In Vivo Invasion of Modified Chorioallantoic Membrane by Tumor Cells: the Role of Cell Surface-bound Urokinase

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Abstract. The ability of the chick embryo chorioallantoic membrane (CAM) to withstand invasion by tumor cells can be intentionally compromised by altering its morphological integrity. Using a newly developed quantitative assay of invasion we showed that intact CAMs were completely resistant to invasion by tumor cells, wounded CAMs did not pose a barrier to penetration, and CAMs that were wounded and then allowed to reseal displayed partial susceptibility to invasion. The invasion of resealed CAMs required catalytically active plasminogen activator (PA) of the urokinase type (uPA); the invasive efficiency of tumor cells was reduced by 75% when tumor uPA activity or tumor uPA production was inhibited. The invasive ability of human tumor cells, which have surface uPA receptors but which do not produce the enzyme, could be augmented by saturating their receptors with exogenous uPA. The mere stimulation of either uPA or tissue plasminogen activator production, in absence of binding to cell receptors, did not result in an enhancement of invasiveness.

These findings suggest that the increased invasive potential of tumor cells is correlated with cell surface–associated proteolytic activity stemming from the interaction between uPA and its surface receptor.

Local infiltration of normal tissue by tumor cells and their dissemination to distant sites involve migration through the stroma of connective tissue as well as the penetration of natural barriers, such as basement membranes. The invasiveness of tumor cells has been studied in a variety of experimental models (Albini et al., 1987; Foltz et al., 1982; Jones et al., 1981; Mignatti et al., 1986; Starkey et al., 1984; Terranova et al., 1986), including the chorioallantoic membrane (CAM)[1] (Armstrong et al., 1982; Leighton, 1964; Ossowski and Reich, 1983; Poste et al., 1980; Poste and Flood, 1979; Scher et al., 1976). It has been shown in a number of these models that tumor cells elaborate enzymes that can degrade components of the extracellular matrix (Danø et al., 1985; Jones et al., 1981; Mignatti et al., 1986; Nakajima et al., 1984; Salo et al., 1982; Sas et al., 1986; Sloane et al., 1986). In most cases enhanced metastatic potential of cells was shown to be correlated with their greater ability to degrade matrix components (Danø et al., 1985; Jones et al., 1981; Liotta et al., 1980; Salo et al., 1982; Sloane et al., 1986; Terranova et al., 1986) but some lack of correlation has also been observed (Sas et al., 1986). It is conceivable that the above discrepancies were due to an inability to mimic in vitro the highly specialized interactions that occur in vivo between tumor cells and the complex and heterogenous extracellular components.

The CAM model, which combines the technical simplicity of an in vitro assay with the advantages of an in vivo environment, has been used in the past by a number of investigators to study the invasiveness of virally transformed cells or tumor cell lines, however, the role of preteolytic enzymes in invasion has not been studied in this model. The results of these studies have been conflicting probably because, with few exceptions (Armstrong et al., 1982), the morphological integrity of CAMs used for inoculations was not carefully assessed. Indeed, histological assessment of CAMs in the present study revealed that the method used for their preparation had a pronounced effect on their morphology. The susceptibility of these CAMs to tumor cell invasion was correlated with their structural integrity. This observation was used in preparation of CAMs that were completely, partially, or minimally resistant to tumor cell invasion.

The use of partially resistant CAMs permitted the examination of the role of plasminogen activator (PA) content, PA type (urokinase-type PA [uPA] vs. tissue-type PA [tPA]), and PA localization (soluble in the medium vs. surface receptor bound) in invasion of CAMs by tumor cells. Plasma membrane receptors for uPA have been recently identified on a number of cells (Vassalli et al., 1985), including tumor cells (Stoppelli et al., 1986). uPA binds to these receptors via the amino-terminal portion of the molecule and remains catalytically active (Appella et al., 1987; Stoppelli et al., 1986; Vassalli et al., 1985). In most uPA-producing cells the receptors are fully occupied by the endogenously produced enzyme (Stoppelli et al., 1986) but, as will be shown, in some cells the receptor is synthesized in the absence of uPA production.

1. *Abbreviations used in this paper*: CAM, chorioallantoic membrane; EGF, epidermal growth factor; HMW-uPA, high molecular weight urokinase-type plasminogen activator; PA, plasminogen activator; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

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Saturation of these receptors with exogenously added uPA, which remains active on the surface of the cells, allowed the testing of its role in enhancing tumor cell invasion. Also, since human receptors only bind human uPA (Appella et al., 1987; Huarte et al., 1987) we were able, by using human cells expressing very high levels of muse uPA, to determine whether overproduction of uPA, which because of its origin cannot bind to human uPA receptors, will equip the cells with an increased invasive potential.

Materials and Methods

Materials

COFAL-negative embryonated eggs were obtained from Spafas, Inc. (Norwich, CT); DME from Gibco Laboratories (Grand Island, NY); FBS from Hazleton Research Products Inc. (Lenexa, KS); ^{125}IUdR (specific activity of 2,200 Ci/mmol) from New England Nuclear (Boston, MA); human urokinase standard from Leo Pharmaceuticals (Ballerup, Denmark), Breokinase (high molecular weight urokinase) from Green Cross Corp. (supplied by Breon Lab. Inc., NY), Abbokinase (low molecular weight urokinase) from Abbott Laboratories (North Chicago, IL); Triton X-100, collagenase type 1A, and DMSO from Sigma Chemical Co. (St. Louis, MO); chromogenic substrate for plasmin (Spectrozyme PL) from American Diagnostica Inc. (New York, NY). Human plasminogen was purified from fresh human plasma as previously described (Deutsch and Mertz, 1970); bovine fibrinogen (Calbiochem-Behring Corp., La Jolla, CA) and rabbit anti-human uPA were prepared as described (Strickland and Beers, 1976; Ossowski and Reich, 1983); rabbit anti-mouse uPA IgG was purified from serum of rabbits immunized with PA purified from conditioned medium of Lewis lung carcinoma cells.

Cell Lines

HEp3 cells (Toolan, 1954) were obtained by collagenase dissociation of HEp3 tumors, serially passaged on CAMs of 10-d-old chick embryos, using previously described techniques (Ossowski and Reich, 1980). HeLa cells maintained in our laboratory were originally obtained from Dr. J. Nevins (Rockefeller University), and Wish-mUK, a human cell line transfected with mouse uPA cDNA (Belin et al., 1985), under the transcrptional control of a human heat shock promoter using a previously described vector (Dreano et al., 1987), was generously donated by Dr. D. Belin (University of Geneva Medical School, Switzerland).

