

Serum albumin is a specific inhibitor of apoptosis in human endothelial cells

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SUMMARY

Excess blood vessels are removed by apoptosis of endothelial cells, however, the signals responsible for this have not been defined. Apoptosis of cultured human umbilical vein endothelial cells is induced by deprivation of serum or adhesion. In this paper, apoptosis in human umbilical vein and microvascular endothelium was induced by deprivation of serum and or adhesion. Apoptosis was confirmed on the basis of morphology, ultrastructure and internucleosomal cleavage of DNA. Loss of endothelial adhesion was found to be an early event in cultured endothelial cell apoptosis and was exploited to quantitate apoptosis. The effect of: bovine serum albumin; human serum albumin; recombinant human albumin; dithiothreitol reduced human and bovine albumin; CNBr treated human and bovine albumin as well as ovalbumin upon endothelial apoptosis was determined. Native bovine and human albumin as well as recombinant human material inhibited apoptosis at physiological concentrations with identical

dose response curves in both umbilical vein and microvascular cells. Dithiothreitol treatment destroyed all protective activity while bovine but not human albumin was partially inactivated by CNBr treatment. The unrelated protein ovalbumin was not protective. Albumin did not inhibit apoptosis if cells were also deprived of adhesion. The data suggest that albumin is a specific inhibitor of human endothelial apoptosis but does not protect cells also deprived of adhesion. Reduced supply of albumin to endothelium in poorly perfused blood vessels may provide a mechanism for the removal of excess blood vessels in remodelling tissues. Also, the failure of albumin to protect endothelial cells deprived of adhesion from apoptosis may reflect the need to remove potentially micro-embolic cells detached due to trauma.

Key words: Endothelium, Human, Apoptosis, Albumin, Adhesion, Remodelling

INTRODUCTION

Excess cells are removed by apoptosis which is a highly regulated form of cell death frequently requiring protein synthesis. Morphologically and ultrastructurally, apoptosis is recognised by cellular fragmentation into apoptotic bodies often containing fragments of condensed nuclear material. Internucleosomal cleavage of DNA into 180 base pair fragments is the principal biochemical marker for apoptosis (Gerschenson and Rotello, 1992; Raff, 1993).

Angiogenesis and vascular restructuring occur throughout growth and development and involve both proliferation and apoptosis of vascular endothelial cells (EC). Apoptosis of EC occurs in: the involuting breast (Walker et al., 1989), pressure atrophy of the parotid gland (Walker and Gobe, 1987) and fibrotic lung lesions (Polunovsky et al., 1993). EC apoptosis probably contributes to the maturation of vascular granulation tissue to avascular scar tissue (Robbins et al., 1981). Although many factors inducing EC proliferation and differentiation are known (Folkman, 1995; Klagsbrun, 1992), surprisingly little is known about regulation of EC apoptosis. Tumour necrosis factor induces apoptosis in bovine EC (Robaye et al., 1991),

while deprivation of serum or matrix adhesion induce apoptosis in human umbilical vein EC (HUVEC) (Araki et al., 1990a,b; Meredith et al., 1993). An important role for $\alpha v \beta 3$ integrins in inhibition of apoptosis in newly formed blood vessels has been demonstrated by Brooks et al. (1994). Fibroblast growth factor and phorbol esters reduce the apoptosis of serum deprived HUVEC (Araki et al., 1990a,b). Also, an alveolar epithelial cell product has recently been found to reduce apoptosis in bovine EC treated with tumour necrosis factor (Wendt et al., 1994) while heat shock and endotoxin act together to increase apoptosis in porcine EC (Buchman et al., 1993). However, none of the known EC apoptosis regulatory factors seem to have any clear relationship with apoptosis in tissues undergoing non-inflammatory vascular remodelling.

The prime role of the vasculature is to deliver blood to the tissues, and a signal for the removal of excess blood vessels may lie in their relative patency. During tissue remodelling, changing vascular patterns result in altered flow through established vessels. The survival of EC in well perfused vessels coupled with apoptosis of EC in less privileged vessels would provide a mechanism for optimising vascular perfusion in changing tissues. This proposition is consistent with the

apoptosis-like changes in EC of the corpus luteum subjected to reduced blood flow (Azmi and O'Shea, 1984).

Within this model, EC must detect vascular perfusion. Shear stress is a potent EC stimulus (Ando and Kamiya, 1993; Reinhart, 1994) and may signal vascular perfusion, however, there is little apoptosis of cultured EC in the absence of fluid flow (Araki et al., 1990a). Apoptosis often follows withdrawal of a critical trophic factor, consistent with HUVEC apoptosis following loss of adhesion or serum deprivation (Araki et al., 1990a; Meredith et al., 1993). A similar inhibitory effect is reported for colony stimulating factors in the case of haemopoietic precursor cells (Williams et al., 1990) and sex steroids for hormone responsive cells (Rennie et al., 1989; Roberts et al., 1992). From this, chemical components of blood may act alone or together with shear stress to signal perfusion.

Cultured human EC have unusually high serum requirements, with maximal growth occurring at concentrations of up to 50% serum (Davison et al., 1980; Jaffe, 1984). Other mesenchymal cells such as fibroblasts or smooth muscle cells are cultured at lower serum concentrations and survive with little or no serum (Medcalf and Hamilton, 1986; Laiho et al., 1986; Libby et al., 1988; Zoellner et al., 1992). From this, the sensitivity to serum with regard to both cell growth and apoptosis is a specific characteristic of EC. The major serum protein albumin (Alb) has often been used as a supplement in serum free cultures of HUVEC (Bussolino et al., 1986; McArthur et al., 1986; Bevilacqua et al., 1986). Furthermore, EC have specific receptors for Alb (Schnitzer et al., 1988, 1992) which have been suggested as mediating maintenance of normal vascular permeability by stimulating reduced EC calcium levels (He and Curry, 1993). Therefore, we considered the possibility that Alb may act as one of the primary anti-apoptotic signals for EC and the aim of the current study was to determine if human EC apoptosis was inhibited by Alb in a protein specific manner.

