Upregulation of Urokinase Receptor Expression on Migrating Endothelial Cells

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Abstract. One of the phenotypic hallmarks of migrating endothelial cells, both in vivo and in vitro, is expression of the urokinase-type plasminogen activator (u-PA), a key mediator of extracellular proteolysis. In the study reported here, we have used an in vitro model of endothelial cell migration to explore the mechanism of this phenomenon. We have found that wounding of an endothelial cell monolayer triggers a marked, rapid and sustained increase in expression of a specific high-affinity receptor for u-PA (u-PAr) on the surface of migrating cells. Migrating cells displayed an increase in the levels of u-PA and u-PAr mRNAs, and this increase was mediated by endogenous basic fibroblast growth factor (bFGF). We also show that the increase in u-PA activity on migrating cells can be accounted for by an increase in receptor-bound u-PA, and that the increase in activity is also dependent on endogenous bFGF. These results demonstrate that the expression of plasmin-mediated proteolytic activity by migrating endothelial cells is a consequence of increased production of both u-PA and its receptor, and that this in turn is mediated by endogenous bFGF. This suggests that u-PA, produced at increased levels by migrating cells, binds to u-PAr whose expression is upregulated on the same cells. These observations are in accord with the postulated role of u-PAr in mediating efficient and spatially restricted extracellular proteolysis, particularly in the context of cell migration.

THE vascular endothelium consists of a highly ordered monolayer of quiescent non-migrating cells, which can be induced to migrate and replicate in a number of physiological and pathological settings. This occurs for example during angiogenesis in which new capillary blood vessels are formed from preexisting vessels in response to angiogenic stimuli. During this process, microvascular endothelial cells locally degrade their basement membrane, and subsequently invade the surrounding interstitial extracellular matrix within which they form a capillary sprout. The sprout develops into a functional vessel after formation of a lumen (reviewed by D'Amore and Thompson, 1987; Zetter, 1988).

To breach the mechanical barriers imposed by the basement membrane and surrounding extracellular matrix, endothelial cells use limited proteolytic degradation of matrix components at regions of contact with the cell surface (reviewed by Moscatelli and Rifkin, 1988; Pepper and Montesano, 1991). Plasminogen activators (PAs)[1] are key mediators in this respect; they convert the widely distributed and proteolytically inactive plasminogen to active plasmin, a protease of tryptic specificity capable of directly degrading certain matrix components and also of activating other matrix-degrading enzymes such as metalloproteases (reviewed by Saksela and Rifkin, 1988).

To mediate efficient and appropriate matrix remodelling during angiogenesis, extracellular proteolysis must be confined to the immediate pericellular environment. This is achieved in part by the spatial restriction of protease activity, which occurs through the binding of urokinase-type plasminogen activator (u-PA) to a specific high affinity cell surface receptor (Vassalli et al., 1985; Stoppelli et al., 1985), and also as a consequence of protease inhibitor production, which prevents excessive matrix destruction (reviewed by Pepper and Montesano, 1990). The human u-PA receptor (u-PAr) is a 313 amino acid residue 55,000–65,000 M_r glycoprotein linked to the cell surface by a carboxy-terminal glycolipid anchor (Behrendt et al., 1990, 1991; Roldan et al., 1990; Plough et al., 1991). u-PA binding to u-PAr occurs via an EGF-like domain in the amino-terminal of u-PA (Appella et al., 1987). The u-PAr identified on cultured endothelial cells is likewise a 55,000-M_r glycoprotein which binds the EGF-like domain of u-PA (Fibbi et al., 1988; Miles et al., 1988; Barnathan et al., 1990*a*,*b*; Mignatti et al., 1991; Haddock et al., 1991). Plasminogen/plasmin-binding sites have also been identified on endothelial cells (Bauer et al., 1984; Hajjar et al., 1986; Miles et al., 1988).

1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial; BME, bovine microvascular endothelial; CPAE, calf pulmonary artery endothelial; DCS, donor calf serum; PA, plasminogen activators; rhbFGF, recombinant human bFGF; tcu-PA, two-chain u-PA; u-PA, urokinase-type plasminogen activator.

The observations that u-PA binding sites are rapidly redistributed to the leading edge of monocytes placed in a chemotactic gradient (Estreicher et al., 1990), and that u-PAr number and affinity can be modulated by cytokines and phorbol esters (reviewed by Vassalli et al., 1991; Blasi, 1993), suggest that receptor expression and function are highly dynamic in nature. However, the important question as to whether u-PAr expression is upregulated on migrating cells has not yet been addressed. Using an in vitro model in which endothelial cells can be induced to migrate by mechanically wounding a confluent monolayer, we have previously demonstrated that u-PA activity is induced in cells migrating from the edges of such an experimental wound (Pepper et al., 1987). The studies described in this paper were performed in order to determine firstly, whether u-PAr expression is upregulated on migrating endothelial cells, and secondly, whether the increase in u-PA activity observed in these cells is due to a regional increase in u-PA synthesis, an increase in u-PA receptor expression, or a combination of both.

Material and Methods

Cell Culture

Bovine microvascular endothelial (BME) cells from adrenal cortex (Furie et al., 1984) were grown in MEM, α modification (Gibco AG, Basel, Switzerland), supplemented with 15% heat-inactivated donor calf serum (DCS; Flow Laboratories, Baar, Switzerland), penicillin (500 U/ml), and streptomycin (100 μg/ml). Cells were used between passages 15 and 20.

Bovine aortic endothelial (BAE) cells isolated from scrapings of adult bovine thoracic aortas according to the procedure of Gajdusek and Schwartz (1983) and cloned by limiting dilution as previously described (Pepper et al., 1992*a*), were cultured in DMEM (GIBCO AG) supplemented with 10% DCS, penicillin (500 U/ml), and streptomycin (100 μg/ml). Cells were used between passages 8 and 11.

Calf pulmonary artery endothelial (CPAE) cells, purchased from the American Type Culture Collection (Rockville, MD), were cultured in DMEM supplemented with 20% FCS serum (Flow Laboratories), penicillin (500 U/ml), and streptomycin (100 μg/ml). Cells were used at passage 24.

Endothelial cells were subcultured at a 1:4 or 1:5 split ratio in 1.5% gelatin-coated tissue culture dishes or flasks (Falcon Labware, Becton-Dickinson Company, Lincoln Park, NJ) or on poly-L-lysine (Sigma Immunochemicals, St. Louis, MO)-coated glass microscope slides (for in situ hybridization studies). Culture media were changed every 2–3 d, and all experimental manipulations, except those with varying cell density, were performed upon reaching confluence (5–7 d). The last medium change was always 24 h before starting the experiment.

Wound-edge Caseinolysis

Seven parallel wounds were created with a 2.0-mm-wide rubber policeman in confluent BME monolayers in 35 mm tissue culture dishes (Falcon Labware), the dish rotated through 90° and an additional set of parallel wounds created perpendicular to the first. Culture medium and detached cells were removed, the monolayers washed twice with PBS, and fresh complete medium added. 24 h later, monolayers were washed twice with PBS containing acid-treated BSA (1 mg/ml), and overlaid with a mixture containing 2% non-fat dried milk, 0.8% agar, and plasminogen (40 μg/ml) in DMEM as previously described (Pepper et al., 1987). Dishes were incubated at 37°C for 3 h and photographed under dark field illumination using a Zeiss ICM 405 inverted photomicroscope (Carl Zeiss, Oberkochen, Germany).

To determine the effect of anti-bFGF antibodies on wound-edge caseinolysis, confluent monolayers of BME cells in 35 mm tissue culture dishes were mechanically wounded with a razor blade to mark the original wound edge (Bürk, 1973), washed twice with serum-free α-MEM, and serum-free α-MEM containing 0.1% gelatin and either normal rabbit γ-globulins (200 μg/ml) or rabbit anti-recombinant human bFGF (rhbFGF) γ-globulins (200 μg/ml) (see below) added to the monolayers. Titration experiments revealed that 200 μg/ml anti-rhbFGF γ-globulins were required for almost complete inhibition of cell-associated PA activity using the overlay technique (results not shown). 24 h after wounding, monolayers were overlaid as described above, and photographed after 3 h at 37°C under dark-field illumination and phase contrast microscopy, using a Zeiss ICM 405 inverted photomicroscope.

