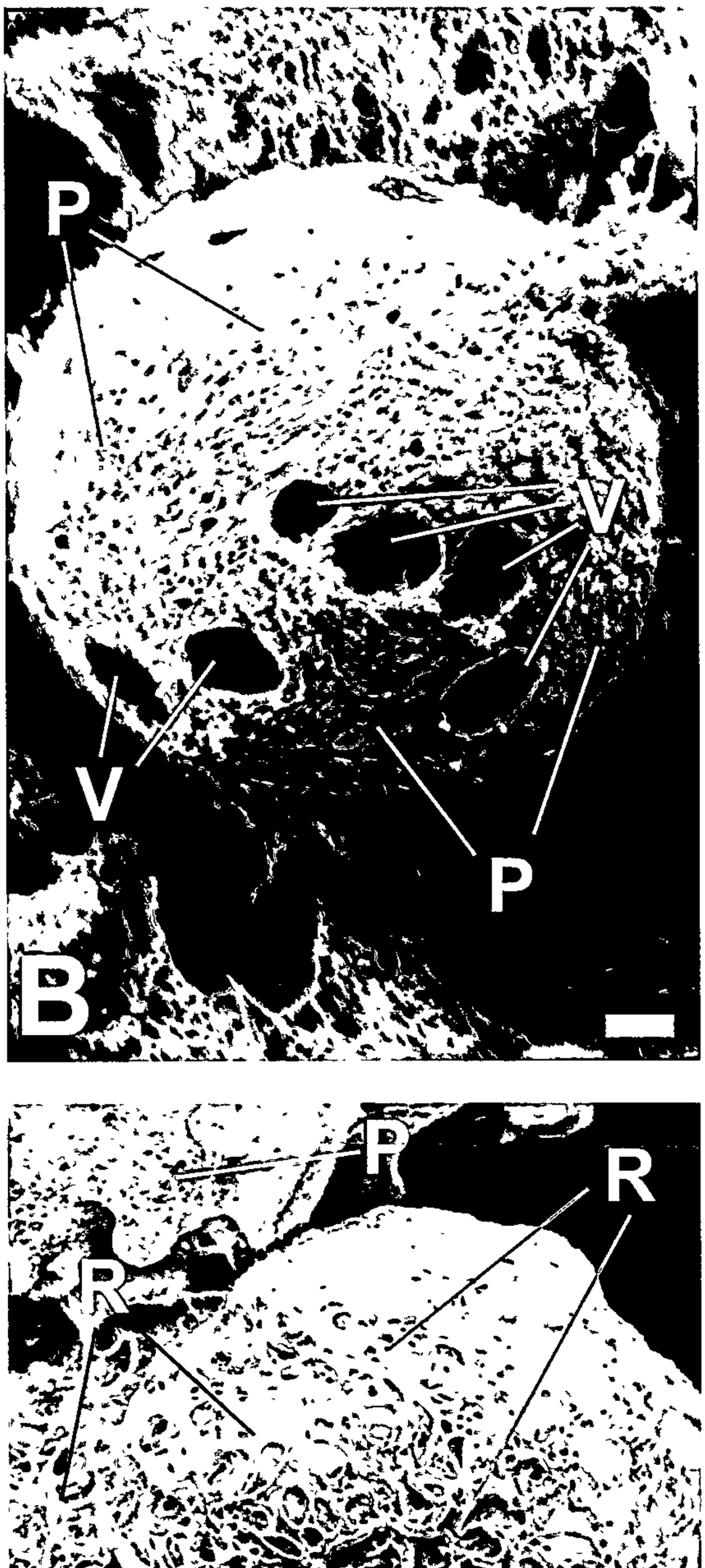
A

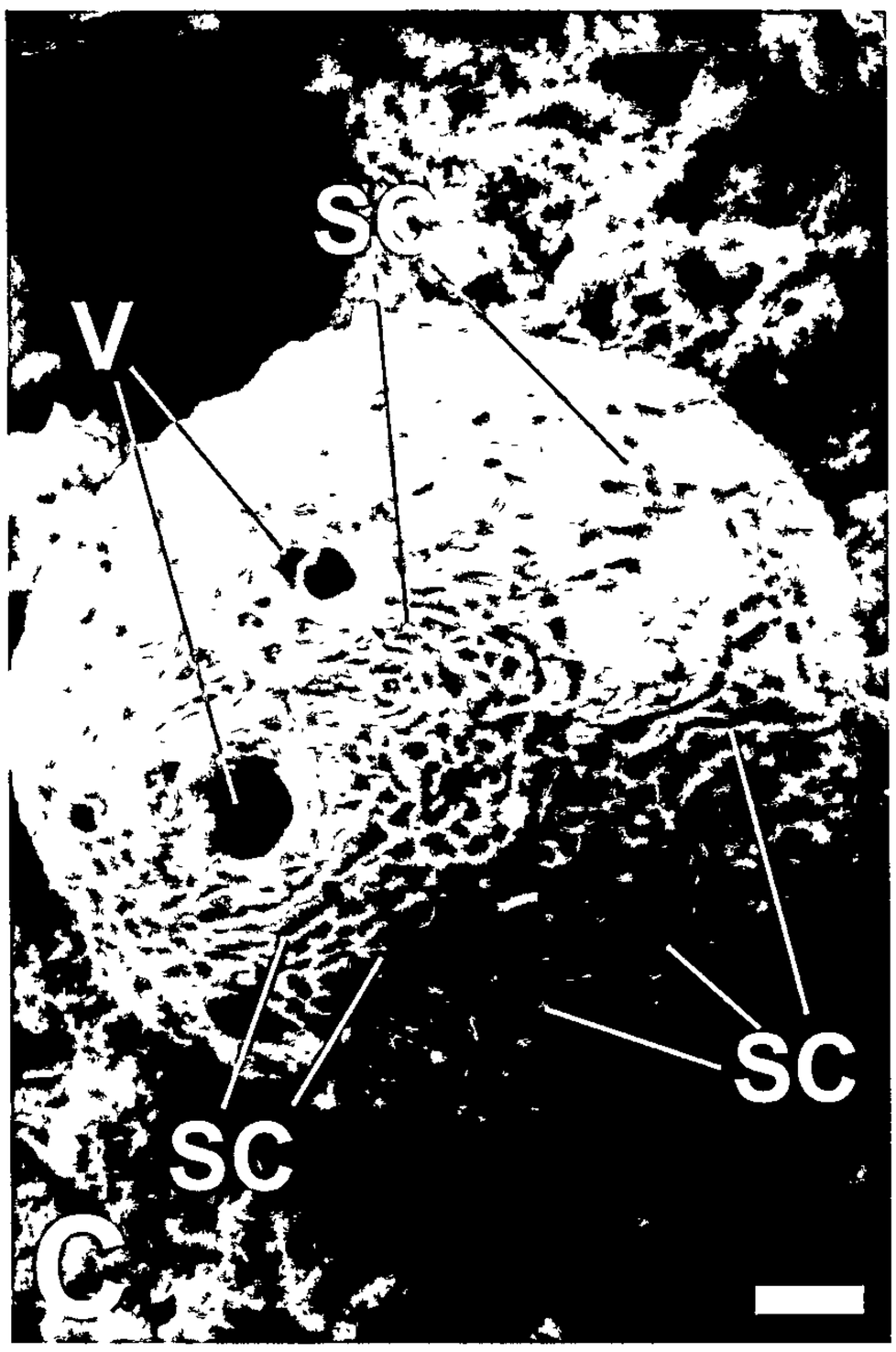


B

Figure 4.13. Scanning electron micrographs of surface features of apoptotic M&SDA-EC. Blebbing (Bl) of cells typical of apoptosis was seen in some HUVEC (*A*). Small pores (P) were seen to open onto the surface of many cells, consistent with the pores communicating with canaliculi seen by TEM. Large crater-like surface voids (V) were displayed on many apoptotic HUVEC (*B* & *C*), which was also consistent with the large vacuoles seen by TEM. The presence of wide trench-like structures on some cells was interpreted as representing the "side on" opening of surface canaliculi (SC) (*C*). In very occasional cells, surface rope like elevations of plasma membrane (R) were seen (*D*), possibly reflecting the impression of underlying cytoskeletal elements. With the exception of the rope like structures (R) which were unexpected, the surface features of apoptotic HUVEC observed were consistent with the appearance of apoptotic endothelium as seen by TEM. (Bar for A = 2 μm, Bar for B = 2 μm, Bar for C = 1 μm, Bar for D = 2 μm)


