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Inhibition of Microvascular Endothelial Apoptosis in Tissue Explants by Serum Albumin

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Plasma factors appear to inhibit endothelial cell (EC) apoptosis *in vivo* so that flow influences microvascular form. The identity of these factors has not, however, been established. Earlier, we reported that apoptosis in isolated, serum-deprived human EC is inhibited by albumin (Alb). Here, we demonstrate likely biological relevance of this to vascular remodelling in experiments with tissue explants. Rat skin explants were incubated in medium M199 with or without serum, bovine Alb, or human Alb. EC in paraffin sections of explants were labelled by lectin histochemistry and the relative proportion of apoptotic was EC determined. Apoptosis was confirmed by transmission electron microscopy and terminal deoxynucleotidyl transferase labelling. Serum-free culture induced EC apoptosis ($P < 0.02$) and this was strongly inhibited by Alb at physiological concentrations ($P < 0.01$). This was not a nonspecific protein effect, as mercaptoethanol denaturation destroyed the activity and ovalbumin was not protective. Also, protection was not due to serum contaminants, as recombinant human Alb had activity identical to that of native material. The dose response was identical for all Alb preparations tested, with maximal activity at physiological concentrations. Protection was not limited to rat tissue as similar results were obtained with human gingival explants. These data support a role for Alb as a plasma antiapoptotic factor for EC in tissues.

Key Words: endothelium; apoptosis; albumin; remodelling; tissue explants.

INTRODUCTION

Apoptosis of endothelial cells (EC) is accepted as the primary event in microvascular remodelling in both physiological and pathological settings (Walker and Gobe, 1987; Walker *et al.*, 1989; Feinberg and Noden, 1991; Lang and Bishop, 1993; Polunovsky *et al.*, 1993; Desmouliere *et al.*, 1995; Meeson *et al.*, 1996; Sgonc *et al.*, 1996).

Morphologically and ultrastructurally, apoptosis is recognised by cellular fragmentation into apoptotic bodies often containing fragments of condensed nuclear material but with intact organellar structures. Internucleosomal cleavage of DNA into 180-bp fragments is a widely accepted biochemical marker for apoptosis which can be detected *in situ* by terminal deoxynucleotidyl transferase labelling (TDTL). This contrasts with necrosis, in which homeostasis is lost and there is disruption of membranous structures,

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degeneration of mitochondria, and ribosomal detachment from rough endoplasmic reticulum (RER). Apoptotic cells may, however, undergo secondary necrosis once biochemical degradation has progressed to the stage that apoptotic particles are unable to maintain homeostasis (Gerschenson and Rotello, 1992; Raff, 1993; Martins and Earnshaw, 1997).

Although broadly similar to apoptosis in other cells, apoptosis in cultured human EC is characterised by canalicular fragmentation, which is an unusual pattern of ultrastructural degradation, with the formation of complex interconnecting canaliculi. This seems to lead to accelerated mechanical fragmentation of apoptotic particles and has been proposed as an endothelial adaptation to minimise the microembolic potential of apoptotic EC (Zoellner *et al.*, 1996a). Despite the important role of EC apoptosis in varied biological processes, little is known about the mechanism through which EC apoptosis is regulated *in vivo*.

A number of cytokine and chemical factors which induce EC apoptosis have been identified (Araki *et al.*, 1990a, b, 1993; Robaye *et al.*, 1991; Nakamura *et al.*, 1994; De Bono and Yang, 1995; Maier *et al.*, 1995; Tsukada *et al.*, 1995; Dabrowska *et al.*, 1996; Dimmeler *et al.*, 1997), while EC become apoptotic if deprived of adhesion (Meredith *et al.*, 1993; Brooks *et al.*, 1994; Zoellner *et al.*, 1996b). The relevance of these factors to *in vivo* events is, however, unclear.

Since the prime function of microcirculation is to deliver blood, it has been argued that blood vessels which are well perfused are permitted to survive while poorly perfused vessels are deprived of signals associated with flowing blood and are then lost through EC apoptosis (Meeson *et al.*, 1996; Zoellner *et al.*, 1996b). This is supported by *in vivo* observations of the rat pupillary membrane, in which reduced flow by macrophage-induced EC apoptosis is followed by further EC apoptosis in affected vessels (Lang and Bishop, 1993; Meeson *et al.*, 1996). Apoptosis follows withdrawal of critical trophic factors in many cell types (Rennie *et al.*, 1989; Williams *et al.*, 1990; Roberts *et al.*, 1992) and the same is suggested to apply to EC deprived of plasma factors (Meeson *et al.*, 1996; Zoellner *et al.*, 1996b). It is important to note that the identity of these factors has not as yet been established.

We recently reported that serum albumin (Alb) strongly inhibits human EC apoptosis under serum-free conditions (Zoellner *et al.*, 1996b). This activity was identical for native bovine Alb (BSA) and human Alb (HSA) from several sources. Maximal activity was seen at physiological levels of the protein, with an identical dose response seen in all experiments. The activity was not due to a serum contaminant of native Alb preparations, as recombinant HSA raised in yeast (rHA) had activity identical to that of native material and the dose response of protection did not vary with preparations of native Alb having differing levels of purity. Also, the protective activity was not due to a nonspecific protein effect, as it was dependent upon the native conformation of the protein and the unrelated protein ovalbumin was not protective. Further, we found that there was a dual requirement for both Alb and adhesion in cultured EC to circumvent apoptosis. We suggested that Alb may be a chemical anti-apoptotic factor in plasma signalling vascular perfusion to EC and in this way plays a role in vascular remodelling (Zoellner *et al.*, 1996b). The current paper supports this possibility by demonstrating regulation of microvascular endothelial apoptosis in rat and human tissues by Alb.

