Urokinase-type Plasminogen Activator Is Induced in Migrating Capillary Endothelial Cells

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Abstract. Cellular migration is an essential component of invasive biological processes, many of which have been correlated with an increase in plasminogen activator production. Endothelial cell migration occurs in vivo during repair of vascular lesions and angiogenesis, and can be induced in vitro by wounding a confluent monolayer of cells. By combining the wounded monolayer model with a substrate overlay technique, we show that cells migrating from the edges of an experimental wound display an increase in urokinase-type plasminogen activator (uPA) activity, and that this activity reverts to background levels upon cessation of movement, when the wound has closed. Our results demonstrate a direct temporal relationship between endothelial cell migration and uPA activity, and suggest that induction of uPA activity is a component of the migratory process.

THE vascular endothelium consists of a highly ordered monolayer of cells which provides a structural and functional barrier between circulating blood and the surrounding tissues. In response to a variety of stimuli, normally quiescent endothelial cells can be induced to migrate. In vivo, this occurs during the regeneration of endothelial lesions in large blood vessels (Fishman et al., 1975; Schwartz et al., 1978; Haudenschild and Schwartz, 1979), and during the formation of new capillary blood vessels in the process of angiogenesis (Ausprunk and Folkman, 1977; Folkman, 1985). In vitro, endothelial cell migration can be triggered by mechanically wounding a confluent monolayer of cells (Sholley et al., 1977; Selden and Schwartz, 1979; Gotlieb and Spector, 1981; Ryan et al., 1982; Madri and Stenn, 1982).

Proteases, and in particular plasminogen activators (PAs),[1] have been correlated with cell migration in a variety of invasive biological processes (Reich, 1978; Mullins and Rohrlich, 1983; Saksela, 1985; Danø et al., 1985; Goldfarb and Liotta, 1986). Using the wounded monolayer model together with a substrate overlay technique, which has the advantage of allowing direct visualization of proteolysis around migrating cells, we demonstrate here that capillary endothelial cells migrating from the edges of an experimental wound display an increase in urokinase-type PA (uPA) activity.

Materials and Methods

Materials

4β-phorbol 12-myristate 13-acetate (PMA), ε-aminocaproic acid, cycloheximide, mitomycin C, and amiloride were purchased from Sigma Chemical

Co. (St. Louis, MO). Trasylol was purchased from Bayer-Pharma AG (Zurich, Switzerland). Affinity-purified anti-human tissue-type plasminogen activator (tPA) antibodies were a generous gift from Dr. W.-D. Schleuning (Laboratoire Central d'Hematologie, CHUV, Lausanne).

BSA (Sigma Chemical Co.) was acid treated as described (Loskutoff, 1978), to remove labile protease inhibitors. Plasminogen was purified from human plasma by lysine-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity chromatography (Deutsch and Mertz, 1970).

### Endothelial Cell Culture

Cloned microvascular endothelial cells from bovine adrenal cortex (BME cells) (Furie et al., 1984), a generous gift from Drs. M. B. Furie and S. C. Silverstein (Columbia University, New York), were routinely subcultured in gelatin-coated tissue culture flasks (Falcon Labware, Becton-Dickinson, Oxnard, CA) in complete medium consisting of MEM, alpha modification (α-MEM) (Gibco AG, Basel, Switzerland), supplemented with 15% heat-inactivated donor calf serum (Flow Laboratories, Baar, Switzerland), penicillin (500 U/ml), and streptomycin (100 µg/ml). Cells were seeded into 35-mm gelatin-coated culture dishes (Falcon Labware), and grown to confluence in complete medium before the experiments were started.

Wounding and Substrate Overlay of Confluent Monolayers

Confluent monolayers of BME cells in 35-mm culture dishes were wounded with a 5-mm-wide rubber policeman. Wounded cultures were washed three times with PBS and twice with α-MEM, and fresh complete medium was replaced. 24 h after wounding (unless otherwise indicated), monolayers were overlaid essentially as previously described (Vassalli et al., 1977). Briefly, monolayers were washed twice with PBS containing acid-treated BSA (1 mg/ml), and overlaid with a mixture containing 2% instant non-fat dry milk, 0.8% agar, and plasminogen (40 µg/ml) in α-MEM. The plates were incubated at 37°C for 60–120 min, and photographed under dark-field illumination. The wound edge was photographed under phase-contrast or dark-field illumination using a Zeiss ICM 405 inverted photomicroscope. In control experiments, plasminogen was omitted from this mixture. In some experiments, Trasylol or ε-aminocaproic acid was added to the overlay mixture at 200 U/ml and 500 µg/ml, respectively.