Methods

Quantitation of Tumor Cells in CAM Mesenchyme. HEp3 cells, prepared by dissociation of serially passaged CAM-HEp3 tumors (Ossowski and Reich, 1980), were grown in culture for 2–4 d and labeled with 0.2 μCi/ml of ^{125}IUdR for 24–48 h. Specific activity of these cells varied from 0.1 to 0.3 cpm/cell. CAMs of 10-d-old embryos, separated from the egg shell by creation of an artificial air chamber (Ossowski and Reich, 1980), were inoculated with 4×10^5 cells per CAM, incubated for 8 h at 37°C, washed in situ five times with PBS (1 ml per wash) and excised. Extreme care was taken not to damage the CAMs during this procedure. The CAMs were incubated for 20 min in 35-mm culture dishes with 2 ml of TD buffer (1 liter of TD buffer contains 16 g NaCl, 0.76 g KCl, 0.2 g $Na_2HPO_4 \times 7\ H_2O$ and 6 g Sigma 7-9; Sigma Chemical Co.) and trypsin-EDTA (0.05% trypsin, 1 mM EDTA) or in 2 ml of PBS (1 liter of PBS contains 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 2.16 g $Na_2HPO_4 \times 7\ H_2O$) with 0.15% collagenase and 2.5% BSA. At the end of incubation the CAMs were carefully removed from the enzyme solutions, rinsed gently in 2 ml of PBS, and counted in a gamma counter. All the PBS washes were centrifuged at low speed and the supernatants and pellets were also counted in the gamma counter. Routinely, >95% of the radioactivity was found in the pellets. To ensure that the incorporated ^{125}IUdR was not released and reused by the surrounding normal cells, CAMs inoculated with radioactive cells were dissociated with collagenase into single cell suspensions 20 and 40 h after inoculation. The specific radioactivity of the tumor cells, which are very large and easily distinguishable from the chick cells, was determined. After correction for the increase in tumor cell number during these periods the specific radioactivity was found to be unchanged.

Preparation of CAMs. Intact CAMs were prepared as described (Scher et al., 1976). Briefly, 2 ml of albumin was removed from 5-d-old embryos, before the development of the CAM. The eggs were incubated on their sides in a stationary incubator. This procedure resulted in the creation of a space between the egg shell and the rest of the egg content and allowed for the development of the CAM unattached to the egg shell membrane. Wounded CAMs were prepared in 10-d-old embryos by puncturing the egg shell on the long side of the egg and applying negative pressure through an opening in the egg shell over the natural air sac. This procedure, which led to the formation of a new air sac by mechanical separation of the CAM from the egg shell membrane, resulted in severe damage to the CAM. Resealed CAMs were prepared by the above procedure except that wounding was followed by incubation of eggs, before inoculation, for periods varying from 7 to 30 h. Histological examinations were done on sections obtained from tissues fixed in buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin.

Plasminogen Activator Determinations. All methods used have been described previously (Ossowski and Reich, 1980; Strickland and Beers, 1976). We found that the radioactive fibrin plate method (Strickland and Beers, 1976) and the assay which used chromogenic substrate for plasmin (Spectrozyme PL; American Diagnostica Inc.) (Mira-y-Lopez and Ossowski, 1987; Verheijen et al., 1982), gave comparable results. Both assays detected as little as 0.5-1 mU of uPA.

Detection of uPA Receptors and Determination of Receptor-bound uPA Activity. HeLa, HEp3, and Wish-mUK cells were plated at 2×10^5 in 24-well tissue culture trays and incubated with iodinated high molecular weigh uPA (HMW-uPA) as previously described (Vassalli et al., 1985; Stoppelli et al., 1986). 20-fold excess of unlabeled HMW-uPA (catalytically active or inactivated by incubation with 20 mM DFP for 60 min at 37°C, followed by extensive dialysis) was used to determine the nonspecific binding. Low molecular weight uPA could neither bind to the receptor nor could it compete, even at a 100-fold molar excess, for the binding of HMW-uPA. The stripping of occupied receptors was performed as previously described (Stoppelli et al., 1986). To determine the functional stability of the receptor-bound uPA, HeLa cells were plated at 2×10^4 per well in a 96-well tray and the following day all the cultures were washed twice with PBS-BSA. Half of the cultures were incubated in PBS-BSA with 0.1 μg/ml of HMW-uPA and the other half, incubated in PBS-BSA alone, served as controls. At different times, eight wells in each group were washed four times with PBS-BSA. Four wells in each group were incubated for 30 min at 37°C with 50 μl of PBS-BSA containing 1 mg/ml of human plasminogen, the other four served as background controls (incubated in PBS-BSA alone). The supernatants of all cultures were aspirated, centrifuged, and their plasmin activity was determined.

Preparation of HeLa Cells with Receptor-bound uPA. Cells (2×10^6/ml) detached with Tris-saline-1 mM EDTA (pH 7.4) were resuspended in Dulbecco's medium with 20 mM Hepes and 1 mg/ml BSA (DB-Hepes-BSA) and incubated for 45 min at 4°C with 0, 0.1, 0.2, or 0.4 μg/ml of HMW-uPA (HeLa cells) and with 0.2 μg/ml (Wish-mUK and HEp3 cells). After three washes with DB-Hepes-BSA the cells were resuspended in PBS containing 1 mg/ml of human plasminogen, dispensed (100 μl) into plastic tubes at 2×10^5 per tube, and incubated for 30 min at 37°C. The supernatants were separated by centrifugation and assayed for plasmin activity by fibrin plate or chromogenic assays. As a control, supernatants of cells that had been incubated without plasminogen were mixed with 1 mg/ml of plasminogen, incubated under identical conditions as the experimental, and assayed for plasmin activity. Only the supernatant from HeLa cells, preincubated with 0.4 μg/ml of uPA, had a small but detectable plasmin activity when subsequently mixed with plasminogen; this activity was subtracted from the experimental value.

Identification of PA Types Produced by Cells. Two methods were used to determine whether the PA produced was of the tPA or the uPA type, and whether it was of mouse or human origin. Lysates and conditioned media of cells were subjected to electrophoresis on SDS-PAGE and simultaneous determinations of molecular weight and activity were performed using zymography (Granelli-Piperno and Reich, 1978). In addition, cell lysates and conditioned media were preincubated with specific, inhibitory antibodies and the residual PA activity of these mixtures was measured using fibrin plate or chromogenic substrate assays.

