



## REGULAR ARTICLE

# Fibrinolytic Proteins in Apoptotic Human Umbilical Vein Endothelial Cells

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## Abstract

Endothelial cells express fibrinolytic proteins including: urokinase (u-PA) and tissue type (t-PA) plasminogen activators, type-1 (PAI-1) and 2 (PAI-2) plasminogen activator inhibitors, and u-PA receptor (u-PAR). Apoptotic endothelial cells detach, potentially forming both local and circulating microthrombi in vivo. In this article, apoptotic human umbilical vein endothelium was obtained by serum starvation and compared with nonapoptotic cells rescued from death with fresh medium containing serum. Antigen levels for t-PA, PAI-1, PAI-2, and u-PAR were reduced greatly in apoptosis ( $p < 0.05$ ). In contrast, u-PA levels were similar in apoptotic as compared with rescued cells ( $p < 0.05$ ). Radioactive amino acids were used to determine absolute levels of protein synthesis and degradation in these cells. Reduced antigen levels likely were due to proteolysis as there was 98% total protein degradation and very little protein synthesis in apoptotic endothelial cells. Also, u-PA levels in apoptotic

endothelial cells were not affected by the protein synthesis inhibitor cycloheximide. Endothelial cells in inflammatory sites are exposed to cytokines, which increase both apoptosis and u-PA levels. Data from this article support the idea that maintained u-PA levels in apoptotic endothelium may protect from micro-thrombosis in inflammatory sites as well as in the circulation. © 1998 Elsevier Science Ltd.

*Key Words:* Endothelium; Apoptosis; Fibrinolysis; Microthrombosis

Apoptosis is a mechanism for the removal of excess cells from the body. During apoptosis, cells fragment into small apoptotic bodies often containing condensed nuclear material. The integrity of the plasma membrane is maintained as is that of organelles. Biochemically, internucleosomal cleavage of DNA is the most widely accepted marker for apoptosis. Apoptosis is thought to be an active process and often requires protein synthesis. The net effect of apoptosis is to produce small cellular fragments, which are phagocytosed by surrounding cells. This differs from necrosis that occurs when homeostasis has failed. In necrosis apoptotic bodies do not form, plasma membrane and organellar integrity are lost and DNA is cleaved randomly [1–4]. In vivo, apoptosis occurs in only occasional cells throughout the tissue, with apoptotic particles being phagocytosed by adjacent cells. Because of this, apoptotic particles in vivo rarely persist long enough to lose

*Abbreviations:* CM, conditioned medium; CL, cell lysate; ECGS, endothelial cell growth supplement; HUVEC, human umbilical vein endothelial cells; PAI-1, plasminogen activator inhibitor type-1; PAI-2, plasminogen activator inhibitor type-2; SCS, supplemented calf serum; t-PA, tissue type plasminogen activator; u-PA, urokinase plasminogen activator; u-PAR, urokinase plasminogen activator receptor.

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homeostatic balance. In culture, however, the relative number of apoptotic particles is the culture medium is often too high for nonapoptotic cells to remove the debris before loss of homeostasis. This results in “secondary necrosis” of apoptotic particles and is recognized readily by electron microscopy [4].

Fibrinolysis is effected by the proteinase plasmin, which is generated by proteolytic activation of inactive plasminogen. This activation step is mediated by plasminogen activators including tissue type plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA). Endothelial cells synthesize both u-PA and t-PA. Also, endothelial cells synthesize the plasminogen activator inhibitors 1 (PAI-1) and 2 (PAI-2), which are recognized as the major natural inhibitors for both t-PA and u-PA [5–9]. Endothelial cells also express the receptor for urokinase (u-PAR) and so are able to focus fibrinolytic activity to the cell surface [10,11]. Some of these fibrinolytic proteins are thought to have activities beyond the regulation of plasmin generation. For example, u-PAR serves as an adhesion molecule for vitronectin, and this is inhibited by specific cleavage of u-PAR by u-PA [12]. Also, a role in smooth muscle cell proliferation [13] and leukocyte chemotaxis [14] has been suggested for these two proteins. In addition, PAI-2 has been suggested as having a role in the regulation of apoptosis [15,16].

Under resting conditions, endothelial cells have profibrinolytic activity, while upon stimulation with cytokines interleukin-1 or tumour necrosis Factor- $\alpha$ , PAI-1 and PAI-2 levels increase relative to other fibrinolytic proteins thus reducing the fibrinolytic potential of the cells [6]. In addition, there is increased u-PA and u-PAR expression by proliferating and migrating endothelial cells [17,18].