MATERIALS AND METHODS

Materials

Tissue culture flasks and multi-well culture plates were obtained from Corning (New York, USA) while bacterial culture plates (60 cm²) were from Greiner (Kremsmünster, Austria). Supplemented calf serum (SCS) was from Hyclone (Logan, Utah, USA) and Heparin was donated by Hoffman LaRoche (Basel, Switzerland). The antibiotics penicillin, streptomycin and fungizone were from Seralab (Crawley Down, Sussex, UK). EC growth supplement was prepared by the method of Maciag et al. (1984). Na laurylsarcosine from Serva (Heidelberg, Germany), proteinase K and RNAase A (Boehringer Mannheim, Mannheim, Germany) were used to prepare DNA.

Highly purified native bovine serum albumin (BSA) and human serum albumin (HSA) were purchased from Boehringer (Marburg, Germany). In two experiments, a less highly processed preparation of BSA was used (Fraction V, Sigma, St Louis, USA). Recombinant HSA (rHA) raised in yeast was donated by Delta Biotechnology (Nottingham, UK). SDS-PAGE of BSA and HSA revealed multiple minor contaminants of native material (Fig. 1A). However, no such bands were found in SDS-PAGE of rHA (Fig. 1B). CNBr treated Alb was prepared as described elsewhere (McMenamy et al., 1971). Alb in a formic acid buffer was treated overnight with CNBr at 4°C. DTT was used to denature both HSA and BSA by heating Alb with 0.7 M DTT at 37°C for 2 hours. Successful CNBr cleavage and reduction of disulphide bonds was confirmed by SDS-PAGE. Treated Alb was exhaus-

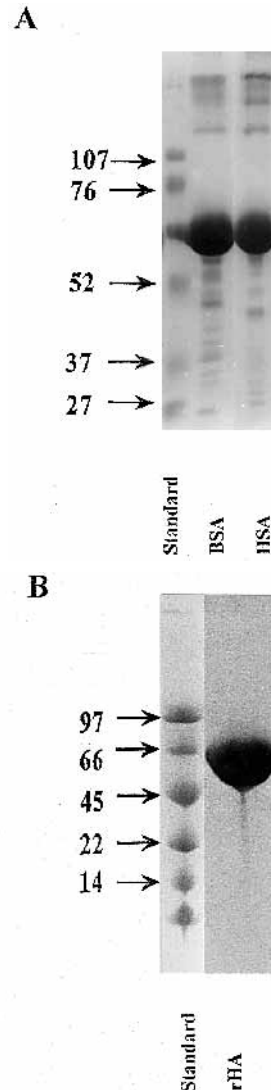


Fig. 1. SDS-PAGE of BSA and HSA stained with Coomassie blue (A) as well as of rHA (B). Bands representing minor protein contaminants are detected in native BSA and HSA, however, no such bands are seen in rHA preparations. Size standards (in kDa) are shown at the left of each panel.

tively dialysed against first PBS and finally M199 with antibiotics before sterile filtration. All other reagents used in this study were purchased from Sigma (St Louis, USA).

Preparation of endothelial cells

HUVEC were obtained by collagenase digestion from umbilical cords (Gimbrone et al., 1974; Zoellner et al., 1993). EC were cultured on gelatine in a growth medium of M-199 with 20% SCS, EC growth supplement (50 µg/ml) and heparin (30 U/ml). Penicillin (100 U/ml), streptomycin (100 µg/ml) and fungizone (2.5 µg/ml) were used as antibiotics. HDMEC were obtained by the method modified by Jackson et al. (1990). Briefly, dermal tissue discarded following surgery was treated with trypsin and passed through a fine nylon mesh. Human dermal microvascular EC (HDMEC) were further purified using *Ulex europaeus* lectin coated Dynabeads (Dyna, Oslo, Norway). Experiments were performed with cells from third to fifth passage. The identity of cells was confirmed by their characteristic cobblestone morphology, staining for FVIII-associated antigen (Jaffe,

1980; Jaffe et al., 1973) and the binding of *Ulex europaeus* lectin (Holthöfer et al., 1982).

Cell culture conditions for studying apoptosis

HUVEC and human dermal microvascular EC (HDMEC) were grown to confluence. Cells were then washed with either HBSS or M199 and further cultured in M199 with antibiotics in the absence of SCS, EC growth supplement or heparin. The effect of test reagents upon EC apoptosis in these washed cultures was determined. Experiments in which DNA fragmentation was studied were performed with HBSS washed cells in either 75 or 225 cm² flasks. DNA fragmentation of HUVEC deprived of adhesion was studied by comparing the DNA of trypsin-EDTA released HUVEC applied to 60 cm² tissue culture plates with that of cells applied to 60 cm² bacterial culture plates. Necrotic cells were prepared by freeze-thawing monolayers twice and further incubation with M199. Experiments to assess adherent cell number were with EC grown to confluence in 12- or 24-well tissue culture plates, washed with M199 and further cultured in triplicate with 1 ml of M199 with or without test reagents.

Detection of apoptosis

Apoptosis of EC was confirmed on the basis of: morphology, ultra-structure and the internucleosomal DNA cleavage characteristic of apoptosis. DNA laddering was demonstrated using a modification of the method of Smith et al. (1989) (Zoellner et al., 1996). In experiments comparing DNA from adherent with detached cells, DNA from both populations was prepared separately and all of the resulting DNA loaded for electrophoresis. In other experiments, detached and trypsin-EDTA released cells were pooled before preparation of DNA. In these experiments, DNA was quantitated by spectrophotometry and equal quantities of DNA loaded per lane. To quantitate the density of bands in these gels, black and white negatives of the gels were analysed using a Molecular Dynamics Personal Densitometer SI and Image QuANT 4.1 software. Triplicate readings of separate zones in each band were made and these are expressed as means of arbitrary units of density.

