CELL REGULATION, Vol. 1, 843–852, October 1990

A high-affinity receptor for urokinase plasminogen activator on human keratinocytes: characterization and potential modulation during migration

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Low passage cultures of normal human keratinocytes produce several components of the plasminogen activator/plasmin proteolytic cascade, including urokinase plasminogen activator (uPA), tissue plasminogen activator (tPA), and two specific inhibitors. Studies here presented demonstrate that these cells also contain a high-affinity (K_d = 3 $\times 10^{-10}$ M) plasma membrane-binding site for uPA. High molecular weight uPA, either as the single-chain precursor or two-chain activated form, bound to the receptor; however, low molecular weight (33 kD) uPA, tPA, or epidermal growth factor did not compete for binding, demonstrating specificity. Acid treatment, which removed endogenous uPA from the receptor, was required to detect maximal binding (45 000 sites per cell). To investigate the possibility that the uPA receptor on keratinocytes may be involved in epithelial migration during wound repair, cultures were wounded and allowed to migrate into the wounded site. Binding sites for uPA were localized by autoradiographic analysis of ^{125}I-uPA binding as well as by immunocytochemical studies using anti-uPA IgG. With both techniques uPA binding sites were detected selectively on the plasma membrane of cells at the leading edge of the migrating epithelial sheet. This localization pattern suggests that uPA receptor expression on keratinocytes may be coupled to cell migration during cutaneous wounding.

Introduction

Turnover of the extracellular matrix is an important aspect of cutaneous wound repair. Shortly after wounding, a provisional matrix consisting primarily of fibrin and fibronectin (Grinnell *et al.,* 1981; Clark *et al.,* 1982) is organized, through which keratinocytes migrate. Controlled degradation of the matrix is required during cell migration and tissue remodeling; the plasminogen activator/plasmin proteolytic cascade (Saksela, 1985) may play an important role in the cleavage of various matrix components. Urokinase plasminogen activator (uPA) converts plasminogen to plasmin, a serine proteinase that can degrade several extracellular molecules including fibrin, fibronectin, and laminin (Saksela, 1985; Liotta *et al.,* 1981). Plasmin also converts procollagenase to its active form (Werb *et al.,* 1977). Urokinase PA at relatively high concentrations has the ability to cleave fibronectin directly (Quigley *et al.,* 1987).

In fibroblasts, uPA has been localized to sites of focal contact between the cells and substratum (Pollanen et al., 1987; Hebert and Baker, 1988), suggesting the possibility that the enzyme is involved in the release of the cells from the substratum during migration. Several observations (Hebert and Baker, 1988) suggest that uPA at the focal adhesions is bound to a specific plasma membrane receptor (Vassalli et al., 1985; Blasi et al., 1987). Urokinase PA bound to this receptor can be in an active form and hence allows cells to generate proteolysis in the pericellular space (Cubellis et al, 1986; Ellis et al, 1989; Stephens et al, 1989).

We have previously shown that human keratinocytes contain and secrete uPA (Morioka et al, 1985). Using an in vitro wound-healing model, we have also presented data that suggest that enhanced expression of this enzyme is a property of migrating keratinocytes (Morioka et al, 1987). In the current study we have shown that human keratinocytes possess a specific, high-affinity receptor for uPA, and we present evidence that the receptor is modulated during cell migration.

Results

Characteristics of ^{125}I-uPA binding to keratinocyte cultures

Keratinocyte monolayers, grown to confluence in serum-free medium, were incubated with

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single-chain, 55 kD ^{125}I-uPA for various times from 10 to 210 min at 23°C (Figure 1). Ligand binding increased with time, reaching a plateau by 120 min. Nonspecific binding, determined by addition of a 100-fold excess of unlabelled uPA, was consistently ≤6%. Brief treatment of the cultures with isotonic buffer at pH 3.0, which is known to strip receptors of their endogenous ligand (Haigler et al, 1980; Bajpai and Baker, 1985; Stoppelli et al, 1986), was required to obtain maximal binding; if this step was omitted, binding of ^{125}I-uPA was decreased by 75–90% (in experiments with two cultures). Measurement of uPA in the acid wash by enzyme-linked immunosorbent assay (ELISA) revealed that 31 000 ± 10 000 uPA molecules per cell were released (mean ± SD of five determinations on cultures from two donors). In contrast, in a neutral wash only 3200 ± 700 uPA molecules per cell were detected. These results demonstrate that the majority of the uPA binding sites were occupied by endogenous uPA; this finding is consistent with the observation that keratinocytes secreted uPA (0.8 ± 0.5 pmole/ml in 48 h, mean ± SD of five cultures) under the culture conditions employed. Based on these data, subsequent binding experiments were performed with acid-stripped cultures, and incubations with ligand were carried out for 150 min to ensure that steady-state conditions were reached.