Endothelial cell apoptosis has been observed in several remodelling tissues, including the parotid gland, the mammary gland, fibrotic lungs, and skeletal muscles in hypertension [19–23]. Cultured human umbilical vein endothelial cells (HUVEC) undergo apoptosis when deprived of serum or adhesion [24–27]. Endothelial apoptosis differs from that of other cells in that the detachment that occurs during apoptosis may result in shedding of apoptotic particles into the circulation to form potentially microembolic particles. Earlier work demonstrated an unusual mechanism for mechanical fragmentation of apoptotic human endothelial cells

through the formation of canalicular structures. In canalicular fragmentation, long branching canaliculi form from invaginations of the plasma membranes of apoptotic cells. These eventually “honeycomb” apoptotic particles, making them very fragile and liable to mechanical disruption, releasing cytoplasmic contents into the surrounding medium. This observation was interpreted as suggesting a specific adaptation to prevent microembolism by detached apoptotic endothelium [28]. Also, it has been reported that there is increased tissue factor expression in endothelial cells undergoing apoptosis [29].

In view of the capability of endothelial cells to either inhibit or promote fibrinolysis, the regulation of fibrinolytic proteins in inflammatory lesions and the possible role of fibrinolytic proteins beyond the activation of plasminogen, it was the aim of this study to analyze the changes in the fibrinolytic system in endothelial cells during apoptosis. Since there is great variation in base-line levels of fibrinolytic proteins between HUVEC from different donors [6–8], it was first necessary to develop a method for the generation of apoptotic and nonapoptotic populations of HUVEC from the same starting population. This is also described in this article.

## 1. Materials and Methods

### 1.1. Materials

Tissue culture flasks, 12-well culture plates and 6-well culture plates were obtained from Corning (Corning, NY). Supplemented calf serum (SCS) was purchased from Hyclone (Logan, UT) and Heparin was donated by Hoffman LaRoche (Basel, Switzerland). The antibiotics penicillin, streptomycin, and fungizone were from Seralab (Crawley Down, Sussex, England). Endothelial cell growth supplement (ECGS) was prepared by the method of Maciag et al. [30] Na Laurylsarcosine from Serva (Heidelberg, Germany), proteinase K and RNAase A (Boehringer Mannheim, Mannheim, Germany) were used in the preparation of DNA. Total protein synthesis and degradation in cells was studied using the tritiated radioactive amino acid mix available from Amersham to label proteins. All other reagents

used in this study were purchased from Sigma (St. Louis, MO).

### 1.2. Preparation of Endothelial Cells

HUVEC were obtained by collagenase digestion from umbilical cords [6,31]. HUVEC were cultured on gelatin culture plates in a growth medium of M199 with 20% SCS, with additional ECGS (50  $\mu\text{g/ml}$ ), and heparin (30U/ml). Penicillin (100 U/ml), streptomycin (100  $\mu\text{g/ml}$ ), and fungizone (2.5  $\mu\text{g/ml}$ ) were used as antibiotics. Experiments were with cells from third to fifth passage. HUVEC from six separate donors were used in these experiments.

### 1.3. Verification and Quantitation of Apoptosis

Electron microscopy and DNA gel electrophoresis were used to confirm the apoptotic status of HUVEC. DNA from apoptotic cells has a ladder appearance in gel electrophoresis. This was performed using a modification of the method of Smith et al. [32]. Methodology for both DNA gel electrophoresis and electron microscopy are described in detail elsewhere [27,28].

Since apoptotic HUVEC rapidly lose adhesion [24,25,27], it is possible to quantitate endothelial apoptosis in cultures by counting remaining adherent cells in triplicate wells by using a haemocytometer. Data are expressed in terms of multiples of  $10^5$  cells per well from triplicate wells counted. Earlier work has determined that the contribution of cell division to apoptosis levels determined in this way is negligible [27].

### 1.4. Preparation of Conditioned Medium and Cell Lysates for Determination of Antigen Levels

Experiments were with confluent HUVEC in 12- or 6-well tissue culture plates. Cells were washed with M199 prior to serum starvation for 12 hours. Detached apoptotic cells were then harvested by washing with M199. The remaining adherent cells were rescued from further apoptosis by culture with fresh M199 containing SCS (20%) while the apoptotic cells were resuspended in an equivalent volume of the same medium. Cells were then incubated for up to 24 hours following rescue.

Conditioned medium (CM) of both rescued and apoptotic HUVEC was obtained by transfer of medium to Eppendorf tubes and gentle centrifugation. CM was then decanted into fresh tubes for storage. Cell lysates (CL) were of M199 washed adherent rescued HUVEC or M199 washed pellets of apoptotic HUVEC. CL were prepared by resuspending cells in PBS containing TX-100 (0.1%). Samples were stored frozen at  $-70^\circ\text{C}$ .