MATERIALS AND METHODS

Materials

Tissue culture plates were obtained from Corning (New York, NY). Supplemented calf serum (SCS) was from CSL (Melbourne, Australia). Proteinase K, RNase A, and terminal transferase were from Boehringer Mannheim (Mannheim, Germany). Highly purified native BSA was purchased from Behring (Marburg, Germany), while HSA was from Behring as well as from Calbiochem (San Diego, CA). rHA raised in yeast was donated by Delta Biotechnology (Nottingham, England). SDS–PAGE of BSA and HSA revealed multiple minor contaminants of native material. However, no such bands were found in SDS–PAGE of rHA (Zoellner *et al.*, 1996b). Heating with mercaptoethanol

(ME) was used to denature BSA. Treated Alb was exhaustively dialysed against first PBS and finally medium M199 with antibiotics before sterile filtration. Whatman No. 1 filter paper (Maidstone, UK) was cut into small pieces and autoclaved for use as explant rafts. All other reagents used in this study were purchased from Sigma (St. Louis, MO).

Preparation of Tissue Explants

The skin of newborn rats is very thin and has little hair. This is an advantage for tissue explants, facilitating penetration of gas and medium into thin tissues, while hairless newborn animals have reduced bacterial contamination compared with adult animals. Inbred Wistar rat pups obtained from the Westmead Hospital Animal Care Facility were sacrificed by decapitation at from 3 to 5 days of age. After decapitation, animals were disinfected by briefly soaking in 70% ethanol and then the bodies were allowed to dry by evaporation over a 5-min period. Skin was removed as a continuous sheet using aseptic technique and washed five times with gentle agitation in 50-ml quantities of medium M199 containing penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (2.5 μg/ml) as antibiotics. A scalpel was used to cut skins into small flat pieces measuring 3 × 3 mm in extent. These were then soaked with gentle agitation in 1 ml of test medium for 5 min and placed onto rafts of autoclaved Whatman No. 1 filter paper which had been presoaked with test reagent. Explants were in 24-well tissue culture plates, with from three to four pieces of tissue in each well. Excess medium was removed from explants, so that there was a thin layer of medium rising as a meniscus over tissue fragments from the filter paper rafts.

A similar protocol was followed for preparation of human gingival explants. Human gingival biopsies were obtained with informed consent from patients having teeth extracted in the Oral Surgery Unit, Westmead Dental Hospital. Biopsies were removed from extraction sites prior to extraction. Specimens were then exhaustively washed with M199 containing antibiotics before preparation of 1-mm-thick slices. These were then soaked in test reagent and explants established in the same way as for rat tissues. Explants obtained in this way were maintained for up to 24 h without the appearance of necrosis as judged by light and electron microscopy.

Stimulation of Tissue Explants

Penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (2.5 μg/ml) were used as antibiotics in all experiments. Tissues were incubated at 37°C under CO_2 (5%) with medium M199 alone or containing HSA (4%), BSA (4%), rHA (4%), ovalbumin (4%), ME–BSA (4%) (w/v) or SCS (20% v/v). In dose–response experiments, HSA, BSA, and rHA were used at concentrations ranging from 0.04 to 7% (w/v) (5.8–580 μM). Stimuli were applied in quadruplicate wells in all rat skin experiments, with triplicate wells being used in human gingival cultures due to the much smaller amount of human tissue available. After 24 h of incubation, tissues were fixed and processed for either light or electron microscopy. Penetration of explants by Alb was confirmed in experiments in which biotin-labelled BSA (0.1 mg/ml) was included with BSA (40 mg/ml, 4% w/v) for the duration of the experiment. Tissues were then fixed and paraffin sections prepared for streptavidin–peroxidase immunohistochemistry.

Paraffin Sections for Lectin Histochemistry

Tissue explants for light microscopy were fixed with 10% neutral buffered formalin for 3 h and then washed three times with PBS before dehydration in graded alcohols and paraffin embedding. Individual blocks contained all tissue explants from each experimental culture well, so that each well corresponded to one paraffin block. Four-micrometer sections were prepared for lectin histochemistry. *Banderia simplicifolia* lectin (BS) is a lectin known to bind EC in many animal species, including rats (Laitinen, 1987). Earlier work has demonstrated retention of surface lectin binding by EC during apoptosis (Zoellner *et al.*, 1996a) and for this reason, lectin binding was used to aid in identification of both apoptotic and nonapoptotic rat EC in paraffin sections. Briefly, sections were deparaffinised before blocking endogenous peroxidase with 3% H_2O_2 in PBS. Sections were then washed with PBS before incubation with biotin-labelled BS (0.01 mg/ml) for 1 h. Slides were then washed three times before further incubation with streptavidin–peroxidase conjugate (0.01 mg/ml). After further washing, bound peroxidase activity was detected using diaminobenzidine (DAB) as a chromogen (Kiernan, 1981). Sections were then stained with haematoxylin and coverslipped for examination. Human EC do not bind BS, but readily bind *Ulex europaeus*-1 (UEA-1) lectin (Holthöfer *et al.*, 1982). Consequently, UEA-1 lectin binding was used for identification of human EC in paraffin sections. A protocol was used for UEA-1 that was similar to that described for BS labelling, with the only differences being that UEA-1 was not biotinylated, but was detected with a rabbit anti-UEA-1 serum followed by a peroxidase-conjugated goat anti-rabbit immunoglobulin preparation. In all lectin staining procedures controls consisted of sections not exposed to lectin, lectins and antisera were diluted in PBS with SCS (10%) and Tween 20 (0.5%), and the wash solution was PBS with Tween 20 (0.5%).