Cycloheximide (0.1 µg/ml) was added to the culture medium 30 min before, and for 24 h after wounding, i.e., until the cells were overlaid. To de-

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1. *Abbreviations used in this paper*: BME, bovine microvascular endothelial cells; PA, plasminogen activator; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.
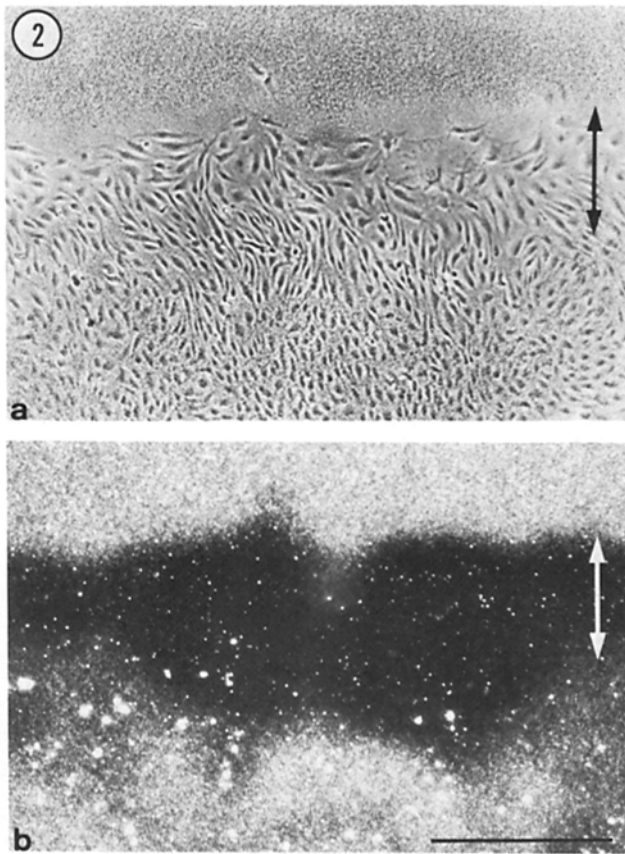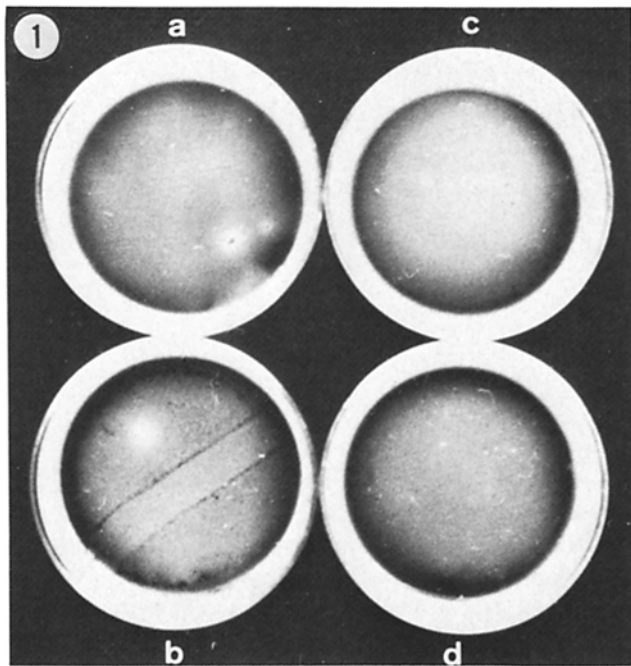
Figures 1 and 2. (Fig. 1) Caseinolytic assay. Cultures were overlaid 24 h after wounding, with a mixture containing (*a*) casein and agar, or (*b*) casein, agar, and plasminogen. Under dark-field illumination, zones of caseinolysis appear as dark bands on a white background, revealing the production of PA by cells lining the wound edge. Caseinolysis is inhibited by the addition of Trasylol (*c*) or ε-aminocaproic acid (*d*) to overlays containing plasminogen. Overlays photographed after 90 min at 37°C. (*Fig. 2*) Wound edge caseinolysis. (*a*) Higher magnification of the wound edge (limits of the region of caseinolysis indicated by the arrows) under phase-contrast microscopy. (*b*) Dark-field illumination of the same region; the area of caseinolysis is seen as a dark band between bands of unlysed casein above and below (limits of the region of caseinolysis indicated by the white arrows). Bar, 500 μm.
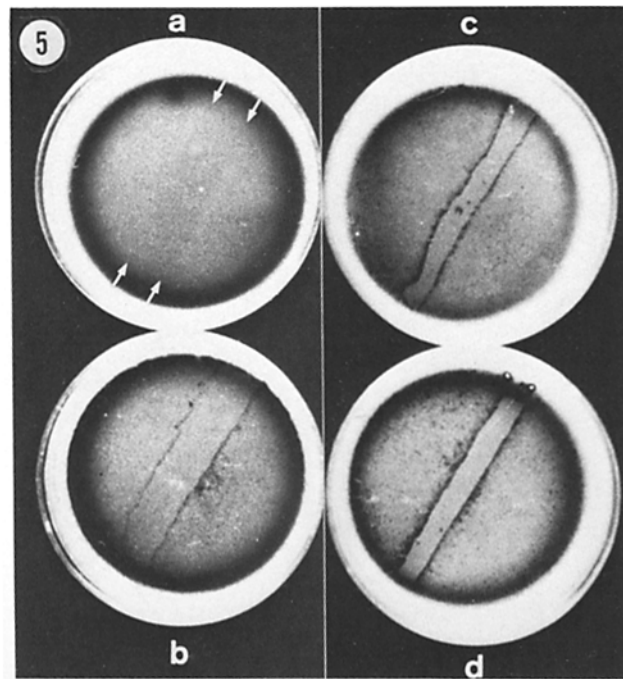
termine whether the effect of cycloheximide was reversible, medium containing the drug was removed after 24 h, the cultures were washed, and fresh complete medium was added. After a further 48 h, the cultures were overlaid. Control wounded cultures that had not been treated with cycloheximide were processed in the same way.