Specific sandwich ELISAs were used to determine antigen levels for PAI-1, u-PA, t-PA (Technoclone, Vienna, Austria), PAI-2, and u-PAR (Biopool, Upsala, Sweden) [6,33,34]. Rescued and apoptotic cell number were determined in parallel triplicate wells by cell counts [27]. Antigen levels are expressed in  $\text{ng}/10^5$  cells/ml.

### 1.5. Determination of Total Protein Degradation and Synthesis

To compare protein degradation between apoptotic and rescued HUVEC, cells in triplicate wells were grown to confluence in growth medium containing radioactive amino acid mix (3  $\mu\text{Ci/ml}$ ). Monolayers were then washed with M199 and challenged with serum-free M199 also containing radioactive amino acids at 3  $\mu\text{Ci/ml}$  for 12 hours. Detached cells were then harvested. Apoptotic and adherent cells were then washed and cultured for 24 hours with M199 20% SCS, a medium rescuing the remaining adherent cells. Levels of TCA precipitable radioactivity were determined for both apoptotic and rescued HUVEC at the time of rescue as well as 24 hours after rescue.

To study protein synthesis, triplicate wells of rescued and apoptotic populations were obtained as described above with the difference that cells were not exposed to radioactive amino acids during growth or serum deprivation. However, upon harvesting apoptotic HUVEC, 3  $\mu\text{Ci/ml}$  of radioactive amino acids was added to the serum supplemented M199 during the “rescue phase” of the experiment to both nonapoptotic rescued HUVEC and apoptotic HUVEC. TCA precipitable radioactivity was determined after 24 hours of culture. Cell counts of parallel triplicate wells confirmed rescue of cells and also determined the number of apoptotic cells in the experiment.