Terminal Deoxynucleotidyl Transferase Labelling

A method modified from that described in the literature was used in this study (Gavrieli *et al.*, 1992). Briefly, tissues were fixed with ice-cold 4% paraformaldehyde in PBS and paraffin sections prepared. These were dehydrated and treated with proteinase K (10 μg/ml) before washing with sterile water. Sections were then treated with terminal transferase together with biotin-14–dATP and dATP. Control sections were treated in the same way, with the exception that terminal transferase was omitted. Streptavidin–peroxidase with DAB labelling was used to detect fragmented DNA. Sections were counterstained with methyl green and coverslipped for examination.

Electron Microscopy

Tissues for electron microscopy were fixed with 2.5% gluteraldehyde in PBS for 3 h before three washes in PBS and further dissection into 1-mm cubes. Further fixation was then performed with 1% osmium in the same buffer for 1 h at 4°C. Tissues were then washed, dehydrated with graded acetones, and embedded in Epon. Ultrathin sections were collected on copper grids and stained with uranyl acetate and lead citrate prior to examination by electron microscopy using a Philips CM10 electron microscope (Zoellner *et al.*, 1996a,b).

Quantitation of Endothelial Cell Apoptosis

Lectin-labelled paraffin sections were used for quantitation of EC apoptosis. Since sections were 4 μm in width, overlap of lectin-labelled EC with nonendothelial nuclei did not pose a problem in interpretation of sections. Sections were examined at a magnification of 1000 using an oil objective with an Olympus Vanox light microscope. It was possible to reliably distinguish between apoptotic and nonapoptotic nuclear morphology in EC. Apoptotic nuclei were fragmented into small spherical nuclear particles which stained intensely and homogeneously with haematoxylin. Detachment is an early event in EC apoptosis (Araki *et al.*, 1990a; Zoellner *et al.*, 1996a,b) and this was also seen in this study, with many lectin-labelled apoptotic EC found in vascular lumina. In contrast, nonapoptotic EC had large, vesicular nuclei, which stained lightly with haematoxylin and had finely stippled chromatin. Similar morphological criteria have been used by other workers for identification of apoptotic EC (Walker and Gobe, 1987; Walker *et al.*, 1989; Lang and Bishop, 1993; Polunovsky *et al.*, 1993; Desmouliere *et al.*, 1995; Sgnoc *et al.*, 1996; Meeson *et al.*, 1996).

Fields within tissue fragments on coded slides were examined at random. Only unambiguous EC nuclei were included in cell counts, with criteria for inclusion being labelling with lectin and location in a vascular structure. All EC nuclei within a given field of view were counted and classified as either apoptotic or nonapoptotic. A minimum of 100 EC nuclei were counted per section examined and the relative percentage of apoptotic EC was calculated for each section studied. Means and standard deviations of relative percentages were calculated for quadruplicate wells, in the case of rat skin explants, or for triplicate wells in the case of human gingival explants. Wilcoxon's rank sign test was used to determine the statistical significance of differences between treatment groups over multiple experiments. Student's *t* test was applied for comparison of means of quadruplicate groups within individual experiments.