To determine if ^{125}I-uPA was binding to the cell surface or rather to one or more components of the extracellular matrix (ECM), several types of experiments were performed. First, binding of ^{125}I-uPA to keratinocyte monolayers was compared with binding to isolated ECM. Less than 2% of the ^{125}I-uPA which bound specifically to the cell monolayers was able to bind to isolated ECM (Table 1, Part A), strongly suggesting that uPA binds to the cell surface and not to a matrix component. In the second series of experiments, keratinocyte monolayers that had bound ^{125}I-uPA were harvested in two ways: with the nonionic detergent Triton X-100, which solubilizes integral membrane proteins, or with SDS, which solubilizes extracellular matrix as well as cellular components. The specifically bound counts per minute (CPM) recovered by both harvesting procedures were very similar, although nonspecifically bound CPM were fewer with Triton X-100 harvest (Table 1, Part B). This result shows that nearly all the specifically bound uPA was released from the keratinocyte monolayer by nonionic detergent, suggesting that uPA is associated with a membrane protein.

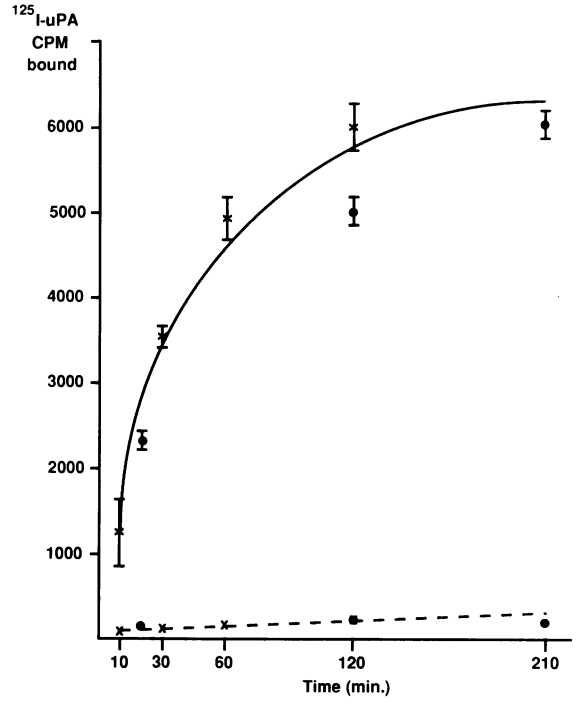

Figure 1. **Time-course of ^{125}I-uPA binding.** Confluent monolayer cultures of human keratinocytes in 12-well plates were treated with acidic buffer, washed, and then incubated with 1.1×10^{-10} M ^{125}I-uPA at 23°C for 10–210 min. Cultures were washed five to six times before solubilization in 0.1% sodium dodecyl sulfate to determine cell-associated radioactivity. The solid line shows total binding. The dashed line shows nonspecific binding, determined in the presence of a 100-fold excess of unlabeled single-chain uPA. Each time point was done in duplicate, and two separate experiments are shown (× and ●).

Finally, we investigated the behavior of culture-bound and -unbound uPA when solubilized in 3% Triton X-114 (Estreicher et al, 1989). At 0°C aqueous solutions of Triton X-114 are homogenous, but they separate into two phases at ≥20°C. After phase separation, integral membrane proteins are mostly recovered in the detergent phase, whereas hydrophilic proteins remain in the aqueous phase (Bordier, 1981). As expected, when soluble ^{125}I-uPA was added to Triton X-114 cell extracts in the presence of a large excess of unlabelled uPA (to saturate the binding sites) and then subjected to phase separation, 88.5 ± 1.0% (average of two experiments) of the label was recovered in the aqueous phase. However, ^{125}I-uPA that had been bound to keratinocyte cultures prior to extraction in Triton X-114 was mostly found in the detergent phase (67.7 ± 1.5%). These findings, as well as the ones described above in Table 1, suggest strongly that uPA binds to an integral membrane receptor protein in the keratinocyte.

Table 1. uPA binds specifically to cells but not to ECM

	CPM bound		
	Total CPM	Non-specific CPM	Specific CPM
Part A*			
Cell monolayer	84 473 ± 2 865	12 650 ± 78	71 823
ECM	14 756 ± 2 487	13 860 ± 1131	896
Part B†			
Cell monolayer			
SDS harvest	103 590 ± 18 024	17 006 ± 277	86 584
Triton X-100 harvest	89 371 ± 3 193	7 109 ± 269	82 262

* ^{125}I-uPA (3×10^{-10} M) was added to keratinocyte monolayers or to extracellular matrix (ECM) from which the cells had been removed. After incubating for 2.5 h and washing to remove unbound uPA, all wells were harvested with SDS to determine total bound CPM. Nonspecifically bound CPM were determined in the same way, except that a 200-fold excess of unlabelled uPA was added along with the radiolabeled ligand.

† ^{125}I-uPA (3×10^{-10} M) in the presence and absence of a 200-fold excess of unlabeled uPA was incubated with keratinocye monolayers for 2.5 h. After washing, the wells were harvested with SDS or with Triton X-100.