Mitomycin C (10 μg/ml) was added 4 h before wounding. Wounded cultures were incubated in the absence of mitomycin C for 24 h thereafter until being overlaid. The effect of mitomycin C on cell multiplication was determined as follows: confluent monolayers were wounded (with a blade) in such a way as to mark the initial wound edge (Bürk, 1973); after 24 h the monolayers were fixed and stained by the Feulgen technique, to facilitate visualization of condensed chromosomes. The number of mitotic figures amongst the cells that had migrated past the initial wound edge was determined in randomly selected photographic fields from treated and nontreated cultures.

For the characterization of the PA's produced by wound edge cells, wounded monolayers were fixed with 95% ethanol for 2 min at room temperature, and amiloride or affinity-purified anti-human tPA antibodies added to the overlay mixture at a concentration of 1 mM or 10 μg/ml, respectively.

Zymographic Assay

Culture media and cell lysates were prepared from PMA-stimulated BME cells as previously described (Montesano et al., 1986). Aliquots (20 μl) of both the culture media and the cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and zymography as described (Vassalli et al., 1984). Where indicated, amiloride was incorporated into the underlay at a concentration of 1 mM, or affinity-purified anti-human tPA antibodies at a final concentration of 10 μg/ml. The zymograms were photographed after 8 h of incubation at 37°C.

Results

Plasminogen-dependent Caseinolysis

Monolayers of BME cells were overlaid with an agar-casein mixture in the presence or absence of plasminogen, 24 h after mechanical wounding. In the presence of plasminogen, zones of caseinolysis along the edges of the wound were macroscopically evident as dark lines against a white background under dark-field illumination (Fig. 1 *b*). Caseinolysis did not occur in the absence of plasminogen (Fig. 1 *a*), or when the protease inhibitors ε-aminocaproic acid or Trasylol were included in the overlay mixture containing plasminogen (Fig. 1, *c* and *d*), thus demonstrating that substrate lysis was catalyzed by plasmin. Under phase-contrast and dark-field illumination at higher magnification, the lysis was seen to be confined to the edge of the wounded monolayer (Fig. 2, *a* and *b*), and to correspond to 5–15 rows of cells from the leading front.