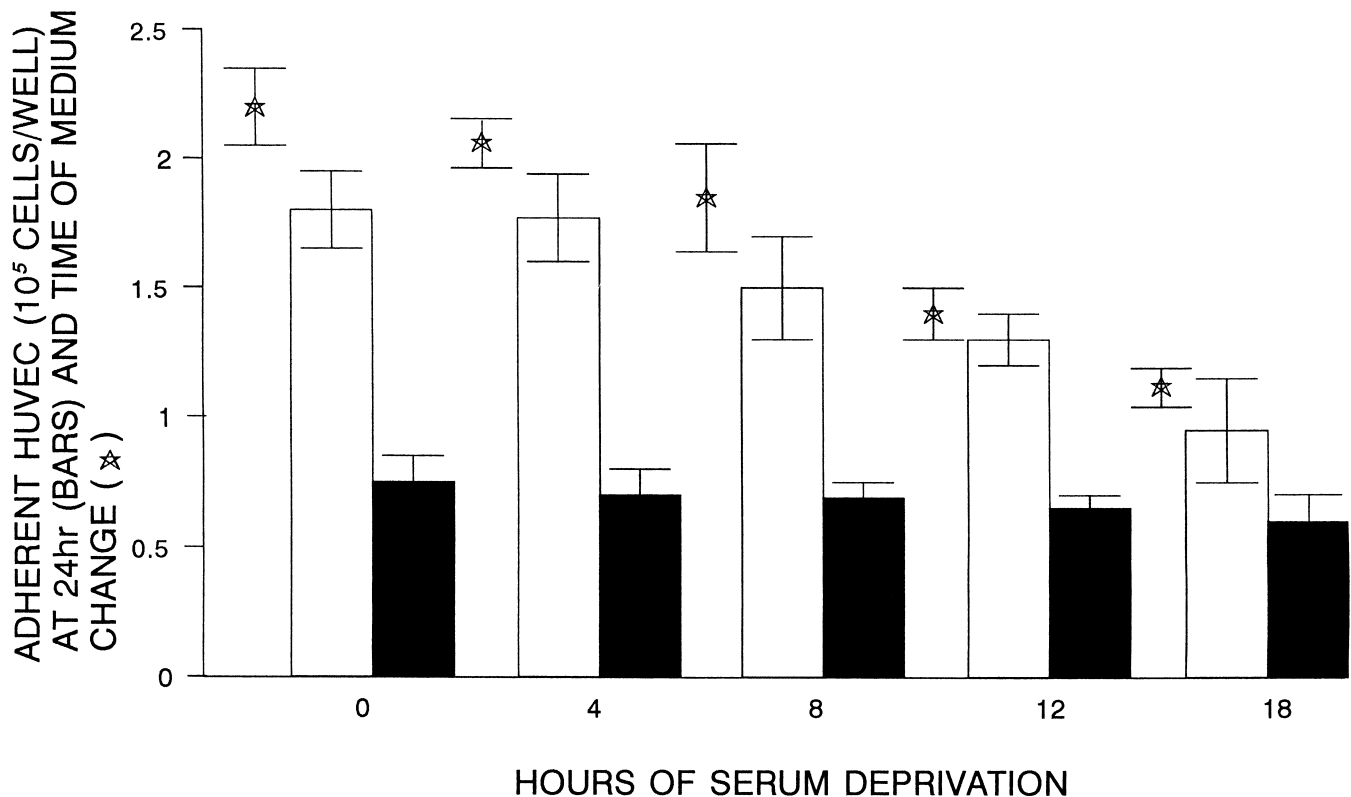


Fig. 1. Rescue by serum from further loss of adhesion in serum starved HUVEC. HUVEC were deprived of serum for from hour 0 to hour 24 before washing and treatment with either M199 (black bars) or M199 with 20% SCS (open bars). Stars indicate the number of cells present at the time that cells were washed and indicate the steady reduction in cell number over time by serum starvation. For example, the star at hour 0 indicates the number of cells before serum starvation, while stars at 4, 8, 12, and 18 hours each indicate the number of cells remaining after these periods of serum starvation, respectively. Bars indicate the cell number present at the 24-hour time point in cultures washed at the indicated time points and provided with fresh medium with (open bars) or without (black bars) 20% SCS. It is clear that serum was able to rescue almost all remaining HUVEC at each time point tested, regardless of how much apoptosis had occurred (open bars). In contrast, when serum starved cells were provided with fresh M199 medium without SCS, there was no effect upon survival so that the same number of cells were present at the 24-hour time point, regardless of the time of medium change (black bars). Earlier work showed that cell division does not contribute significantly to cell number under these experimental conditions [11].

## 2. Results

### 2.1. Rescue of Surviving Adherent HUVEC in Serum-free Cultures from Further Apoptosis by Fresh Medium Containing Serum

Figure 1 shows that serum deprivation resulted in a steady decrease in adherent cell number. However, almost all of the remaining adherent cells were rescued from further apoptosis by providing fresh M199 with SCS (20%). Changing medium alone had no effect upon HUVEC survival (Figure 1). Electron microscopy of detached apoptotic particles revealed the nuclear fragmentation, retained

organellar structure, intact rough endoplasmic reticulum and canalicular fragmentation typical of endothelial cell apoptosis (micrographs not shown). Also, DNA gel electrophoresis revealed the ladder pattern typical of apoptosis in detached cells, while rescue of cells with serum strongly inhibited this (Figure 2). These data show that challenge of HUVEC with serum-free culture followed by rescue with medium containing serum, generates apoptotic and rescued cells from the same starting population of HUVEC.

The objective of these experiments was to establish a method to generate sufficient apoptotic and nonapoptotic HUVEC from the same starting pop-