γ-globulin fractions were prepared from normal rabbit serum or rabbit anti-rhbFGF antiserum (kindly provided by Drs. Y. Sato and D. B. Rifkin, New York University School of Medicine, New York) by two precipitations with 50% ammonium sulfate, dialyzed against distilled water or PBS and stored at −20°C. The preparation and characterization of the anti-rhbFGF antibodies has previously been described (Dennis and Rifkin, 1990).

Zymography and Reverse Zymography

Confluent monolayers of BME cells in 35 mm petri dishes were washed twice with serum-free α-MEM, and 1.5 ml serum-free α-MEM containing 200 KIU/ml Trasylol (Bayer-Pharma AG, Zurich, Switzerland) was added to each dish. Monolayers were multiple wounded as described above and the medium not changed after wounding. 15 h later cell extracts and culture supernatants were prepared as described (Vassalli et al., 1984; Pepper et al., 1990); Trasylol (200 KIU/ml) was added to cell extracts. Cell number was determined in a separate series of control and multiple-wounded dishes in parallel, and sample volume normalized accordingly. Zymography and reverse zymography were performed as previously described (Pepper et al., 1990).

To determine whether the increase in u-PA activity in multiple-wounded cultures was due to receptor-bound u-PA, multiple-wounded monolayers prepared in serum-free medium containing Trasylol (200 KIU/ml) as described above, were processed 18 h after wounding as follows: wounded monolayers were washed twice with PBS, and 1.0 ml, pH 3.0, buffer (0.1 M NaCl/50 mM glycine-HCl, pH 3.0), was added for 2 min at room temperature with gentle shaking. 500 μl, pH 7.4, buffer (0.15 M NaCl/0.5 M Hepes, pH 7.4) was added to neutralize the acid buffer, and monolayers were washed three times with PBS containing 1 mg/ml acid-treated BSA (PBS/BSA) on ice. 10 nM DFP-treated 55,000-M_r human two-chain u-PA (tcu-PA) in binding medium (serum-free α-MEM containing 20 mM Hepes, pH 7.4, 1 mg/ml acid-treated BSA, and 200 KIU/ml Trasylol) was added for 1 h at 4°C. Where relevant, a 400-fold molar excess of a peptide corresponding to the receptor-binding region of amino terminus of mouse u-PA (amino acids 13-33) (Appella et al., 1987) was added in binding medium 15 min before the addition of human tcu-PA. Monolayers were washed four times with PBS/BSA on ice and cell extracts prepared for zymography as described above.

RNA Preparation from Multiple-wounded Cultures

Approximately 50 parallel wounds were created with a 1.0-mm-wide pointed rubber policeman in confluent monolayers in 100 mm tissue culture dishes (Falcon Labware), the dish rotated through 90° and an additional set of parallel wounds created perpendicular to the first. The medium was not changed after wounding. Total cellular RNA was extracted at the indicated times according to a modification of the method of Glisin et al. (1974) as previously described (Pepper et al., 1990).

To determine the effect of anti-bFGF antibodies on wound-induced mRNA expression, two procedures were followed. In the first (designated "pre"), confluent monolayers were washed once with serum-free α-MEM and serum-free α-MEM containing 0.1% gelatin and either normal rabbit γ-globulins (200 μg/ml) or rabbit anti-rhbFGF γ-globulins (200 μg/ml) added to the monolayers. Monolayers were then multiple-wounded and the medium not changed thereafter. In the second (designated "post"), monolayers were multiple-wounded, washed once with serum-free α-MEM, and serum-free α-MEM containing 0.1% gelatin and either normal rabbit γ-globulins or rabbit anti-rhbFGF γ-globulins (200 μg/ml) added. In both cases, total cellular RNA was extracted 4 h after wounding as described above.

To determine the effect of exogenous bFGF on u-PAR mRNA expression, rhbFGF (3 ng/ml) (provided by Dr. P Sarmientos, Farmitalia Carlo Erba, Milan, Italy) was added to confluent monolayers of BME cells in 100 mm tissue culture dishes, and total cellular RNA extracted at various time points thereafter as described above.

Density and Replication Experiments

Low-density cultures of BME cells were prepared by splitting confluent

monolayers in 100 mm tissue culture dishes (~6 × 10^6 cells/dish) 1:3 or 1:6 in fresh medium. Fresh medium was added in parallel to confluent monolayers. Total cellular RNA was extracted 28 h later from low density and confluent cultures. In some experiments, hydroxyurea (5 mM) was added to 1:3 split cultures after overnight spreading and attachment, and total cellular RNA extracted 24 h later.

For inhibition of cell division in low density BME cultures, cells were seeded at 1 × 10^5 cells per gelatin-coated 15 mm well of a 24-well plate (Costar, Cambridge, MA). After overnight attachment and spreading, medium was changed and hydroxyurea (5 mM) added. 15–18 h later, [^{3}H]thymidine (2 μCi/ml) was added to each well. 4 h later, medium was removed, cells were washed with ice cold PBS, incubated on ice for 30 min in 10% TCA, washed with PBS, solubilized in 0.5 M NaOH for 30 min at room temperature, and 150-μl aliquots counted in 3 ml Aquassure scintillation fluid (DuPont, Boston, MA). Percent inhibition of the mean of duplicate cultures was calculated as 100 − (hydroxyurea − background × 100/control − background).

For inhibition of cell division in wounded monolayers, hydroxyurea (5 mM) was added to confluent monolayers in 100 mm tissue culture dishes (Falcon Labware), the monolayers were multiple wounded as described above, and the medium not changed thereafter. RNA was prepared as described above after a further 4 h.

Plasmid Construction and In Vitro Transcription

pSP64-mUK. A 625 bp PstI-HindIII fragment (positions 427-1078) of mouse u-PA cDNA clone pDB15 (Belin et al., 1985) was subcloned into pSP64 (Melton et al., 1984).

pBSbUK. A 2.5-kb pair EcoRI-XhoI fragment of bovine u-PA cDNA (Krätzschmar et al., 1993) was subcloned into pBLUESCRIPT SK (Stratagene Cloning Systems, Heidelberg, Germany).

pBSbUPAr. A 1.2-kb pair EcoRI-XhoI fragment of bovine u-PA receptor cDNA (Krätzschmar et al., 1993) was subcloned into pBLUESCRIPT SK.

pSP65-hTA. A 614-bp BglII-EcoRI fragment (positions 188-801) isolated from pW349F, a plasmid containing a 2.6-kb pair tissue-type PA (t-PA) cDNA insert (Fisher et al., 1985), was subcloned between the EcoRI and BamHI sites of pSP65 (Melton et al., 1984).

pSP64-mUK, pBSbUK, pBSbUPAr, and pSP65-hTA were linearized, respectively, with HincII, EcoRI, EcoRI, and HindIII, and used as templates for bacteriophage SP6 (pSP64-mUK, pSP65-hTA) and T7 (pBSbUK, pBSbUPAr) RNA polymerases (Melton et al., 1984). Transcription was performed exactly as described by Busso et al. (1986).

Northern Blot Hybridization

Total cellular RNA was denatured with glyoxal, electrophoresed in 1.2% agarose gels (5 μg RNA per lane), and transferred overnight onto nylon membranes (Hybond, Amersham, Arlington Heights, IL) as described by Thomas (1980). Filters were baked under vacuum at 80°C for 2 h, exposed to UV light (302 nm) for 30 s, and stained with methylene blue to reveal 18S and 28S rRNA markers for determination of RNA integrity and even loading. Prehybridation, hybridization, and posthybridization washes were performed as previously described (Pepper et al., 1990). Filters were exposed to Kodak XAR-5 films (Eastman Kodak Co., Rochester, NY) at −80°C between intensifying screens. Autoradiographs were scanned with a GenoScan laser scanner (Genofit, Geneva, Switzerland).

In Situ Hybridization

Confluent monolayers of BME or BAE cells on poly-L-lysine–coated Color Frost glass microscope slides (Menzel-Gläser, Germany) were mechanically wounded by scraping away half of the monolayer with a razor blade. The medium was not changed after wounding. Wounded monolayers were fixed 4 or 24 h after wounding in 4% paraformaldehyde in PBS for 15 min at 4°C, rinsed in PBS, and stored in 70% ethanol at 4°C until analyzed. 1-3 × 10^6 cpm of ^{3}H-labeled antisense RNA probe, prepared as described by Sappino et al. (1989), was applied to each slide in 20–70 μl of hybridization mixture. Prehybridization, hybridization and posthybridization washes were performed as previously described (Sappino et al., 1989). After graded ethanol dehydration, slides hybridized to ^{3}H-labeled RNAs were immersed in NTB-2 emulsion (Eastman Kodak Co.) diluted 1:1 in deionized water. After 1–4-mo exposure, slides were developed in Kodak D-19 developer, fixed in 30% Na thiosulfate, and counterstained in methylene blue. Monolayers were photographed under dark-field illumination and transmitted light using an Axiophot photomicroscope (Carl Zeiss).