Results

Quantitation of Tumor Cells in the CAM Mesenchyme

To quantitate the efficiency of penetration of CAMs by tumor cells, chick embryos were inoculated on CAMs with labeled

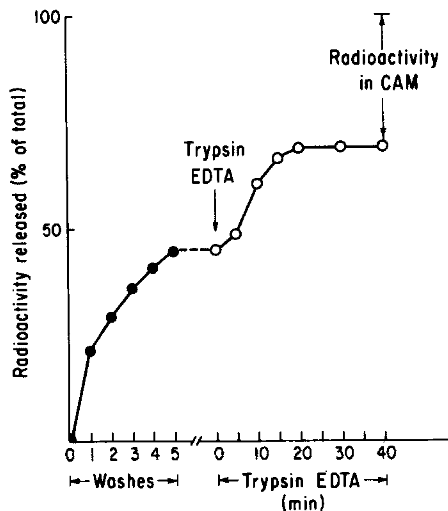

Figure 1. Quantitation of tumor cells inside the CAM mesenchyme. Two CAMs were inoculated with 2 × 10^5 ^{125}IUdR-labeled HEp3 cells (0.23 cpm/cell) and, after 8 h of incubation, the CAMs were washed in situ five times with 1 ml of PBS. Care was taken not to damage the CAMs during washing. The CAMs were excised and incubated at 37°C in 2 ml of 0.05% trypsin and 1 mM EDTA. At indicated times, the CAMs were removed, counted in a gamma counter, and reincubated in fresh enzyme solutions. The results shown are the average of two CAMs and are expressed as percent of total radioactivity recovered in washes, released by trypsin, and found associated with the CAMs. The total recovered radioactivity was 80% of inoculum. (*Solid circles*) Radioactive cells released in PBS washes; (*open circles*) radioactive cells released by trypsin-EDTA treatment.

tumor cells. At the end of 8 h of incubation the CAMs were subjected to repeated washing with PBS which removed nearly half of the total radioactive tumor cells. Subsequently, the washed CAMs were excised and incubated in trypsin-EDTA solution; an additional 25% of all cells was released in the first 20 min of this treatment, the rest of the radioactive cells (30% of the total number of recovered cells) was completely resistant to further treatment with the enzyme (Fig. 1), and remained associated with the CAMs even after 1 h of incubation (not shown). CAMs of embryos inoculated with tumor cells and incubated for periods of up to 72 h, contained greater proportions (up to 80%) of cells protected from the action of trypsin. To identify the trypsin-protected compartment by histology, CAMs incubated for 40 h after inoculation with tumor cells were excised and sectioned before and after trypsin-EDTA treatment (Fig. 2). The comparison of these sections showed that the enzyme treatment primarily released tumor cells attached to the outer surface of the CAM (Fig. 2), leaving the cells which had penetrated the CAM mesenchyme in place. These cells could only be released by enzymes, such as collagenase, which caused a complete disintegration of the CAM structure (not shown). Similar results were seen with CAMs examined 8 h after inoculation but, in this case, the number of tumor cells in the CAM mesenchyme was too small for efficient visualization in histological sections.

Effect of Structural Integrity of CAMs on the Susceptibility to Invasion by Tumor Cells

Different experimental procedures used to prepare CAMs for

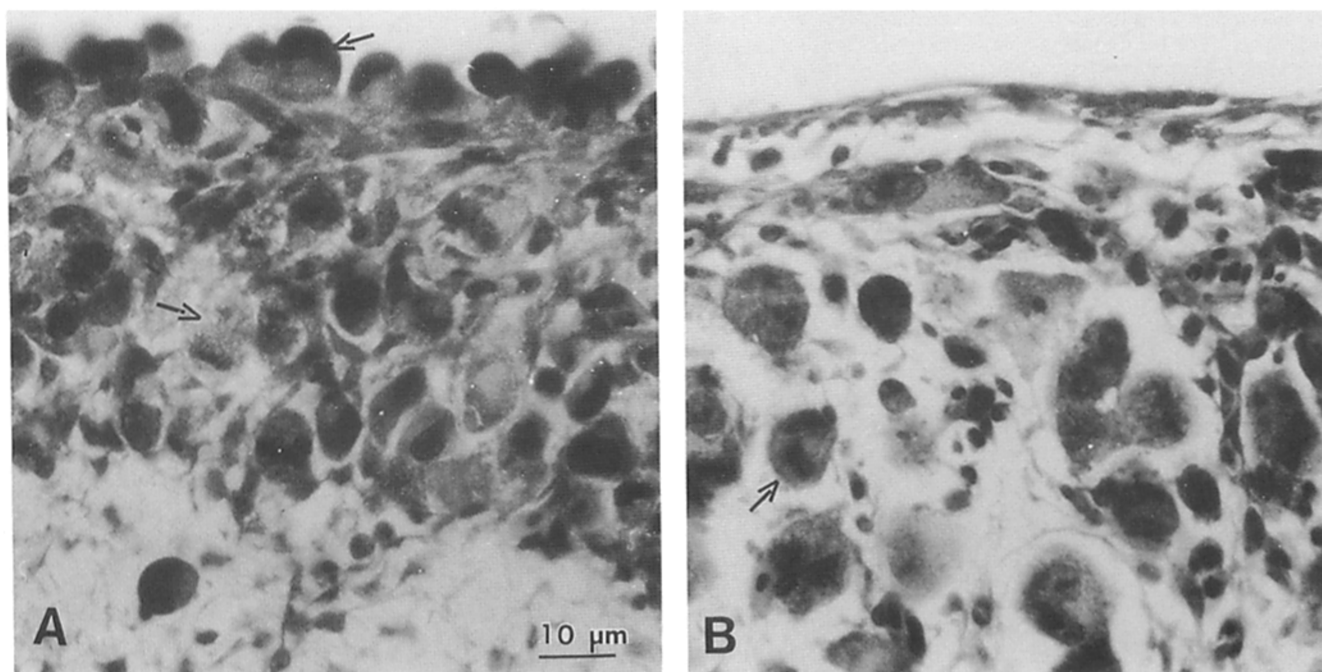

Figure 2. The effect of trypsin-EDTA treatment on the CAM surface-attached HEp3 cells. CAMs were inoculated with 5 × 10^5 HEp3 cells, incubated for 40 h, washed in situ with PBS, and excised. The CAM shown in *A* was immediately fixed. The CAM shown in *B* was incubated for 20 min with trypsin-EDTA, rinsed gently with PBS, and then fixed. Both CAMs were sectioned and stained with H&E. Arrows indicate tumor cells.