RESULTS

Serum Deprivation Induces Endothelial Apoptosis in Rat Skin Tissue Explants

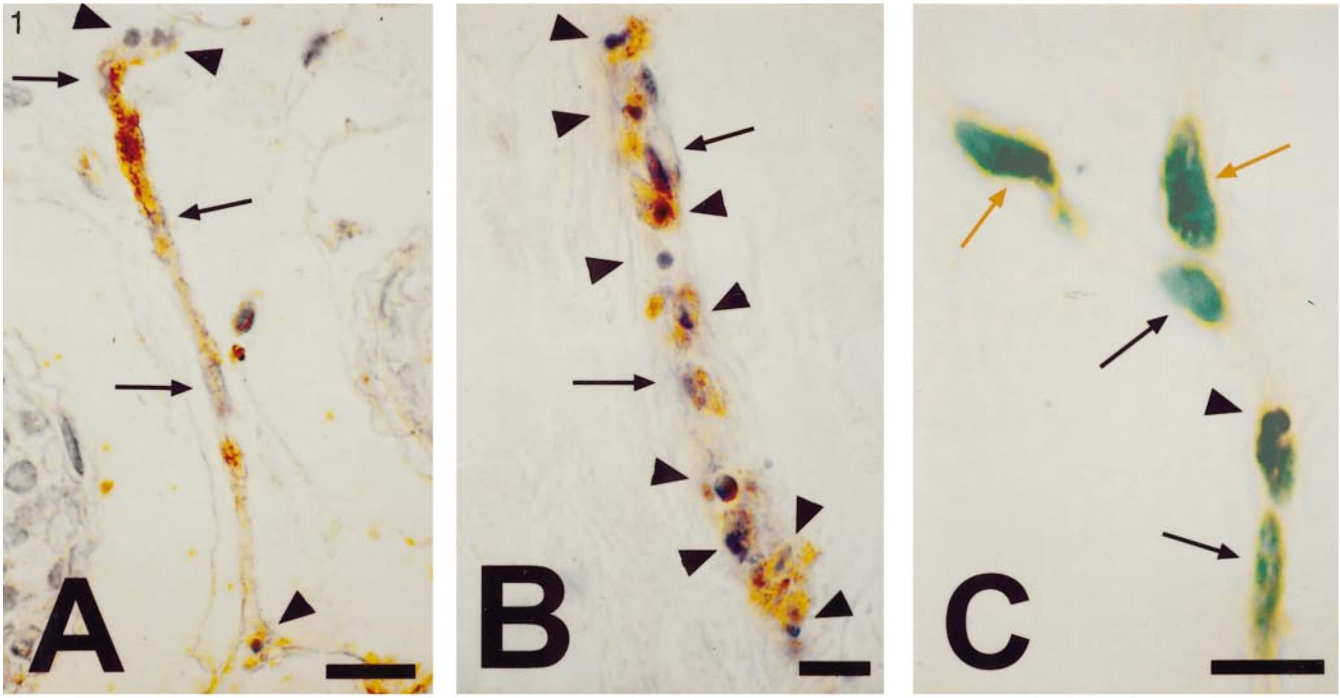
Examination of paraffin sections of rat skin explants to M199 alone for 24 h revealed the presence of morphologically apoptotic EC. These were often present as isolated lectin-positive cells with apoptotic nuclear morphology in otherwise normal vessels (Fig. 1A). In some vessels, however, almost all EC had an apoptotic nuclear morphology (Fig. 1B). TDTL demonstrated DNA fragmentation in vascular nuclear particles (Fig. 1C). These TDTL-positive particles had the same appearance as nuclear particles counted as apoptotic in lectin stained sections (Figs. 1A and 1B). The demonstration of DNA fragmentation in these particles was consistent with their identity as apoptotic EC.

Electron microscopy of rat skin explants revealed the presence of both normal and apoptotic EC. Apoptotic EC were recognised through the formation of small apoptotic particles often containing fragments of condensed nuclear material as well as canalicular fragmentation (Fig. 2). Detachment of apoptotic EC resulted in the location of many apoptotic cells within the lumina of blood vessels. The vascular location of these detached cells was often confirmed through the presence of erythrocytes (Fig. 2) and occasional leukocytes admixed with apoptotic particles. Also, nonapoptotic EC were often present, clearly defining the luminal wall and often displaying the presence of multiple transcytotic vesicles typical of normal microcirculatory EC. Further supporting an apoptotic rather than necrotic origin for these particles is that putative apoptotic fragments had intact organellar structures including RER, mitochondria, and Golgi apparatus (Fig. 2). There was, however, evidence for secondary necrosis in some apoptotic EC which varied greatly in extent in different apoptotic particles. In some cells, the only sign of secondary necrosis was disruption of mitochondrial integrity, but with retention of cristae (Fig. 2B). Occasionally, however, there was complete loss of cytoplasmic contents from apoptotic particles, indicating late secondary necrosis. Fibroblasts, epithelial cells, smooth muscle cells, pericytes, and mast cells were identified and did not reveal the presence of either apoptosis or necrosis. These cells as well as both apoptotic and nonapoptotic EC did, however, occasionally contain lipid vacuoles suggestive of reversible cell injury. These ultrastructural observations verify the apoptotic status of cells seen in paraffin sections as well as the endothelial origin of these apoptotic particles.

Serum deprivation greatly increased the number of

FIG. 1. Paraffin sections of apoptotic EC in rat skin explant cultures deprived of serum and albumin. Sections were stained by peroxidase histochemistry for BS lectin binding (A, B) and TDTL (C). EC are BS positive as evidenced by the red-brown stain. Apoptotic cells present as dense, round, haematoxiphilic particles (arrowheads), while nonapoptotic EC have more pale elongated nuclei (arrows) (A, B). EC with an apoptotic nuclear morphology are mostly detached and located within the vascular lumen. In most vessels, only isolated apoptotic EC were found (A); in some vessels, however, almost all EC were apoptotic (B). DNA fragmentation is demonstrated as red-brown stain in small rounded particles of nuclear material by TDTL (arrow head) (C). This contrasts with the methyl green counterstain of nonapoptotic EC (black arrows). In some cells without an apoptotic nuclear morphology, isolated areas of TDTL labelling are seen (orange arrows), suggesting an early stage of apoptosis. Bar for A is 20 μm; bars for B and C are 10 μm.