P
BII
A

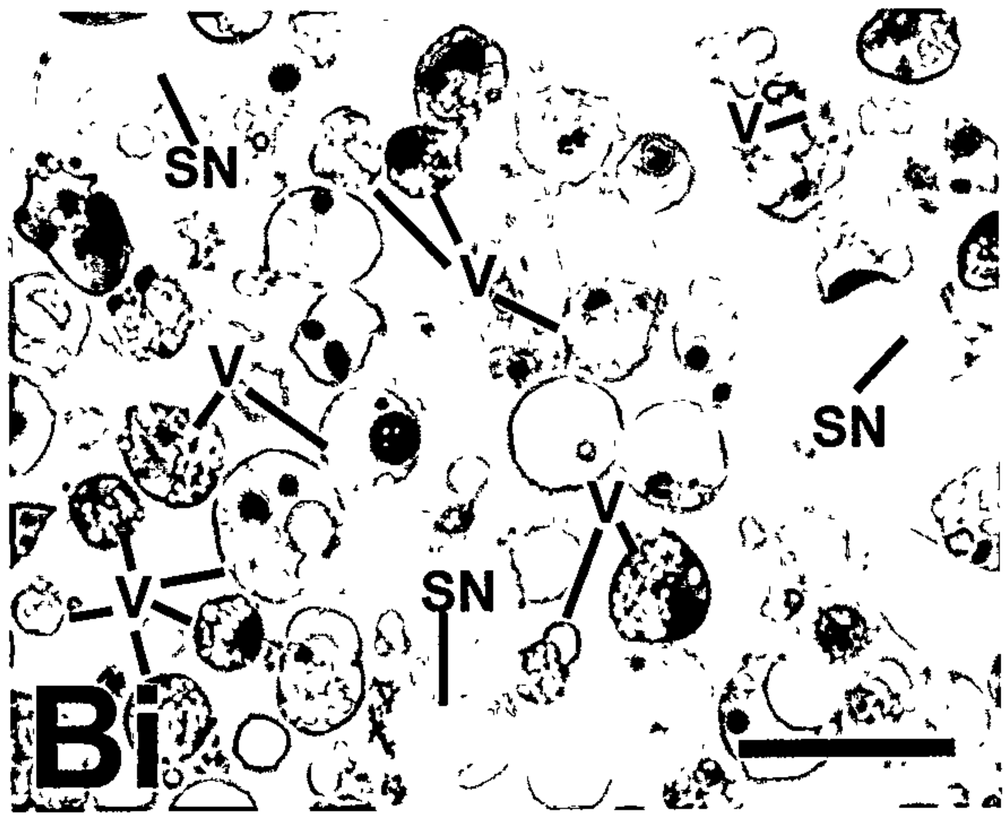

P
V
V
P
B

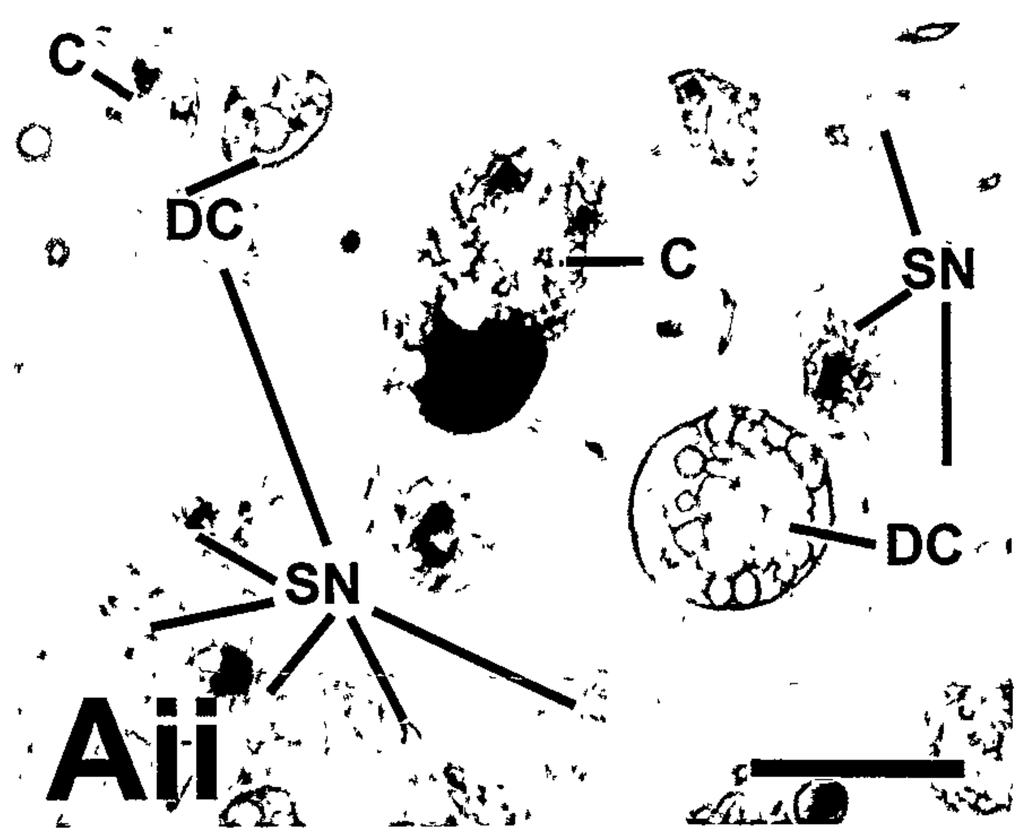

SC
V
SC
SC
C


R
R
R
R
R
P
SC
D
P

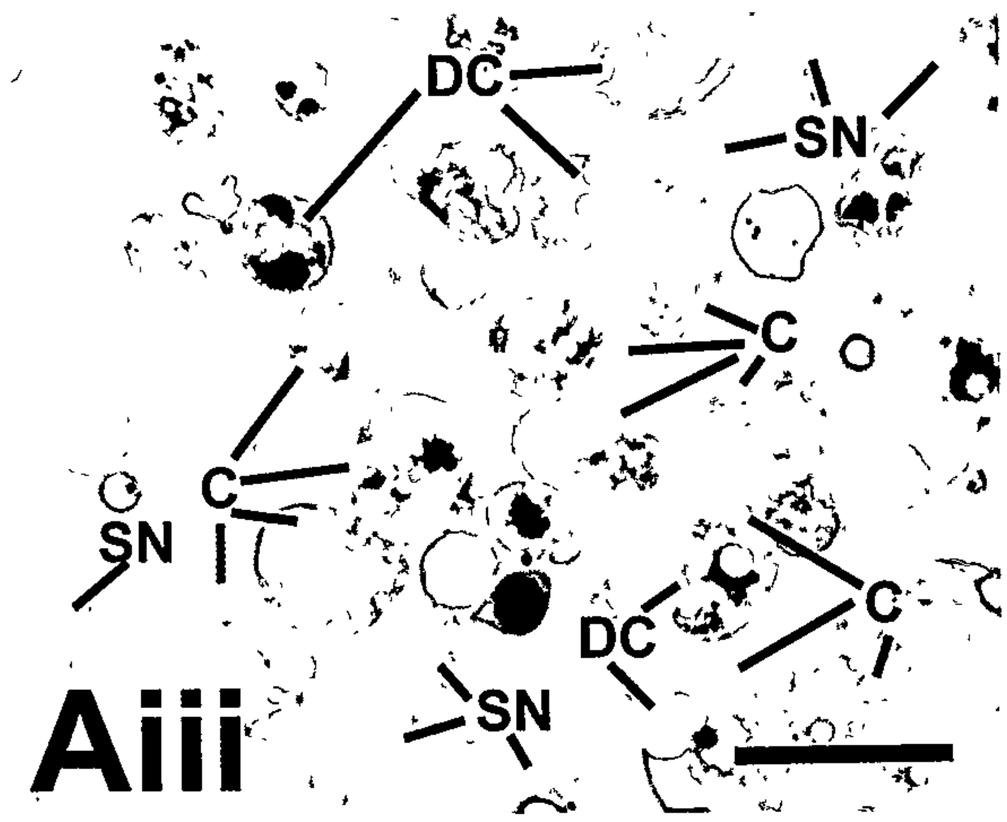
Figure 4.14. Photomicrographs of semi-thin sections of M&SDA-EC (A) and HL-60 cells (B), after 12 hr of apoptosis in static culture conditions (i) and 3 hr of further static culture (ii) or culture in conditions of low mechanical stress (iii). All of the cells had the light microscopic features of apoptosis including fragmentation and the formation of condensed nuclear particles. In addition, canalicular fragmentation was seen as vacuole-like structures in HUVEC while the vacuoles of HL-60 cells (V) were also discerned. Canaliculi appeared more dilated at later time points (DC) (*Aii*, and *Aiii*). Secondary necrosis (SN) was also apparent, as irregular poorly defined cellular debris. Cells appeared generally smaller after 15 hr of apoptosis as compared with 12 hr of apoptosis, consistent with earlier SEM observations. Also, there was the impression that M&SDA-EC were more irregular in shape in the starting population (*Ai*) and static advanced apoptotic population (*Aii*) as compared with cells which had been exposed to low levels of mechanical stress (*Aiii*). These changes were not readily seen by visual inspection of the photomicrographs of HL-60 cells (*B*). (Bars = 20 μm)


C
C
C
C
C
Ai


SN
V
V
V
SN
SN
Bi


C
DC
C
SN
DC
SN
Aii


SN
V
V
V
SN
Bii


DC
SN
C
C
SN
DC
C
SN
Aiii

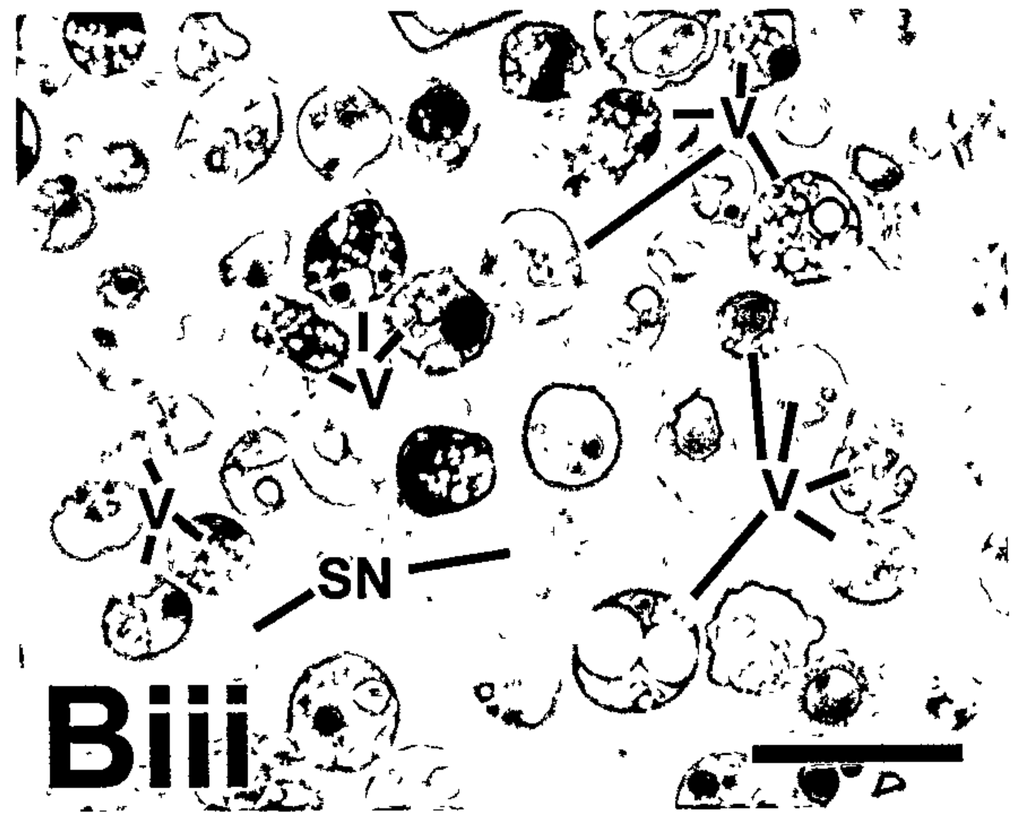
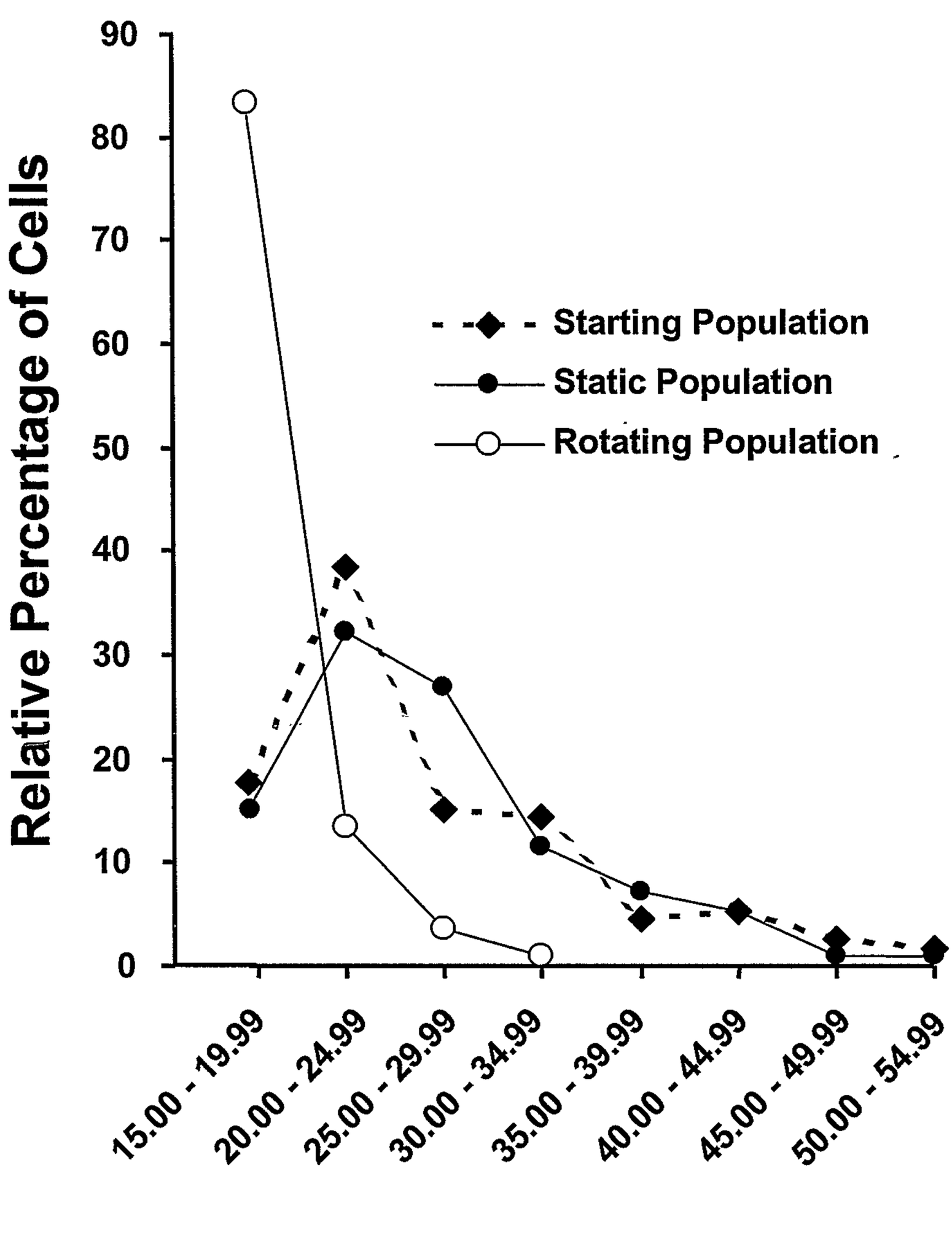