Experiments were performed to determine if bound uPA remained on the surface of the keratinocyte or was internalized. After acid stripping and incubation with ^{125}I-uPA for 180 minutes, cultures were washed to remove unbound ligand and then incubated with fresh medium (containing no additional uPA) for 2 h at 37°C. At the end of the incubation period, cells were treated with isotonic buffer at pH 3.0 to remove surface bound ligand and then were solubilized to recover internalized ligand. Seventy-five percent of the total cell-associated radioactivity remained on the cell surface, as determined by its sensitivity to removal with acidic buffer (Table 2). These results show that the interaction of uPA with its binding site on the keratinocyte does not lead to rapid ligand internalization. To eliminate the possibility that acid treatment itself damaged the cells and hence prevented internalization of any ligand, we performed control experiments (see Methods) with ^{125}I-EGF. Epidermal growth factor (EGF) was rapidly internalized into acid-pretreated keratinocytes upon incubation at 37°C, as expected from previously published studies (O'Keefe et al, 1982).

Specificity of ^{125}I-uPA binding

To investigate the specificity of the uPA binding site on the keratinocyte, competition experiments were performed (Figure 2). Unlabelled single-chain and two-chain (WinKinase) 55 kD uPA competed for binding of ^{125}I-uPA. However, the low molecular weight (33 kD) form of uPA, which retains enzymatic activity but lacks the receptor-binding domain (Blasi et al, 1987), did not. Neither tissue PA, an enzyme that catalyzes the same reaction as uPA but has a distinct structure, nor EGF, which has partial homology to the receptor-binding domain of uPA (Appella et al, 1987), competed for the uPA binding site

Table 2. Effect of acid treatment on cell-associated ^{125}I-uPA

CPM	
Surface-bound (i.e. released by acid treatment)	Internalized (i.e. insensitive to acid treatment)
45 305 ± 7081	14 982 ± 1921

Cultures were acid-treated to remove endogenous ligand and incubated with 5×10^{-10} M ^{125}I-uPA for 180 min to allow steady-state binding to be attained. They were then washed to remove unbound ligand and incubated with fresh medium (containing no additional uPA) for 2 h at 37°C. At the end of the incubation period, the medium (containing 18 604 ± 1892 released CPM) was removed. Cells were then treated with isotonic buffer at pH 3.0 for 15 min at 37°C to remove surface-bound ligand; this acidic wash was removed and released CPM were determined. Finally, the cells were solubilized in SDS to recover internalized ligand and the CPM in this lysate were determined.

All values are the mean ± SD of 3–4 replicate culture wells. All data are corrected for nonspecific binding, determined in replicate wells by the addition of a 100-fold excess of unlabeled uPA.

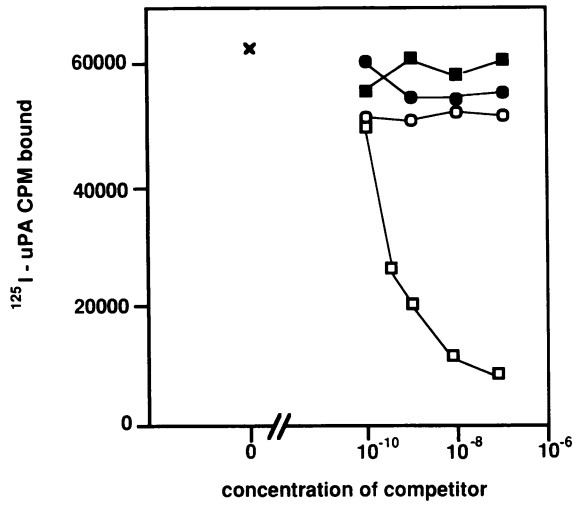

Figure 2. **Specificity of ^{125}I-uPA binding.** Acid-stripped cultures were incubated for 150 min with 9×10^{-11} M ^{125}I-uPA either alone (×) or in the presence of varying concentrations of unlabeled single chain (55 kD) uPA (— □ —), the 33 kD form of uPA (— ● —), epidermal growth factor (— ■ —), or tissue PA (— ○ —). Total binding was determined as described in Figure 1.

(Figure 2). These findings agree with previous characterizations of the uPA receptor on human monocyte and carcinoma lines (Vassalli et al, 1985; Stoppelli et al, 1986; Appella et al, 1987).