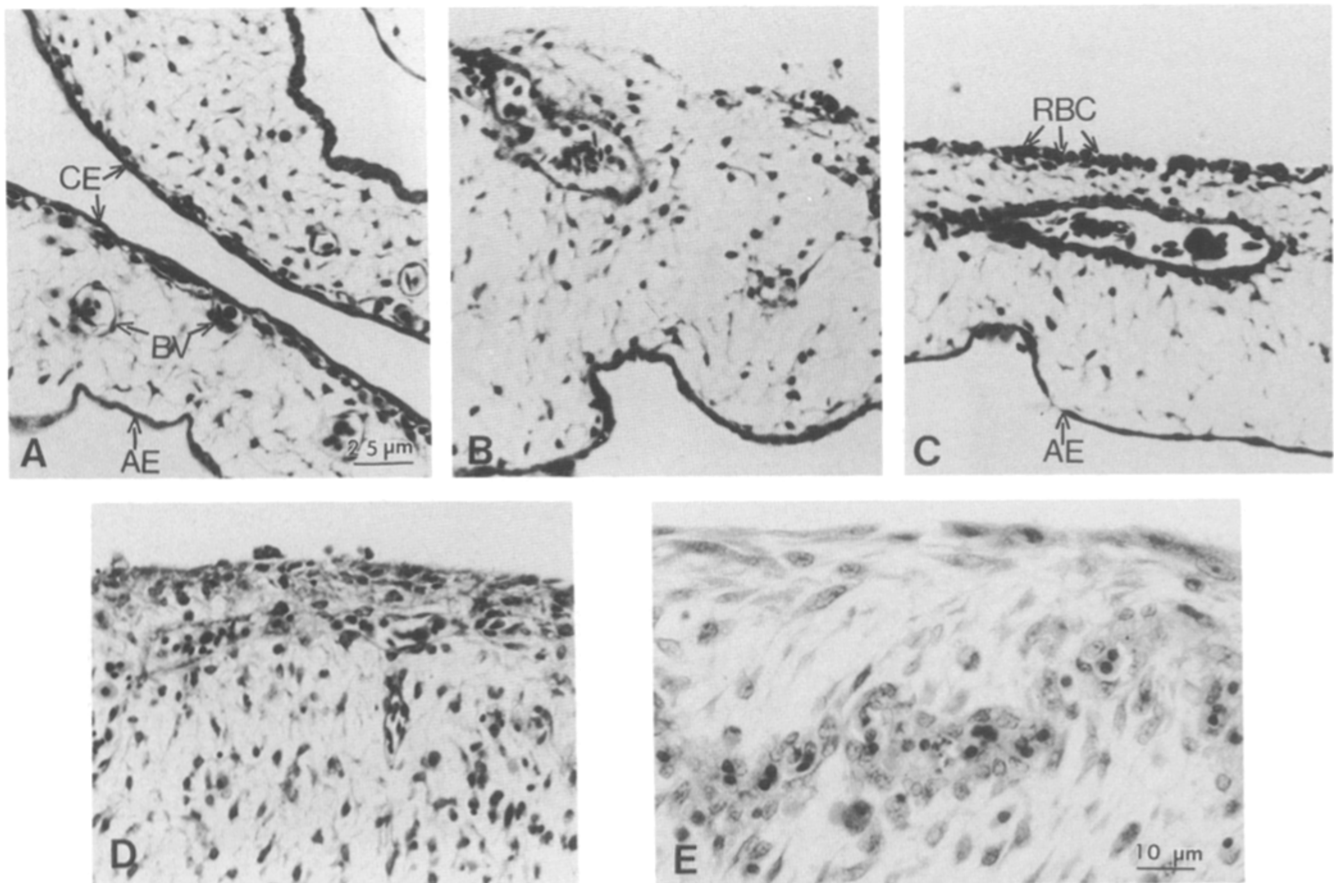

Figure 3. Morphology of intact, wounded, and resealed CAMs. (*A*) Intact CAM from a 10-d-old embryo, prepared as described in Materials and Methods, was excised, folded to fit more surface area into a single histological section, and fixed. The photomicrograph shows a section through CAM in which, due to folding, two chorionic epithelia face each other. Note the continuity of the epithelium and the richness of the vasculature immediately adjacent to the epithelium. (*B* and *C*) Wounded CAMs were excised from 10-d-old embryos (see Materials and Methods), fixed immediately, and used for preparation of histological sections. Note that this method of CAM preparation resulted in some areas in the disappearance of the entire chorionic epithelium from the surface of the CAM (*B*), and that extensive bleeding was evident in other areas of the CAM (*C*). (*D* and *E*) Resealed CAMs were prepared by incubating embryos with wounded CAMs for 30 h, followed by excision and fixation of the CAMs. Note that the fibroblasts in the mesenchyme are aligned perpendicularly to the surface, but that on the surface they form an almost continuous multilayered (*D*) or double-layered (*E*) sheet. *CE*, chorionic epithelium; *BV*, blood vessel; *RBC*, red blood cells; *AE*, allantoic epithelium.

tumor cell inoculation yielded CAMs with a variable degree of epithelial injury. We examined the morphology of the CAMs prepared by three methods and compared their sensitivity to tumor cell invasion. We found that in an intact CAM (see Materials and Methods for preparation) the chorionic epithelium, which is known to be composed of one to four layers of cells resting on a basement membrane, remained in its original state (Fig. 3 *A*); a large number of capillary blood vessels was present in the mesenchyme immediately underlying the epithelium. In contrast, in the wounded CAMs, prepared by a routinely used method of an artificial air sac formation, the chorionic epithelium was completely missing in many areas of the CAM (Fig. 3 *B*), and extensive hemorrhage was often observed immediately after wounding (Fig. 3 *C*). Incubation of embryos with wounded CAMs at 37°C led to formation of continuous, single, or multilayered sheets of cells on the wounded surfaces (resealed CAM; Fig. 3, *D* and *E*). Examination of sections of CAMs prepared at 8–12 h after wounding revealed the presence in the mesenchyme of fibroblast-like cells, positioned vertically, with their long axes perpendicular to the wounded surface (not shown). Approximately 30 h after wounding the CAM surface was resealed with a continuous layer of fibroblast-like cells (Fig. 3, *D* and *E*). Lateral migration from the edge of the wounded chorionic epithelium was not seen in any of the examined sections.

Intact, wounded, and resealed CAMs were inoculated with radioactive HEp3 cells and, after 20 h of incubation, the number of tumor cells that had penetrated the mesenchyme was quantitated using the trypsin-EDTA incubation. In the wounded CAMs, 50% and in the CAMs resealed for 7 h 40% of the inoculum was found in the mesenchyme. In contrast, only 23% of the inoculated tumor cells was found in the mesenchyme of CAMs wounded and resealed for 28 h. Intact CAMs were completely resistant to invasion by HEp3 cells (Fig. 4).

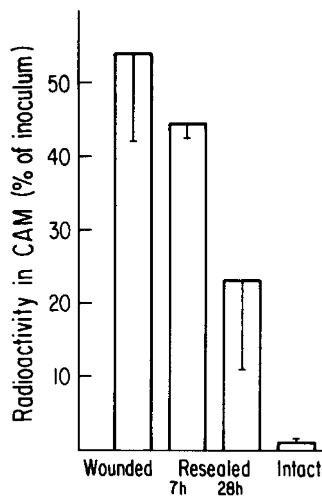

Figure 4. Correlation between the structural integrity of the CAM and its susceptibility to invasion by tumor cells. Radioactive HEp3 cells (0.27 cpm/cell, 2 × 10^5 cells per CAM) were inoculated on six of each of the following: intact, wounded, and resealed CAMs. The latter were allowed to reseal for either 7 or 28 h before inoculation with tumor cells. After 22 h of incubation, all CAMs were washed, excised, treated with trypsin-EDTA for 20 min, and counted in a gamma counter. The mean number of tumor cells in the mesenchyme of the different CAMs was compared to that of wounded CAMs by Student *t* test. The number of cells in the CAM resealed for 7 h did not differ significantly from the wounded CAM; the intact CAM and CAM resealed for 28 h were significantly different ($P < 0.01$). The vertical bars indicate standard deviations.