V
V
V
V
SN
Biii

Figure 4.15. The distribution of M&SDA-EC according to circularity as measured from semi-thin sections of M&SDA-EC after 12 hr of apoptosis in static culture conditions (starting population), and 3 hr of further static culture (static population) or culture in conditions of low mechanical stress (rotating population).

The measure of circularity used in this study is such that the lower the number, the more circular the structure measured is. From this, it is seen that there was no significant difference in circularity between starting populations and populations cultured for a further 3 hr in static culture conditions. However, there was a significant increase in the circularity of populations exposed to low levels of mechanical stress by rotation ($p < 0.01$). Similar results were obtained in a further three experiments with cells from separate donors.



Circularity

Figure 4.16. The distribution of HL60 cells according to circularity as measured from semi-thin sections of HL-60 cells, after 12 hr of apoptosis in static culture conditions (starting population) and 3 hr of further static culture (static population) or culture in conditions of low mechanical stress (rotating population). HL60 cells were already highly circular at the beginning of experiments while no further significant increase in circularity was seen with further culture in either static or rotating conditions. Similar results were obtained in a further three experiments.

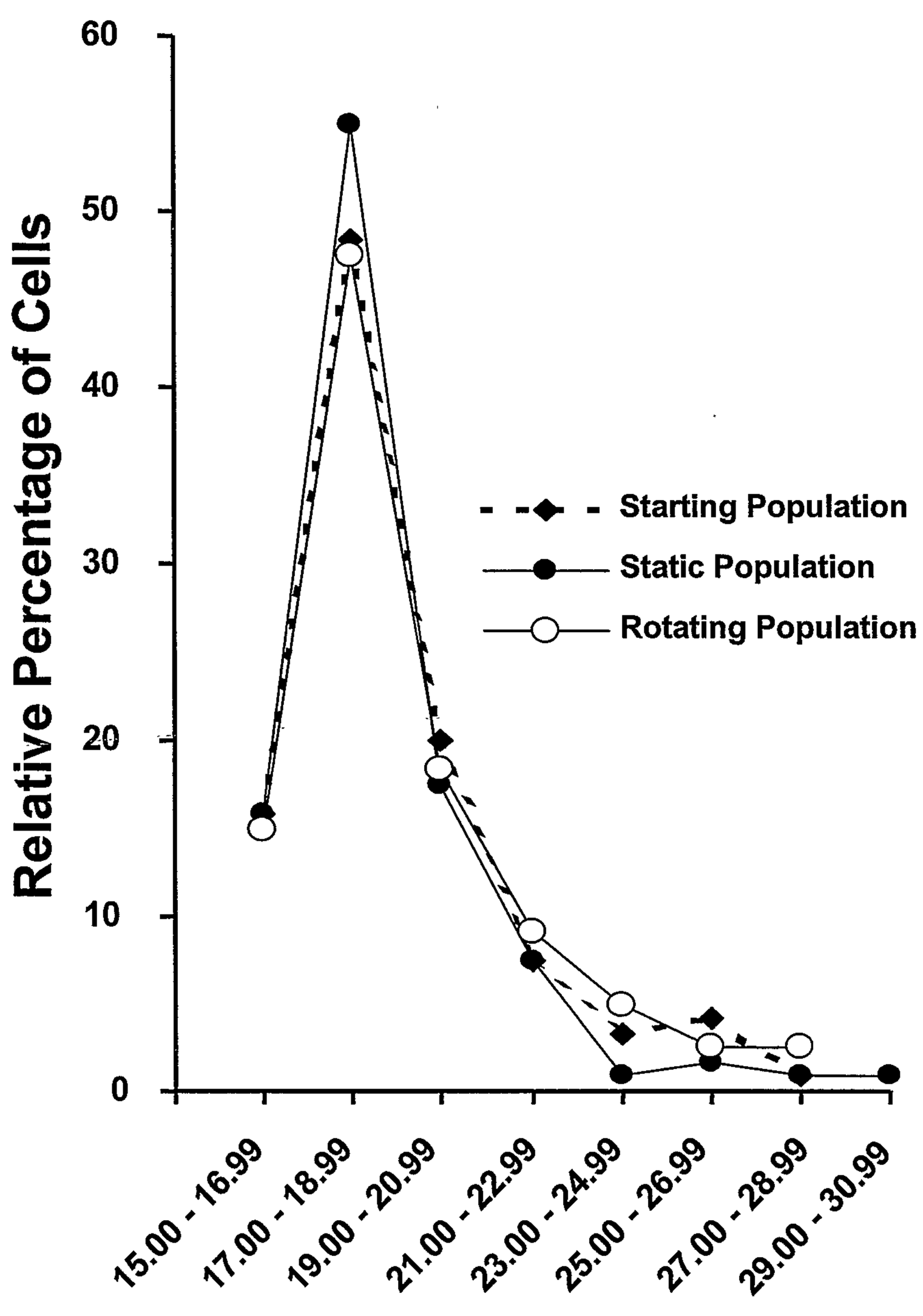
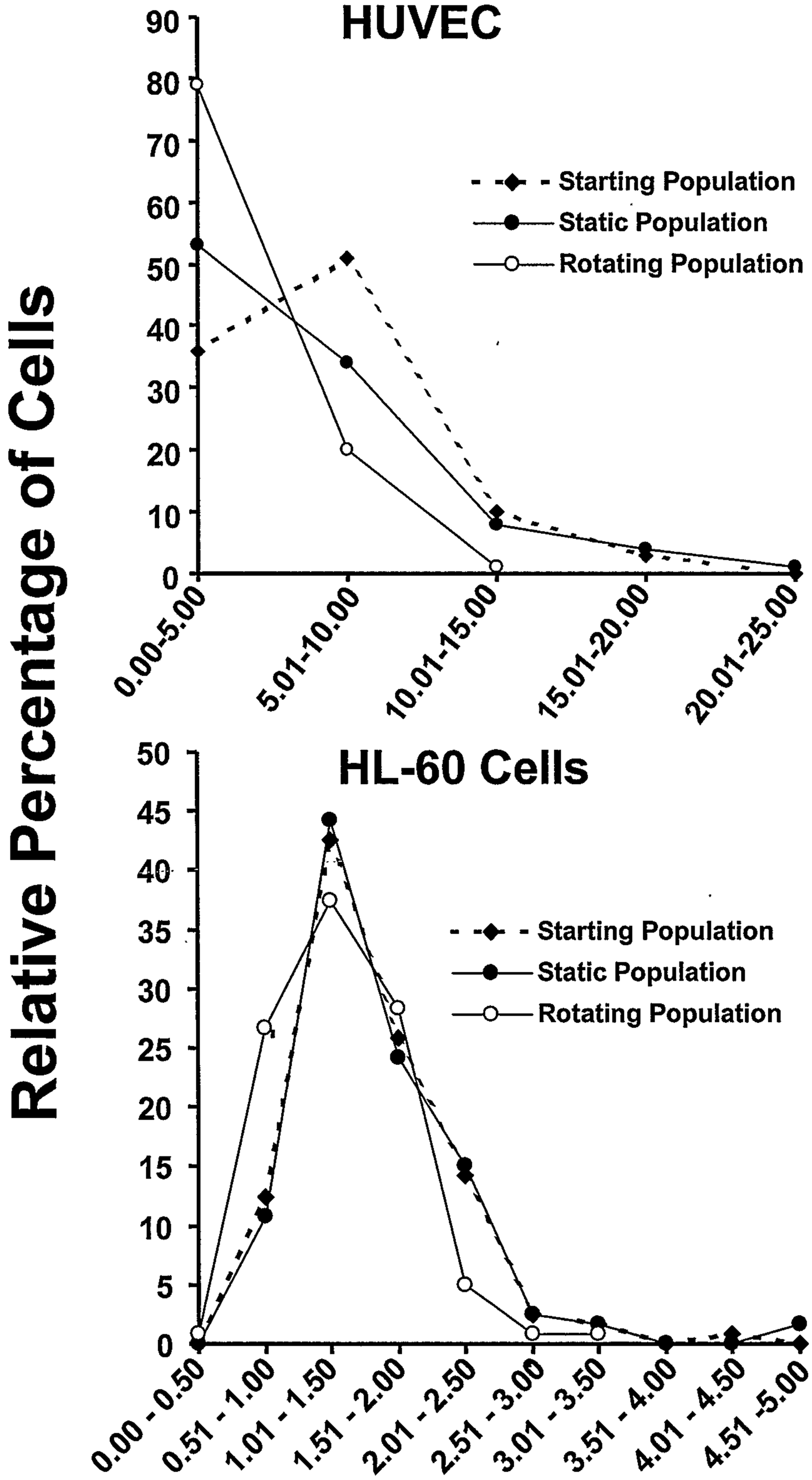