Quantitation of in situ hybridization was performed on positive prints of dark-field images. Since it was rarely possible to mark the initial wound edge at the time of wounding, the migrating front of cells was used as the standard point of reference. The number of autoradiographic grains was counted in eight consecutive 100-μm-deep fields extending backwards from the migrating front into the remaining confluent monolayer, and the number of grains per cell in each field calculated as follows: (total number of grains − background)/number of cells in that field. Background was calculated from regions of the slide devoid of cells.

^{125}I-labeled u-PA Binding to Wounded Endothelial Cell Monolayers

Confluent monolayers of BME or BAE cells in gelatin-coated 35 mm petri dishes were wounded with a rubber policeman, dead and detached cells were removed and 2 ml of fresh complete medium added. 4 or 24 h later wounded monolayers were washed twice with PBS, and 1.5 ml, pH 3.0 buffer added for 2 min at room temperature with gentle shaking. The acid buffer was neutralized by adding 750 μl, pH 7.4 buffer, and monolayers were washed three times with PBS/BSA on ice. 10 nM ^{125}I-labeled DFP-inactivated 55,000-M_r human tcu-PA in binding medium was added for 1 h at 4°C. Where relevant, a 10–400-fold molar excess of a peptide corresponding to the receptor-binding region of the amino terminus of mouse u-PA (see above) was added in binding medium 15 min before the addition of ^{125}I-labeled u-PA. Monolayers were washed four times with PBS/BSA on ice and fixed with 3.5% paraformaldehyde in PBS for 30 min at room temperature. Fixed monolayers were washed three times with PBS, and overlaid with Ilford L4 emulsion. Autoradiographs were developed after 1 or 2 wk. Quantitation was performed on positive prints of dark-field images, and the migrating front of cells was used as the standard point of reference. The number of autoradiographic grains was counted in six consecutive 100-μm-deep fields extending backwards from the migrating front into the remaining confluent monolayer, and the number of grains per cell in each field calculated as follows: (total number of grains − background)/number of cells in that field. Background was calculated from regions of the dish devoid of cells.

For iodination of u-PA, 55,000 M_r human tcu-PA (Serone, Denens, Switzerland) was radiolabeled using iodogen (Pierce Chemical Co., Rockford, IL) and Na-^{125}I (Amersham International, Amersham, UK) as described (Vassalli et al., 1984), and was used within 6 wk of iodination. ^{125}I-labeled u-PA had a specific activity of 2.6–3.4 × 10^6 cpm/μg.

Peptide synthesis. A 21mer corresponding to amino acids 13–33 of mouse u-PA (Appella et al., 1987) was synthesized by the solid phase technique using standard Boc/benzyl strategy on a model 430A machine (ABI Inc., Foster City, CA). Purified peptide eluted as a single peak on analytical HPLC and was further characterized by fast atom bombardment mass spectrometry. The peptide was synthesized according to the correct sequence of mouse u-PA as described by Belin et al. (1985), in which amino acid number 12 of the peptide was a lysine (K) and not a leucine (L) as published by Appella et al. (1987).

Results

The Increase in PA Activity on Migrating Cells Is Mainly Due to Receptor-bound u-PA

Mechanical wounding of a confluent quiescent monolayer of endothelial cells induces cells lining the wound-edge to migrate and replicate (Sholley et al., 1977). We have previously demonstrated that u-PA activity is induced in migrating BME cells (Pepper et al., 1987). In the present studies, multiple-wounding experiments were devised to maximize the yield of protein and mRNA from migrating and replicating cells. When multiple-wounded monolayers of BME cells were overlaid 24 h after wounding, increased PA activity was observed over wound-edge cells (Fig. 1). In these experiments the width of the resulting wounds (created with a 2-mm-wide rubber policeman) was chosen to avoid wound closure and maximize cell migration at the time the

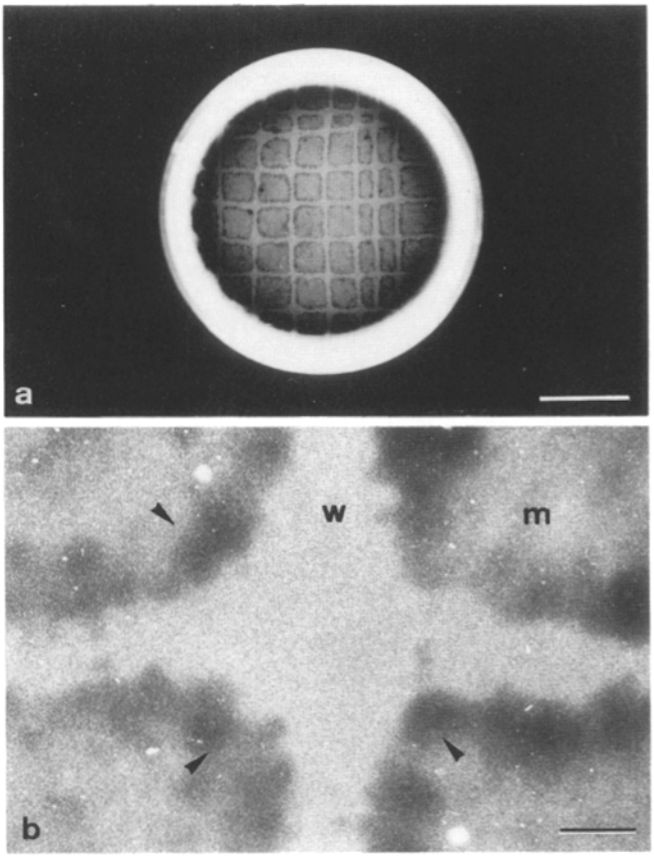


Figure 1. In situ analysis of PA activity in a multiple-wounded BME monolayer. A confluent monolayer of BME cells was multiple wounded, washed, overlaid after 24 h with a thin layer of agar containing casein and plasminogen, and photographed under dark-field illumination after 3 h incubation at 37°C. (*b*) A higher magnification of the intersection of two perpendicular wounds shown in *a*. The remaining monolayer (*m*), the wound devoid of cells (*w*), and plasminogen-dependent caseinolytic bands over cells at the wound edge (*arrowheads*) are indicated in *b*. Bars: (*a*) 1 cm, (*b*) 500 μm.

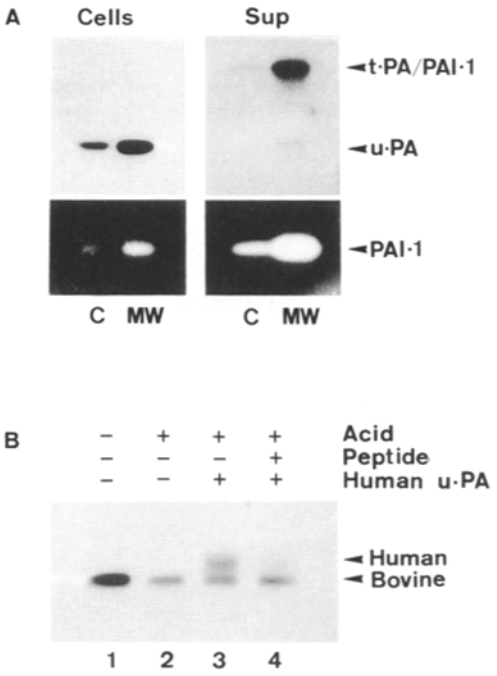


Figure 2. Zymography and reverse zymography of multiple-wounded BME monolayers. (*A*) Zymography revealed an increase in u-PA in cell extracts (*cells*) and t-PA/PAI-1 complexes in culture supernatants (*sup*) of multiple-wounded monolayers (*MW*). *C*, controls. Reverse zymography revealed an increase in PAI-1 in both cell extracts and culture supernatants of multiple-wounded cultures. (*B*) To determine whether the increase in cell-associated u-PA activity (lane *1*) was due to receptor-bound u-PA, monolayers were acid treated to elute receptor-bound u-PA. Acid treatment removed most of the cell-associated u-PA activity (lane *2*). Human tcu-PA bound efficiently to acid-treated monolayers (lane *3*), and this could be inhibited by preincubating the cells with a 400-fold molar excess of a peptide corresponding to the receptor binding region of mouse u-PA (lane *4*). This indicates that acid treatment had unmasked u-PA receptors previously occupied by endogenous bovine u-PA. Human and bovine u-PAs can easily be distinguished from one another due to differences in electrophoretic mobility.

monolayers were overlaid. No apparent difference was observed in the extent of caseinolysis over non-migrating cells when compared to cells in a non-wounded monolayer overlaid in parallel; this was true whether or not the medium was changed after wounding (results not shown).

