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.

Materials and Methods

Materials

48-phorbol 12-myristate 13-acetate (PMA), L-arginine, cyclic AMP, 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, Upsala, 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 (MEM), and antibiotic (FBS, 10% heat-inactivated, alpha, calf serum, FBS, 20% heat-inactivated, alpha, serum, penicillin, 500 U/ml, streptomycin, 100 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 L-arginine was added to the overlay mixture at 200 U/ml and 500 U/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-
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 lines against a white background, revealing the production of PA by cells lining the wound edge. Caseinolysis is inhibited by the addition of Trasylol (c) or e-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.

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 e-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).
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.

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 Danó 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
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 < 0.001).

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 (Ossowsk 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 did not inhibit wound-edge caseinolysis in fixed (Fig. 9 b) or nonfixed cultures (not shown).
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 antiproliferative activity of anti-tPA antibodies do not inhibit wound edge cascinolysis 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 Go/G1 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 G0. Thus, although our findings demonstrate that cell division is not required for induction of uPA activity, it is conceivable that a wound-induced Go/G1 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; Mascarado 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 cytoskeletonally 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 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 observations) 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.

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.

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References

Aggeler, J., L. N. Kapp, S. C. G. Tseng, and Z. Werb. 1982. Regulation of protein secretion in Chinese hamster ovary cells by cell cycle position and cell density. Exp. Cell Res. 139:275-283.

Ausprunk, D. H., and J. Folkman. 1977. Migration and proliferation of endothelial cells in perfused and newly formed blood vessels during tumour angiogenesis. Microvasc. Res. 14:53-65.

Bissell, M., J. H. Hall, and G. Parry. 1982. How does the extracellular matrix direct tissue organization? J. Theor. Biol. 99:31-68.

Bürk, R. 1973. A factor from a transformed cell line that affects cell migration. Proc. Natl. Acad. Sci. USA. 70:369-372.

Dann, K., P. A. Andreassen, J. Grandahl-Hansen, P. Kristensen, L. S. Nielsen, and T. B. Eng连云港. 1984. Angiotensin converting enzyme and transforming growth factor in cultured endothelial cells. J. Cell Biol. 99:66-90.

Danzel, M., D. Presta, J. Joseph-Silverstein, and D. B. Rifkin. 1986a. Both normal and tumor cells produce basic fibroblast growth factor. J. Cell Physiol. 129:273-276.

Danzel, M., D. Presta, and D. B. Rifkin. 1986b. Purification of a factor from human placenta that stimulates capillary endothelial cell proliferation. DNA synthesis and migration. Proc. Natl. Acad. Sci. USA. 83:2091-2095.

Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen: purification from human plasma by affinity chromatography. Science (Wash. DC). 170:1095-1096.

Fishman, J. A., G. B. Ryan, and M. J. Karnovsky. 1975. Endothelial regeneration in the rat carotid artery and the significance of endothelial denudation in the pathogenesis of myointimal thickening. Lab. Invest. 32:339-351.

Folkman, J. 1985. Tumour angiogenesis. Lab. Invest. 53:27-41.

Furie, M., M. B. Furie, B. L. Napravnik, and S. J. Bertozzi. 1984. cultured endothelial cell monolayers that restrict the transendothelial passage of large molecular weight molecules. J. Cell Biol. 98:1033-1041.

Gabbiani, G., F. Gabbiani, R. L. Heimark, and S. M. Schwartz. 1984. Organization of the actin cytoskeleton during early endothelial regeneration in vitro. J. Cell Biol. 99:66-90.

Goldfarb, L. H., and L. A. Liotta. 1986. Proteolytic enzymes in cancer invasion and metastasis. Semin. Thromb. Hemostasis. 12:294-307.

Gotlieb, A. I., W. Spector, M. K. K. Wong, and C. Lacey. 1984. In vitro reenactment of tumor invasion in an in vitro experimental system. J. Cell Biol. 98:237-247.

Gotlieb, A. I., L. M. May, L. Subrahmanyan, and V. I. Kalnins. 1981. Disruption of microtubule organizing centers in migrating sheets of endothelial cells. Mol. Biol. Cell. 2:601-611.