Relative Percentage of Cells
60
50
40
30
20
10
0
Starting Population
Static Population
Rotating Population
15.00 - 16.99
17.00 - 18.99
19.00 - 20.99
21.00 - 22.99
23.00 - 24.99
25.00 - 26.99
27.00 - 28.99
29.00 - 30.99
Circularity

Figure 4.17. The distribution of cell size as measured from semi-thin sections of M&SDA-EC and HL-60 cells, after 12 hr of apoptosis in static culture conditions (starting population) and 3 hr of further static culture (static population) or culture in conditions of low mechanical stress (rotating population). Cell size reduced significantly for HUVEC with time ($p < 0.01$), while this was much more pronounced for HUVEC exposed to mechanical stress ($p < 0.01$). A slight but statistically insignificant reduction in cell size was seen in HL-60 cells exposed to conditions of low mechanical stress. These data are broadly consistent with the observations made by SEM and support the idea that HUVEC become smaller during apoptosis and this is exacerbated by low levels of mechanical stress.


HUVEC
90
80
70
60
50
40
30
20
10
0
Starting Population
Static Population
Rotating Population
0.00-5.00
5.01-10.00
10.01-15.00
15.01-20.00
20.01-25.00
HL-60 Cells
50
45
40
35
30
25
20
15
10
5
0
Starting Population
Static Population
Rotating Population
0.00 - 0.50
0.51 - 1.00
1.01 - 1.50
1.51 - 2.00
2.01 - 2.50
2.51 - 3.00
3.01 - 3.50
3.51 - 4.00
4.01 - 4.50
4.51 -5.00
Relative Percentage of Cells
Visible Surface Area (μm2)

4.4 Discussion

Because of the very time intensive nature of experiments described in this chapter, it was not possible to perform experiments with each of the three apoptotic HUVEC populations described in Chapter 2. However, since data obtained in Chapter 2 suggested there was no significant difference between any of these populations, while it was nonetheless desirable to study cell size changes in apoptotic cells deprived of both matrix and serum, it was decided to perform the experiments described in this chapter using M&SDA-EC.

To obtain apoptotic populations of HL-60 cells, the cells were exposed to calcium ionophore in serum deprived medium. Serum provides cultured cells with survival and growth factors and its withdrawal is reported to induce apoptosis in HL-60 cells (Barroso *et al.*, 1997; Durrieu *et al.*, 1999). Despite this, in the current study when HL-60 cells were subjected to serum deprivation alone, only few cells displayed the transmission electron microscopic features of apoptosis while no laddering typical of apoptosis was seen by DNA gel electrophoresis. Also, although the calcium ionophore A23187 at a concentration of 10 μM has been used to induce apoptosis in HL-60 cells (Ramachandra and Studzinski, 1995), in the current study, this also failed to induce significant apoptosis while increasing concentrations of this ionophore to 30 μM alone also failed to achieve sufficient levels of apoptosis. It was only when cells were subjected to both high concentrations of the calcium ionophore and serum deprivation that apoptosis of all cells was apparent as determined by TEM and confirmed by internucleosomal DNA fragmentation. By exposing HL-60 cells to

these culture conditions, it was possible to reliably and conveniently obtain large numbers of apoptotic cells for comparison with apoptotic endothelium.

HL-60 cells afforded the further advantage in the current study of containing extensive intracellular membranous structures of a similar dimension to the canaliculi forming in endothelium during apoptosis. These consist of lysosomal structures, which unlike apoptotic endothelial canaliculi, do no communicate with one another or the cell surface. One disadvantage, however, of HL-60 cells, was that these cells were considerably smaller and more rounded in shape than HUVEC, and it is possible that this may have had some effect upon the ability of the cells to achieve further reduction in size or change in shape with apoptosis and mechanical stress. However, significant changes were seen in cell size with time and apoptosis in HL-60 cells, suggesting that while the HL-60 cell model may not comprise an optimal comparison for apoptotic HUVEC, that these cells can at least undergo qualitatively similar size changes and so afford a reasonable comparison to HUVEC.

Although significant deformity of cells was seen in scanning electron micrographs, presumably due to distortion of cells during application to supporting membrane filters, it was nonetheless possible to obtain quantitatively meaningful data using this model. This was in part facilitated by the exclusion of cells which were partially obscured from view in scanning electron micrographs, as well as of cells which were apparently degraded. These may have represented cells undergoing secondary necrosis, or alternatively, may have been cells ruptured during cell processing. Although it could be argued that this selection may have affected the data and conclusions drawn, data obtained using a very different method for assessment of cell

size, examination of semi thin sections was essentially similar. This suggested that despite the crude artifacts observed by scanning electron micrographs, the approach used was reasonable. Importantly, examination of scanning electron micrographs permitted assessment of the whole cell, if only from one direction, while the alternative used in studying semi-thin sections had the disadvantage of providing a single section alone through each cell. This may have accounted for the observation of modest reductions in cell size of HL-60 cells studied by SEM as compared with the absence of any clear change in size of static apoptotic HL-60 cells examined in semi-thin sections.

An alternative methodology not used in this study is FACS analysis with assessment of forward-scatter and side-scatter as measures of cell size and internal complexity respectively (Thompson *et al.*, 1983; Worthington *et al.*, 1984; Clement *et al.*, 1986; Santiago-Schwarz and Fleit, 1988; Mukwedeya *et al.*, 1996). This approach was not used in the current study, although preliminary experiments were performed with FACS analysis and are described in the Appendix.

Rotation of cultured cells in suspension was used to provide low levels of mechanical stress. Because this resulted in turbulent mixing of the culture medium, it was not possible to meaningfully quantitate or model the presumably shearing stresses experienced by cells suspended in these culture media. One consequence is that it is not possible to compare the stresses exerted upon cells in this model system with those experienced by cells *in vivo* when circulating through micro and macro-vessels. Also, it was not possible to avoid some degree of mechanical stress in starting and static populations of cells, as any handling of cell suspensions inevitably results in

some essentially unquantifiable degree of mechanical stress. Nonetheless, it was possible to achieve highly reproducible and quantifiable effects of mechanical stress using this simple experimental system so that the method used seems a reasonable approach to the problem studied.

In the current study, reduction in the size of M&SDA-EC with time appeared significantly greater as compared with that experienced by HL-60 cells and this was more pronounced when the cells were exposed to low levels of mechanical stress. This supports the idea that endothelial cells are capable of very rapid reduction in cell size during apoptosis and also indicates that this is facilitated by mechanical stress. Although it is conceded that HL-60 cells may be uncommonly slow to reduce size or fragment with mechanical stress, and that further experiments with other apoptotic cell populations may be valuable, the data are nonetheless consistent with the idea that endothelium is particularly adapted for rapid fragmentation during apoptosis.

The surface morphology of apoptotic endothelial cells has not been previously described and it was surprising to observe rope-like structures snaking across the surface of some apoptotic endothelial cells. Importantly, no such structures were ever observed in HL-60 cells, while the surface pores, linear channels and complex open fragmented surfaces consistent with canalicular fragmentation were often seen amongst apoptotic HUVEC. The identity of the rope-like structures seen by SEM is undefined, but it seems likely that they represent surface ridges formed by cytoskeletal elements during progressive condensation and shrinkage in apoptosis. The absence of these structures in HL-60 cells however, does not support this, as these cells also underwent reduction in size, although to a lesser extent as compared

with endothelium. Also, these structures are not described in other cells during apoptosis where cellular condensation occurs (Maruyama *et al.*, 2001; Romih *et al.*, 2001; Scott *et al.*, 2001).

One feature of canalicular fragmentation is that the canaliculi open to the cell surface and this may help to explain the presence of the surface rope like structures. Since fluids are not compressible, any fluid in membranous structures not open to the cell surface can not escape from the cell. Because of this, cells are essentially non-compressible and have a limited ability to distort when exposed to compressive stresses. This is the case with all cells other than endothelium during apoptosis, with endothelial cells being an exception because the fluid in canaliculi can escape the interior of the compressed or mechanically stressed cell by simply flowing along canaliculi and out of the surface pore. This would seem to provide apoptotic endothelium with a very much greater capacity to change shape in response to stress than other cellular particles, as canaliculi could be emptied or filled according to the forces exerted upon the cell. It is possible that cells fortuitously experiencing particularly rapid compression through emptying of canaliculi have prominent surface markings from cytoskeletal elements, accounting for the rope-like structures seen.

Further ultrastructural features of endothelial apoptosis not previously reported are the apparent fusion of canaliculi with small vesicles as well as with each other. A further feature not previously noted is the occasional emptying of canaliculi into larger irregular and regular vacuole-like structures. It is not clear if these irregular vacuoles evolve into the large spherical vacuoles, also first noted in this study. Nonetheless, it is interesting that large spherical vacuoles appear to open to the cell surface,

particularly at later time points and that this seems to be reflected by the presence of crater-like voids in scanning electron micrographs of apoptotic endothelium. These large vacuoles became most prominent at late time points during endothelial apoptosis, while in some cells, canaliculi appeared uncommonly dilated at these later time points. These data suggest that canaliculi may form not only through invagination of plasma membranes as previously suggested (Zoellner *et al.*, 1996a), but also may be extended by fusion of smaller intracellular vesicular structures and that the eventual fate of canaliculi may be to dilate and or fuse to form large vacuoles which rupture at the cell surface.