No lysis was observed when monolayers were overlaid immediately after wounding, demonstrating that lysis was not due to preformed enzyme released by dead or damaged cells lining the wound edge (Fig. 3, *a* and *b*). Wound-associated lysis was clearly evident when the cells were overlaid 24 h after wounding (Fig. 3, *c* and *d*), and persisted until the wound had completely closed (see for example Fig. 5 *d*, overlaid 72 h after wounding). No lysis was observed in monolayers overlaid 8 d after wounding, at which time the wound had been closed for a period of 24–48 h (Fig. 3, *e* and *f*).
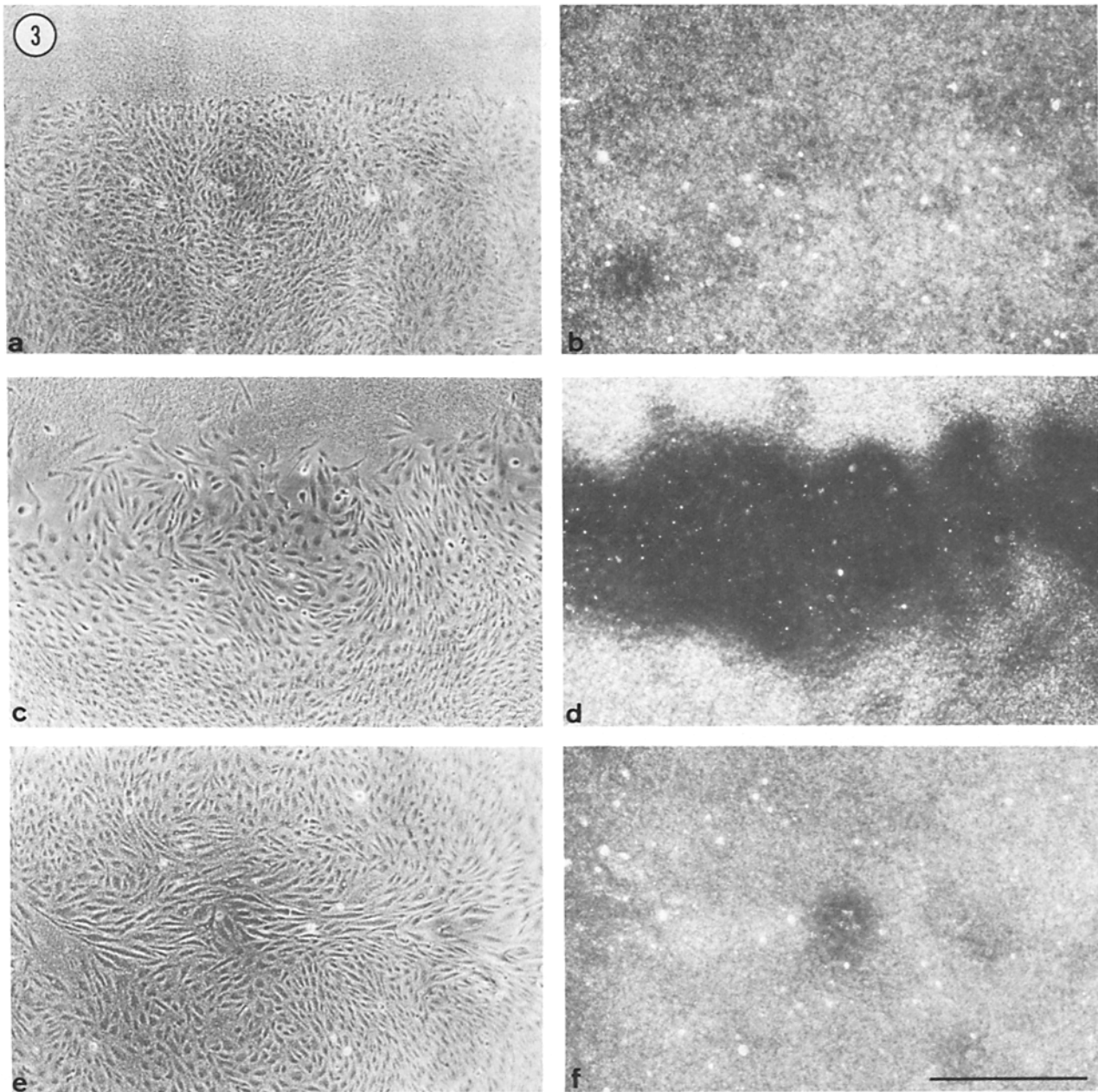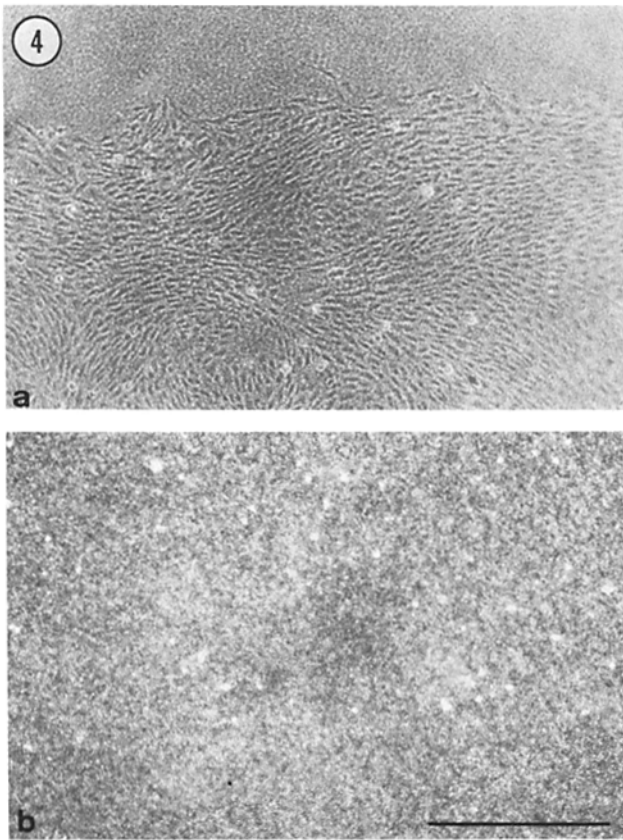
Figure 3. Time course of wound edge caseinolysis. Wounded cultures were overlaid immediately (*a, b*), 24 h (*c, d*), and 8 d (*e, f*) after wounding. (*a, c, e*) The wound edge seen under phase-contrast illumination; (*b, d, f*) the same fields under dark-field illumination. Overlays photographed after 120 min at 37°C. Bar, 500 μm.