Involvement of PAs in CAM Invasion

The role of uPA in CAM invasion was examined by comparing the invasive ability of HEp3 cells expressing high levels of uPA activity to HEp3 cells in which uPA activity was inhibited by anti-uPA IgG (Ossowski and Reich, 1983), or in which enzyme production was blocked by preincubation with 1.3% DMSO (Ossowski and Belin, 1985; Ossowski, 1988), a treatment that reduced the HEp3-uPA activity by >90%.

Wounded or resealed CAMs were inoculated with control, anti-uPA IgG-treated or DMSO-treated HEp3 cells, and the number of tumor cells in CAM mesenchyme was determined 20 h later. The inhibition of uPA activity or production only slightly reduced the number of tumor cells found in the mesenchyme of CAMs which were susceptible to invasion, i.e., wounded CAMs and CAMs resealed for 8 h (Table I). However, in CAMs resealed for 30 h, which, as shown above (Fig. 4), were more resistant to penetration by tumor cells, the inhibition of tumor cell uPA reduced the cell number found in the CAM mesenchyme by ∼75% (Table I).

Table I. The Effect of Inhibition of uPA Activity or Synthesis on HEp3 Cell Invasion of Wounded or Resealed CAMS

Time between CAM preparation and inoculation of cells	Radioactivity in CAM (percent of inoculum)		
	Control mean (SD)	DMSO-treated mean (SD)	Anti-uPA IgG mean (SD)
h			
0	43 (± 11)	37 (± 5)	35 (± 12)
8	45 (± 7)	34 (± 14)*	ND
30	22 (± 11)	6 (± 3)‡	5 (± 3)‡

HEp3 cells, obtained by dissociation of HEp3 tumor transplanted serially on CAMs, were plated at 1.5 × 10^6 per 100-mm dish in medium with 10% serum. The next day, serum concentration was lowered to 5% and one group of cultures received 1.3% DMSO. The next day ^{125}IUdR (0.2 mCi/ml) was added and cultures were incubated for another 24 h. Control cells incorporated 0.3 cpm/cell and DMSO cells 0.24 cpm/cell. Control or DMSO treated, radioactive cells (4 × 10^5 per CAM) were inoculated on wounded CAMs or on CAMs resealed for 8 or 30 h. 12 eggs were used for each experimental group. Also, an aliquot of the control cells was resuspended in rabbit anti-uPA IgG (1.5 mg/50 µl) and inoculated on either wounded or resealed CAMs which received 1.5 mg/CAM of the same IgG 1 h before inoculation. The embryos were incubated for 20 h, at which time the CAMs were excised and the number of tumor cells in the CAM mesenchyme was determined. Student *t* test was used to compare the invasion by control and anti-uPA and control and DMSO-treated cells.
*, $P < 0.05$.
‡, $P < 0.005$.

Can Invasiveness Be Enhanced by Stimulation of tPA Production?

HeLa cells, which endogenously produce only low levels of tPA, were preincubated with epidermal growth factor, a treatment which resulted in a 39-fold increase in the tPA activity released by the cells over a 24-h period (Table II); the cell-associated activity rose only 4-fold (Table II). This enhanced production was maintained for at least 24 h after the removal of the growth factor from the incubation medium (Table II) indicating that, once the production was initiated, it proceeded in the absence of an exogenous stimulus. Both the released and the cell-associated activity were completely inhibited by anti-tPA IgG (not shown). As is evident from Fig. 5, the ability to produce greatly enhanced levels of tPA did not increase the efficiency of penetration of wounded or resealed CAMs.

Is Binding of uPA to Cell Surface Receptors a Prerequisite for uPA Enhancement of Invasion?

To test whether stimulation of production and release of uPA is sufficient to enhance tumor cell invasiveness, we used a human cell line (Wish) transfected with a mouse uPA-cDNA under the transcriptional control of a human heat shock promoter. Wish-mUK cells were induced to synthesize mouse uPA by 2 h of exposure to 43°C. As seen in Fig. 6, between 6 and 24 h after the heat shock, PA production in these cells rose from undetectable levels to almost 1,000 mU/10^6 cells. Most of the PA activity was found in the conditioned medium (Fig. 6). The mouse origin of the cell associated and the secreted PAs was ascertained by zymography (Granelli-Piperno and Reich, 1978) and by inhibition of activity with specific rabbit anti-mouse uPA IgG (not shown). It has been previously established that the binding of uPA to its receptor

Table II. Stimulation of tPA Production in HeLa Cells by EGF

EGF		tPA	
Day 1	Day 2	Cell associated	In conditioned medium
ng/ml	*ng/ml*	*mU/2 ×10^5 cells*	*mU/2 ×10^5 cells*
0	0	17.0	44.0
2	2	67.0	1699.0
2	0	47.0	2709.0

HeLa cells were plated at 1 × 10^5 per 35-mm dish in medium with 5% FBS. On day 1, four cultures received 2 ng/ml of EGF and two served as controls; on day 2, all cultures were washed and two of the cultures, preincubated in EGF, were left without the growth factor; on day 3, media and cells were collected and used to determine PA activity. The numbers represent the mean PA in two cultures. Each PA determination was done in duplicate. The difference between assay duplicates was <14% and between duplicate cultures, <22%. The activity of cell-associated and cell-free PA was completely inhibited by preincubation with 200 µg/ml of rabbit anti-tPA IgG and not inhibited by 200 µg/ml of rabbit anti-uPA IgG.

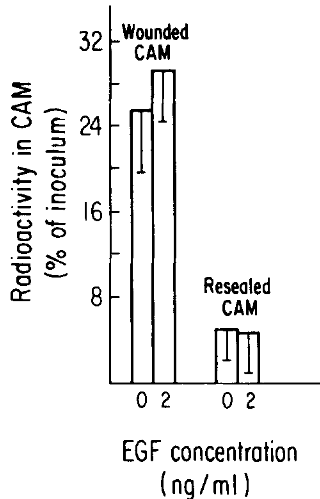

Figure 5. The lack of tPA effect on cell invasiveness. HeLa cells treated with 2 ng/ml of EGF exactly as described in the legend to Table II, except that they were labeled with ^{125}IUdR to a specific activity of 0.17 cpm/cell, and untreated, labeled HeLa cells were inoculated (5 × 10^5 cells per CAM) on wounded CAMs or CAMs that were allowed to reseal for 30 h. After 20 h of incubation the CAMs were washed, excised, treated with trypsin-EDTA, and counted in a gamma counter. Each experimental point is the mean of six CAMs. The vertical lines show standard deviations.