Zymographic analysis of multiple-wounded cultures revealed an increase in cell-associated u-PA activity (Fig. 2 *A*), confirming our results previously obtained with the overlay technique (Pepper et al., 1987). Zymography also revealed an increase in t-PA, bound to PAI-1 (Loskutoff et al., 1986), in the culture supernatant of multiple-wounded cultures (Fig. 2 *A*). t-PA was distinguished from u-PA on the basis of inhibition of u-PA catalytic activity by amiloride (Vassalli and Belin, 1987; Pepper et al., 1987). The t-PA/PAI-1 complex was characterized by Loskutoff et al. (1986) on the basis of its recognition by antibodies to either t-PA or PAI-1. t-PA is usually secreted into the culture medium (Moscatelli, 1986), which explains why it is not detected by the overlay technique (Pepper et al., 1987), which primarily assays for cell-associated activity. Reverse zymography revealed an increase in PAI-1 in multiple-wounded cultures (Fig. 2 *A*), extending our previous observation that PAI-1 mRNA is increased in migrating endothelial cells (Pepper et al., 1992*b*). We have previously demonstrated that the PAI produced by BME cells which is detectable by reverse zymography is PAI-1 (Pepper et al., 1991*a*).

To determine whether the increase in cell-associated u-PA activity in multiple-wounded monolayers (Fig. 2 *A*) was due to receptor-bound u-PA, monolayers were acid treated. Acid treatment removed most of the cell-associated u-PA activity (Fig. 2 *B*, lane *2*) which could be recovered in the acid wash (not shown). To verify that acid treatment had unmasked previously occupied u-PA binding sites, acid-treated monolayers were incubated with DFP-treated 55,000 M_r human tcu-PA. Partial inactivation by DFP treatment was necessary to allow for simultaneous detection of human 55,000 M_r tcu-PA (added at a 10-fold higher concentration (10 nM) than the K_d for human u-PA binding to bFGF-stimulated BME cells (0.8 nM) (Mignatti et al., 1991)) and bovine u-PA activities, which can be distinguished from one another by differences in electrophoretic mobility (bovine u-PA migrates faster). Human tcu-PA bound efficiently to acid-treated

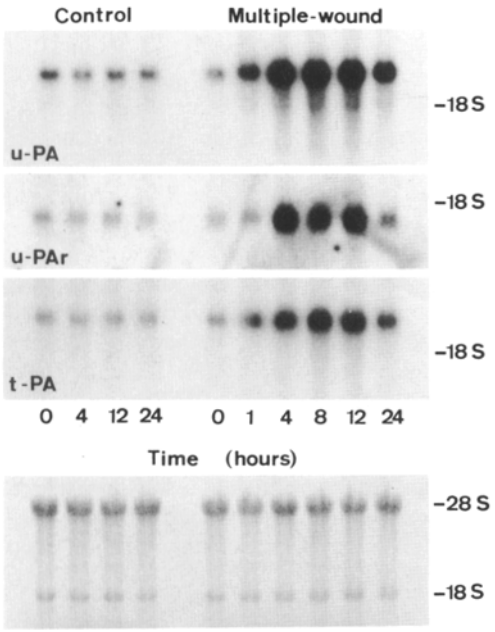


Figure 3. Induction of u-PA, u-PAr, and t-PA mRNAs in multiple-wounded BME monolayers. Confluent monolayers of BME cells were multiple wounded, and the medium not changed thereafter. Total cellular RNA was extracted from non-wounded (*Control*) and multiple-wounded monolayers at the times indicated. Northern blots were hybridized with ^{32}P-labeled u-PA, u-PAr, and t-PA cRNA probes. Methylene blue staining revealed uniform loading of RNAs and intact 28S and 18S ribosomal markers after transfer and UV cross-linking to nylon filters (*bottom panel*).

monolayers (Fig. 2 *B*, lane *3*), and this could be inhibited by preincubating the cells with a 400-fold molar excess of a peptide corresponding to the receptor binding region of the NH_2 terminus of mouse u-PA (amino acids 13-33; Appella et al., 1987) (Fig. 2 *B*, lane *4*). The murine peptide has previously been shown to be an efficient competitor of human u-PA binding to BME cells (Mignatti et al., 1991). Bovine u-PA differs from murine and human sequences in the same region by two and three amino acids, respectively (Krätzchmar et al., 1993). Human tcu-PA did not bind to BME cells in the absence of acid elution (not shown), demonstrating that most u-PA binding sites on these cells were occupied by endogenous bovine u-PA.

u-PA and u-PA Receptor mRNAs Are Increased in Multiple-wounded Microvascular and Large Vessel Endothelial Cell Monolayers

For mRNA analysis, the width of the initial wounds (created with a 1-mm-wide pointed rubber policeman) was chosen to allow for closure of the majority of wounds after 24 h (see Fig. 1 in Pepper et al., 1992*b*). Northern blots of total cellular RNA from multiple-wounded BME cell monolayers were hybridized with ^{32}P-labeled murine or bovine u-PA, bovine u-PAr and human t-PA probes. (Where duplicate hybridizations were performed with the murine and bovine u-PA probes, identical results were obtained). This revealed a transient increase in u-PA, u-PAr and t-PA mRNA levels (Fig. 3). The decrease in u-PAr mRNA was linked to the time of wound closure (Fig. 3). u-PA and t-PA mRNAs were maximally increased 16.9- and 6.1-fold, respectively, after

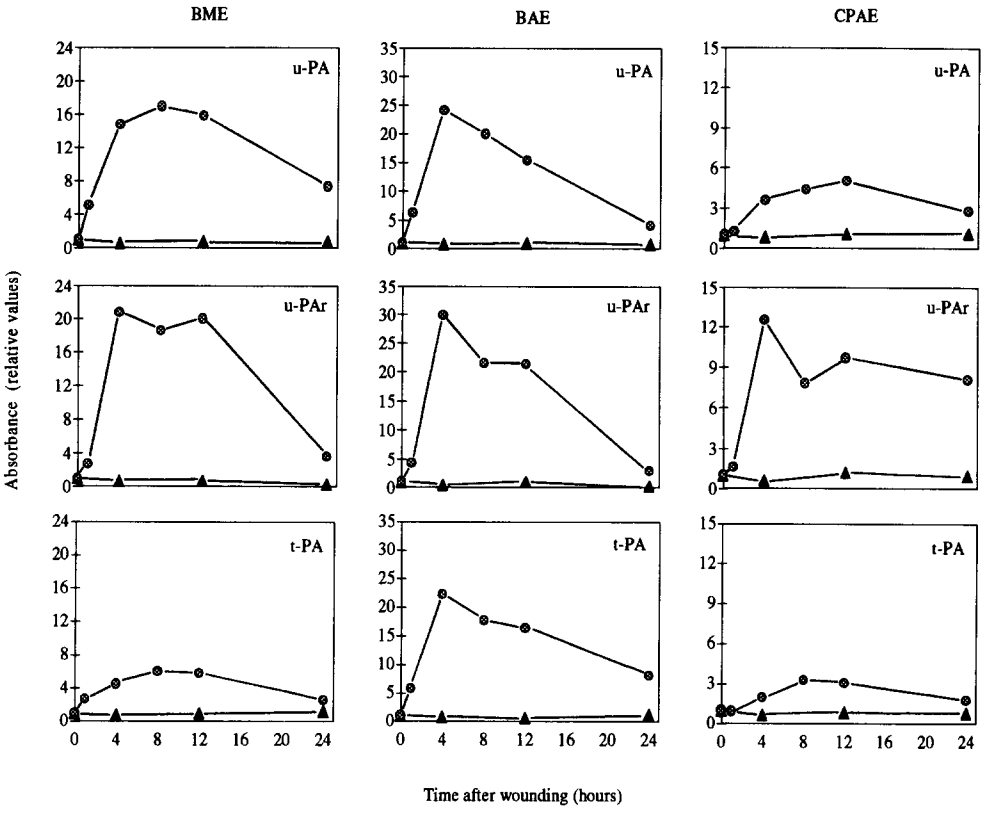


Figure 4. Quantitative analysis of u-PA, u-PA receptor, and t-PA mRNA induction in multiple wounded monolayers of BME, BAE, and CPAE cells. Endothelial cell monolayers were multiple-wounded, and the medium not changed thereafter. Total cellular RNA was extracted from non-wounded (▲) and multiple-wounded (⊛) monolayers at the times indicated. Northern blots were hybridized with ^{32}P-labeled u-PA, u-PAr, and t-PA cRNA probes. Autoradiographs were quantitated by densitometric scanning, and values are expressed relative to controls at 0 h.