FIG. 3. Light micrographs demonstrating penetration of biotin-labelled BSA into rat skin (A) and human gingival (C) explant cultures by streptavidin–peroxidase histochemistry. Controls consisted of rat skin (B) and gingival tissues (D) which were incubated with unlabelled BSA. Biotin-labelled BSA presented as red-brown stain throughout the connective tissues (CT), demonstrating the ability of Alb to penetrate into both rat skin and gingival explant cultures. Biotin-labelled BSA was largely excluded by the epithelium (Ep) and rat skin muscle fibres (M) (A). Basal epithelial cells of the gingival epithelium as well as of rat hair follicles and epidermis also contained biotin (arrows), while label was detected between individual epithelial cells in gingival tissues (arrow heads) (C). Bars are 60 μm.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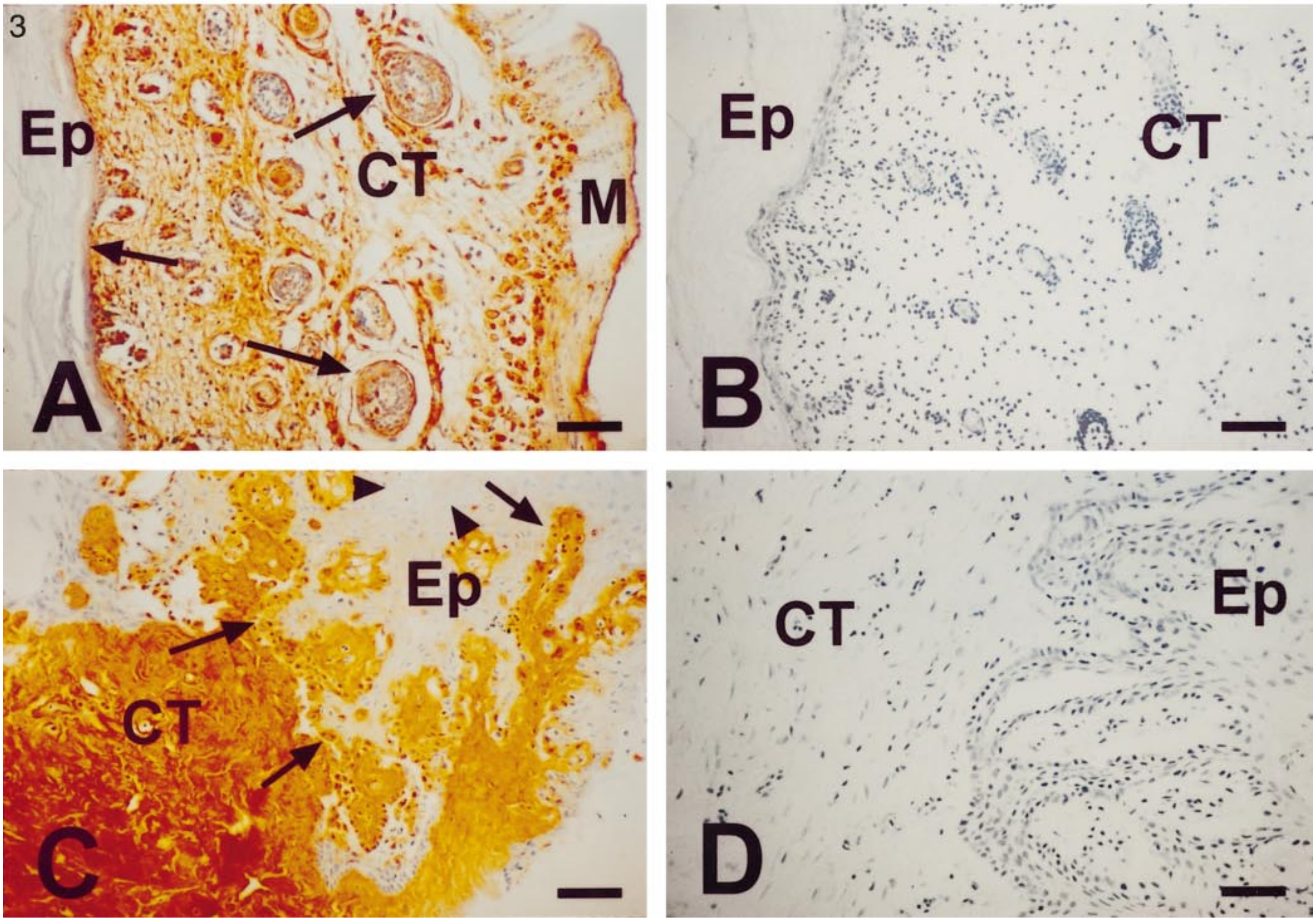
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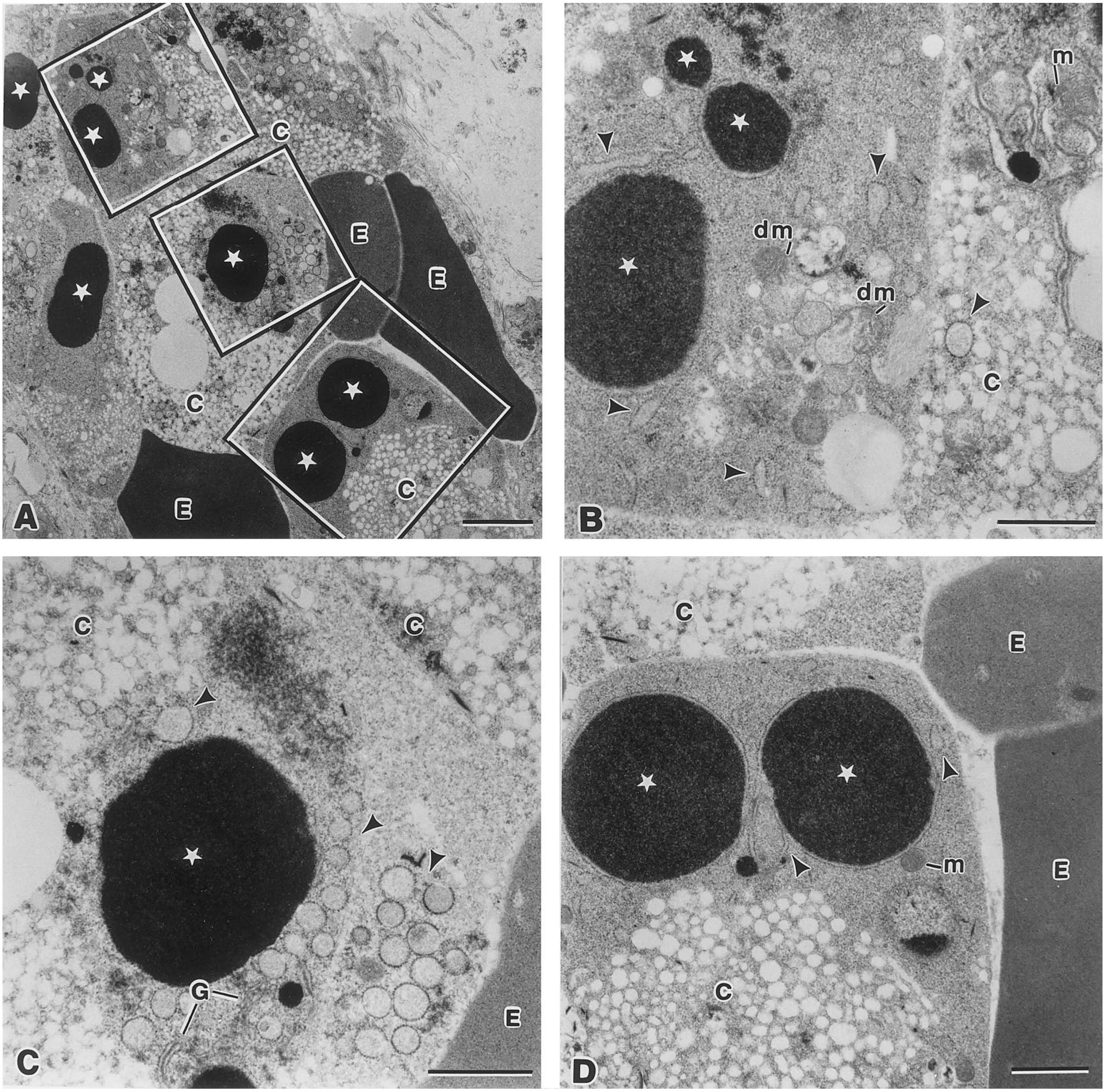