Parameters of the keratinocyte uPA receptor

To investigate further the characteristics of uPA binding to keratinocytes, saturation binding curves were generated using various concentrations of ^{125}I-uPA; a representative experiment is shown in Figure 3. The data were analyzed by Scatchard transformation (Figure 3B) as well as with nonlinear least-squares curve fitting (Accufit, Beckman Instruments, Fullerton, CA). Based on Scatchard analyses of seven experiments with confluent monolayer cultures from five donors, we determined that the K_d for the uPA receptor was $3.0 \pm 1.6 \times 10^{-10}$ M; there were 45 000 ± 15 000 receptors per cell. The corresponding parameters determined by nonlinear least-squares curve fitting analyses were $K_d = 2.5 \pm 1.2 \times 10^{-10}$ M and 42 000 ± 18 000 receptors per cell.

uPA receptor in migrating keratinocytes

To consider a possible role for uPA and its receptor during keratinocyte migration, we localized both endogenous uPA antigen, as well as ^{125}I-uPA binding in wounded cultures. Stratification and differentiation were induced in confluent monolayer cultures by incubation in medium with elevated calcium and no EGF (Shipley and Pittelkow, 1987). These stratified cultures were then "wounded" by cutting with a scalpel; the cells retracted from the wound, leaving a denuded area. Upon further incubation, the

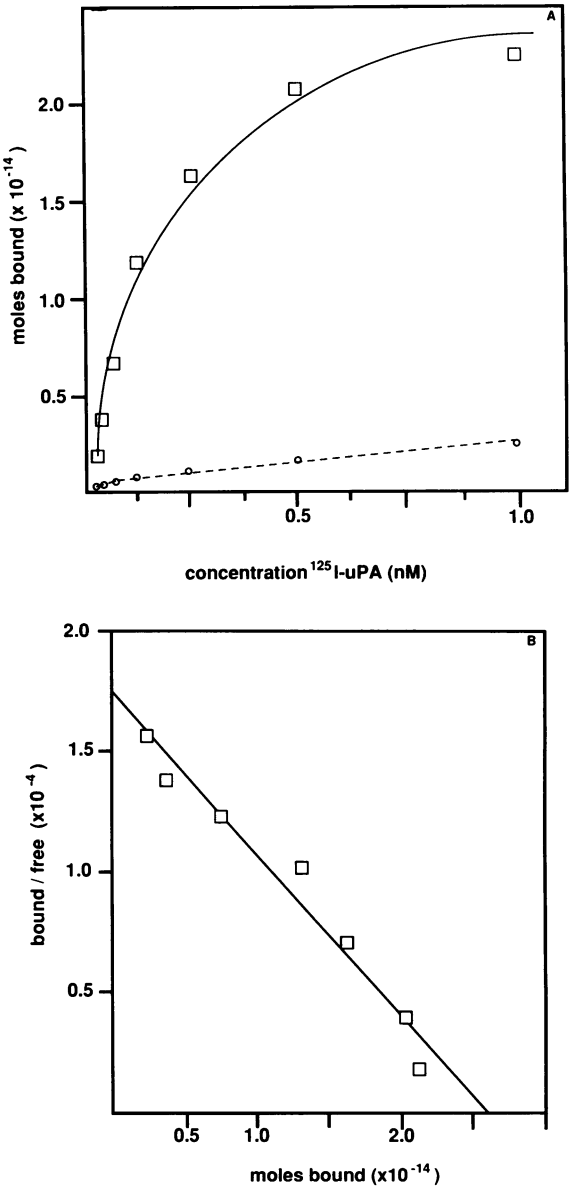

Figure 3. **Characterization of ^{125}I-uPA binding.** Cultures were acid-stripped and then incubated for 150 min at 23°C with ^{125}I-uPA at concentrations from 1.6×10^{-11} to 1.0×10^{-9} M. Cell-associated radioactivity was determined as described in Figure 1. (A) Total cell-associated radioactivity is shown with the solid line. Nonspecific binding, determined in the presence of a 100-fold excess of unlabeled uPA (WinKinase), is shown with the dashed line. (B) Scatchard transformation (r = 0.98) of the data, yielding a $K_d = 1.8 \times 10^{-10}$ M and 43 500 receptors per cell.

keratinocytes gradually migrated into the denuded area (Morioka et al, 1987).

When migrating cultures were stained with anti-uPA IgG in the absence of permeabilizing agent to examine surface-bound antigen, uPA was selectively detected on cells at the leading edge of the migrating epithelium (Figure 4, A and D). If cultures were acid-treated before incubation with antibody, staining for uPA was not observed (Figure 4, B and E). However, if the acid-treated cultures were washed and then incubated with exogenous uPA, uPA antigen was again selectively detected on cells at the leading edge (Figure 4F). These studies show that endogenous uPA binds to the surface of migrating keratinocytes, most likely through the uPA receptor.

To test further this hypothesis, experiments were performed to investigate the binding of ^{125}I-uPA to migrating cultures. When the cultures were acid-treated, incubated with ^{125}I-uPA, and then analyzed with autoradiography, a dramatic increase in ^{125}I-uPA binding was observed at the leading edge of the culture (Figure 5A), consistent with the immunocytochemical results shown in Figure 4. Inclusion of a 100-fold excess of unlabelled uPA blocked binding of ^{125}I-uPA, demonstrating specificity. When the acid pretreatment was omitted, no detectable binding of ^{125}I-uPA was observed (Figure 5B); this finding was expected since the immunocytochemical results shown in Figure 4 indicate that the uPA binding site is occupied with endogenous ligand.