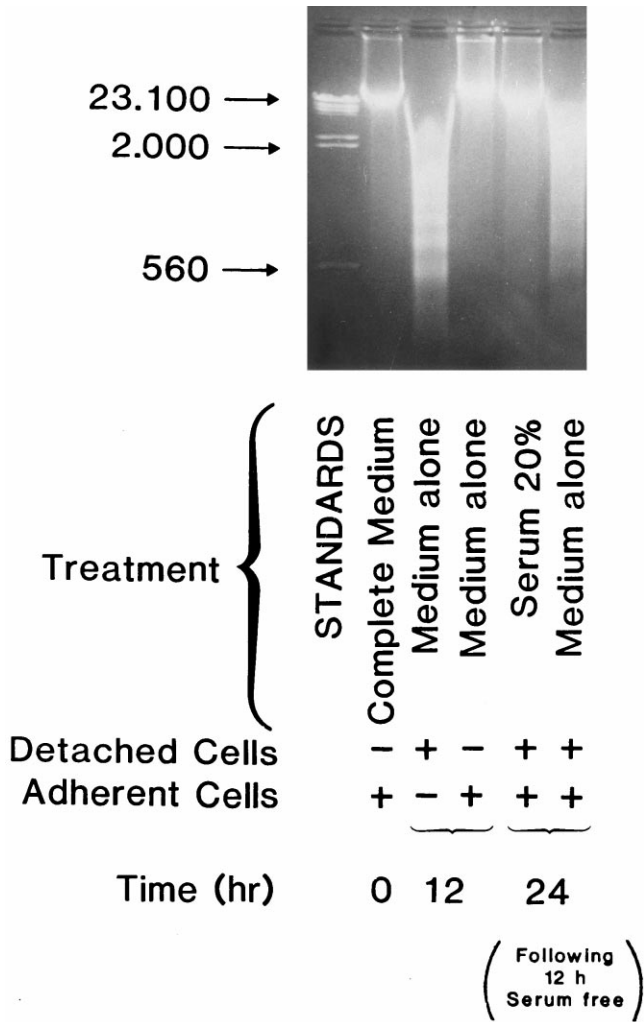


Fig. 2. Ethidium bromide stained agarose gel electrophoresis of DNA of rescued and apoptotic HUVEC. The intact large molecular weight DNA of nonapoptotic adherent HUVEC is shown in the starting cell population (hour 0). After 12 hours of serum starvation with M199 alone (medium alone), detached cells showed the DNA laddering typical of apoptosis while the remaining adherent cell population contained mostly high molecular weight DNA. Remaining adherent cells were treated for a further 12 hours with either M199 containing 20% SCS (serum 20%) or M199 alone (medium alone). Total DNA from these two populations was applied to the gel from pooled adherent and any floating cells (24 hours). Serum rescued the cells from further apoptosis as evidenced by greatly reduced DNA laddering as compared with M199 alone. This experiment coupled with that illustrated in Figure 1 indicates that serum starvation followed by rescue with fresh medium and serum can be used to obtain apoptotic and rescued nonapoptotic HUVEC from the same starting population of cells.

ulation. By the 12-hour time point, there were sufficient apoptotic cells present for experimentation. To minimize the impact of secondary necrosis upon experiments, the 12-hour time point was selected for the routine preparation of apoptotic and rescued populations of HUVEC.

2.2. u-PA Levels in Apoptotic HUVEC are Comparable to Levels in Rescued Cells Despite Great Reduction in Levels of Other Fibrinolytic Proteins

Table 1 shows the result of a typical experiment comparing levels of fibrinolytic antigen in cultures of both rescued and apoptotic HUVEC at hour 0 and hour 24 after rescue with serum. u-PA was always present in the CL of rescued cells at the time of rescue. The amount of u-PA found in CL of apoptotic HUVEC at the time of rescue was comparable with that in rescued cells. Although u-PAR was detected in CL of rescued HUVEC, none was found in the apoptotic population at hour 0. From this, u-PA levels in apoptotic HUVEC were unlikely to be maintained by u-PAR binding. In some experiments, u-PA antigen levels in CL of apoptotic HUVEC reduced over the period of study, but increased to the same extent in the CM, suggesting release from the cytosol of cell associated u-PA. t-PA, PAI-1, and PAI-2 were present in cultures of rescued HUVEC, while levels of these proteins were either very low or undetectable in cultures of apoptotic HUVEC. Similar results were obtained with HUVEC from a total of six separate donors, although in one experiment, there was a slight increase in PAI-1 antigen levels in the apoptotic population over time. Inclusion of the protein synthesis inhibitor, cycloheximide (1 µg/ml) in experiments indicated that maintained u-PA levels in apoptotic HUVEC were not dependent upon protein synthesis (Table 1).

Figure 3 shows the relative percentage of total antigen present in cultures of apoptotic HUVEC as compared with that in the rescued population in experiments with cells from six separate donors. Although there was great variability in total and relative levels of fibrinolytic proteins between experiments, within each experiment the relative amount of fibrinolytic antigen in apoptotic cells as compared with rescued cells was always greater for u-PA than for other fibrinolytic proteins ( $p < 0.05$ ).

Table 1. Antigen levels of fibrinolytic proteins in apoptotic and rescued HUVEC at the time of rescue and after 24 hours of further culture

HUVEC	Time and condition	Sample	Antigen (ng/ml/10 <sup>5</sup> cells)				
			u-PA	u-PAR	t-PA	PAI-1	PAI-2
Rescued	0 Hr	CM	UD	UD	UD	UD	UD
		CL	1.2±0.1	0.6±0.1	0.4±0.1	5.4±0.1	5.0±1.1
	24 Hr	CM	UD	0.2±0.0	1.1±0.0	>500	UD
		CL	1.3±0.1	0.5±0.1	0.4±0.1	8.0±0.4	5.5±0.7
	24 Hr+Cyclo	CM	UD	0.2±0.0	UD	110±5	UD
		CL	1.1±0.2	0.4±0.1	0.3±0.0	1.9±0.1	5.2±1.3
Apoptotic	0 Hr	CM	UD	UD	UD	UD	UD
		CL	1.3±0.1	UD	0.3±0.0	UD	UD
	24 Hr	CM	UD	UD	UD	UD	UD
		CL	1.4±0.1	UD	UD	UD	UD
	24 Hr+Cyclo	CM	UD	UD	UD	UD	UD
		CL	1.4±0.2	UD	UD	UD	UD