Gotlieb, A. I., W. Spector, M. K. K. Wong, and C. Lacey. 1984. Migration into an in vitro experimental system of the actin cytoskeleton. J. Cell Biol. 99:66-90.

Grimaldi, G., P. Di Fiore, E. K. Locatelli, J. Falco, and F. Blasi. 1986. Modulation of actin cytoskeleton during early endothelial regeneration in vitro. J. Cell Biol. 106:180--186.

Gross, J. L., D. Moscatelli, and D. B. Rifkin. 1983. Increased capillary endothelial cell motility: DNA synthesis and migration. Proc. Natl. Acad. Sci. USA. 80:2633-2637.

Hausdorffer, C. C., and S. M. Schwartz. 1979. Endothelial regeneration. II. Restitution of endothelial continuity. Lab. Invest. 41:407-418.

Hornia, M., R. A. Badley, V. P. Leito, and J. Virtanen. 1985. Actomyosin organization in stationary and migrating sheets of cultured human endothelial cells. Exp. Cell Res. 157:116-126.

Iyer, V. N., and W. Szybalski. 1963. A molecular mechanism of mitomycin action: linking of complementary DNA strands. Proc. Natl. Acad. Sci. USA. 50:355-362.

Levin, E. G., and D. J. Loskutoff. 1979. Comparative studies of the fibrinolytic activity of cultured vascular cells. Thromb. Res. 9:159-169.

Levin, E. G., and D. J. Loskutoff. 1982. Increased capillary endothelial cell motility in response to angiogenic stimuli in vitro. Proc. Soc. Exp. Biol. Med. 172:468-472.

Loskutoff, D. J. 1978. Intracellular plasminogen activator expression during angiogenesis. Exp. Cell Res. 117:5-16.

Loskutoff, D. J. 1978. Effects of acidified fetal bovine serum on the fibrinolytic activity and growth of cells in culture. J. Cell. Physiol. 96:361-370.

Loskutoff, D. J. 1978. Intracellular plasminogen activator expression in growing and quiescent cells. J. Cell Physiol. 97:9-16.

Loskutoff, D. J., T. Ny, M. Sawdey, and D. Lawrence. 1986. Fibrinolytic system of cultured endothelial cells: regulation by plasminogen activator inhibitors. J. Cell Biol. 103:271-284.

Madri, J. A., and K. S. Stenn. 1982. Aortic endothelial cell migration. I. Matrix requirements and composition. Am. J. Pathol. 106:180--186.

Mascaro, R. N., and P. Sherline. 1984. Inulin and multilamination-stimulating activities induced by very rapid centrifugal orientation response to wounding in endothelial cell monolayers. Diabetes. 33:1099-1105.

Mignatti, P., E. Robbins, and D. Rifkin. 1986. Tumour invasion through the amniotic membrane: requirement for a protease cascade. Cell. 47:487-498.

Miskin, R., and E. Reich. 1980. Plasminogen activator: induction of synthesis by DNA damage. Cell. 19:217-224.

Montesano, R., and L. Orci. 1985. Tumor promoting phorbol esters induce angiogenesis in vitro. Cell. 41:673-681.

Montesano, R., J.-D. Vassalli, A. Baird, R. Guillemin, and L. Orci. 1986. Basic fibroblast growth factor induces angiogenesis in vitro. Proc. Natl. Acad. Sci. USA. 83:7297-7301.

Montesano, R., M. S. Pepper, J.-D. Vassalli, and L. Orci. 1987. Phorbol esters induce cultured endothelial cells to invade a fibrin matrix in the presence of fibrinolytic inhibitors. J. Cell. Physiol. 132:509-516.

Morikawa, S., G. S. Lazarus, J. L. Baird, and P. J. Jensen. 1987. Migration kininotransferase express urokinase-type plasminogen activator. J. Invest. Dermatol. 88:418-422.

Moscatelli, D. 1986. Urokinase-type and tissue-type plasminogen activators have different distributions in cultured bovine capillary endothelial cells. J. Cell. Biol. 103:19-29.

Moscatelli, D., E. Jaffe, and D. B. Rifkin. 1980. Tetradeacyls ceramide activate stimulates latent collagenase production by cultured human endothelial cells. Cell. 20:343-351.

Pepper et al. 1987. Urokinase in Migrating Endothelial Cells 2541