It was noted that apoptotic EC rapidly lost adherence and data demonstrating this are provided. This was exploited as an indirect measure to quantitate the extent of apoptosis by counting the number of adherent cells remaining after 24 hours treatment in 12- or 24-well tissue culture plates. Adherent cells were washed once with M199 to remove floating cells before release with trypsin-EDTA. Released cells were counted using a Burkert-Turk cell counting chamber. An apoptosis protection index ranging from 0 to 100 was defined. A value of 100 was assigned to the number of adherent cells remaining after treatment with SCS (20%) so that the effect of other agents upon EC survival is expressed as a percentage of protection by serum. This method gave good reproducibility both within triplicates and between experiments, permitting application of Student's *t*-test to the data.

Because EC apoptosis was quantitated on the basis of the number of adherent cells present in cultures after 24 hours of treatment, it was possible that cell division contributed to final adherent cell counts and that this had a significant impact upon the calculated apoptosis protection index. To exclude this possibility, an experiment was performed in which triplicate well cultures of HUVEC were exposed to experimental conditions with medium containing either SCS (20%), BSA (4%), HSA (4%) or with M199 alone in the presence or absence of nocodazole (1 µM), an agent which blocks cell cycle during division (O'Connor and Jackman, 1996). After 24 hours of incubation, cells were fixed with formal buffered saline and stained with haematoxylin. The number of mitotic figures at the conclusion of the experiment was determined in cell counts in which all mitotic and non-mitotic cells were counted in three randomly selected fields in each well at a magnification of 200 using an Olympus CK2 inverted microscope. More than 100 cells were counted in each well and the relative percentage of mitotic figures per well calculated. It was found that the contribution of cell division to final adherent cell number in

the experimental conditions used was: 3.1±1.4% in the presence of SCS (20%); 1.9±0.6% in the presence of HSA (4%); 1.8±0.5% in the presence of BSA (4%) and 1.1±0.4% in M199 alone (data expressed as relative percentage of adherent cell number after 24 hours of treatment). This was consistent with the observation that full growth medium containing ECGS and heparin was required for significant growth of cultured human EC. Because the contribution of cell division to final adherent cell number was much less than the standard deviations of cell counts encountered in experiments, it was decided that the contribution of cell division to the apoptosis protection index was negligible.

The effect of albumin upon EC apoptosis

The effect of native BSA and HSA upon EC apoptosis was determined. Dependence of the activity of Alb upon the native conformation of the protein was studied in experiments with dithiothreitol (DTT) reduced HSA and BSA as well as with CNBr treated HSA and BSA. Further experiments with ovalbumin and recombinant HSA (rHA) were performed to exclude a non-specific protein effect as well as mediation of activity by serum contaminants of native Alb preparations. Having studied the effect of Alb upon apoptosis in adherent EC, the effect of Alb upon apoptosis in non-adherent EC was determined in experiments comparing the extent of internucleosomal DNA cleavage between adherent HUVEC and those deprived of adhesion by seeding on bacterial culture plates.

Electron microscopy

Floating-detached HUVEC were collected and concentrated by centrifugation before further processing. Adherent HUVEC are spread across a two-dimensional surface obscuring differences between adherent and floating cells not due to cell spreading. To overcome this, adherent HUVEC were first released from the tissue culture surface with trypsin-EDTA. Cell pellets were resuspended in PBS with 0.25% glutaraldehyde. The cells were then further processed by fixation with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) for 1 hour at 4°C. Cells were then washed three times with phosphate buffer before fixation with 1% osmium in the same buffer for 1 hour at 4°C, further washing, dehydration with graded alcohols and embedding in Epon. Ultrathin sections were collected on copper grids and stained with uranyl acetate and lead citrate prior to examination by electron microscopy (Zoellner et al., 1996).

RESULTS

Loss of adhesion by apoptotic endothelium

In serum free conditions, many cells started to fragment into small apoptotic bodies and detach. Apoptotic bodies were often spread along adherent cell processes to create a beaded appearance (Fig. 2A). Cells undergoing such fragmentation rapidly lost adhesion to leave holes in the monolayer, and appeared to continue fragmentation while floating. It is important to note that few cells with this apoptotic morphology could be found in EC cultures before serum deprivation.

Fig. 2B shows the pattern of DNA fragmentation of both adherent and floating-detached HUVEC cultured in the absence of serum. There was internucleosomal DNA cleavage in both the adherent and floating cell populations. Cycloheximide inhibited the appearance of ladder DNA in both the floating and adherent cell populations. It is assumed that detachment of cells represented a toxic effect of the cycloheximide. In adherent cells, cycloheximide did not stop all internucleosomal fragmentation, and this may represent that population of cells already undergoing apoptosis at the start of the

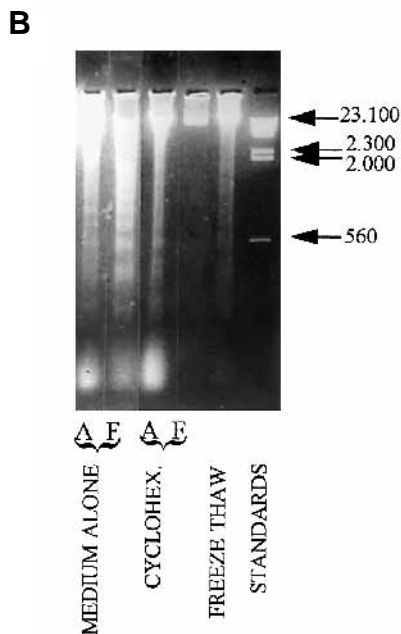
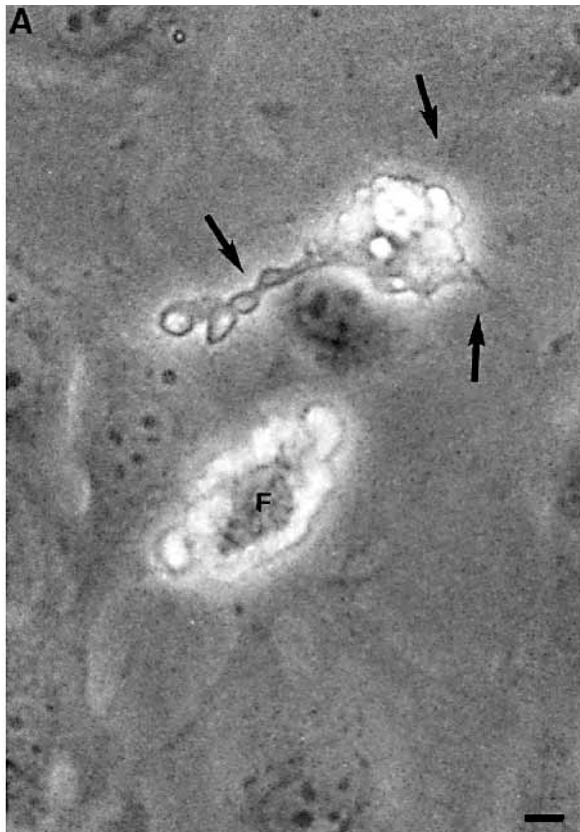