Note that many cells in the migrating epithelium that are just interior to the leading edge do *not* have detectable surface uPA detectable by either immunostaining (Figure 4, A, D, and F) or ^{125}I-uPA autoradiography (Figure 5A). This pattern indicates that the binding site for uPA is selectively expressed on cells at the leading edge of the migrating epithelium.

Discussion

Early passage human epidermal keratinocytes produce urokinase and tissue-type plasminogen activators (Jensen et al, 1990) as well as PA inhibitors type 1 and 2 (Hashimoto et al, 1989). The present report demonstrates that these cells also possess a high affinity plasma membrane receptor for uPA. Furthermore, plasminogen has been detected in the basal layer of epidermis (Isseroff and Rifkin 1983). The functions of this complex plasminogen activating cascade in epithelial tissue are currently unclear. However, the present finding that keratinocytes bear a uPA receptor whose expression appears to be enhanced on migrating cells supports the hypothesis (Morioka et al, 1987; Grøndahl-Hansen et al, 1988) that the uPA proteolytic cascade may function in keratinocyte migration during cutaneous wound repair.

The keratinocyte uPA receptor has biochemical properties (Figures 2 and 3) analogous to a receptor previously characterized on several transformed cell lines (Blasi *et al.,* 1986), including the monocyte line U937 (Vassalli *et al.,* 1985; Stoppelli *et al.,* 1985), the carcinoma line A431 (Stoppelli *et al.,* 1986), an epithelial line with HeLa markers (CCL 20.2) (Kirchheimer *et al.,* 1989), and the fibrosarcoma line HT1080 (Nielsen *et al.,* 1988). For each line, the K_d was $\approx 10^{-10}$ M, and there were 20 000–80 000 binding sites per cell. Furthermore, in each case, binding of uPA was not through the catalytic domain, but rather through the amino terminal fragment of the enzyme; hence, single chain precursor and two-chain uPA both bound to the receptor, but the enzymatically active, 33-kD catalytic subunit did not. Several normal cells, including human monocytes (Vassalli *et al.,* 1985), a bovine capillary endothelial line (Fibbi *et al.,* 1988), and an established epidermal line (Del Rosso *et al.,* 1990), have also been reported to bear such a receptor.

Urokinase PA binding sites with different properties were detected in fibroblasts. Human foreskin fibroblasts bound two-chain uPA ($K_d \approx 2 \times 10^{-9}$), but not single-chain precursor uPA (Bajpai and Baker, 1985). Mouse 3T3 fibroblasts bound active, but not inhibited uPA, suggesting that the catalytic site of uPA was involved in the interaction (Del Rosso *et al.,* 1985). At least some of the fibroblast binding capacity for uPA was due to complex formation with protease nexin (Baker *et al.,* 1980; Low *et al.,* 1981; Eaton *et al.,* 1984). For several reasons, it is unlikely that the keratinocyte binding sites for uPA detected in the present study were related to protease nexin: protease nexin bound active two-chain uPA and the 33-kD catalytic subunit, but not single-chain precursor uPA (Eaton *et al.,* 1984); protease nexin-enzyme complexes were rapidly internalized and degraded (Low *et al.,* 1981); protease nexin formed complexes not only with uPA, but also with tPA and other proteinases (Eaton *et al.,* 1984). Hence through secretion of protease nexin, fibroblasts appear to generate a mechanism for control of active serine proteinases through cellular binding, uptake, and degradation (Low *et al.,* 1981). This is in distinct contrast to the type of uPA receptor reported here for the keratinocyte and else-