Lysis did not occur when the cells had been incubated in the presence of cycloheximide (0.1 μg/ml), an inhibitor of protein synthesis (Fig. 4). This effect was completely reversible when the drug was removed and the cells overlaid after a further 48-h incubation in complete medium without cycloheximide (Fig. 5, *a–d*).

Wounding has been demonstrated to induce both cell migration and cell division (Sholley et al., 1977; Selden and Schwartz, 1979; Ryan et al., 1982). Wound-edge caseinolysis was observed in cultures treated with mitomycin C (10 μg/ml) (Fig. 6). The efficacy of mitomycin C treatment was confirmed by the complete absence of mitotic figures amongst the migrating cells in treated cultures (Fig. 7 and Table I).

Together these results demonstrate that the lysis is not simply a consequence of cell proliferation, and allow us to separate cell migration from cell division. Although mitomycin C–treated and nontreated cells had migrated approximately the same distance into the wound after 24 h (Fig. 7, *a* and *b*), the number of cells in the wound in treated cultures was always less than in controls (Table I). This might account for the slower rate of lysis seen in mitomycin C–treated cultures (compare Figs. 2 and 6; although lysis is clearly evident under dark-field illumination [Figs. 2 *b* and 6 *b*], the caseinolysis seen by phase-contrast microscopy over mitomycin C–treated migrating cells [Fig. 6 *a*] is not as complete as that seen over control cells [Fig. 2 *a*]). Background lysis in non-

Figure 6. Inhibition of cell division does not prevent caseinolysis. Monolayers were treated with mitomycin C (10 µg/ml) for 4 h before wounding, and then incubated in the absence of the drug for 24 h thereafter, until being overlaid. (*a*) Phase-contrast and (*b*) dark-field views of the wound edge, indicating the presence of wound edge–associated lysis in treated cultures. Photographed after 90 min at 37°C. Bar, 500 µm.

Figures 4 and 5. (*Fig. 4*) Cycloheximide inhibits induction of wound-associated caseinolysis. Cultures were treated with cycloheximide (0.1 µg/ml) for 30 min before and for 24 h after wounding. (*a*) The wound edge seen under phase-contrast illumination. (*b*) Dark-field illumination of the same region showing complete absence of caseinolysis. Overlays photographed after 120 min at 37°C. Bar, 500 µm. (*Fig. 5*) Reversibility of cycloheximide inhibition. (*a*) Cells incubated continuously in the presence of cycloheximide (0.1 µg/ml) for 30 min before wounding and until overlaid, i.e., 24 h after wounding. Note the complete absence of caseinolysis; posi-
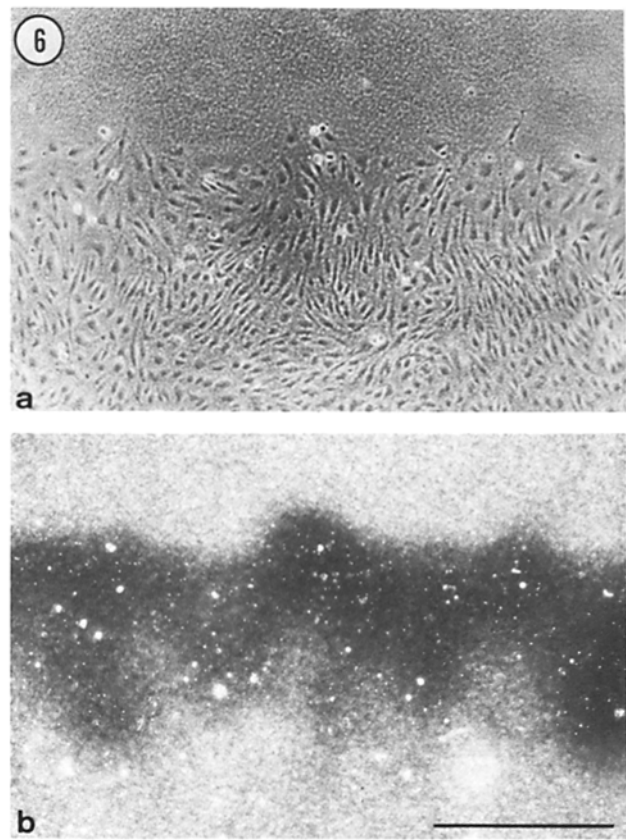
wounded regions of the mitomycin C–treated monolayer was increased above that observed in nontreated cultures (not shown); this is consistent with the report that PA synthesis is induced by exposure to mitomycin C and other agents that cause DNA damage (Miskin and Reich, 1980).