Fig. 2. The morphology and DNA cleavage pattern of apoptotic HUVEC. (A) An adherent EC undergoing fragmentation into smaller apoptotic bodies. The cell is attached to the culture surface by adherent cell processes (arrows). A floating-detached cell (F) is above the plane of focus, and has the irregular appearance of a fragmenting apoptotic EC. Bar, 5 μ m. (B) Size fractionated DNA from adherent (A) and floating-detached (F) HUVEC after 6 hours treatment with medium alone or cycloheximide at 10 μ g/ml as compared with DNA from necrotic freeze-thawed cells. Size standards (in bp) are shown at right.

experiment. DNA from cells treated with cycloheximide and the DNA laddering of apoptosis contrasted with the smear of DNA from necrotic freeze-thawed cells.

Detached cells were smaller as compared with trypsin released adherent cells (Fig. 3A,B). Also, the large nuclei of adherent cells with loose chromatin and prominent nucleoli, were transformed to condensed and fragmented electron-dense nuclear rests in floating cells. Intracellular organelles such as mitochondria, endoplasmic reticulum and Weibel Palade bodies were intact in all floating cells, while some cells had vesicular structures confluent with the plasma membrane. Some cells in the adherent cell population had the ultrastructural appearance of apoptotic floating-detached cells although these were very few in number. The ultrastructure of floating and adherent cells remained constant, regardless of whether the cells were obtained from serum free cultures or from cultures treated with serum (20%) or Alb. The morphological, DNA and ultrastructural changes suggest that apoptosis commences with adherent cells and is completed following detachment. The rapid loss of adhesion of apoptotic EC permitted the use of adherent cell number as a measure of the extent of EC apoptosis.

Albumin inhibits HUVEC apoptosis

HUVEC cultured for 24 hours in medium alone became apoptotic while serum and Alb greatly reduced this (Fig. 4). Also shown in Fig. 4 is the effect of CNBr and DTT treatment of BSA and HSA upon the protective effect. CNBr treated BSA had reduced protective activity while HSA was not affected. Also, denaturation with DTT completely destroyed the ability of Alb to inhibit apoptosis. Quantitation of this experiment is shown in Fig. 5.

Alb inhibited apoptotic DNA laddering (Fig. 6 and Table 1). An important point to note with regard to this figure is that although there is less 'DNA laddering' in the presence of serum or Alb as compared with cultures incubated with M199 alone, there is nevertheless appreciable laddering in the presence of both Alb and serum. There is an apparent inconsistency between the extent of internucleosomal cleavage and the extent of apoptosis as determined on the basis of adherent cell number. This is explained by the constant basal rate of apoptosis in cultured EC and the rapid degradation of DNA by non-internucleosomal cleavage once internucleosomal cleavage has occurred. Because of this, surviving cell number provides a far more meaningful measure of the extent of apoptosis than internucleosomal DNA cleavage. The possibility that the activity of Alb was due to a serum contaminant was excluded in experiments with rHA which was as effective as HSA in inhibiting DNA laddering and loss of adhesion with both albumins protecting the cells ($P < 0.001$) (Figs 6 and 7, Table 1). Importantly, the unrelated protein ovalbumin was not protective (Fig. 7).

Native and recombinant albumin protect umbilical vein and microvascular endothelium with equal efficiency

Fig. 8A shows the result of a dose response experiment, in which both BSA and HSA increased the HUVEC apoptosis protection index at concentrations above 5.8 μ M and reaching maximum by 440 μ M. Similar results were obtained with rHA (Fig. 8B).