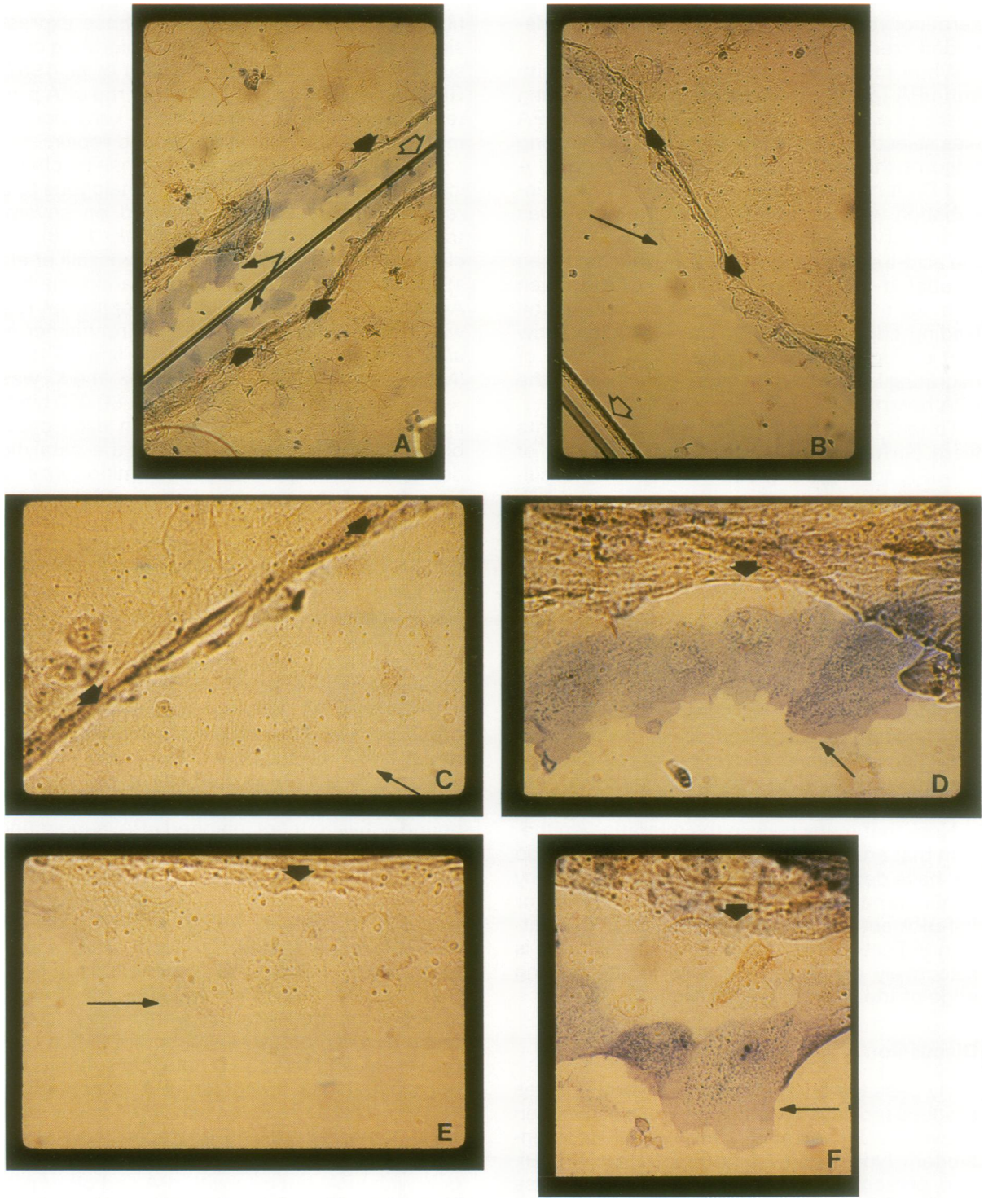

Figure 4. **Immunocytochemical localization of uPA on wounded keratinocyte cultures.** Stratified keratinocyte cultures were wounded with a scalpel and then incubated at 37°C for 24 h to allow cell migration into the denuded area. Staining with primary antibody was done without permeabilizing agent to examine only surface antigen. Antibody deposition was visualized with blue substrate for alkaline phosphatase. (A) Stained with anti-uPA IgG (63×); (B) pretreated with acid, then stained with anti-uPA IgG (63×); (C) stained with normal rabbit IgG (250×); (D) stained with anti-uPA IgG (250×); (E) pretreated with acid, then stained with anti-uPA IgG (250×); (F) pretreated with acid, reincubated with uPA, then stained with anti-uPA IgG (250×). In all panels, the closed arrowheads indicate the point to which the cultures have retracted and the folded epidermal sheet; the long arrows indicate areas of outgrowth from the wound edge; and the open arrowheads show the scratch in the plastic dish made by the scalpel upon wounding.