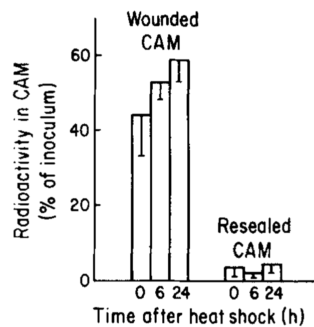

Figure 7. Invasion of CAM by control and mouse uPA producing Wish-mUK cells. ^{125}IUdR-labeled (0.2 cpm/cell) control and heat-shocked cells, used immediately, 6, and 24 h after the exposure to 43°C, were inoculated onto wounded and resealed CAMs (3 × 10^5 cells per CAM). After 20 h of incubation the CAMs were excised and tumor cell invasion was quantitated as described in Materials and Methods.

is species restricted (Appella et al., 1987; Huarte et al., 1987). By using a published procedure (Stoppelli et al., 1986) we examined the binding kinetics of human ^{125}I-labeled HMW-uPA to Wish-mUK cells and, from a Scatchard plot, calculated the receptor site number for uPA to be 100,000 per cell. The uPA receptors were found to be unoccupied in both control cells, which do not produce uPA, and in heat shock–induced cells which produce 1,000 mU per 10^6 cells of mouse uPA. This indicated that the endogenously produced uPA did not bind to receptors present on human cells (results not shown).

The invasive ability of these cells (controls and heat shocked, taken immediately after exposure to 43°C or incubated for an additional 6 or 24 h) was examined by inoculation onto wounded and resealed (30 h) CAMs. As shown in Fig. 7, cells stimulated to produce very high levels of mouse uPA, which is unable to bind to human uPA receptors, were not more invasive than control cells, suggesting that membrane localization of uPA may be crucial for the enhancement of invasion.

To test this possibility more directly, Wish-mUK and HeLa cells were preincubated with uPA in vitro, a treatment that equipped them with surface-bound enzyme. The number of uPA receptors on HeLa cells was calculated from Scatchard analysis to be 37,000; the dissociation constants for uPA on both HeLa and Wish-mUK cells were found to be in the subnanamolar range. In the native state the receptors in both types of cells were unoccupied (not shown); Wish-mUK cells did not produce detectable PA, while the line of HeLa cells used produced tPA exclusively. Incubation of HeLa cells with increasing concentrations (0.1–0.4 μg/ml) of human HMW-uPA yielded cells with greatly enhanced, surface-bound plasminogen–activating activity (Fig. 8); HeLa cells exposed to 0.2 μg/ml of uPA (a concentration 10 times in excess of the calculated K_d value for uPA binding) were most effective in activation of plasminogen to plasmin (Fig. 8). More than 50% of the surface-bound enzyme remained active 8 h after it was bound to the receptors (Fig. 9).

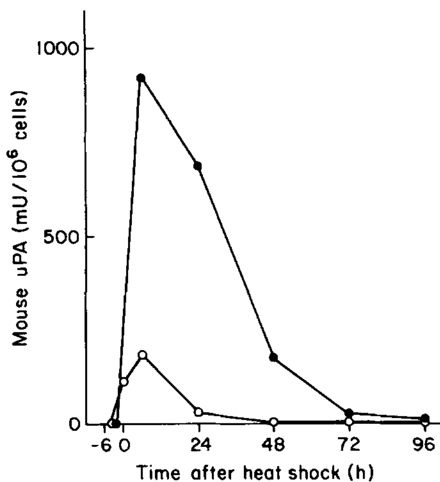

Figure 6. Stimulation of uPA activity in human cells (Wish-mUK) transfected with mouse uPA DNA. Wish-mUK cells were plated in 25-cm^2 flasks at 2.5 × 10^5 cells per flask. When semiconfluent, they were filled with medium containing 5% FBS and heat shocked by submerging in a 43°C water bath for 2 h. 6 h after exposure to heat, and every 24 h thereafter, the medium and cells from two flasks were collected and used to determine PA activity. Medium was replaced in all remaining flasks. The results given are the averages of two flasks and four PA determinations. The values did not differ by >7%. All of the PA activity was shown by zymography (Granelli-Piperno and Reich, 1978) to comigrate with standard mouse uPA. The activity was completely inhibited by specific, rabbit anti-mouse uPA IgG. (*Open circles*) Cell-associated PA; (*solid circles*) PA in conditioned medium.

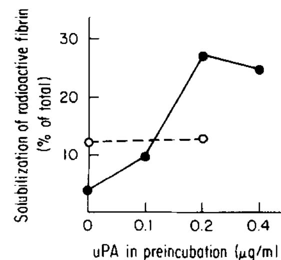

Figure 8. PA activity of HeLa and HEp3 cells incubated with uPA. HeLa and HEp3 cells (2 × 10^6/ml) were incubated in suspension with 0, 0.1, 0.2, or 0.4 μg/ml HMW-uPA (HeLa), or 0, and 0.2 μg/ml HMW-uPA (HEp3). After three washes, aliquots of cells (2 × 10^5 per 100 μl) were incubated for 30 min at 37°C with or without 1 mg/ml of human plasminogen. At the end of incubation, the cells were removed by centrifugation and the supernatants were assayed for plasmin activity by a fibrin plate assay (Strickland and Beers, 1976). The results are the average of two determinations which did not differ by >4%. (*Open circles*) HEp3 cells; (*solid circles*) HeLa cells.

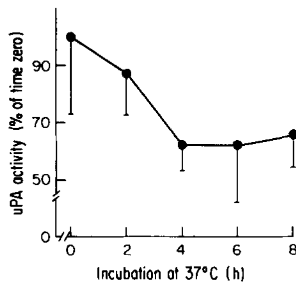

Figure 9. The stability of plasminogen-activating activity of receptor-bound uPA. HeLa cells were plated at 2 × 10^4 cells per well in a 96-well tray and incubated overnight in medium with 5% FBS. The next day the cultures were incubated for 45 min at 4°C, half of the cultures in 0.1 μg/ml HMW-uPA in PBS with 1 mg/ml of BSA and the other half in PBS with BSA alone. At the indicated times, eight control wells and eight wells that were preincubated with uPA were washed extensively with PBS/BSA; four wells in each group were incubated for an additional 30 min with 50 μl PBS containing 1 mg/ml of human plasminogen, the other four incubated with PBS/BSA, served as background controls. The supernatants were collected, centrifuged, and their plasmin content was determined on a fibrin plate assay (Strickland and Beers, 1976). The results shown are the mean of four wells, the bars indicate standard deviation.