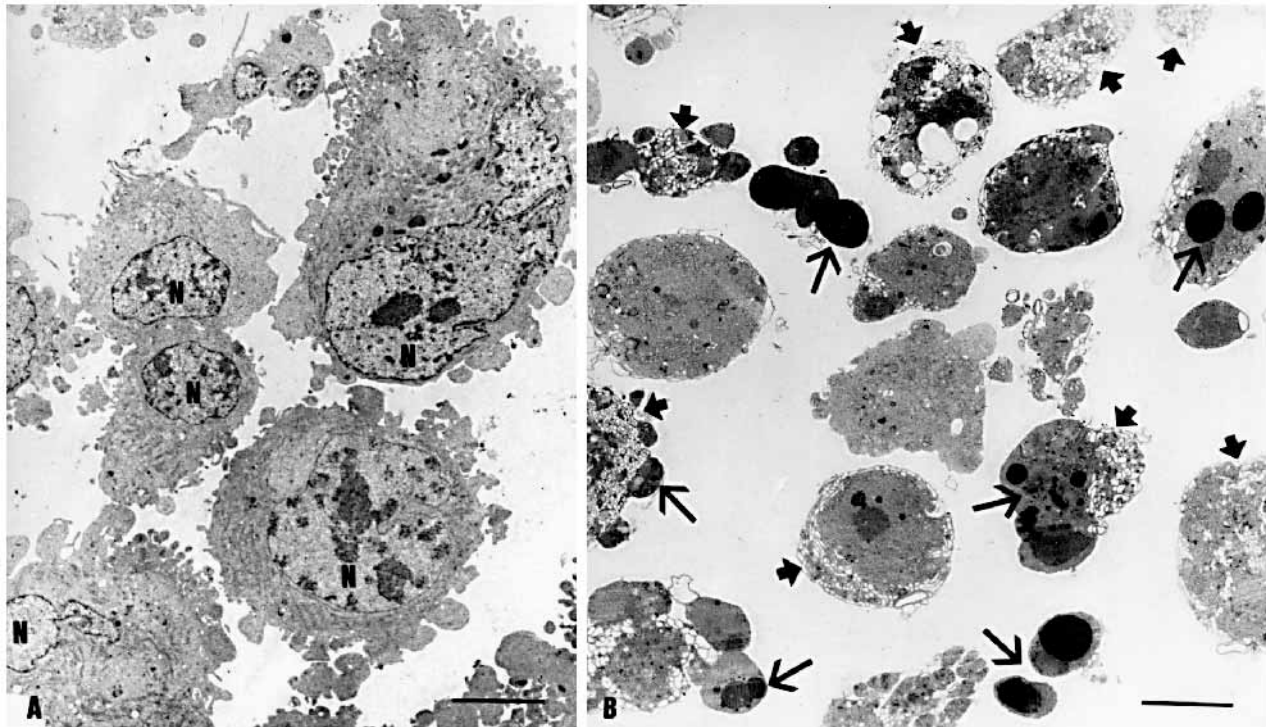


Fig. 3. Ultrastructure of trypsin-EDTA released adherent HUVEC (A) compared with (B) detached-floating cells. (A) The nuclei of adherent cells (N) have loose chromatin and prominent nucleoli, while floating-detached cells (B) are smaller with condensed and fragmented nuclear material (thin arrows). Some of the floating-detached cells are highly vesiculated (thick arrows). Bars, 5 μ m.

The morphology of human dermal microvascular EC (HDMEC) deprived of serum was similar to that of HUVEC. HSA and rHA were protective ($P < 0.01$), while ovalbumin failed to significantly improve HDMEC survival in serum free conditions (Fig. 8C). Also, the dose response of protection for rHA was similar in HDMEC to that seen in HUVEC (Fig. 8D).

Albumin does not overcome HUVEC apoptosis due to lack of adhesion

Serum, BSA and HSA were ineffective in protecting HUVEC from apoptosis in conditions of adhesion deprivation (Fig. 9 and Table 2), demonstrating that HUVEC require both adhesion and albumin in order to circumvent apoptosis.

DISCUSSION

All floating-detached HUVEC studied had the ultrastructural

characteristics of apoptosis while only occasional adherent cells were apoptotic. This was reflected by the extent of DNA laddering in both of these populations. Some ladder DNA was also found in the adherent cycloheximide treated population (Fig. 1B), supporting the morphological observation of the presence of occasional apoptotic cells at the start of experiments. The data indicate that EC apoptosis commences with adherent cells, and is completed following detachment of the cells, consistent with earlier work (Zoellner et al., 1996). This permitted the use of the remaining adherent cell number to quantitate the extent of apoptosis in EC cultures. Apoptotic cell number always increased in a linear fashion over time, regardless of whether serum was present or not. From this, serum reduced the rate of apoptosis rather than the absolute number of cells capable of entering apoptosis (data not shown) so that our data support the earlier report that there is a basal rate of HUVEC apoptosis which is increased with withdrawal of serum (Araki et al., 1990a). Data shown in the current paper

Table 1. Intensity of internucleosomal cleavage bands of HUVEC cultured with medium alone, BSA, HSA, rHA or cycloheximide

Base pairs	Medium	BSA 4%	HSA 4%	rHA 4%	Cycloheximide 10 μ g/ml
180	37 \pm 1	8 \pm 2	16 \pm 2	16 \pm 3	15 \pm 1
360	124 \pm 5	51 \pm 7	94 \pm 3	102 \pm 6	36 \pm 4
540	167 \pm 1	116 \pm 9	138 \pm 3	150 \pm 10	57 \pm 2

HUVEC were cultured for 12 hours in serum free conditions. Adherent and any detached-floating cells were harvested and pooled prior to the preparation of DNA. Although appreciable apoptosis occurred in the presence of albumin, the intensity of internucleosomal cleavage bands of DNA was greater from cultures deprived of this protein. Cycloheximide strongly inhibited internucleosomal cleavage. Error bars indicate s.d.

Table 2. Intensity of internucleosomal cleavage bands of HUVEC permitted or deprived of adhesion in the presence of serum, medium alone, BSA or HSA

Treatment	Serum (20%)	Medium	Serum (20%)	Medium	BSA (4%)	HSA (4%)
Matrix	Adhesion permitted	Adhesion permitted	Adhesion denied	Adhesion denied	Adhesion denied	Adhesion denied
540 bp	2.9±0.3	30±2	7.4±0.1	58±5	32±5	44±4
720 bp	2.4±0.4	49±5	15±2	70±3	59±3	68±10
900 bp	5.3±1.8	65±3	32±4	79±2	76±2	77±1

HUVEC were either permitted to adhere to the normal gelatine matrix, or denied adhesion by plating onto non-adhesive bacterial culture plates. Both adhesion and serum were required to inhibit internucleosomal DNA cleavage, while neither BSA or HSA inhibited DNA cleavage in the absence of adhesion. Error bars indicate s.d.

suggest that Alb is an important factor in serum protecting EC from apoptosis.

It could be argued that the effect of Alb in protecting EC from apoptosis is a non-specific protein effect and not due to specific activation of the cells by Alb. However, it was possible to inactivate the protein by denaturation. Also, the difference in the ability of CNBr cleavage to inactivate BSA as compared with HSA is consistent with the different cleavage sites for CNBr in these two species of Alb (McMenamy et al., 1971; Brown, 1977). Further, the structurally unrelated protein ovalbumin did not protect either HUVEC or HDMEC from apoptosis.