The plasticity of apoptotic endothelial cells was evident in the increased circularity of apoptotic endothelium exposed to low levels of mechanical stress, and the close packing of canaliculi within these rounded particles is consistent with the idea that canalicular fragmentation facilitates this change in shape. Like boulders being rounded by a glacier, it is not surprising that the cells became essentially spherical with mechanical stress. Significantly, this plasticity supports the suggestion that apoptotic endothelial cells are readily deformed by mechanical stress and is consistent with the idea that canalicular fragmentation may facilitate this. Unfortunately, the high degree of circularity of HL-60 cells before apoptosis makes meaningful comparison of this quality during apoptosis difficult, and further experimentation with more irregular cells during apoptosis is required to compare the deformation of apoptotic endothelium with that of other apoptotic cells. It is interesting to note that rounded particles would be expected to pass more readily through small vessels, so that the greater circularity of mechanically stressed apoptotic endothelium may reflect a previously unsupposed physiological function.

Although the changes in cell size and shape seem related to canalicular fragmentation, no causal relationship between canalicular fragmentation and these structural changes can be claimed. This awaits further experiments in which canalicular fragmentation is inhibited and the effect of this upon cell size and shape determined.

In conclusion, in addition to identifying previously unknown ultrastructural features of endothelial apoptosis, the data presented are consistent with the idea that endothelial cells are adapted to become mechanically smaller and fragment into highly spherical particles. This is in turn consistent with the earlier suggestion that canalicular fragmentation is an adaptation to minimise the micro-embolic potential of apoptotic endothelial cells (Zoellner *et al.*, 1996a), and is also consistent with the anti-thrombotic properties observed in earlier chapters. This is suggested as having biological significance in protecting the host from pulmonary micro-embolism after detachment of endothelial cells by trauma or during vascular remodeling. It is accepted, however, that a direct causal relationship between canalicular fragmentation and the shape and size changes studied has not been established by the current data.

CHAPTER FIVE

General Disscussion

5.1. Principal Findings of This Thesis

The fundamental issue addressed in this thesis is the micro-thrombotic and micro-embolic potential of endothelium during apoptosis. As described in 1.2.b, endothelial apoptosis occurs in tissues during vascular remodelling (Walker *et al.*, 1989; Meeson *et al.*, 1996; Tatarczuch *et al.*, 1997), and cells released during this process into the circulation may act as sites for micro-thrombosis. A separate mechanism through which apoptotic endothelium may enter the circulation is traumatic detachment of cells (George *et al.*, 1991; Lai *et al.*, 1996), followed by apoptosis due to matrix deprivation (Meredith *et al.*, 1993; Zoellner *et al.*, 1996a). Circulating endothelial cells have also been observed in thrombotic thrombocytopenic purpura and sickle cell anemia (Lefevre *et al.*, 1993; Solovey *et al.*, 1997), and it seems likely that these cells become apoptotic in response to matrix deprivation.

Because of the central role played by platelets in thrombosis, much of this thesis has been focused upon the effect of apoptotic endothelial cells upon platelet activation. It was found that apoptotic endothelial cells retain the native ability of non-apoptotic endothelial cells to inhibit platelet aggregation. Further investigation revealed that this was reduced by using inhibitors which block the synthesis of PGI_2 and NO. Also, the stable end products of PGI_2 and NO degradation were detected in the culture supernatants of both apoptotic and non-apoptotic endothelium. From this, it was concluded that PGI_2 and NO were important in mediating the anti-aggregatory activity studied.

Although difficulties in obtaining sufficient numbers of SDDA-EC prevented direct detection of PGI_2 and NO from this apoptotic endothelial population, metabolic

inhibitors of PGI_2 and NO synthesis reduced the anti-aggregatory activity of these cells and no clear difference was apparent between apoptotic endothelial cells derived by either serum or matrix deprivation. Also, there did not appear to be any synergy between these two stimuli for apoptosis with regard to their effect upon platelet aggregation. This suggests that interactions between platelets and apoptotic endothelial cells are similar regardless of the biological setting from which the apoptotic cells are derived.

Although anti-aggregatory activity was observed, SEM revealed that platelets bound to apoptotic endothelial particles via the extensive surface processes formed by activated platelets. This occurred even in the absence of platelet agonists and was consistent with findings recently reported by others (Bombeli *et al.*, 1999), although it appeared that once bound, the anti-aggregatory activity of endothelial cells inhibited the formation of larger aggregates. The maintained anti-aggregatory activity may be important not only for apoptotic cells shed into the circulation, but also perhaps to protect from thrombosis in the vessel wall at early stages of endothelial apoptosis when cells are still attached.

This work seems to support the idea that apoptotic endothelial cells are adapted to minimize the formation of platelet thrombi. Although consistent with the maintained fibrinolytic profile of apoptotic endothelium (Zoellner *et al.*, 1998), this seems inconsistent with the pro-coagulant activity observed by others (Bombeli *et al.*, 1997). It may not, however, be necessary for all anti-thrombotic properties of endothelium to be maintained in order to inhibit microthrombus formation around apoptotic endothelial particles. As discussed in 1.1.b.iii, platelet activation is an important and

early event for effective blood clotting. Even though some thrombin and fibrin may be formed in the absence of individual elements of the clotting system, biologically effective clotting requires platelets and components of the coagulation system. Low level activation of platelets after binding to apoptotic endothelium was recently reported (Bombeli *et al.*, 1999) and this was supported by SEM observations in the current thesis. It seems possible that the anti-aggregatory activity from apoptotic endothelium suppresses the activation of bound platelets sufficiently well to greatly limit clot propagation around apoptotic endothelial particles, and that any clot which is formed is removed by u-PA released by the apoptotic particles (Zoellner *et al.*, 1998). Nonetheless, since clotting is the end result of a balance between pro and anti-thrombotic forces, there may be occasions when these anti-thrombotic mechanisms fail with the result that microthrombi are able to form around apoptotic endothelial particles. With regard to this, it is interesting to note the increased tissue factor expressed by apoptotic endothelium only after LPS stimulation (Bombeli *et al.*, 1997) while septic shock is associated with microthrombi in the lungs and elsewhere (Cotran *et al.*, 1999).

The main biological impact of any microthrombi forming about apoptotic endothelial particles is suggested as being micro-embolism with possible micro-infarction of vessels. Also, because endothelial cells are larger than capillaries, detached apoptotic endothelial cells could act as micro-emboli. Earlier work suggested a possible role for canalicular fragmentation in minimizing the physical size of apoptotic endothelial particles (Zoellner *et al.*, 1996a). Since micro-embolism is a fundamentally mechanical event dependent upon cell size and also perhaps cell shape after mechanical impaction in vessels, it was considered important to know something of

the size and shape of apoptotic endothelial cells exposed to mechanical stress. Apoptotic endothelial cells were found to reduce in size rapidly as compared with a control population of HL-60 cells during apoptosis. Mechanical stress appeared to facilitate this reduction in size in apoptotic HUVEC, although there was no clear effect of mechanical stress upon the size of apoptotic HL-60 cells. It was also found that low levels of mechanical stress greatly increased the circularity of apoptotic endothelial cells, a structural feature seemingly well suited for passage of apoptotic endothelial particles through small vessels. It was felt possible that the increased circularity may have reflected increased cellular flexibility, possibly facilitated by the passage of fluid in and out of canaliculi during cellular deformation. Taken together, these observations were consistent with the idea that endothelial cells are adapted to minimize their micro-embolic potential during apoptosis. However, despite the interest in canalicular fragmentation, a causal relationship between changes in cell size and shape and canaliculi was not established, although such a relationship may yet be found in further experiments.

In performing this work, previously unknown ultrastructural features of endothelial apoptosis were discovered. TEM revealed the dilation of canaliculi over time as well as the apparent fusion of canaliculi to form large vacuolar structures. These often appeared to move to the surface of apoptotic cells and were reflected by large surface voids in SEM images. TEM also revealed the fusion of small vesicles with canaliculi, suggesting that canaliculi form not only by invagination of the plasma membrane, as suggested by the binding of UEA-1 coated gold particles in earlier studies (Zoellner *et al.*, 1996a), but also through fusion with small vesicles (4.3.d). SEM also revealed surface pores, lateral surface opening of canaliculi, membrane rests and highly

fragmented surface domains expected from earlier TEM studies of apoptotic endothelium (Zoellner *et al.*, 1996a). An unexpected finding was the presence of rope-like plasma membrane elevations in some apoptotic endothelial cells, and the origin of these remains unclear.

5.2. The Direction of Future Work

Although the work described in this thesis identifies anti platelet aggregatory activity and structural changes in apoptotic endothelium, there are a number of interesting and important questions which have not been addressed. This reflects a combination of limitations in time and resource for experimental work, as well as the decisions made during experimentation as to which particular aspects of work seemed most promising. Additional experiments relating either to: aspects of the current study which could not be addressed due to time or resource limitations, or work which would seem interesting to begin in light of the findings of this thesis, are discussed below.

Although many properties of endothelium appear common to all endothelial cells, there are significant differences in endothelium from different tissues and sites (Gerritsen, 1987; Kumar *et al.*, 1987; Page *et al.*, 1992). Microvascular and macrovascular endothelial cells are accepted as essentially different, so that it is desirable to study isolated cultured microvascular cells where essentially microvascular events are under consideration (Kumar *et al.*, 1987; Bicknell, 1993; Couraud, 1994). Nonetheless, the number of cells required for individual experiments

may sometimes exceed availability from individual microvascular sources. As described in 2.1.b.i. HUVEC are often used for cell culture experiments because umbilical cords are easily obtained and recovery and growth of the cells is technically simple. Importantly for this study, HUVEC could be obtained in very large numbers from individual donors, while it was important to be able to work with sufficient numbers of cells and replicate experiments to be able to make statistically meaningful conclusions. This was clearly a compromise, as on the one hand the processes under investigation were suggested as primarily microvascular in nature, while on the other hand, it was not possible to obtain sufficient numbers of microvascular cells to perform the large platelet aggregatory experiments described. Nevertheless, having established the experimental model using readily accessible HUVEC and established the anti-aggregatory activity of apoptotic HUVEC, it would now be interesting to expand these studies into work with cultured microvascular cells. Similarly, the generality of findings for other species would be interesting to determine in experiments with endothelial cells derived from animal tissues. Although it is expected that essentially similar data would be obtained as with HUVEC, differences may be found which compel reevaluation of the conclusions drawn in this thesis.

Most experiments investigating the effect of indomethacin and L-NAME upon the ability of apoptotic and non-apoptotic endothelium to inhibit platelet aggregation were performed using thrombin as the platelet agonist. Only one such experiment was performed using ADP as the platelet agonist in the presence of indomethacin or L-NAME treated endothelium, while no such experiments were performed using collagen as an agonist. This seemed justified on the assumption that endothelial cells do not respond significantly to ADP or collagen. Since platelet activation is a direct

response of platelets to the agonist rather than endothelial products, the single experiment with ADP was interpreted as confirmatory of the expected result rather than the first of a separate series of experiments. At the time, this appeared a reasonable compromise between the value of the data to be obtained and the resources required to carry out these additional experiments. In retrospect, however, it may be worthwhile confirming the assumptions made were justified by performing further experiments using ADP as an agonist in the presence of indomethacin and L-NAME treated endothelial cells.