Triplicate cultures of both rescued and apoptotic HUVEC were incubated with M199 containing 20% SCS for 0 to 24 hours with or without cycloheximide (1 µg/ml). CM and CL were prepared and antigen levels determined by ELISA. UD indicates samples where no antigen was detected. Much lower total antigen levels of u-PAR, t-PA, PAI-1, and PAI-2 are present in apoptotic cells as compared with rescued adherent HUVEC. u-PA levels are similar in apoptotic cells as compared with rescued HUVEC. Cycloheximide did not affect u-PA levels although levels of PAI-1 and t-PA were reduced.

Also, in the case of u-PAR, t-PA, PAI-1, and PAI-2, total amounts of antigen were always less in apoptotic cells as compared with rescued cells ( $p < 0.05$ ). Although PAI-2 levels reduced to a greater extent in apoptosis than did u-PA levels, the reduction in PAI-2 was less extreme than that in PAI-1, t-PA, and u-PAR in experiments with cells from five out of six donors. Data suggest that u-PAR, t-PA, PAI-1, and to a lesser extent PAI-2 undergo degradation during HUVEC apoptosis.

### 2.3. HUVEC Apoptosis is Accompanied by Significant Protein Degradation

Table 2 shows that apoptotic HUVEC contained only 1.8% of the labeled protein present in the rescued population ( $p < 0.001$ ) immediately following serum starvation. This extensive protein degradation may have contributed to the low levels of u-PAR, t-PA, PAI-1, and PAI-2 seen in apoptotic HUVEC relative to rescued cells in seen in Table 1 and Figure 3. Over the 24 hours of incubation following the separation of apoptotic from rescued cells, however, protein degradation in rescued cells was greater than that seen in apoptotic cells ( $p < 0.05$ ) suggesting loss of degradative mechanisms in advanced stages of apoptosis. Protein synthesis by apoptotic HUVEC was considerably less than in rescued cells ( $p < 0.001$ ; Table 2).

### 3. Discussion

The increased apoptosis found in HUVEC under serum-free conditions was confirmed in this study as was the detachment of apoptotic HUVEC from the culture surface [24,25,27,28]. These cells displayed the classic ultrastructural hallmarks of apoptosis including: fragmentation into apoptotic bodies, nuclear fragmentation, and retention of organellar and plasma membrane integrity [1–4, 27,28]. In contrast to these apoptotic cells, very few rescued cells had the morphological and ultrastructural characteristics of apoptosis, and it is assumed that these few cells represent the basal rate of apoptosis in the presence of serum reported elsewhere [24,27,28]. The “laddering” of DNA widely accepted as a biochemical marker for apoptosis [1–3,24–28] corresponded with the morphological and ultrastructural changes. Because of the early loss of attachment by apoptotic HUVEC, it was possible to separate the apoptotic from the adherent cell population containing very few apoptotic cells and to study the two populations separately.

The rapid loss of adhesion by apoptotic HUVEC permits accurate quantitation of the extent of apoptosis in serum-free cultures by performing cell counts of the remaining adherent cell population [27]. The contribution of newly divided cells to

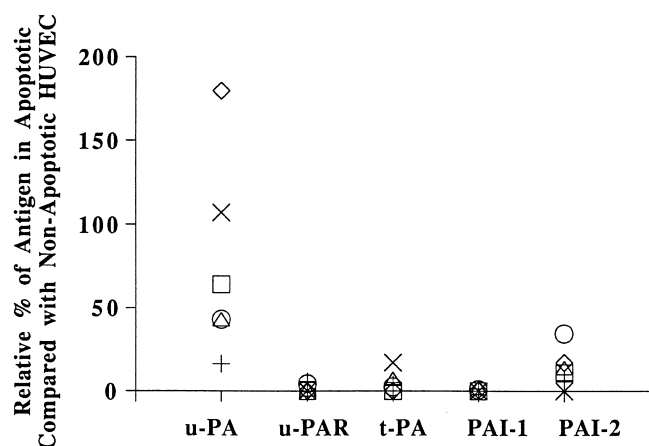


Fig. 3. Total fibrinolytic antigen levels in cultures of apoptotic HUVEC relative to rescued HUVEC. The symbols represent values for HUVEC derived from six individual umbilical cords. Total antigen levels in rescued HUVEC cultures were assigned a value of 100% so data shown are percentage values of antigen from apoptotic cells of the same donor relative to rescued cells. Where no antigen was detected in apoptotic cultures, the value of "0" was assigned. Relative levels of u-PA in cultures of apoptotic cells were always higher than for other fibrinolytic proteins within the same experiment. In most experiments, PAI-2 appeared to be partially protected in a similar way to u-PA, but this protection was less complete than for u-PA. Other fibrinolytic proteins had either undetectable or comparatively low levels of expression in apoptotic cells compared with rescued HUVEC.