Alb is known to bind many serum components (Brown, 1977), however, the possibility that protection was due to a serum contaminant was excluded in experiments showing that yeast rHA had the same activity as native material. Native BSA and HSA preparations appeared to contain small amounts of contaminant protein as seen in SDS-PAGE, however, no such contaminant proteins were detected in rHA. It is stressed that similar dose responses would not be expected with two separate native Alb preparations from two species as well as with rHA if the protective effect was mediated by a contaminant. Similarly, the adsorption by Alb of pro-apoptotic lipids

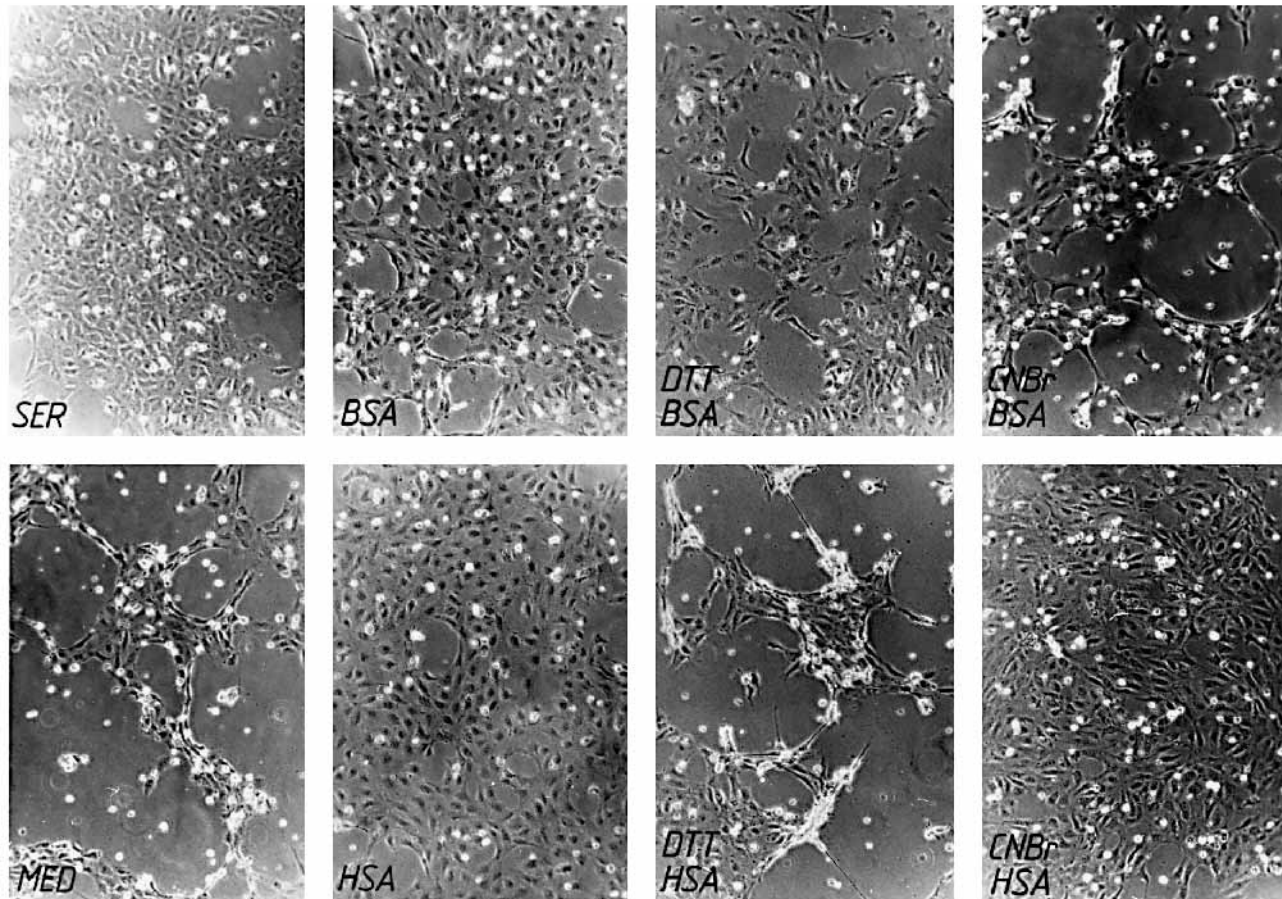


Fig. 4. The effect of Alb (580 µM or 4%, w/v) upon HUVEC morphology. Confluent HUVEC treated for 24 hours in the presence of M199 alone (MED), BSA, HSA, as well as with BSA and HSA at the same concentration but pre-treated by reduction with DTT or cleavage with CNBr. Bar, 150 µm.

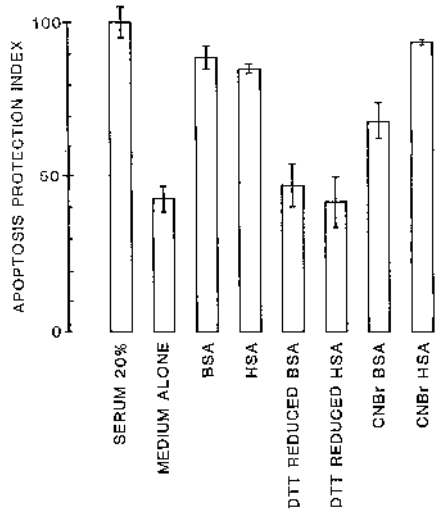


Fig. 5. The effect of Alb (580 μ M or 4%, w/v) upon HUVEC adhesion. Apoptosis protection indexes for the experiment illustrated in Fig. 3; error bars indicate s.d. Both HSA and BSA greatly reduced the effect of serum deprivation ($P < 0.01$). DTT treatment destroyed this protective effect ($P < 0.01$) while CNBr treatment reduced protection by BSA ($P < 0.02$) but did not affect HSA.

or other factors is unlikely to explain the activity, as such adsorptive capacity would vary with preparations of Alb with differing purity while the anti-apoptotic activity always displayed the same dose response. The ability of fibroblast growth factor and phorbol esters to inhibit HUVEC apoptosis in serum free conditions (Araki et al., 1990a,b) also strongly argues against the possibility that Alb exerts its protective effect by binding a toxic or apoptosis inducing factor in medium. This possibility would also be inconsistent with the highly regulated and active nature of HUVEC apoptosis demonstrated in this and other papers. Further, the production of an Alb dependent EC derived factor mediating the anti-apoptotic effect seems unlikely.