Also, although the anti-aggregatory activity of five separate populations of endothelial cells were studied in Chapter 2, it was not possible to reliably detect PGI_2 or NO production by SDDA-EC. It was felt that this was due to the difficulty in obtaining sufficient experimental material for direct detection of these cell products. However, an alternative possibility is that this reflected much lower levels of PGI_2 and NO released by these cells as compared with the other apoptotic populations studied. However, this alternative interpretation seems unlikely in view of the ability of indomethacin and L-NAME to reduce the anti-aggregatory activity of SDDA-EC. Nonetheless, it would be desirable to expand the study by testing for PGI_2 and NO levels in experiments with much larger numbers of SDDA-EC. Similarly, it would seem sensible to also test SDnA-EC for NO and PGI_2 production, although this would only confirm the production of these well known endothelial products by non-apoptotic endothelium.

Both thrombin and ADP are thought to be physiologically important activators of platelet aggregation and for this reason, most of the work in this thesis has focused

upon these two agonists. Apoptotic endothelial cells inhibited platelet aggregation in response to thrombin and ADP and as discussed above, in most cases it seems reasonable to assume that the release of anti-aggregatory substances by endothelium is independent of the platelet agonist used. Nonetheless, it would be interesting to confirm this assumption in further experiments using other platelet agonists including serontonin, TXA_2, calcium and thrombin receptor activating peptide (Section 1.1.b.iii).

The expression of TM, TF, heparin sulphate, TFPI, u-PA, t-PA, PAI-1, PAI-2 and uPA receptor has been studied in apoptotic endothelium, while the increased surface expression of phosphatidyl serine and tenase activity has also been demonstrated (Bombeli *et al.*, 1997; Zoellner *et al.*, 1998; Bombeli *et al.*, 1999). However, no reports seem available investigating levels of VWF or protein S in apoptotic endothelium, and this seems an important omission in view of the role of these endothelial proteins as pro and anti thrombotic factors. Related to this, is that these studies have used HUVEC without detailed examination of microvascular endothelium so that further work with microvascular cells seems important.

A small number of preliminary experiments were performed in this thesis, investigating levels of VWF in apoptotic and non-apoptotic endothelium and these are described in the Appendix (A.III). VWF antigen was detected in apoptotic endothelium in several experiments, although further work is required to verify these preliminary results and determine the biological significance of these findings.

In as much as aggregometry experiments were highly consumptive of both time and cells, experiments investigating the size and shape of apoptotic endothelium were also very time consuming, with large numbers of cells being individually analysed for size and shape. Because of this, in order to perform sufficient work for statistically meaningful interpretation it was necessary to select a single population of apoptotic endothelial cells for study. It was decided to use M&SDA-EC for this, as these cells were felt to reflect endothelial apoptosis in response to both serum and matrix deprivation, while no clear differences between MDA-EC, SDDA-EC and M&SDA-EC had been noted in earlier work. Although perhaps a reasonable compromise, it would now be interesting to repeat this work with MDA-EC and SDDA-E to confirm that the assumption of the absence of a meaningful difference between these apoptotic endothelial populations is justified.

Apoptotic HL-60 cells were used in the current study for structural comparison with apoptotic HUVEC. These cells were selected because of the presence of multiple membrane bound structures of a similar size to the canaliculi seen in apoptotic endothelium, as well as the comparative ease with which apoptosis could be induced. One disadvantage, however, was that these cells were quite small as compared with HUVEC while HL-60 cells were also very round in shape before the onset of apoptosis. It is possible that the small size of HL-60 cells made them much less liable to apoptotic fragmentation than larger cells while the initial circularity of HL-60 cells made observation of any further increase in circularity impossible to detect. For these reasons, it would be desirable to now repeat these experiments using much larger and more irregular apoptotic cells than HL-60 cells. It would probably be most convenient to work with immortalized cell lines while it would seem wise to study

several different cell types to ensure that any changes seen are applicable to large cells in general. Also, it may be interesting to study cells, which unlike HL-60 cells, are matrix dependent.

Although this thesis does provide some new information about structural events during canalicular fragmentation, the mechanism responsible for canaliculi formation remains unknown. Earlier work demonstrated the binding of UEA-1 coated gold particles to the membrane surface of canaliculi in pre-fixed apoptotic endothelial cells. This together with the obvious connection of canaliculi with the cell surface via surface pores was interpreted as evidence that canaliculi form through invagination of the apoptotic plasma membrane (Zoellner *et al.*, 1996a). In the current study, canaliculi appear to fuse with small membranous vesicles, suggestive of further growth by membrane fusion. To further investigate this process, it is suggested that TEM of apoptotic endothelium exposed to a range of metabolic inhibitors blocking cytoskeletal or membrane biology should be performed. Examples of agents which might be appropriate for this are: cytochalasin B and D, latrunculin A and B, phallodin, vinblastine, and azide which interfere with actin polymerization, actin stability, tubulin polymerization and membrane fusion. Identification of agents specifically inhibiting canaliculi formation would also permit further investigation of the specific effect of canalicular fragmentation upon apoptotic endothelial size and shape in experiments similar to those described in Chapter 4, but using non-canalicular apoptotic endothelial cells as the control population for comparison with apoptotic endothelial cells able to form canaliculi. This would finally establish whether or not canalicular fragmentation contributes to reducing cell size and increasing circularity during endothelial apoptosis.

All of the work in this thesis has been performed with isolated cultured cells so that there is a need to extend these studies into suitable animal models. One of the assumptions made in investigation of the anti-thrombotic and anti-embolic properties of apoptotic endothelium is that non-apoptotic endothelial cells are difficult to negotiate through the microcirculation. Although the large size of endothelium as compared with small vessels seems to make this assumption self-evident, it is accepted that there have been no experiments verifying that this is the case. One simple approach to testing this would be to remove the mesenteric tissues from rats and determine the effect of HUVEC infusions upon flow rate through the mesenteric circulation under constant pressure. Apoptotic cells could also be infused and the effect of these as compared with non-apoptotic cells determined. Preferably, experiments would be performed using metabolic inhibitors to prepare apoptotic endothelium capable and incapable of canalicular fragmentation. The further question of the extent to which trauma releases endothelium from the microcirculation could be investigated studying heparinized anaesthetised rats. The left and right iliac veins would be canulated separately while fluid would be distributed equivalently to both legs via the canulated descending aorta. One leg could be subjected to a controlled level of trauma and the flow rate as well as the number of shed cells could be determined for each leg receiving an identical amount of perfusate from the canulated aorta. These experiments would confirm both the effect of trauma upon endothelial shedding as well as the micro-embolic potential of such cells.

Since the lungs receive the circulating blood from the entire body and are the most likely site of biologically significant micro-embolism from detached endothelium, these experiments could then form the basis for further work with nude mice. In these

experiments, these immune deficient animals would be infused with known quantities of apoptotic and non-apoptotic endothelial cells. Any effect upon pulmonary efficiency would be detected by reduced circulating oxygen saturation as determined using a pulse oxy-metre clipped to the hind leg. Recovery of animals over time would be monitored and the lungs and other tissues investigated by paraffin histology and TEM at increasing times to characterize changes in both the endothelium and the pulmonary circulation. Again, it would be desirable if populations of endothelium capable and incapable of canalicular fragmentation were used. This would best be achieved using cells pre-treated with metabolic inhibitors identified as outlined above, or perhaps more crudely using fixed cells.

By pursuing these additional experiments, it should be possible to address the most pressing questions arising from work in this thesis.

APPENDIX

A.I. Introduction

In order to maintain continuity and focus upon the more important aspects of the thesis work which was either preliminary or unsuccessful was not described in the earlier chapters. This appendix describes these previously excluded experiments in the form of brief notes which can be read in conjunction with the relevant thesis chapters, or alternatively may be read in isolation.

A.II. Establishment of Experimental Procedures for Investigating the Effect of Apoptotic Endothelium Upon Platelet Activity

As mentioned earlier (2.1.a), this model was established with the intention of studying the effect of apoptotic endothelial cells upon platelet aggregation. In early aggregometry experiments SDnA-EC and SDDA-EC at a concentration of 10^6 cells / ml were used applying collagen as the platelet agonist. Neither apoptotic nor non-apoptotic endothelial cells displayed a clear effect upon platelet aggregation, and this was interpreted as reflecting inclusion of insufficient numbers of endothelial cells. This interpretation was later supported in further experiments where the cell concentration was increased to 10^7 cells / ml, where clear effects upon platelet aggregation were seen and are described in Chapters 2 and 3. Experiments then began using M&SDA-EC and UnA-EC as further controls for apoptotic and non-apoptotic endothelial cells respectively. Having established the experimental model using collagen as the agonist, the study was extended to include thrombin and ADP as physiologically important agonists. A series of experiments was necessary to optimize concentrations of both of these platelet agonists. As seen in Chapters 2 and 3, the simple methodology developed was effective for studying anti-aggregatory interactions between platelets and apoptotic endothelial cells.

A.III. Assessment of von Willebrand Factor Levels in Apoptotic Endothelial Cells

A.III.1. Introduction

As outlined in 1.1.b.iii, VWF is involved in the adhesion of platelets to the damaged vessel wall (Ruggeri, 1997; Sadler, 1998), which is the first step in thrombus formation. Synthesis of VWF is confined to endothelial cells and megakaryocytes (de Leeuw *et al.*, 2001). It was felt that it would be interesting to know if any changes occurred in the level of VWF in endothelium during apoptosis. However, there do not appear to be any reports studying the expression of VWF by endothelium during apoptosis. Preliminary experiments were performed investigating VWF antigen levels in apoptotic and non-apoptotic endothelial populations.

A.III.2. Materials and Methods

A.III.2.a. Materials

Rabbit anti-human VWF and peroxidase labelled rabbit anti-human VWF were purchased from Dako (Sydney, Australia). Collagen was obtained from Nycomed Horm (Germany) while ICN Biomedical (Australia) supplied 96-well EIA microtitre plates. Commercial reference plasma was used as a standard for VWF (Helena Laboratories, USA).

A.III.2.b. Preparation of Endothelial Cells

MDA-EC, SDA-EC, M&SDA-EC, SDnA-EC and UnA-EC were prepared as indicated in 2.2.b and 2.2.c. Endothelial cell populations were adjusted to 10^7 / ml in HBSS and lysed using triton-X 100 at a final concentration of 0.5 % (v/v).