FIG. 2. Electron micrographs of an apoptotic vessel in a rat skin explant culture deprived of serum and albumin. Fragmented condensed nuclear material is seen in apoptotic particles (stars) within the vascular lumen. Canalicular fragmentation (C) and erythrocytes (E) support interpretation of this as a vascular structure with apoptotic EC (A, B, C, D). Intact RER (arrowheads), mitochondria (m), and Golgi (G) are present in cellular fragments (B, C, D). Some mitochondria are disrupted, although cristae are intact (dm) (B). These changes are typical of apoptosis in EC. Bar for A is 1 μm; bars for B, C, and D are 0.5 μm.

EC in rat skin explant cultures with an apoptotic nuclear morphology (seven experiments, $P < 0.02$). There was considerable variation between individual experiments in the relative percentage of apoptotic EC in tissues treated with M199 alone ranging from 22 ± 5 to 86 ± 8% with the median value being 78 ± 9%. In all experiments, however, serum (20%) significantly reduced the relative percentage of apoptotic EC, with levels of apoptosis ranging from 6 ± 1 to 24 ± 8% and with a median value of 16 ± 4%. On the basis of these data, it was concluded that serum deprivation induces EC apoptosis in rat skin explants.

Serum Albumin Penetrates into the Connective Tissues of Tissue Explants

Penetration of biotin-labelled BSA was seen in both rat skin and human gingival explant cultures (Fig. 3). Biotin label was found throughout connective tissues, but was largely excluded by the epithelium. Where biotin label was in the epithelium, it was confined to spaces between epithelial cells (Fig. 3C) or within the basal cell layer (Figs. 3A and 3C). Control sections (Figs. 3B and 3D) verified that labelling was due to penetration of tissues by biotin-labelled BSA and did not reflect endogenous peroxidase activity or background streptavidin–peroxidase binding.