8 h, and u-PAr mRNA was maximally increased 20.8-fold after 4 h (Fig. 4). A similar increase in u-PA, u-PAr, and t-PA mRNAs was also observed in multiple-wounded monolayers of BAE and CPAE cells (Fig. 4).

Cells migrating from the edges of a wounded BME cell monolayer are moving to a state of low density (Pepper et al., 1992*b*). In the present studies, we observed an increase in u-PA and u-PAr mRNAs in low-density BME cell cultures (results not shown). Addition of hydroxyurea (5 mM) to low-density cultures for 24 h, which inhibits [^{3}H]thymidine incorporation by 98% (mean of two separate experiments), did not reduce the increase in u-PA, u-PAr, or PAI-1 mRNA levels (results not shown). Similarly, addition of hydroxyurea (5 mM) to multiple-wounded BME cell monolayers for 4 h after wounding did not affect the wound-induced increase in u-PA, u-PAr, or t-PA mRNAs (results not shown). We have previously demonstrated that 4-h exposure to hydroxyurea (5 mM) inhibits [^{3}H]thymidine incorporation into low density BME cell cultures by 81% (Pepper et al., 1992*b*).

The increase in u-PA and u-PA Receptor mRNAs Is Localized to Cells at the Wound Edge

The localization of the increase in u-PA and u-PAr mRNAs was determined by in situ hybridization. Wounded monolayers of BME and BAE cells were hybridized with ^{3}H-labeled anti-sense bovine u-PA and u-PAr RNA probes. In these experiments, BME or BAE cell monolayers on poly-L-lysine–coated glass microscope slides were wounded by scraping away half of the monolayer with a razor blade; this resulted in the absence of an opposing migrating front and hence of wound closure. An increased number of autoradiographic grains was observed with both u-PA and u-PAr probes over cells at the edge of wounded BME and BAE cell monolayers (Figure 5 and results not shown). This increase was observed both at 4 hours (Fig. 5) and 24 h (results not shown) after wounding. The increase at 4 h was quantitated in BME cells by determining the number of autoradiographic grains per cell in eight consecutive 100-μm-deep

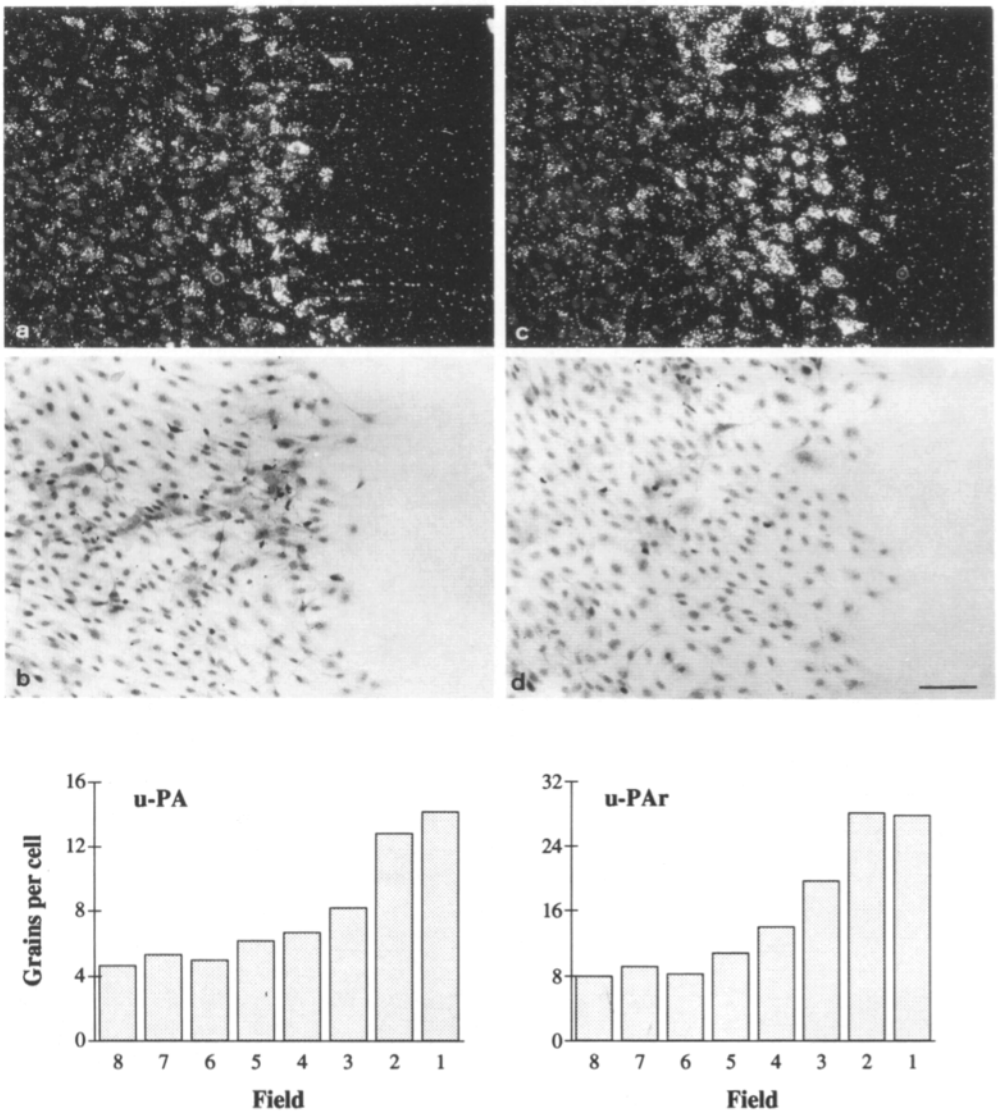


Figure 5. In situ hybridization of wounded monolayers of BME cells. Confluent monolayers of BME cells on poly-L-lysine–coated glass microscope slides were wounded by scraping away half of the monolayer. Cultures were fixed after 4 h, subjected to in situ hybridization using ^{3}H-labeled anti-sense bovine u-PA (*a* and *b*) and u-PAr (*c* and *d*) RNA probes. Cells were counterstained with methylene blue after in situ hybridization. *a* and *c* Are dark-field views of *b* and *d*. Quantitation was performed in eight consecutive 100-μm-wide fields beginning at the leading edge (*field 1*) as described in Materials and Methods. Results are expressed as the mean number of autoradiographic grains/cell from six series of eight consecutive fields in cultures fixed 4 h after wounding, and are pooled from two experiments. Bar, 100 μm.