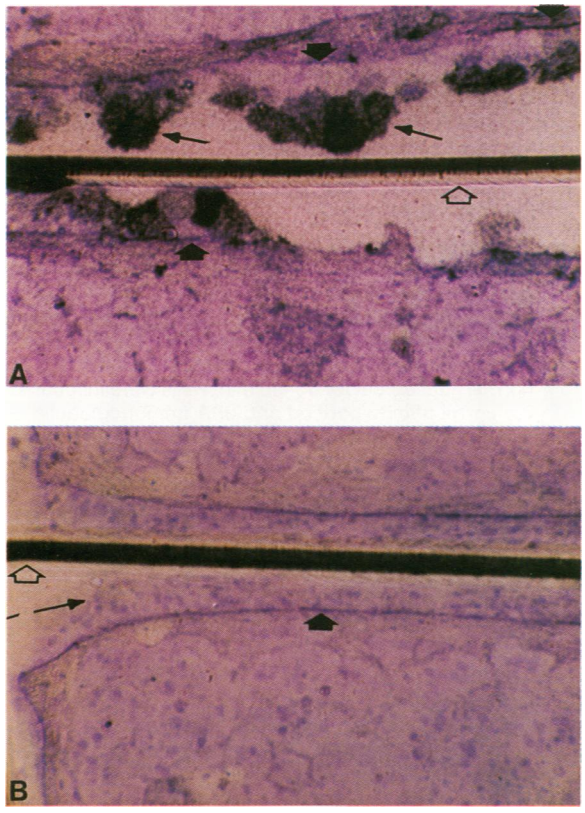

Figure 5. **Modulation of ^{125}I-uPA binding upon wounding.** (A) Stratified keratinocyte cultures were wounded and reincubated as in Figure 4. To localize binding of ^{125}I-uPA, cultures were acid-stripped and then incubated at 23°C for 150 min with 2×10^{-10} M ^{125}I-uPA. After extensive washing, cultures were fixed with formalin and processed for autoradiography. Counterstain was hematoxylin. Open arrowheads indicate the scratch in the plastic dish made by the scalpel upon wounding; closed arrowheads show areas of retraction and folding of the cell sheet; and long arrows illustrate areas of outgrowth from the wound edge. (B) Cultures were treated as in A, but the pretreatment with acidic buffer was omitted. No detectable binding was observed.

where for several other cell types (Blasi *et al.,* 1986).

The finding that receptor-bound uPA in the keratinocyte is internalized only very slowly is consistent with an extracellular function for bound uPA. The receptor-uPA complex is part of a cell surface proteolytic system with the capacity to generate proteolytic activity limited to the pericellular space (Cubellis *et al.,* 1986; Ellis *et al.,* 1989; Stephens *et al.,* 1989). This ability to localize and concentrate proteolysis at the cell surface may be essential for keratinocyte migration through the provisional matrix during wound repair. The migration of keratinocytes from the wound edges or from the hair follicles into the wound bed is an important early event in the reepithelialization process. Cell migration, which begins within hours after wounding, precedes the enhanced cell proliferation required to restore epidermal architecture (Potten and Allen, 1975; Krawczyk, 1971; Marks and Nishikawa, 1973).

Using an in vitro model of epidermal wounding, we have previously published evidence (Morioka *et al.,* 1987) that migrating keratinocytes have enhanced expression of uPA, detectable both in the cytoplasm and along the plasma membrane. By demonstrating enhanced binding of uPA to the surface of keratinocytes at the leading edge of a migrating epithelial sheet, the present report provides strong evidence for the enhanced expression of a uPA receptor in these migrating cells. Hence, it is intriguing to speculate that the expression of uPA and of its receptor may be coordinately regulated in migrating keratinocytes.

A greater concentration of uPA at the cell surface may be required to aid the keratinocyte in its movement through the complex extracellular matrix of a wound bed. Several events in this process require extracellular proteolysis which might be mediated by cell-bound uPA: e.g., cleavage of a path through the matrix, breakage of cell-substratum bonds to allow cell movement, and/or generation of biologically active fragments of fibrin or fibronectin (Barnhart, 1968; Seppa *et al.,* 1981; Thiery, 1984). The possible involvement of receptor-bound uPA in these events is under study.

Methods

Materials

Iodogen was obtained from Pierce (Rockford, IL); Na^{125}I, from Amersham (Arlington Heights, IL). MCDB153 basal medium was purchased from Irvine (Santa Ana, CA); epidermal growth factor, from Collaborative Research (Bedford, MA); insulin, hydrocortisone, and bovine serum albumin (#8022) from Sigma Chemical Co. (St. Louis, MO). Bovine pituitaries were purchased from Pelfreez Biologicals (Rogers, AK). K-2 emulsion was from Ilford Scientific (Paramus, NJ). Low molecular weight (33 kD) uPA was from Mochida Pharmaceuticals (Tokyo, Japan). Urinary uPA in the two-chain form (WinKinase) was obtained from Sterling-Winthrop (Rensselaer, NY) through NIH. Single-chain uPA was a generous gift from Dr. Donald Moir of Collaborative Research.

Iodination of uPA

Single-chain uPA was iodinated using 50 μg of Iodogen and 1 mCi Na^{125}I per 11 μg protein, as previously described (Baker *et al.,* 1980). The concentration of recovered uPA was determined by ELISA of duplicate uniodinated samples. Specific activity of iodinated uPA was 1400–3800 Ci/mmole.

Cell culture

Human keratinocytes from neonatal foreskin were propagated in MCDB 153 base medium with 0.03 mM Ca^{2+} and

the following additives: bovine pituitary extract, insulin, EGF, hydrocortisone, and high amino acids, as previously described (Shipley and Pittelkow, 1987). Cells grown under these conditions remained in monolayer form and had a basal-like phenotype. All biochemical analyses of binding were done on third-passage keratinocytes grown to confluency in 12-well plates.

Autoradiography experiments were done on cultures grown in six-well plates and in some experiments on coverslips. Upon reaching confluency, the cultures were incubated for 5–6 d in MCDB medium with 2.0 mM Ca^{2+} and no EGF to induce differentiation and stratification (Shipley and Pittelkow, 1987). Wounding of the cultures by cutting with a scalpel led to retraction of the cell sheet and subsequent migration of the keratinocytes into the denuded area, similarly to our previous findings in serum-containing cultures (Morioka *et al.*, 1987).