Albumin Is a Specific Inhibitor of Endothelial Apoptosis in Serum-Free Rat Skin Tissue Explants

The results of a typical experiment with rat skin explants are shown in Fig. 4. Both HSA and BSA at physiological concentrations (4%) inhibited EC apoptosis. This observation was made in eight separate experiments ($P < 0.01$). The possibility that this was due to contamination of native Alb preparations with serum contaminants was excluded by the observation that rHA had the same activity as native material. ME–BSA and ovalbumin were not active ($P < 0.001$), suggesting that activity was dependent upon the native configuration of the protein and not due to a nonspecific protein effect. Electron microscopy of tissues exposed to HSA and BSA revealed identical changes in tissues as seen under serum-free conditions (data not shown), with the exception that there were far fewer apoptotic EC.

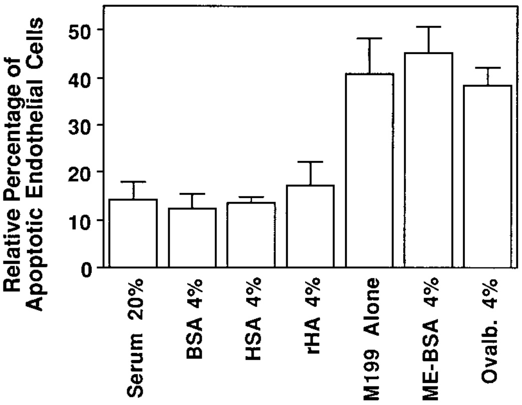

FIG. 4. The effect of serum (20%), BSA (4%), HSA (4%), rHA (4%), ME–BSA (4%), and ovalbumin (Ovalb.) (4%) compared with medium M199 alone upon the relative percentage of apoptotic EC in rat skin explant culture. Serum-free incubation of explant cultures increased EC apoptosis. This was inhibited by BSA, HSA, and rHA. ME–BSA and ovalbumin had no protective activity. These data suggest that Alb inhibits EC apoptosis in rat skin explant cultures and that this is dependent upon the native conformation of the molecule and not a nonspecific protein effect.

In Fig. 5A, it is seen that the dose response of BSA was identical to that of HSA, with maximal protection seen at physiological levels of the protein ($P < 0.001$). Similarly, Fig. 5B illustrates an identical dose response of protection for rHA as was seen for HSA with maximal protection at physiological concentrations of the proteins ($P < 0.001$). The identical dose response with different preparations of Alb further supports the conclusion that protection was not due to contaminants of Alb preparations.

Albumin Inhibits Endothelial Apoptosis in Human Gingival Explants, Similar to the Effect in Rat Skin Explants

EC in human gingival tissues displayed morphological apoptosis at both the light and the electron microscope level, with light microscopic and ultrastructural changes that were identical to those in rat skin explants being seen (data not shown). Some inflammatory cells, including lymphocytes, neutrophils, and plasma cells, were present in these specimens, reflecting low levels of

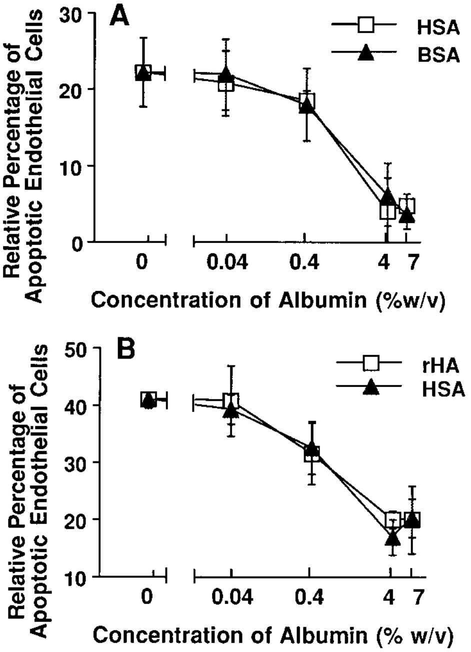

FIG. 5. The dose response of protection against EC apoptosis by HSA and BSA (A) compared with HSA and rHA (B) in serum-free rat skin explant cultures. In A, the dose response of protection was identical for both BSA and HSA and protection was maximal at physiological levels of the proteins, while in B, rHA had the same dose response as native HSA. The identical dose response of all preparations tested suggests that protection is not due to a contaminant of native Alb preparations.

chronic inflammation typical of gingival tissues. The data for human gingival tissue were similar to those obtained for rat skin explants. For example, in one experiment the following relative percentage values of apoptotic EC were obtained after stimulation with the corresponding stimuli for 24 h: M199 alone, 44 ± 5%; HSA (4%), 20 ± 1%; BSA (4%), 15 ± 4%; rHA (4%), 13 ± 5%; ovalbumin (4%), 52 ± 16%; mercaptoethanol-reduced BSA (4%), 45 ± 8%; and serum (20%), 24 ± 5%. There was specific inhibition of EC apoptosis by HSA, BSA, and rHA ($P < 0.01$) and absence of protection by ME–BSA and ovalbumin ($P < 0.05$).