Caseinolysis Is Due to Cell-associated uPA

Since it has been suggested that the invasive phenotype is associated with uPA rather than tPA activity (see Danø et al., 1985, and references therein), we considered it important to determine whether the caseinolysis observed at the wound edge was due to either or both of these enzymes. Anti-catalytic antibodies against uPA from different species cross-react poorly, and to our knowledge, antibodies to bovine uPA are at present unavailable. However, it has recently been

tions of the wound edges are indicated by arrows. (*b*) Nontreated control overlaid 24 h after wounding. (*c*) Cells incubated in the presence of cycloheximide as in *a*, and overlaid after a further 48-h incubation in the absence of the drug (i.e., overlaid 72 h after wounding). Note the presence of caseinolysis, indicating reversibility of cycloheximide inhibition. (*d*) Nontreated control overlaid 72 h after wounding. Overlays photographed after 120 min at 37°C.
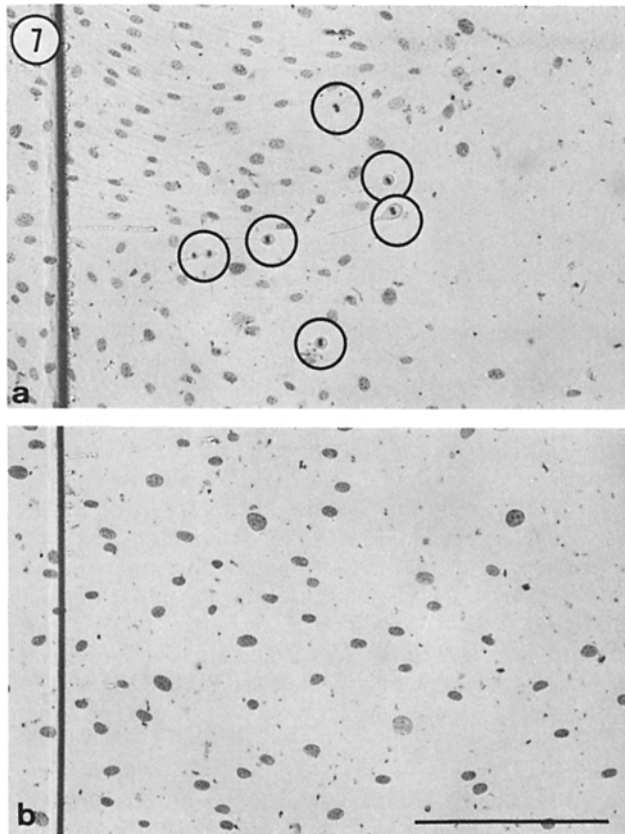
Table I. Wound-edge Cell Division: Effect of Mitomycin C

	No. of migrating cells per mm of wound edge	% Mitotic figures
Control	320.0 ± 13.1*	1.7 ± 0.3
Mitomycin C	201.9 ± 11.7*	0

Control and mitomycin C–treated monolayers were wounded with a blade to mark the original wound edge, and 24 h later were fixed and stained by the Feulgen technique. Photographic fields measuring 640 μm × 430 μm, in which the wound edge was perpendicular to the long axis of the fields (see Fig. 7), were randomly selected from both control and treated monolayers. A total of 24 fields representing four fields from each of three petri dishes in two separate experiments were counted from both mitomycin C–treated and non-treated cultures. The total number of cells in the wound and the number of cells in mitosis amongst the migrating cells (as judged by the presence of stained condensed chromosomes) were determined for each field. The number of migrating cells is expressed per millimeter of wound edge, and the number of mitotic figures as a percentage of total migrating cells. Results represent the mean ± standard error of the mean (* $P \ll 0.001$).

Figure 7. Mitomycin C inhibits mitosis at the wound edge. Wounded monolayers with or without preincubation in mitomycin C (10 μg/ml for 4 h before wounding) were fixed and stained by the Feulgen technique 24 h after wounding. (*a*) Control culture; several mitotic figures (*circled*) are visible amongst migrating cells. (*b*) Mitomycin C–treated culture; note the complete absence of mitotic figures amongst migrating cells. The original wound edge is indicated by the dark line on the left. Bar, 200 μm.

did not inhibit wound-edge caseinolysis in fixed (Fig. 9 *b*) or nonfixed cultures (not shown). We thus conclude that the plasminogen-dependent caseinolysis observed in association with migrating endothelial cells at the wound edge is due to cell-associated uPA activity.