the adherent cell number was minimal, as cultured HUVEC require additional growth factors for cell division and these agents were not present during serum starvation or rescue. The minimal contribution of cell division to final cell number in these experimental conditions has been established in earlier work, where dividing cells were quantitated

by using the cell cycle inhibitor nocodazole [27]. Most importantly for the current study was the observation that adherent serum starved HUVEC could be rescued from further apoptosis through the provision of fresh medium with serum. This was reflected by the morphology, ultrastructure, intact DNA, and adherent cell number of rescued HUVEC cultures. Microcirculatory remodeling through endothelial apoptosis is observed to occur preferentially in poorly perfused microvessels [19]. It has been suggested that endothelial cells exploit fluid flow as a signal for survival, so that only those vessels which are well perfused survive while functionally inactive vessels are lost by apoptosis [19,27]. Serum deprivation and laminar shear stress have both been suggested as factors, which may signal vascular perfusion to endothelial cells in vivo [35,27]. From this, it is argued that serum deprivation represents a biologically relevant means of producing apoptotic endothelium for study. Since the objective of this study was to compare apoptotic with nonapoptotic HUVEC and the fibrinolytic profile of HUVEC is otherwise well described in the literature [5–11], no assessment of fibrinolytic proteins in unchallenged HUVEC was performed in this study.

Rescue of remaining adherent cells in cultures of serum starved HUVEC occurred following up to 18 hours of serum starvation, so that the standard 12-hour period of serum starvation routinely used to obtain apoptotic and rescued populations was well within the limit of tolerance of the experimental system. Accurate determination of both apoptotic and rescued HUVEC number was possible by performing cell counts throughout experiments [27] so that antigen levels could be related to cell

Table 2. Protein levels, degradation, and synthesis in rescued adherent as compared with apoptotic HUVEC

HUVEC	Counts per minute/10 <sup>5</sup> cells			
	Cells labeled before separation of rescued from apoptotic cells			Labeled after separation of rescued from apoptotic cells
	0 hour at separation	24 hours after separation	Net reduction (CPM/%)	24 hours after separation
Rescued	55500±400	47000±900	-10500/17%	32800±1800
Apoptotic	960±20	860±60	-100/11%	227±3

Counts of TCA precipitates are corrected for cell number. Both rescued and apoptotic cells were washed prior to counting at hour 0, while data from both 24-hour counts are of pooled CM and CL. At hour 0, the apoptotic population had much less labeled protein than the rescued population ( $p < 0.001$ ), suggesting massive proteolysis during apoptosis.

number and sample volume. The 12-hour time point was selected for separation of apoptotic and rescued populations, as this was the earliest time at which sufficient numbers of apoptotic cells could be obtained for experimentation. It was found that attempts to obtain increased numbers of apoptotic particles by prolonging serum starvation beyond 12 hours was counterproductive, as apoptotic fragments started to undergo secondary necrosis as evidenced by electron microscopy.

There are significant quantitative differences in the levels of fibrinolytic and other proteins present in cultures of HUVEC from different donors [6,7], and this was seen in the current study. This donor variability poses problems in the interpretation of experiments with cells from single donors. One approach to this would be to pool HUVEC from different donors for experimentation. However, HUVEC from different donors also differ in their rate of proliferation resulting in the overgrowth of cells from some donors. The view taken in this article is that separate experiments should be performed with cells from separate donors, and that the inter-donor variability may reflect differences of biological importance within the population.