A.III.2.c. von Willebrand Factor Antigen ELISA Assay

This was a standard in-house ELISA assay, similar to that described by Bartlett *et al.* (1976) and has been used in the Department of Haematology, Westmead Hospital for more than 15 years. Briefly, 96-well EIA plates were coated with 1: 1000 diluted rabbit anti-VWF and left in a wet box overnight at 4 °C. Following three washes in ELISA buffer (0.12 M NaCl, 0.02 M imidazole, 0.005 M citric acid, 0.1 % BSA; pH 7.3), plates were incubated with BSA (5%) in ELISA buffer for 1 hour at 22 °C to reduce non-specific background binding. Plates were rewashed twice and then incubated in triplicate with 200 µl volumes of samples at suitable dilutions. Calibration curves were established using pooled normal plasma form at least 40 individuals as well as a commercial reference plasma as a secondary standard. After 2 hours at 22 °C, plates were washed four times, and then allowed to incubate for 2 hours at 22 °C with 200 µl volumes of horseradish peroxidase labelled rabbit antisera raised to human VWF (1: 1000 in ELISA buffer). Plates were rewashed five times, and then allowed to incubate with 200 µl volumes of the peroxidase substrate tetramethylbenzidine dihydrochloride (0.42 mM) and H_2O_2 (0.3 mM) in buffer to allow colour development. The reaction was stopped once adequate colour was evident, usually after 10 minutes, by addition of 50 µl / well of H_2SO_4 (4 M). Absorbances were read at 450 nm using an automated plate reader (Biotek Instruments, model 312, USA) incorporating a data management software package.

Values for VWF antigen were then calculated with reference to the standards, and converted to a value of units / 10^7 cells, where 1 unit is equivalent to that amount of VWF present in 1 ml of normal plasma.

A.III.3. Results

UnA-EC had the highest levels of VWF antigen amongst all five populations of endothelium studied while VWF antigen reduced in apoptotic endothelial cells. No strong differences were seen between apoptotic populations although SDnA-EC seemed to have slightly lower levels of VWF antigen. Results of three experiments expressed as absolute levels of VWF antigen are shown in Figure A.III.1.

A.III.4. Discussion

VWF plays an important role in haemostasis and thrombosis. Following injury to the blood vessel, platelets adhere to exposed subendothelium where VWF serves as a bridge between platelets (Wagner, 1990). Although VWF is derived from endothelium, there are no reports describing levels of VWF during endothelial apoptosis. VWF antigen levels were generally lower in apoptotic populations as compared with untreated cells. The low levels in SDNA-EC may reflect preparation for apoptosis by the as yet non-apoptotic cells in serum deprived conditions. These preliminary data are consistent with the idea that apoptotic endothelial cells are adapted to minimize micro-thrombotic potential. However, in the absence of functional data such as collagen binding capacity, it is not possible to make clear statements regarding the possible biological significance of the observed changes in antigen level. Also, it is accepted that the reduction in antigen levels seen are so small that they may have little functional significance.

A.IV. Indomethacin Reduced Basal Production of Prostacyclin by Apoptotic Endothelium

A.IV.1. Introduction

The experiments performed in Chapter 3 revealed that apoptotic endothelial cells produced PGI_2. Several additional experiments were performed and are described in this appendix investigating the effect of indomethacin upon basal production of PGI_2 by apoptotic endothelium.

A.IV.2. Materials and Methods

A.IV.2.a. Materials

The reagents and materials used in the study were as described in 3.2.a.

A.IV.2.b. Incubation of apoptotic and non-apoptotic endothelial cells with indomethacin

MDA-EC, M&SDA-EC and UnA-EC were obtained from four separate donors as indicated in 2.2.b. Identical procedures as described in 3.2.b.ii were used in this study with the only difference being that cells were pre-incubated with indomethacin (250 μM) for 100 minutes at room temperature.

A.IV.3. Results

Figure A.IV.1 illustrates the results of 4 separate experiments with HUVEC from separate donors in which supernatants from M&SDA-EC, MDA-EC and UnA-EC had

greatly decreased levels of antigen at the 24 hr time point as compared with cells not pre-treated with indomethacin.

A.IV.4. Discussion

Indomethacin is a widely accepted inhibitor of cyclooxygenase activity. This was exploited in Chapter 3, in which pretreatment with indomethacin reduced the anti-aggregatory activity of all five HUVEC populations for thrombin stimulated platelets, indicating a role for cyclooxygenase products. The experiments described in this appendix provide further and consistent data indicating that indomethacin reduces PGI_2 production by both apoptotic and non-apoptotic endothelium. Difficulties with the quality of standards as well as the relatively small number of experiments performed compel these data to be viewed as preliminary so that additional experiments are required.

It would be interesting to perform similar experiments determining the effect of L-NAME upon NO production by apoptotic endothelium. Several preliminary experiments were performed, but unfortunately there was insufficient time to obtain data of sufficient quality for a proper assessment.

A.V. Fluorescence Activated Cell Scanning Analysis for Apoptotic Endothelium

A.V.1. Introduction

As indicated in 4.4, an alternative methodology for determining cell size apart from SEM, is FACS analysis with the assessment of forward-scatter and side-scatter as measures of cell size and internal complexity. FACS analysis has the further advantage that much larger populations of cells can be quantitated in a single experiment, and this provides a further incentive to extend current work in this way. This approach was not used in the current study, as it was felt that identification of particles undergoing secondary necrosis would be more difficult and previous experience indicated that the alcohol fixation protocols necessary for apoptotic labeling of nuclear material with propidium iodide would likely cause precipitative shrinkage and distortion of cells. Nonetheless, preliminary experiments were performed with FACS analysis to determine if FACS data is at least consistent with the direct morphological observations.

A.V.2. Materials and Methods

A.V.2.a. Materials

Propidium iodide was obtained from Sigma (St. Louis, USA) while sodium citrate was supplied by Ajax Chemicals (Auburn, NSW, Australia). Triton X-100 was purchased from BDH Laboratory Supplies (Poole, England). Falcon FACS tubes were supplied by Becton Dickinson Labware (New Jersey, USA).

A.V. 2.b. Fluorescence Activated Cell Scanning Analysis

M&SDA-EC and HL-60 cells in both static and rotating culture conditions were prepared as described in 4.2.g. and pelleted by centrifugation at 2,000 rpm for 5 minutes at 4 °C. Cell pellets were then resuspended in 1 ml of HBSS and added dropwise to 5 ml of ice cold 70 % ethanol while gently vortexing to ensure adequate mixing of the cells before storage at -20 °C until required. One day prior to FACS analysis, 3 ml volumes were transferred to FACS tubes and centrifuged at 2,000 rpm for 5 minutes at 4 °C while the remaining 3 ml cell suspensions were stored at -20 °C for further analysis if required. Cell pellets were air dried at room temperature and then resuspended in 400 µl volumes of HBSS containing propidium iodide (50 µg / ml), RNAse A (0.1 mg / ml), sodium citrate (0.1 % w / v) and triton X - 100 (0.1 %). Cell suspensions were left in the dark for 24 hours at room temperature before analysis with a Becton Dickinson FACScan Device using Cell Quest version 3.2.f1 software. The data was analysed using Windows Multiple Document Interface (WinMDI) Flow Cytometry Application, Version 2.8 (available at www.facs.scripps.edu/).

A.V.3. Results

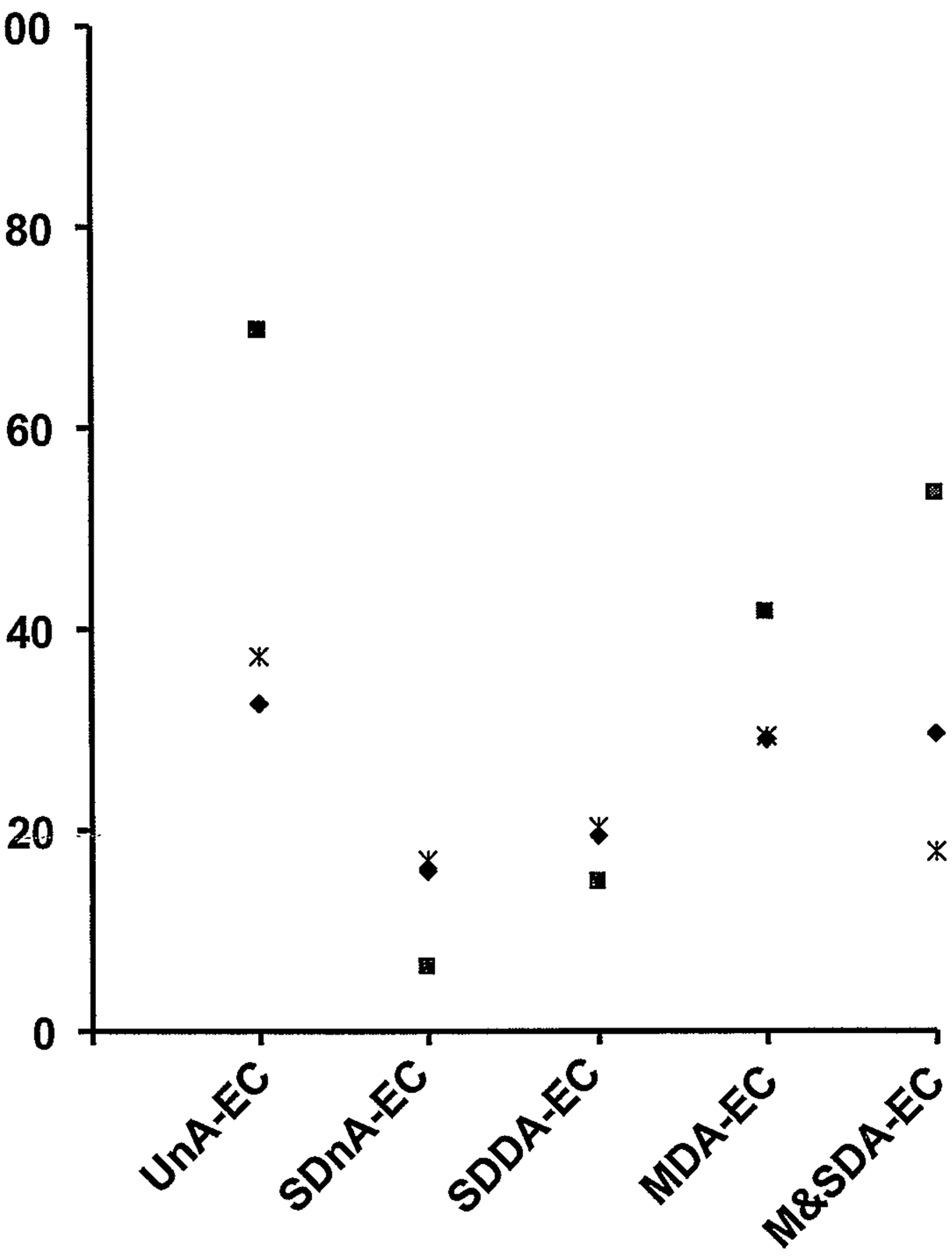
FACS analysis was performed on cells in both rotating and static conditions as well as the starting population, and indicated decreased DNA content of cells with time. DNA profile histograms revealed a significant increase in the magnitude of the sub-diploid peak in both rotating and static populations as compared with starting populations and this was only slight more pronounced in rotating populations, as demonstrated by a modest left shift (Figure A.V.1). No significant difference was found between M&SDA-EC and apoptotic HL-60 cells.