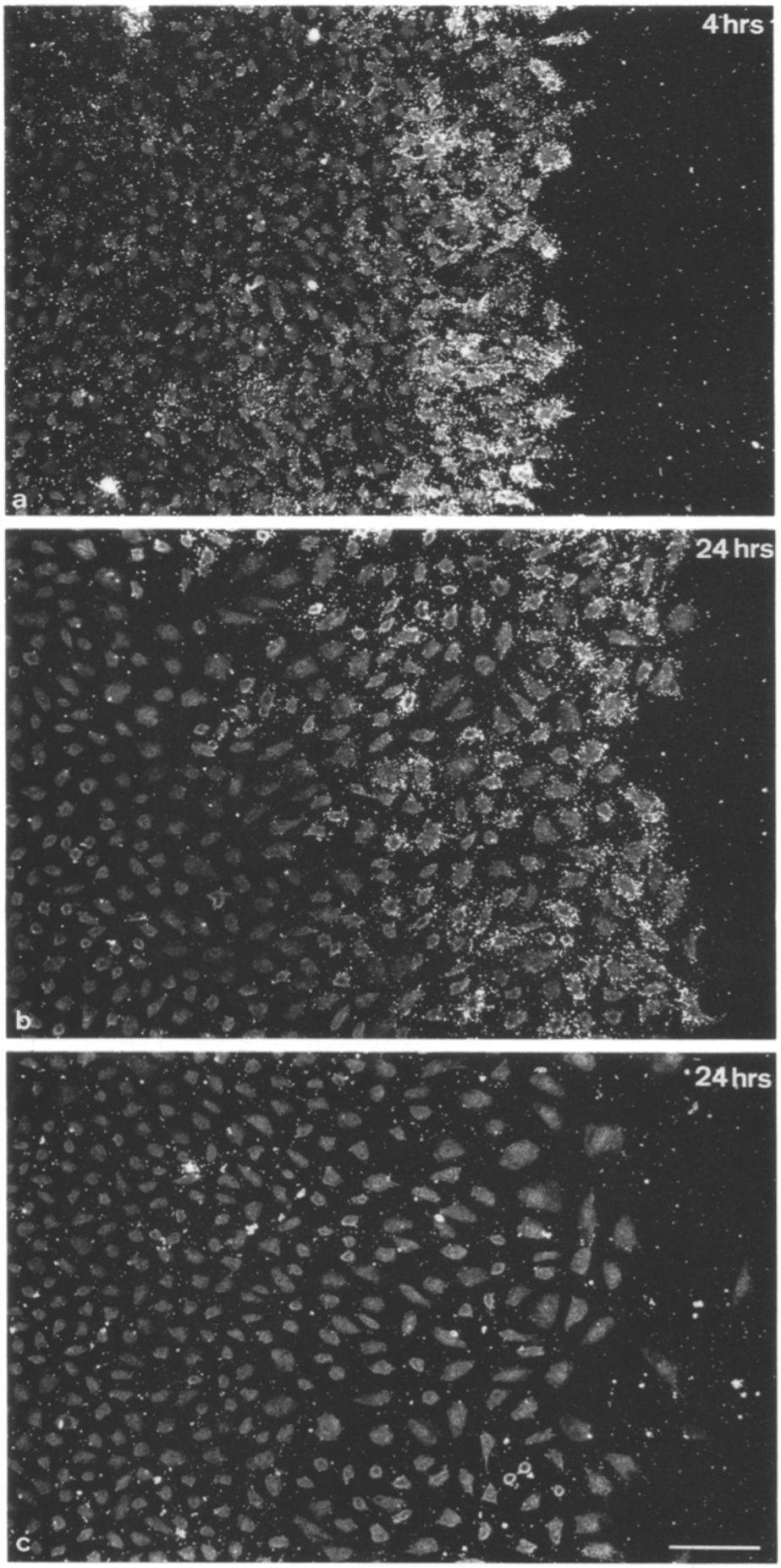


Figure 6. Binding of ^{125}I-labeled u-PA to wounded BAE monolayers. Wounded monolayers of BAE cells were incubated with ^{125}I-labeled DFP-treated 55,000 M_r human tcu-PA (10 nM) 4 h (*a*) and 24 h (*b*) after wounding, and subjected to autoradiography. In *c*, before the addition of ^{125}I-labeled u-PA, wounded monolayers were preincubated with a 400-fold molar excess of a peptide corresponding to the receptor binding region of the NH_2 terminus of mouse u-PA. Bar, 100 μm.

fields beginning at the leading front. This revealed a 3.0- and 3.5-fold increase in u-PA and u-PAr mRNA levels, respectively, in cells in the field closest to the leading edge, when compared to cells in the field furthest from the migrating front (Fig. 5). No difference was observed in the number of autoradiographic grains per cell in non-migrating cells when compared to cells in a non-wounded monolayer hybridized in parallel in the same experiment (results not shown). The reasons for the apparent discrepancy between the levels of u-PA and u-PAr mRNA as quantitated by in situ hybridization (3.0- and 3.5-fold increase, respectively, after 4 h), compared to the levels seen in multiple-wounded cultures (14.8- and 20.8-fold increase, respectively, after 4 h), are not known. It is however possible that differences in the conditions (e.g., stringency) used for Northern blot and in situ hybridization may account for the apparent quantitative differences observed between these two techniques.

u-PA Binding Sites Are More Abundant on Cells at the Wound Edge

To determine whether the increased expression of u-PAr mRNA in migrating cells could be translated into an increase in the number of binding sites for u-PA, wounded monolayers were incubated with ^{125}I-labeled DFP-treated 55,000-M_r human tcu-PA. ^{125}I-labeled u-PA was used at a 10-fold higher concentration (10 nM) than the K_d previously reported for human u-PA binding to bFGF-stimulated BME cells (0.8 nM) (Mignatti et al., 1991). This revealed an increase in the number of u-PA binding sites as determined by autoradiography, on BME and BAE cells at the wound-edge, both 4 and 24 h after wounding (Fig. 6,*a* and *b*, and results not shown). The increase at 4 h was quantitated in BME and BAE cells by determining the number of autoradiographic grains per cell in six consecutive 100-μm-deep fields beginning at the leading front. This revealed a 3.9- and 5.1-fold increase in u-PAr binding sites in BME and BAE cells at the leading edge, respectively, when compared to cells in the field furthest from the migrating front (Fig. 7). Similar results were obtained using ^{125}I-labeled u-PA which had not been pretreated with DFP (results not shown). A large degree of heterogeneity was observed in the binding of ^{125}I-labeled u-PA to individual migrating cells (results not shown).

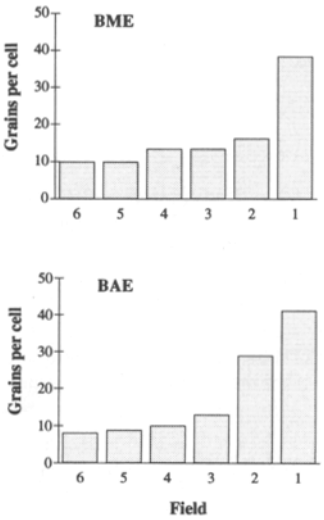


Figure 7. Quantitation of the binding of ^{125}I-labeled u-PA to wounded BME and BAE monolayers. Wounded monolayers of BME or BAE cells were incubated with ^{125}I-labeled DFP-treated human 55,000 M_r tcu-PA (10 nM) 4 h after wounding, and subjected to autoradiography. Quantitation was performed in six consecutive 100-μm-wide fields beginning at the leading edge (*Field 1*) as described in Materials and Methods. Results are expressed as the mean number of autoradiographic grains/cell from nine series of six consecutive fields for both cell types, and are pooled from two experiments.

The specificity of the ^{125}I-labeled u-PA binding to wound-edge endothelial cells was verified by preincubating wounded monolayers with 10–400-fold molar excess of a peptide corresponding to the receptor binding region of mouse u-PA (Appella et al., 1987). Preincubation with the mouse peptide at a 10-fold molar excess partially prevented ^{125}I-labeled u-PA binding to wound-edge cells (results not shown); complete inhibition of binding was achieved by preincubation with a 40-fold or greater excess of peptide (Fig. 6 *c*). These results demonstrate that u-PA binding to migrating endothelial cells is mediated by the u-PAr.

It has previously been demonstrated that u-PA binding sites are rapidly redistributed to the leading edge of monocytes placed in a chemotactic gradient (Estreicher et al., 1990). In our present studies, u-PA did not appear to bind preferentially to the leading edge of migrating cells (Fig. 6, *a* and *b*), which may be due to the absence of a chemotactic gradient in wounded endothelial cell monolayers.