Although the levels of u-PA in apoptotic HUVEC cultures varied from donor to donor, the amount of u-PA in apoptotic HUVEC relative to that in the rescued population was always higher than for other fibrinolytic proteins. This did not seem to be related to protection of u-PA by binding to the apoptotic cell surface via its receptor, as u-PAR levels did not correspond to those of u-PA. The possibility can not be discounted, however, that there are changes in u-PAR during endothelial apoptosis, which may obscure the presence of u-PAR in ELISA assays. This seems unlikely, however, as in some experiments, u-PA antigen appeared to leach out of apoptotic HUVEC into the CM. This would be more consistent with presence of u-PA in the cytosol of apoptotic HUVEC, separate to u-PAR. This would also be consistent with the earlier observation of canalicular fragmentation in apoptotic HUVEC, a process that results in the release of intracellular material [28]. Also, maintained u-PA levels in apoptotic HUVEC did not seem to be due to new protein synthesis in the apoptotic population as the protein synthesis inhibitor cycloheximide did not affect u-PA levels in apoptotic HUVEC. An inconsistent but similar

observation was made with PAI-2, which was less severely reduced in amount in apoptotic as compared with rescued cells than were PAI-1, t-PA, and u-PAR antigens.

When comparing absolute protein levels in the apoptotic population with that in the rescued population immediately after serum starvation, apoptotic cells contain less than 2% of the protein present in the rescued population. This suggests that detachment of the cells is accompanied by massive proteolysis early in HUVEC apoptosis, but that once detached, comparatively little protein degradation occurs in the apoptotic cells. This conclusion is consistent with changes in antigen levels seen in the current study. Most fibrinolytic proteins were present at very much lower levels in apoptotic HUVEC as compared with rescued cells, and these proteins may have been lost by proteolytic degradation. However, once detached, further degradation of fibrinolytic proteins was minimal, perhaps reflecting loss of the proteolytic apparatus during the earliest phase of apoptosis. u-PA and to a lesser extent PAI-2 appeared to be comparatively protected from degradation during the detachment phase of apoptosis.

Despite the proteolysis, however, some new protein was made by the apoptotic cells as evidenced by the incorporation of radioactive amino acids and the small increase in PAI-1 antigen in apoptotic HUVEC from one donor. The amount of protein made by apoptotic HUVEC was significantly less than that in the rescued population, reflecting a general loss of synthetic capacity by the apoptotic fragments. This protein synthesis emphasises the active nature of apoptosis in HUVEC.

In vivo, apoptotic bodies are phagocytosed by adjacent cells, but in the current model system, apoptotic HUVEC are permitted to continue degradation to the point where they may lose homeostasis and undergo secondary necrosis. This, together with canalicular fragmentation [28] may account for the observation that in some experiments, cell associated antigen appeared to leach into the CM over time.

The data shown indicate that u-PA levels in apoptotic HUVEC are maintained despite massive proteolysis, and that this is not due to compensatory synthesis of new u-PA during apoptosis or

binding to u-PAR. Endothelial cells can express significant quantities of u-PA in inflammatory lesions [36] while some cytokines induce apoptosis in endothelium [37,38].

The mechanism through which the bulk of proteolysis occurs in apoptosis is not known, so that mechanisms for protecting u-PA from this are also unclear. The biological relevance of maintained u-PA levels is difficult to ascertain; however, it is possible that maintained u-PA represents a specific adaptation in endothelium to minimize the development of micro-thrombi by apoptotic endothelial cells both at sites of apoptosis as well as in circulating endothelial apoptotic particles. This idea is consistent with the induction of u-PA by tumour necrosis factor- $\alpha$  [7], an agent that also causes endothelial apoptosis [37]. This interpretation is also consistent with the suggested role of canalicular fragmentation in minimizing the impact of apoptotic endothelial micro-emboli [28]. In this model, cytoplasmic u-PA would be released during canalicular fragmentation, facilitating fibrinolysis. Procoagulant activity is reported for apoptotic endothelium [29], although the assembly of platelet-fibrin aggregates around apoptotic endothelial particles has not been described, and this may in part be explained by maintained u-PA. An alternative possibility, is that u-PA may have a role in mediating endothelial apoptosis, either through its enzymatic activity or perhaps through nonenzymatic sites within the molecule. PAI-2 has been proposed as having an antiapoptotic role [15,16], and it is clear from the current study that this protein is reduced in amount in the apoptotic population relative to the rescued nonapoptotic population. It is tempting to speculate that the new balance established between PAI-2 and u-PA in the apoptotic population reflects a role for these proteins in the apoptotic process itself. This possibility would be consistent with the role of serine and cysteine proteinases in mediating apoptosis in other cell types [3,39]. In an earlier study of PAI-2 in adherent serum deprived HUVEC, PAI-2 was mostly cell associated and did not reduce in amount over a 12-hour period of incubation [6]. In the current study, however, PAI-2 levels 24 hours after rescue of adherent cells were usually reduced to half that present at the end of serum starvation. It is possible that this reduction in PAI-2 reflects a rebound phenome-

non, in which the anti-apoptotic activity of PAI-2 is sensed by the cell as being no longer required.

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