Plasma levels of Alb range from 470 μ M to 750 μ M (Märkl,

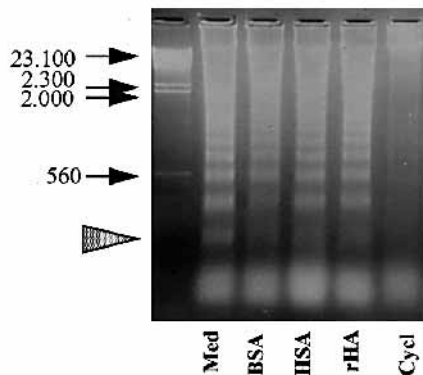


Fig. 6. DNA from pooled adherent and floating-detached HUVEC treated with medium alone (Med) as compared with BSA, HSA or rHA as well as with cycloheximide (Cycl) (10 μ g/ml). There was significantly less apoptosis in the presence of Alb as compared with medium alone. This is especially evident when comparing the intensity of the smallest 180 bp and 360 bp bands (arrowhead at 180 bp) (see Table 1). Size standards (in bp) are shown at left.

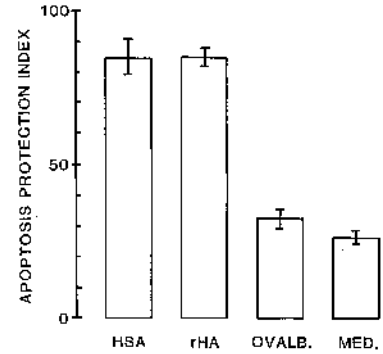


Fig. 7. The effect of rHA and ovalbumin (OVALB) at 800 μ M (or 4%, w/v) as compared with HSA and medium alone (MED) on the apoptosis protection index. Both rHA and HSA protected HUVEC ($P < 0.001$), while ovalbumin had little if any effect.

1979), similar to that at which EC were protected. This supports the suggestion that a physiological function of Alb may be to signal vessel patency to EC. One of the most elegant examples of trophic regulation of angiogenesis is the increased release of the potent angiogenic protein, vascular endothelial growth factor, by hypoxic cells. It is argued that this represents a simple mechanism whereby tissues are able to signal the vasculature of the need for increased tissue perfusion and hence angiogenesis (Shweiki et al., 1992). We propose a similar but reversed role for Alb. Tissue remodelling and the accompanying angiogenesis is assumed to result in new patterns of vascular flow, depriving some established vessels of their previous supply of Alb. Alb is bound and transcytosed by EC (Antohe et al., 1992), and reducing local concentrations of Alb in poorly perfused vessels would permit the cells to engage in apoptosis and thus effect removal of non-functional blood vessels. The possibility that newly formed blood vessels are particularly sensitive to apoptosis is raised by the work of Brooks et al. (1994) illustrating apoptosis of newly formed vessels due to specific inhibition of the $\alpha v \beta 3$ integrin.

Alb is present at appreciable although lower levels in the extravascular compartment (Wiig et al., 1991) and it could be argued that extravascular Alb levels would rarely be sufficiently low to effect Alb starvation of EC in poorly perfused blood vessels. There are several mechanisms, however, which may work against saturation of EC Alb receptors by extravascular Alb. Exclusion by charge effects of Alb from the glomerular basement membrane has been demonstrated (Del-Vechhio et al., 1987; Groggel et al., 1988) and a similar mechanism may greatly reduce Alb levels at the 'extravascular' EC plasma membrane surface. Also, a protein antigenically related to the major EC Alb receptor is present in the extravascular compartment and seems to inhibit binding of Alb to EC (Schnitzer and Oh, 1992). It is possible that one role of this protein is to protect EC from inappropriate inhibition of apoptosis by extravascular Alb. An exception to this, however, may be in chronic inflammatory and early reparative granulation tissue where blood vessels are unusually permeable (Robbins et al., 1981). The high extravascular Alb levels resulting from this high permeability may be important in maintaining the vascularity of these tissues until either the inflammatory stimulus is removed or the wound has closed.