Discussion

Production of proteases, and in particular of PAs, has been implicated in cell migration in a variety of invasive biological processes. It has been proposed that the two different PAs, uPA and tPA, may have different biological functions, uPA being primarily involved in invasive processes and tPA exerting its effect primarily in the circulatory system (see Danø et al., 1985, and references therein). Although most of the evidence linking uPA activity to cellular invasion has to date been indirect, two reports have demonstrated an inhibition of cell invasiveness in the presence of antibodies to uPA (Ossowski and Reich, 1983; Mignatti et al., 1986). By wounding
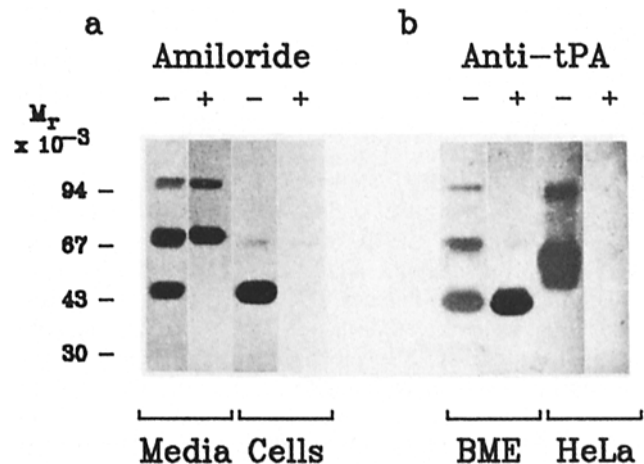
reported that the drug amiloride competitively inhibits the catalytic activity of uPA from a variety of species (Vassalli and Belin, 1987), without affecting the activity of tPA or of plasmin. To determine whether amiloride selectively inhibits bovine uPA, the drug was incorporated into a zymographic underlay which separates PAs on the basis of their molecular weight. Using this technique, we have found that amiloride inhibits the catalytic activity of M_r 47,000 bovine uPA, without affecting that of the M_r 72,000 bovine tPA (Fig. 8 *a*).

For the specific inhibition of tPA activity, we have taken advantage of the excellent cross-species reactivity of anti-catalytic anti-tPA antibodies. Fig. 8 *b* demonstrates that M_r 72,000 bovine tPA and the M_r 100,000 tPA-inhibitor complex (Loskutoff et al., 1986) are inhibited by affinity-purified anti-human tPA antibodies.

Using our wounded monolayer model, we explored the effect of amiloride and anti-catalytic anti-tPA antibodies on wound-associated caseinolysis. Experiments were performed on fixed cells to avoid the possible effects of amiloride related to inhibition of the Na+/H+ antiport system (Seifter and Aronson, 1986). Amiloride incorporated into the overlay completely inhibited caseinolysis along the wound edge (Fig. 9 *a*). In contrast, affinity-purified anti-human tPA antibodies

Figure 8. Zymographic assay. (*a*) Samples of conditioned media (*Media*) and cells (*Cells*) from PMA-treated BME cells were separated by SDS-PAGE and subjected to zymography with (+) or without (−) incorporation of amiloride into the underlay. (*b*) Samples of conditioned media from PMA-treated BME or HeLa cells were prepared as above with (+) or without (−) the incorporation of anti-tPA antibodies into the underlay. See text for details. (HeLa cell supernatant was used as a source of human tPA.)
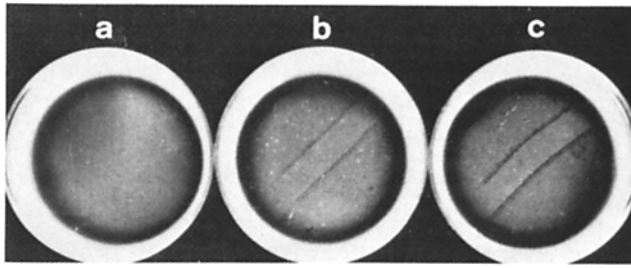
Figure 9. Wound edge cells produce urokinase. Cultures were fixed 24 h after wounding, and overlaid in the presence of plasminogen and either amiloride (*a*) or anti–tPA antibodies (*b*). (*c*) Control. Overlays photographed after 60 min at 37°C.

a confluent monolayer of endothelial cells and overlaying the monolayer with a casein-agar mixture containing plasminogen, we demonstrate here that there is an increase in uPA activity specifically associated with cells migrating from the edge of the wound, and that this activity ceases when the wound has closed. To our knowledge, this is the first direct demonstration of an increase in uPA activity in cells that are in the process of migration. uPA immunoreactivity has recently been localized to the leading edge of migrating keratinocytes (Morioka et al., 1987).