DISCUSSION

Cells with the characteristic nuclear features and TDTL-labelling characteristic features of apoptosis were present in explant cultures (Walker and Gobe, 1987; Walker *et al.*, 1989; Gavrieli *et al.*, 1992; Gerschenson and Rotello, 1992; Lang and Bishop, 1993; Raff, 1993; Desmouliere *et al.*, 1995; Sgnoc *et al.*, 1996; Meeson *et al.*, 1996; Zoellner *et al.*, 1996a; Martins and Earnshaw, 1997). Lectin labelling, location in linear and luminal vascular structures, the presence of surrounding pericytes and smooth muscle cells, as well as luminal erythrocytes and leukocytes, all suggest that apoptotic particles were endothelial in origin (Cliff, 1976; Holthöfer *et al.*, 1982; Laitinen, 1987). Canalicular fragmentation appears to be a feature of apoptosis unique to endothelium (Zoellner *et al.*, 1996a), so that the canalicular fragmentation observed in this study strongly supports the endothelial origin of apoptotic particles seen. The presence of canalicular fragmentation has additional significance, as it has not been previously described in animal EC or tissues. Detachment is an early event in EC apoptosis (Araki *et al.*, 1990a; Zoellner *et al.*, 1996a,b) so that the intravascular location of apoptotic particles described in this paper is typical of EC apoptosis.

Electron microscopy is accepted as a very sensitive and specific means of determining the apoptotic status of cells, so that electron microscopy was used in this study as the principle means of verifying the apoptotic condition of endothelial cells. Lipid vacuoles were noted in some EC, pericytes, fibroblasts, and smooth muscle cells. This likely reflects sublethal cellular injury in the current study, as such lipid vacuoles are widely accepted as a consequence of reversible cellular damage. Necrosis is accompanied by detachment of ribosomes from the RER and loss of organellar integrity and plasma membrane integrity (Cotran *et al.*, 1996). These changes were not seen, suggesting that cells were apoptotic rather than necrotic. However, secondary necrosis was seen in some apoptotic particles. Early secondary necrosis was evidenced by degeneration of mitochondria with retention of mitochondrial cristae in otherwise typically apoptotic cells. This was interpreted as suggesting that degenerative changes were secondary to the onset of apoptosis rather than an early sign of necrosis. Also, necrosis is usually thought to occur simultaneously across most cells in a region of tissue (Cotran *et al.*, 1996). In this study, only isolated individual particles showed signs of necrosis, more consistent with secondary necrosis of apoptotic particles.

Explant cultures have been used by other workers (Watson *et al.*, 1995), and in this paper it is suggested that rat skin explants provide a convenient and reproducible model for the study of EC apoptosis induced by serum deprivation, with human gingival explants providing a means of verifying in human tissues observations made in rat skin experiments. Since apoptotic EC are lost into the circulation in perfused tissues, explant culture has the advantage that apoptotic EC are retained in their location, greatly assisting quantitation. Variation in baseline levels of apoptosis may reflect differences between litters of animals used in individual experiments. Similar differences are seen when studying isolated EC (Zoellner *et al.*, 1996b).

Both BSA and HSA inhibited EC apoptosis with an identical dose response and had maximal activity at physiological concentrations. Activity was not likely a nonspecific protein effect, as neither ME–BSA or ovalbumin had protective activity. Also, loss of activity following reduction by mercaptoethanol supports the suggestion that Alb contains an active site responsible for mediating the antiapoptotic effect. Since rHA had identical activity to native material, activity is unlikely to be due to contamination of Alb preparations with any as yet unidentified serum factor. If a serum contaminant was responsible for protecting EC, then the dose response would be expected to shift either left or right, dependent upon the level of antiapoptotic contaminant present. Since this did not occur, protection is unlikely to be due to a contaminant of either native or recombinant Alb preparations. These observations parallel those reported earlier for isolated cultured human EC (Zoellner *et al.*, 1996b). The current study suggests that earlier observations with isolated human EC (Zoellner *et al.*, 1996b) were not due to isolation of the cells and that animal EC respond to Alb in a fashion similar to that of human cells.

The rationale for investigating the possible role of Alb in regulating EC apoptosis has been that plasma factors may signal EC regarding their location in perfused blood vessels, with poorly perfused vessels being lost by EC apoptosis (Zoellner *et al.*, 1996b). The greatly increased EC apoptosis seen in serum-free explants in this study is consistent with the proposed importance of serum factors as antiapoptotic agents for EC.

Apart from chemical plasma factors, shear stress is also suggested as an important functional signal inhibiting EC apoptosis (Dimmeler *et al.*, 1996; Kaiser *et al.*, 1997). One difficulty, however, with acceptance of shear stress as a critical antiapoptotic factor is that isolated EC are readily cultured in the absence of fluid flow. If shear stress was critical for EC survival, it would be expected that EC would undergo apoptosis rather than proliferate under normal culture conditions. Further undermining an important role for shear stress is the observation in the current study that EC in tissue explants are protected from apoptosis by serum and Alb in the absence of tissue perfusion. Because of this, it is argued that chemical plasma factors are likely to be more important in regulation of EC apoptosis than shear stress.

Alb appears to be the only currently defined plasma factor which inhibits EC apoptosis at physiological concentrations. The mechanism through which Alb may exert this effect is unclear. It seems unlikely to involve a well-described high-affinity receptor for Alb (Schnitzer *et al.*, 1988; Peters, 1996) as the high levels of Alb required for antiapoptotic activity are inconsistent with high-affinity binding. Alternative possibilities are that Alb interacts with a low-affinity antiapoptotic receptor or perhaps neutralizes an EC-derived proapoptotic factor. Further work is required to define the mechanism responsible for this activity.

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