Unfortunately, it was not possible to see convincing shifts in forward and side-scatter between the three populations studied. This appeared to reflect the limits of sensitivity of the FACS analysis system employed, as the size of particles involved was very small.

A.V.4. Discussion

Although it was not possible to observe the expected changes in forward and side scatter, seemingly due to the technical limitation of the system used, the preliminary experiments outlined in this appendix did reveal a modest left shift in DNA content with apoptosis and rotation. This was consistent with changes in cell size observed in chapter 4 by SEM and analysis of semi-thin sections. It may be possible to in some way adapt the protocols used in these preliminary experiments to exploit forward and side-scatter to determine cell size as initially hoped for. If this does become possible, it will be necessary to use particulate material of known size as a standard for determination of absolute cell size.

Figure A.III.1 VWF:Ag was present in all five populations of endothelium studied. UnA-EC had the highest levels of VWF antigen amongst all five populations of endothelium studied. No strong differences were seen between apoptotic populations although SDnA-EC seemed to have slightly lower levels of VWF antigen. Results of three experiments expressed as absolute levels of VWF antigen.



Cell Populations Studied

Figure A.IV.1 The relative change in optical density seen in competitive immune assay for 6-keto $PGF_{1\alpha}$ of supernatants collected over 24 hr from apoptotic (M&SDA-EC, MDA-EC) and non-apoptotic (UnA-EC) endothelium. A clear increasing in optical density was seen in supernatant of all samples over 24 hr period studied, relative to supernatant of all samples not pre-incubated with indomethacin (value of 1 indicated by horizontal line). These data reveal the reduction of PGI_2 by these endothelial populations after pre-treated with indomethacin.

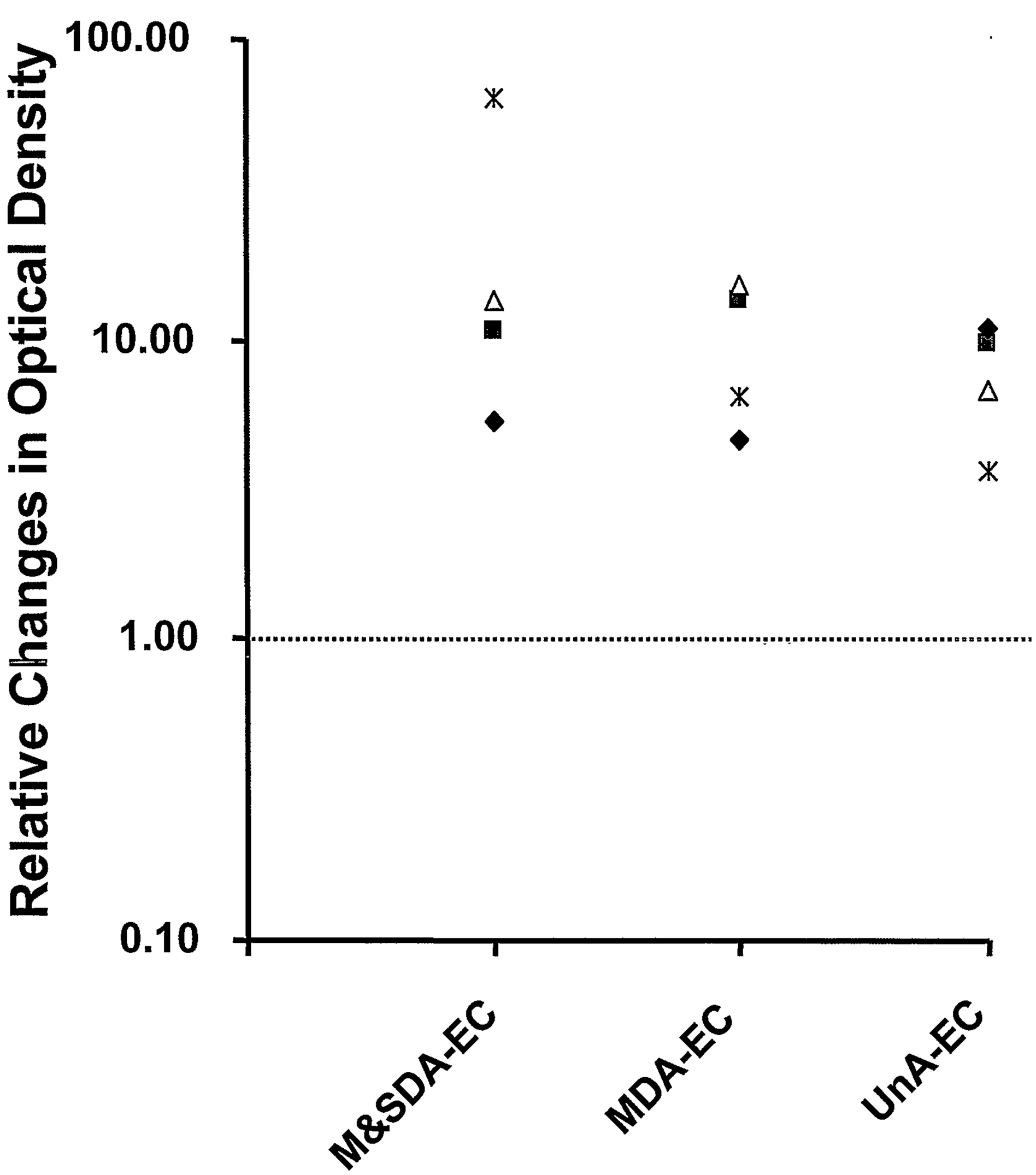
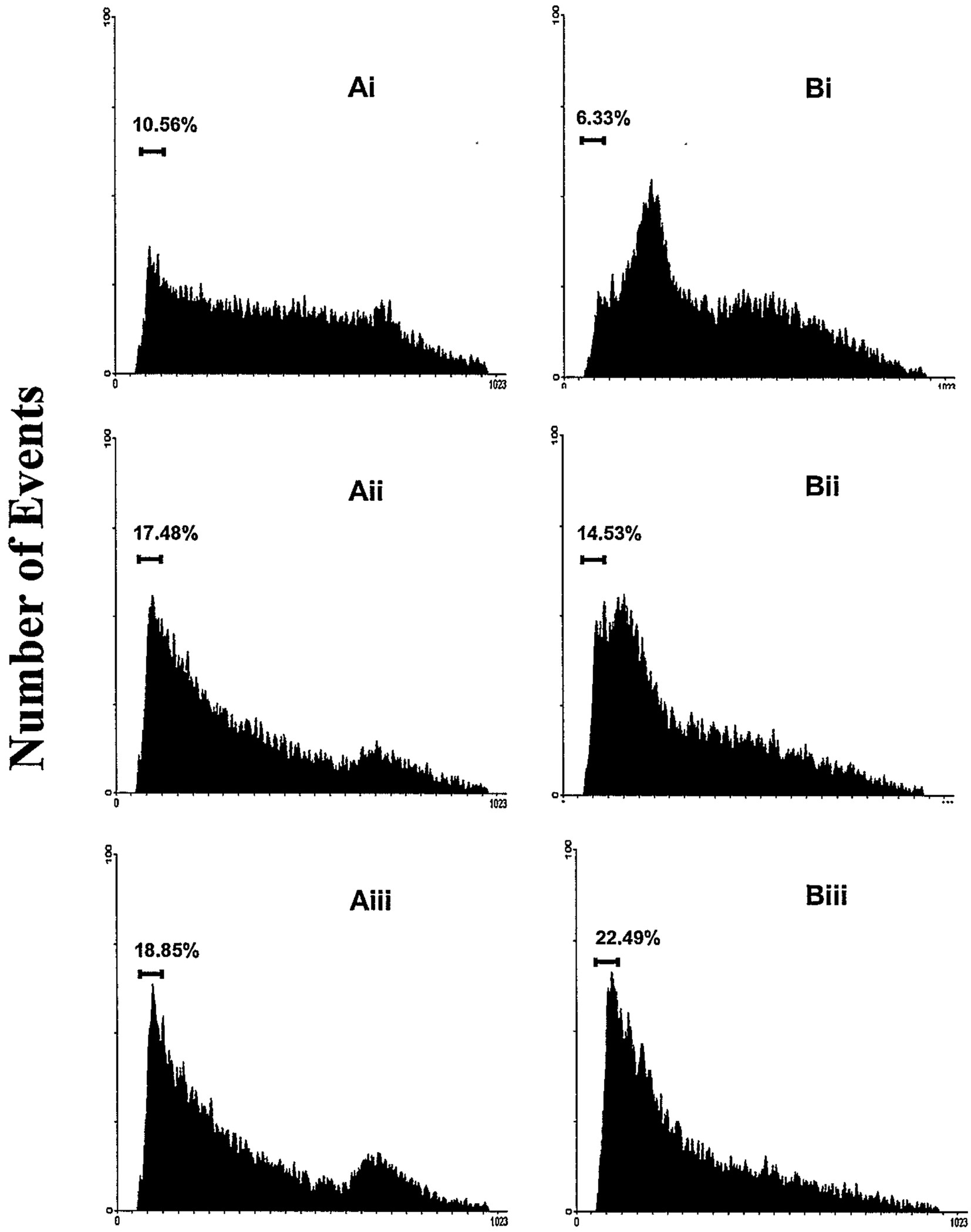

Relative Changes in Optical Density
100.00
10.00
1.00
0.10
M&SDA-EC
MDA-EC
UnA-EC
Cell Populations Studied

Figure A.V.1 FACS analysis indicated decreased DNA content of both HUVEC (A) and HL-60 cells (B) with time. DNA profile histograms revealed a significant increase in the magnitude of the sub-diploid peak in both rotating (*Aiii* and *Biii*) and static populations (*Aii* and *Bii*) as compared with starting populations (*Ai* and *Bi*) and this was slight more pronounced in rotating populations. No significant difference was found between M&SDA-EC and apoptotic HL-60 cells.



DNA Content (PI Fluorescence)

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