To compare the invasiveness of the native HeLa cells to those in which the receptors were saturated with uPA, the cells were inoculated onto wounded CAMs, or CAMs resealed for 30 h. 26% of the inoculated control HeLa cells were found in the mesenchyme of the wounded CAMs; the presence of receptor-bound uPA did not significantly increase this number (Fig. 10 *A*). In contrast, only 1.3% of the inoculated control HeLa cells was found in the mesenchyme of the resealed CAMs. Binding of uPA to its receptor by preincubation of HeLa cells with 0.2 μg/ml of uPA increased their invasive ability eightfold (Fig. 10 *B*); higher concentration of uPA was slightly less effective (Fig. 10 *B*).

Similar results were obtained with Wish-mUK cells preincubated with 0.2 μg/ml of HMW-uPA; this treatment increased the surface plasminogen-activating activity of these cells from an undetectable level to ∼40% of the level found in HeLa cells preincubated with a similar concentration of uPA (Fig. 8). In three separate experiments the average invasion rate of the control cells was 4.2% (± 1.4) of the inoculum. Binding of uPA increased the efficiency of invasion to 14.7% (± 3.4) (not shown).

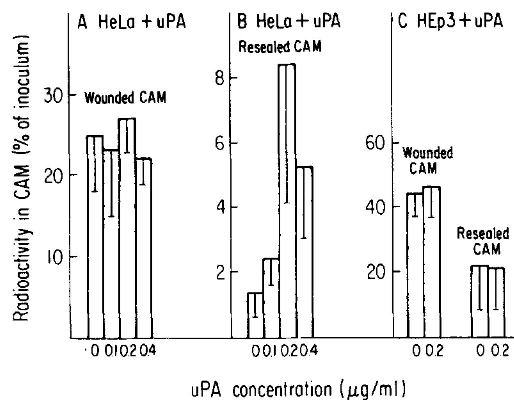

Figure 10. The effect of receptor-bound uPA on the invasiveness of HeLa cells. Radioactive (0.14–0.17 cpm/cell), untreated HeLa cells, or HeLa cells with receptor-bound uPA (the experimental procedures used to obtain these cells were exactly as described in legend to Fig. 8) were inoculated (5 × 10^5 cells per CAM) on wounded CAMs or CAMs which, after wounding, were allowed to reseal for 30 h. Radioactive, untreated HEp3 cells and HEp3 cells preincubated with 0.2 μg/ml of uPA served as controls. After 20 h of incubation, the CAMs were washed, excised, treated with trypsin-EDTA, and counted in a gamma counter. Each experimental point is the mean of results from 6 to 10 CAMs. The vertical lines represent standard deviation.

As expected, preincubation of HEp3 cells, in which approximately 90% of the receptors was occupied by endogenously produced uPA (unpublished observations); exogenous enzyme neither increased their plasminogen-activating activity (Fig. 8) nor affected their invasive potential (Fig. 10 *C*).

Discussion

Two aspects of the results presented here deserve discussion: the usefulness of the CAM as a model for studying invasion of normal tissues by tumor cells, and the role of proteolysis, initiated by plasminogen activators produced by tumor cells, in this process.

The search for an universally useful model in which to study invasion, and particularly the invasion of connective tissue, has not been entirely satisfactory; in vivo studies of organ invasion are difficult to quantitate (Easty and Easty, 1974; Mareel et al., 1975; Noguchi et al., 1978) and most in vitro models, although simple and rapid, use acellular and avascular membranes that do not mimic the in vivo conditions.

The CAM has been extensively used for serial passage of heterologous tumors (Harris, 1958–1959; Karnofsky et al., 1952; Leighton, 1964) as well as for studying some aspects of invasion (Armstrong et al., 1982; Ossowski, 1988; Scher et al., 1976). However, the fact that in many studies the state of morphological integrity of the CAMs used in invasion assays was not rigorously determined contributed to a diversity of experimental results (Poste et al., 1979; Poste and Flood, 1979; Scher et al., 1976).

We found that intentional alteration of structural integrity of CAMs used for tumor cell inoculation had a profound effect on their susceptibility to invasion. Even highly malignant human tumor cells (HEp3), which were invasive and metastatic in the patient (Toolan, 1954), the chick embryo (Harris, 1958–1959; Ossowski and Reich, 1980), and the nude mouse (Ossowski et al., 1987) were unable to penetrate CAMs in which the epithelium was left intact (Figs. 3 *A* and 4). In view of the fact that during the natural course of malignant dissemination the need to breach intact epithelia rarely exists, this may not be surprising.

A completely different outcome was observed in wounded CAMs. The areas of CAMs used for tumor cell inoculation were mostly denuded of epithelium and, as judged by the presence of hemorrhage from the underlying capillaries, this treatment also disrupted the basal laminae (Fig. 3, *B* and *C*). Although some difference was observed between the different cell lines (HEp3: metastatic [Ossowski and Reich, 1980], Wish and HeLa: tumorigenic [Dreano et al., 1987; Noguchi et al., 1978]), all three cell lines showed relatively high efficiency (25–50% of inoculum) of mesenchyme invasion (Figs. 1, 2, 4, 7, and 10). This very high rate of invasion suggests that, in addition to facilitation of tumor cell entry resulting from the physical damage to the CAM epithelium,

wounding may release compounds such as fibronectin or plasminogen which may stimulate migration or exert chemotactic effects (Lacovara et al., 1984; McCarthy et al., 1986; Morioka et al., 1987; Ossowski et al., 1975; Valinsky et al., 1981).

The response of the CAM to wounding, which resulted in the formation of a surface layer of fibroblast-like cells and, most likely, extracellular matrix, (resealed CAM) provided a selective model in which highly malignant cells like HEp3 were found to be more invasive than less malignant cells like HeLa and Wish (Figs. 7, 8, and 10, and unpublished results). It cannot be determined at present, however, whether the increased resistance to invasion of the resealed CAMs is the result of the newly formed physical barrier, the gradual reabsorption of bioactive compounds released during wounding, or the result of the changing compositions of the inflammatory cells and their products, or a combination of the above.

The resealed CAM allowed us to examine, in a quantitative way, whether PA was crucial for efficient invasion of the CAM stroma by tumor cells and, if so, which of the enzyme properties was indispensable for this process. We had previously presented histological evidence indicating that the infiltration of the CAM stroma by single tumor cells requires catalytically active uPA (Ossowski, 1988), an observation that was confirmed in the resealed CAM model. This model also provided an opportunity for testing whether the mere production of plasminogen activator by tumor cells is sufficient to endow them with invasive properties, or whether specific characteristics, such as the type of enzyme and its localization in the cell, are important determinants.