It has been demonstrated that endothelial cells produce both uPA and tPA (Levin and Loskutoff, 1982; Moscatelli, 1986), and that in bovine capillary endothelial cell cultures uPA remains primarily cell-associated whereas tPA is mostly secreted (Moscatelli, 1986). Our experimental system involves cell fixation for the identification of the PAs expressed by migrating cells, which allows us only to characterize cell-associated enzyme(s). We have however observed that anti-catalytic anti–tPA antibodies do not inhibit wound edge caseinolysis in nonfixed cells, thereby providing evidence that secreted tPA does not account for the increased catalytic activity. The use of amiloride and anti–tPA antibodies therefore demonstrates that wound-associated proteolysis is due to increased levels of uPA in or on migrating endothelial cells.

The wounded monolayer model has been used to study the mechanisms and kinetics of endothelial regeneration, and it has been shown that wounding initiates both cell migration and division (Sholley et al., 1977; Selden and Schwartz, 1979; Ryan et al., 1982). Cell-cycle dependent variations in PA activity and uPA mRNA levels have been reported (Rohrlich and Rifkin, 1977; Loskutoff and Paul, 1978; Aggeler et al., 1982; Grimaldi et al., 1986; Scott et al., 1987). We have found that wounding induces uPA activity both in the presence and absence of cell division. However, it has been observed that a cell-cycle specific increase in uPA mRNA occurs during G_0/G_1 transition (Grimaldi et al., 1986). Since the antiproliferative effect of mitomycin C is thought to result from its ability to cross-link complementary DNA strands (Iyer and Szybalski, 1963; Tomasz et al., 1987), treatment with this drug may allow cells to be recruited into G_1. Thus, although our findings demonstrate that cell division is not required for induction of uPA activity, it is conceivable that a wound-induced G_0/G_1 transition might contribute to wound edge–associated proteolysis.

The precise mechanisms responsible for the observed increase in uPA activity in response to mechanical wounding remain to be determined. It has been proposed that signal

transduction to the nucleus may be accomplished via the cytoskeleton (Bissell et al., 1982). Since wounding is accompanied by a reorganization of the endothelial cell cytoskeleton (Gotlieb et al., 1981, 1983, 1984; Mascardo and Sherline, 1984; Gabbiani et al., 1984; Pratt et al., 1984; Hormia et al., 1985; Young and Herman, 1985), the effect we observe may result from a cytoskeletally mediated signal to the cell nucleus.

Although it has been proposed that the increase in PA production associated with cellular invasion is necessary for the degradation of the basement membrane and extracellular matrix components (Mullins and Rohrlich, 1983; Saksela, 1985; Danø et al., 1985; Goldfarb and Liotta, 1986), the precise role(s) of PAs in the invasive process remain to be established. Using the wounded monolayer model, it has been reported that migration of normal and transformed fibroblasts (Ossowski et al., 1973, 1975) and smooth muscle cells (Schleef and Birdwell, 1982) is dependent on the presence of plasminogen. However, this was not the case for bovine aortic endothelial cells, whose migration was unaffected by the removal of plasminogen from the serum (Schleef and Birdwell, 1982). The addition of a variety of serine protease inhibitors to the medium after wounding also did not affect endothelial cell migration (Schleef and Birdwell, 1982). This is in keeping with our previous findings that the addition of serine protease inhibitors or the removal of plasminogen does not affect PMA- or basic fibroblast growth factor-induced endothelial cell invasion of a three-dimensional collagen (Montesano and Orci, 1985; and unpublished observation) or fibrin (Montesano et al., 1987) matrix. Therefore, although wounding, PMA (Levin and Loskutoff, 1979; Moscatelli et al., 1980; Gross et al., 1982; Moscatelli, 1986), basic fibroblast growth factor (Montesano et al., 1986; Moscatelli et al., 1986a, b), and other angiogenic preparations (Gross et al., 1983) stimulate PA production, there is presently no evidence for a causal role of these enzymes in the processes of endothelial cell migration or invasion in our simplified in vitro systems. Plasminogen activation is, however, likely to represent only one member of a cohort of enzymatic and other cellular events required for migratory or invasive processes; the absence of a single member, for example, plasminogen activation, need not therefore necessarily perturb these processes. Furthermore, whether plasminogen activation is necessary for endothelial cell migration and invasion in the more complex in vivo environment remains to be established.

In conclusion, we have devised a novel approach that involves overlaying a wounded monolayer of cells with a casein-agar mixture containing plasminogen, to determine whether migrating cells display an increase in PA activity. Using this model, we have observed an induction of uPA activity in endothelial cells migrating from the edges of an experimental wound, and a reversion of this activity to background levels upon cessation of movement, when the wound has closed. These results provide strong support for the association between migratory behavior and increased expression of uPA activity.

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