To prepare ECM for the experiments shown in Table 1 (Part A), cells were removed by incubation in 0.5% Triton X-100 for 10 min, followed by washing three times with distilled H_2O. Removal of cell debris was verified by phase microscopy. This procedure has previously been used to demonstrate that ECM from endothelial cells contains PA inhibitor 1 (Schleef *et al.*, 1990).

Binding experiments

To strip receptors of endogenous ligand, cultures were treated with an isotonic acidic buffer (50 mM glycine-HCl, 0.1 M NaCl, pH 3) for 5 min (Stoppelli *et al.*, 1986; Haigler *et al.*, 1980). Cells were then neutralized with an equal volume of neutralization buffer [0.5 M *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.1 M NaCl, pH 7.5] and rinsed three times with binding medium (MCDB 153 without insulin, EGF, bovine pituitary extract, or hydrocortisone and with the addition of 28 mM HEPES and 1.0 mg/ml acid-treated bovine serum albumin (Loskutoff, 1978)). This treatment had no effect on cell viability as determined by trypan blue exclusion. Furthermore, control experiments demonstrated that acid pretreatment did not prevent internalization of ^{125}I-EGF, a ligand previously shown to be taken up by many cell types, including the keratinocyte (O'Keefe *et al.*, 1982). When ^{125}I-EGF was allowed to bind to acid-pretreated keratinocytes for 4 h at 4°C, very little (4.0%) internalization of bound label was observed. However, further incubation of the cells at 37°C led to rapid internalization; after only 15 min at 37°C, 60.0 ± 1.0% of the bound ^{125}I-EGF was insensitive to either trypsin or acid treatment of the cells.

To quantify uPA binding, cultures were incubated with ^{125}I-uPA in binding medium at 23°C. Nonspecific binding was determined by the addition of a 100-fold excess of unlabeled uPA [either single-chain uPA or two-chain uPA (WinKinase)]. To harvest, the cultures were rinsed five to six times with phosphate buffered saline (PBS) containing 1 mg/ml bovine serum albumin; 0.5 ml of 1.0% sodium dodecyl sulfate was used to solubilize the cells before determination of cell-associated radioactivity. Data were analyzed with computer-assisted nonlinear least-squares curve fitting (Accufit, Beckman Instruments) and with Scatchard transformation.

In some experiments (Table 1, Part B), cells were solubilized for CPM determination by incubating in 0.5% Triton X-100 in PBS for 10 min.

Wounding and autoradiography

Stratified cultures were wounded by cutting with a scalpel, then further incubated at 37°C for 24–48 h. For binding experiments, cultures were acid-treated and then incubated with ^{125}I-uPA (2×10^{-10} M) for 150 min as described above; control wells were not acid-treated, but otherwise processed identically. After washing, the cultures were fixed for 15 min in 10% formalin, allowed to air-dry overnight, coated with K-2 emulsion, and exposed for 2–3 wk at 4°C. Cultures were counterstained with hematoxylin.

Immunocytochemistry

Stratified cultures were wounded and reincubated as described above. Some wells were acid-stripped as described above and some of these were reincubated with WinKinase (10^{-8} M) for 30 min. After washing in PBS, all wells were fixed in 4% paraformaldehyde for 10 min at room temperature and then washed. After blocking with PBS containing 10% normal goat serum for 60–90 min, wells were incubated with 2.5 μg/ml affinity-purified anti-uPA immunoglobulin G IgG) or nonimmune rabbit IgG in PBS for 45 min at room temperature. The preparation of the antibody and the remainder of the staining protocol have been previously published (Morioka *et al.*, 1985, 1987), except that binding was visualized using avidin-biotin-alkaline phosphatase and substrate kit 3 according to specifications of the manufacturer (Vector Laboratories, Burlingame, CA).

ELISA

ELISA for uPA was performed as previously described for radioimmunoassay (Jensen *et al.*, 1990), except that peroxidase-labeled goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used as the final antibody and 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) peroxidase substrate (Kirkegaard and Perry) was used for color development. As a control for the measurements of uPA released by acid treatment of keratinocytes, we showed that incubation at pH 3.0 for 5 min did not alter the antigenicity of standard uPA.

Acknowledgments

The authors are grateful to Dr. Jeffrey Stadel (SmithKline-Beecham) for helpful discussions, advice, and assistance with data analyses. We also thank Drs. Norman Schechter and Gerald Lazarus (University of PA) for critical reading of the manuscript. Dr. Donald Moir (Collaborative Research) generously donated purified uPA, which made this study possible. Dr. Anne P. Ball of NIH was very helpful in procuring WinKinase. Mr. William Witmer provided expert assistance in preparation of the figures. The assistance of Mrs. Indira David with cell culture and autoradiography is gratefully acknowledged. This study was supported by the National Institutes of Health grant PO1-AR39674-3.

Received: January 8, 1990.
Revised and accepted: August 20, 1990.

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