The Wound-related Increase in u-PA Activity and u-PA and u-PA Receptor mRNA Expression Is Inhibited by Antibodies to bFGF

Using the overlay technique which reveals an increase in u-PA activity associated with BME cells migrating from the wound-edge (Fig. 1), we found that anti-bFGF antibodies completely abolished the increased u-PA activity normally seen over migrating cells (Fig. 8). To assess the mechanisms of this inhibition, u-PA and u-PAr mRNA levels were measured in multiple-wounded monolayers exposed to anti-rhbFGF antibodies. Two protocols were used: in the first, (designated "pre"), antibodies were added to cultures before wounding, and not removed thereafter; in the second, (designated "post"), monolayers were wounded, washed, and antibodies added thereafter. With both protocols, the wound-induced increase in u-PA and u-PAr mRNA levels was markedly reduced; u-PA mRNA was decreased by 62 and 83%, while uPAr mRNA was described by 52 and 90% in "pre" and "post" experiments, respectively (Fig. 9). In addition, the wound-induced increase in u-PA and u-PAr mRNAs was 84 and 60% greater when the medium was not changed after wounding ("pre") than when it was ("post"). Exogenous bFGF, which increases u-PA mRNA in confluent monolayers of BME cells (Pepper et al., 1990), also increased u-PAr mRNA levels in these cells, with a detectable increase in response to 300 pg/ml bFGF, and a maximal response at 1 ng/ml bFGF with no further increase at higher concentrations (results not shown). A time course analysis with 3 ng/ml revealed a maximum 15.3-fold increase after 12 h (Fig. 10). Taken together, these results demonstrate that the increase in u-PA activity and in u-PA and u-PAr mRNA levels in migrating BME cells, is mediated by endogenous bFGF.

Discussion

Although it has consistently been observed that u-PA is absent from resting endothelial cells in the intact organism (Rijken et al., 1980; Kristensen et al., 1984; Larsson et al., 1984), this enzyme is induced in endothelial cells during neovascularization of ovarian follicles, corpus luteum, and

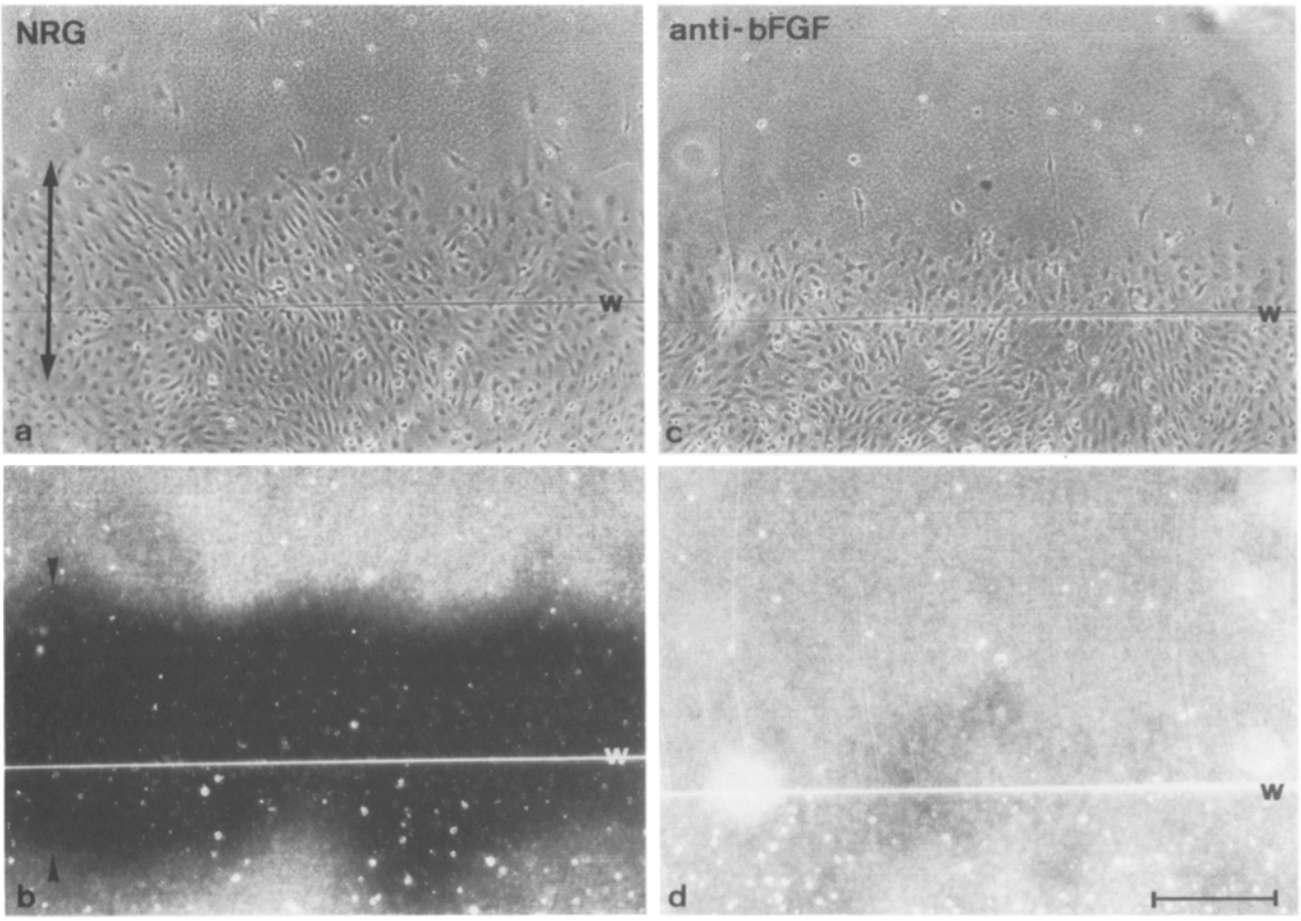
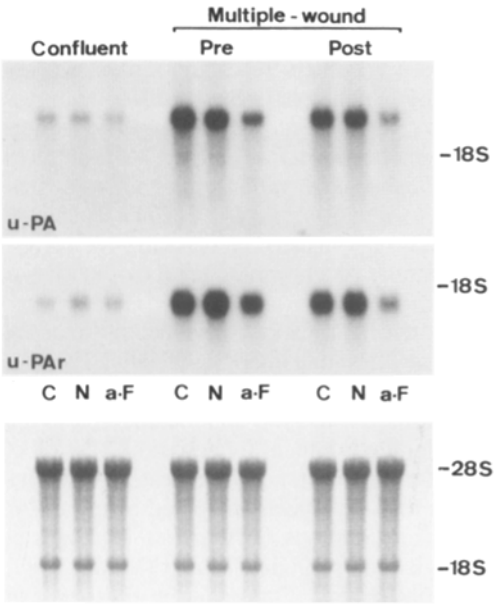


Figure 8. Effect of anti-bFGF antibodies on wound-edge BME cell u-PA activity. Confluent monolayers of BME cells were wounded with a razor blade to mark the original wound edge (*w*), incubated in the presence of normal rabbit γ-globulins (*NRG*; 200 μg/ml) (*a* and *b*) or anti-rhbFGF γ-globulins (200 μg/ml) (*c* and *d*) for 24 h, and overlaid with a thin layer of agar containing casein and plasminogen. Phase contrast (*a*) and corresponding darkfield views (*b*) of the wound-edge region reveal an increase in u-PA–mediated caseinolytic activity as previously described (Pepper et al., 1987), which extends to a few cell rows behind the original wound (*w*). Limits of caseinolysis are indicated by arrows in *a* and by arrowheads in *b*. Anti-rhbFGF γ-globulins markedly inhibit the increased caseinolysis over cells at the wound edge (*d*), and reduce the distance of migration as well as the number of migrating cells which have crossed the original wound edge (*c*). Bar, 300 μm.



maternal decidua (Bacharach et al., 1992), and also during inflammation (Grøndahl-Hansen et al., 1989), situations in which endothelial cell migration is induced. Consistent with these observations, we have previously demonstrated that

Figure 9. Effect of anti-bFGF antibodies on u-PA and u-PAr mRNA levels in multiple-wounded BME monolayers. Two procedures were used. In the first, designated "pre," confluent monolayers were washed and serum-free α-MEM containing 0.1% gelatin (*C*), and either normal rabbit γ-globulins (*N*) (200 μg/ml) or rabbit anti-rhbFGF γ-globulins (*a–F*) (200 μg/ml) were added; monolayers were then multiple wounded and the medium not changed thereafter. In the second, designated "post," monolayers were multiple-wounded, washed, and serum-free α-MEM containing 0.1% gelatin and either normal rabbit γ-globulins or rabbit anti-rhbFGF γ-globulins were added. In both cases, total cellular RNA was extracted 4 h after multiple wounding. Confluent, non-wounded monolayers were processed in the same way in parallel. Northern blots were hybridized with ^{32}P-labeled u-PA and u-PAr cRNA probes. Methylene blue staining revealed uniform loading of RNAs and intact 28S and 18S ribosomal markers after transfer and UV cross-linking to nylon filters (*bottom panel*).