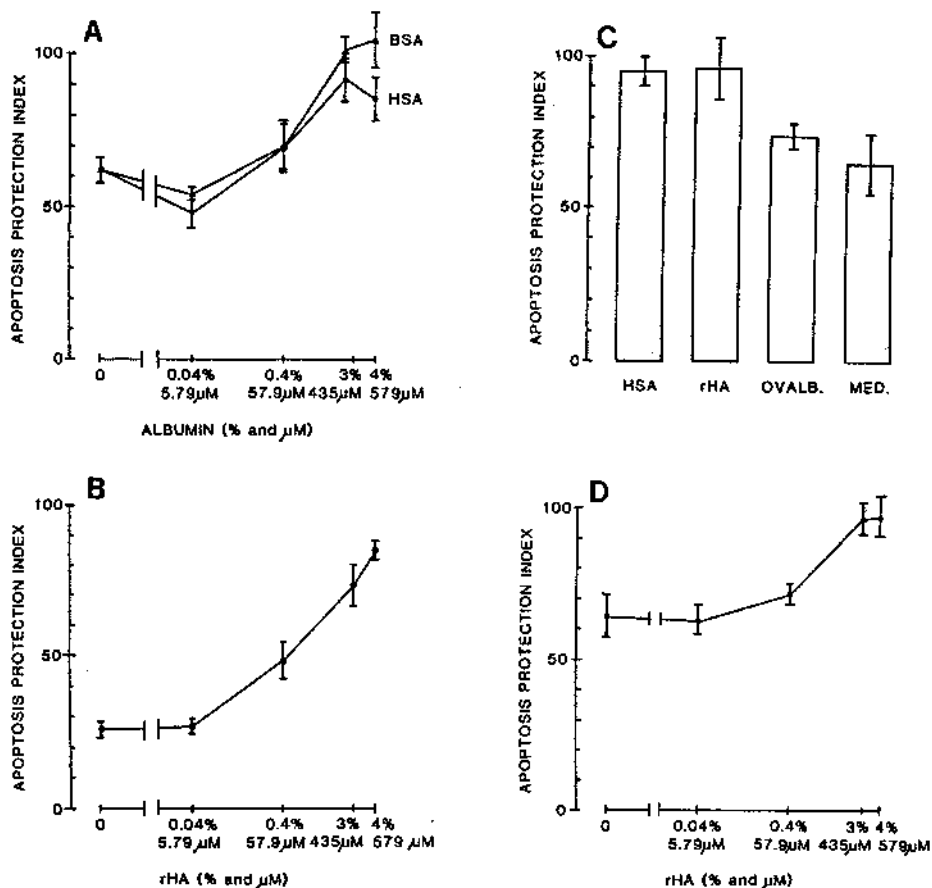


Fig. 8. Apoptosis protection index of HUVEC (A,B) and HDMEC (C,D) with or without Alb or ovalbumin; error bars indicate s.d. (A) HUVEC were cultured for 24 hours in the absence of serum with increasing concentrations of BSA (▲) and HSA (●) or (B) rHA. (C) rHA (580 μM or 4%, w/v) was equally effective at protecting HDMEC over a 48 hour period of serum deprivation as HSA at the same concentration ($P < 0.01$), while ovalbumin (OVALB) (800 μM or 4%, w/v) gave a result similar to medium alone (MED). (D) The dose response of protection for HDMEC was the same using rHA as for HUVEC.

Although the data shown in this paper support a specific anti-apoptotic role for Alb, the possibility remains that there are other as yet unidentified plasma proteins having a similar and perhaps equally powerful anti-apoptotic effect upon endothelium. The health of analbuminemic humans and animals with very low to undetectable levels of Alb (Buehler, 1978) permits speculation of the existence of other plasma proteins with a similarly specific anti-apoptotic role for EC at physiological concentrations.

The effect of Alb occurs at the near milli-molar concentrations seen in blood, in contrast to the activation of cells by nano-molar quantities of cytokines. This difference may reflect the role of Alb as a locational signal, instructing the cell of its correct orientation and maintained function in patent blood vessels. The robust levels of Alb found in blood may be better suited for signalling vascular flow than low concentrations of local or circulating cytokines.

Regarding the mechanism of the anti-apoptotic activity of Alb, the high levels of Alb required to protect EC from apoptosis suggest the presence of low affinity Alb receptors on EC which mediate the anti-apoptotic effect. The putative anti-apoptotic receptors are unlikely to be the same as the EC Alb receptors already identified (Schnitzer et al., 1988, 1992), as these display a high affinity for their ligand and would be expected to protect EC at much lower levels of Alb than seen in experiments. Some precedent is established for anti-apoptotic receptors with low affinity in that integrins are low affinity receptors some of which protect EC from apoptosis

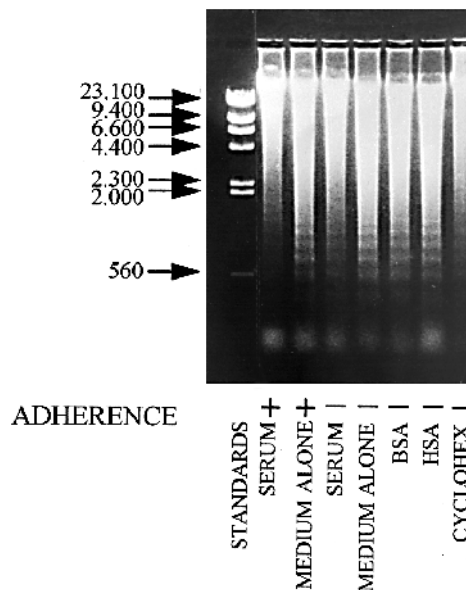


Fig. 9. Agarose gel electrophoresis of DNA from adherent (+) HUVEC in M199 alone or M199 with 20% serum as compared with HUVEC deprived of adherence (-) after 7 hours of treatment with serum (20%), medium alone, HSA (580 μM), BSA (580 μM), or cycloheximide 10 μg/ml. Apoptosis occurred in the absence of adherence, and this was not inhibited by the presence of serum. Size standards (in bp) are shown at left.

(Meredith et al., 1993). The co-existence of high with low affinity receptors for Alb on EC poses significant difficulties in the characterisation of the low affinity anti-apoptotic receptor and work continues towards this end. It is interesting to note that Alb reduces intracellular calcium levels (He and Curry, 1993). Elevation of intracellular calcium is associated with initiation of apoptosis (Gerschenson and Rotello, 1992; Raff, 1993) and it is possible that control of intracellular calcium is part of the pathway through which Alb inhibits apoptosis in EC.

The proposed role for Alb is also consistent with the inability of this protein to protect HUVEC deprived of adhesion. If Alb alone was sufficient to prevent apoptosis, detached EC would survive in the circulation where they would be non-functional and potentially dangerous micro-emboli. With regard to this, it is interesting to note that apoptotic EC undergo an accelerated fragmentation through the formation of complex canaliculi (Zoellner et al., 1996). It is suggested that EC require the twin signals of Alb and matrix adhesion in order to signal vascular patency and tissue integration, respectively. Cells not receiving these stimuli are by definition not part of a functional vessel and must die by apoptosis. Similar rules may apply for other cell types and it is possible that this is a specific example of a general mechanism for preventing the aberrant survival and growth of tissues in inappropriate settings.

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