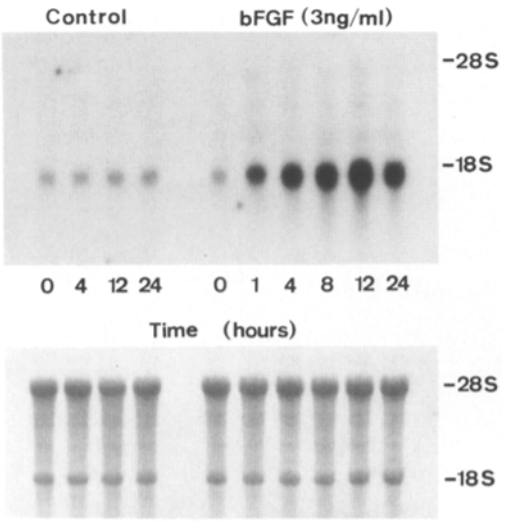


Figure 10. Effect of endogenous bFGF on u-PAr mRNA expression in BME cells. rhbFGF (3 ng/ml) was added to confluent monolayers of BME cells, and total cellular RNA extracted at the indicated time points thereafter. The northern blot was hybridized with a ^{32}P-labeled u-PAr cRNA probe. Methylene blue staining revealed uniform loading of RNAs and intact 28S and 18S ribosomal markers after transfer and UV cross-linking to nylon filters (*bottom panel*).

u-PA activity is increased in migrating endothelial cells in response to mechanical wounding in vitro (Pepper et al., 1987). In this paper, we demonstrate that this increase is mainly due to receptor-bound u-PA. We also demonstrate that u-PA and u-PAr mRNA levels are increased in multiple-wounded endothelial cell monolayers, and by in situ hybridization we show that this increase is localized to migrating cells. u-PA binding sites are also increased on migrating cells. Taken together, these results demonstrate that the induction of PA activity on migrating endothelial cells is due to increased production of u-PA, which binds to u-PAr whose expression is upregulated on the same cells.

What are the mechanisms responsible for the increase in u-PA and u-PAr expression in migrating endothelial cells? Wounding induces a number of alterations in cell functions in cells lining the wound edge, including cell division and cell migration. In this and a previous study (Pepper et al., 1987), we have observed that inhibition of BME cell division neither inhibits the induction of u-PA activity at the wound edge nor affects the increase in u-PA or u-PAr mRNA levels in multiple-wounded monolayers. Similarly, in low density cultures, in which endothelial cells both proliferate and migrate, the increase in u-PA, u-PAr, and PAI-1 mRNAs as compared to confluent cultures was not prevented by inhibition of endothelial cell proliferation. Taken together, these results indicate that the observed changes in expression of different components of the PA system are likely to be associated with cell migration rather than with proliferation.

Sato and Rifkin (1988) have reported that wound-induced BME or BAE cell migration is decreased by addition of anti-bFGF antibodies to cultures after wounding, and Odekon et al. (1992) found that these antibodies decreased wound-edge PA activity in wounded BAE cell monolayers. It has also been demonstrated that u-PA activity is increased in endothelial cells in response to bFGF (Moscatelli et al., 1986; Montesano et al., 1986), and that bFGF increases the number of u-PA binding sites on BME cells (Mignatti et al., 1991). It therefore seemed appropriate to assess whether anti-bFGF antibodies might affect the increase in u-PA activity and u-PA and u-PAr mRNA levels in migrating BME cells. Addition of the antibodies to cultures immediately after wounding completely inhibited the increase in u-PA activity associated with these cells, confirming similar observations by Odekon et al. (1992) with BAE cells. In addition, the increase in u-PA and u-PAr mRNAs in multiple-wounded monolayers was markedly inhibited both when the monolayers were wounded in the presence of anti-bFGF antibodies, and also when these antibodies were added immediately after wounding. That bFGF is released into the medium as a consequence of wounding is suggested by the observation that the wound-induced increase in u-PA and u-PAr mRNAs was greater when the medium was not changed after wounding, and by the finding that the anti-bFGF antibodies were more efficient in reducing this increase when medium containing dead and damaged cells was removed after wounding. The results of this and other studies in which wound-edge phenomena can be inhibited by antibodies to bFGF (Sato and Rifkin, 1988; Odekon et al., 1992; Pepper et al., 1992*b*; Pepper and Meda, 1992), suggest two possible mechanisms of bFGF release. Firstly, bFGF is released from dead or damaged cells as a consequence of wounding (McNeil et al., 1989; Gadjusek and Carbon, 1989; Muthukrishnan et al., 1991), and secondly, bFGF is released from migrating cells (McNeil et al., 1989). Although none of the observations from this and previous studies allow us to exclude either possibility, our results clearly suggest that bFGF released as a consequence of wounding is required for the increase in u-PA, u-PAr, and PAI-1 expression in migrating endothelial cells.

u-PA has been implicated in processes of cell migration and tissue remodeling, while t-PA is believed to be involved mainly in intravascular thrombolysis (reviewed by Moscatelli and Rifkin, 1988). Since our studies are concerned with endothelial cell migration, we have focused on u-PA and u-PAr expression. However, in this paper we have demonstrated that t-PA is also increased in endothelial cell monolayers in response to multiple wounding. The mechanisms for this increase are likely to differ from those discussed above, since t-PA induction in BME cells in response to bFGF is minimal (Pepper et al., 1990, 1991*b*). In addition, t-PA mRNA is decreased in low-density BME cultures (M. S. Pepper, unpublished observation), in contrast to what we observed for u-PA and u-PAr mRNA. Further studies on the localization of the wound-induced increase in t-PA mRNA must await the availability of a homologous (bovine) cDNA for in situ analysis.

What is the functional significance of the increase in u-PA receptor expression on migrating endothelial cells? The first and currently most apparent function is that of increasing the efficiency of extracellular proteolysis and localizing it to the immediate pericellular environment (reviewed by Vassalli et al., 1991; Blasi, 1993). However, additional catalytically independent functions have also been attributed to the u-PA/u-PAr interaction. These include mitogenesis (Rabbani et al., 1990, 1992), chemotaxis (Gudewicz and Gilboa, 1987; Del Rosso et al., 1990), and differentiation (Nusrat and

Chapman, 1991). Similar non-proteolytic functions including chemotaxis (Fibbi et al., 1988) and chemokinesis (Odekon et al., 1992) have also been observed in endothelial cells. Our findings on the co-induction of u-PA and u-PAr provide a molecular basis for the u-PA/u-PAr interaction responsible for both proteolytic and possible non-proteolytic functions of the u-PAr in migrating endothelial cells. They also point to the possible existence of an autocrine loop in which u-PA, through interactions with its receptor, increases its own synthesis and possibly modulates the synthesis of other components of the PA-plasmin system in migrating cells. Indeed, Fibbi et al. (1990) have demonstrated that the catalytically inactive A chain is capable of stimulating u-PA release from human keratinocytes.

Finally, in this paper we also demonstrate an increase in PAI-1 activity in multiple-wounded BME cell cultures, which extends our previous observation that PAI-1 mRNA is increased in migrating endothelial cells (Pepper et al., 1992*b*). However, the kinetics of the PAI-1 mRNA increase were different from those observed for u-PA and u-PAr mRNA; the PAI-1 increase was ephemeral, with an early decrease independent of wound closure (Pepper et al., 1992*b*). We suggest that expression of protease inhibitors at very early stages of cell migration might provide a mechanism which confines extracellular matrix degradation to the immediate pericellular environment. PAI-1 bound to fibrin may protect blood clots from premature dissolution (Reilly and Hutzelmann, 1992). This is particularly important in angiogenesis, which frequently occurs in situations of fibrin deposition such as wound healing.

In summary, we have demonstrated that the increase in u-PA activity on migrating endothelial cells is mainly due to receptor-bound u-PA, which in turn can be accounted for by an increase in both u-PA and u-PAr expression by these cells. Furthermore, the increase in u-PA activity and u-PA and u-PAr mRNA levels in migrating cells can be inhibited by antibodies to bFGF. The demonstration that the u-PAr is increased during cell migration strengthens the hypothesis that cell-associated protease activity is an important element in the cohort of functions expressed by migrating and invading cells.

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