It became evident from our results that even greatly enhanced (39-fold) tPA activity, achieved by incubation of HeLa cells with EGF, did not increase their invasive potential (Fig. 7). This lack of effect could not be easily explained by the need of fibrin to fully express tPA activity since, even in the absence of fibrin, plasminogen activation by EGF-stimulated cells was nearly equal to that generated by the highly invasive HEp3 cells (not shown). Also, evidence of hemorrhage was noted in CAMs after wounding (Fig. 3 *C*), suggesting that fibrin may have been present.

Receptors for tPA, unlike the widely distributed uPA receptors, have been shown to be present on endothelial cells (Hajjar et al., 1987) but not on tumor cells. We found that preincubation of HeLa cells with exogenous tPA did not enhance their PA activity (unpublished data). It appeared, therefore, that the most likely explanation for the lack of effectiveness of tPA in enhancing invasion may be its inability to bind to cell membranes. Additional support for this conclusion was obtained through the use of human Wish-mUK cells, which were stimulated to produce and secrete very high levels of mouse uPA which remained in the medium because it could not bind to the human uPA receptors. Stimulation of mouse uPA production by these cells did not result in augmentation of their invasive ability. This ineffectiveness was not, most likely, due to the secretion of uPA in a proenzyme form since the highly malignant and invasive HEp3 cells produce exclusively pro-uPA (Wun et al., 1982).

In vitro conversion of chicken plasminogen to plasmin by human uPA, tPA, and mouse uPA in solution was found to be very inefficient (not shown), suggesting that, most likely, soluble plasmin cannot account for the observed enhancement of tumor cell invasion. Chicken plasmin generation was slightly enhanced when cells (HEp3 and others) that have receptor bound uPA, were plated directly on fibrin (not shown). That surface-bound proteases may create more favorable conditions for localized proteolysis has been suggested previously (Chapman et al., 1984; Plow et al., 1986; Stoppelli et al., 1986). The results in this paper provide experimental support for this notion: saturation of HeLa cell receptors with exogenous uPA resulted in an eightfold enhancement of their invasiveness. This effect was not seen when tPA production was equally stimulated. A similar result was noted in Wish-mUK cells: secretion of mouse uPA, unable to bind to the human receptors on these cells, did not affect their invasiveness, while a 3.5-fold enhancement of invasiveness was observed when human uPA was bound to their surface receptors.

The reasons for the effectiveness of the cell-bound activity remain to be explored; the enhancing effect of uPA may be due to the generation of proteolysis in critical sites of contact between cells and substratum (Hebert and Baker, 1988; Pöllanen et al., 1988), or because of increased interaction between the enzyme and the substrate due to higher local enzyme concentration (Plow et al., 1986; Stoppelli et al., 1986), or, possibly, as suggested (Campbell and Campbell, 1988; Chapman et al., 1984), because receptor-bound uPA is protected from inhibition by serum and/or cellular PA inhibitors.

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References

Albini, A., Y. Iwamoto, H. K. Kleinman, G. R. Martin, S. A. Aaronson, J. M. Kozlowski, and R. M. McEwan. 1987. A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res.* 47:3239–3245.

Appella, E., E. A. Robinson, S. J. Ulrich, M. P. Stoppelli, A. Corti, G. Cassani, and F. Blasi. 1987. The receptor-binding sequence of urokinase. *J. Biol. Chem.* 262:4437–4440.

Armstrong, B. P., J. P. Quigley, and E. Sidebottom. 1982. Transepithelial invasion and intramesenchymal infiltration of the chick embryo chorioallantoic membrane by tumor cell lines. *Cancer Res.* 42:1826–1837.

Belin, D., J.-D. Vassalli, C. Combepine, F. Godeau, Y. Nagamine, E. Reich, H. P. Kocher, and R. M. Duvoisin. 1985. Cloning, nucleotide sequencing and expression of cDNAs encoding mouse urokinase-type plasminogen activator. *Eur. J. Biochem.* 148:225–232.

Campbell, E. J., and M. A. Campbell. 1988. Pericellular proteolysis by neutrophils in the presence of proteinase inhibitors: effect of substrate opsonization. *J. Cell Biol.* 106:667–676.

Chapman, H. A., O. L. Stone, and Z. Vavrin. 1984. Degradation of fibrin and elastin by intact human alveolar macrophages in vitro. *J. Clin. Invest.* 73:806–815.

Danø, K., P. A. Andreasen, J. Grondahl-Hansen, P. Kristensen, and L. S. Nielsen. 1985. Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.* 44:139–266.

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

Dreano, M., X. Fouillet, J. Brochot, J.-M. Vallet, M.-L. Michel, D. Rungger, and P. Bromley. 1987. Heat-regulated expression of the hepatitis B virus surface antigen in the human Wish cell line. *Virus Res.* 8:43–59.

Easty, D. M., and G. C. Easty. 1974. Measurement of the ability of cells to infiltrate normal tissue in vivo. *Br. J. Cancer.* 29:36–49.

Foltz, C. M., R. G. Russo, G. P. Siegal, V. P. Terranova, and L. A. Liotta. 1982. Interactions of tumor cells with whole basement membrane in the presence or absence of endothelium. *In* Interaction of Platelets and Tumor Cells.

Alan R. Liss, Inc. NY. 353-371.
Granelli-Piperno, A., and E. Reich. 1978. A study of proteases and protease-inhibitor complexes in biological fluids. *J. Exp. Med.* 148:223-234.
Hajjar, K. A., N. M. Hamel, P. C. Harpel, and R. L. Nachman. 1987. Binding of tissue plasminogen activator to cultured human endothelial cells. *J. Clin. Invest.* 80:1712-1719.
Harris, J. 1958-1959. The human tumor grown in egg. *Ann. NY Acad. Sci.* 76:764-774.
Hebert, C. A., and J. B. Baker. 1988. Linkage of extracellular plasminogen activator to human fibroblasts cytoskeleton: colocalization of cell surface urokinase with vinculin. *J. Cell Biol.* 106:1241-1248.
Huarte, J., D. Belin, D. Bosco, A. P. Sappino, and J.-D. Vassalli. 1987. Plasminogen activator and mouse spermatozoa: urokinase synthesis in the male genital tract and binding of the enzyme to the sperm cell surface. *J. Cell Biol.* 104:1281-1289.
Jones, P. A., H. B. Neustein, F. Gonzales, and E. Bogenmann. 1981. Invasion of an artificial blood vessel wall by human fibrosarcoma cells. *Cancer Res.* 41:4613-4620.
Karnofsky, D. A., L. P. Ridgway, and P. A. Patterson. 1952. Tumor transplantation to the chick embryo. *Ann. NY Acad. Sci.* 55:313-329.
Lacovara, J., E. B. Cramer, and J. P. Quigley. 1984. Fibronectin enhancement of directed migration of B16 melanoma cells. *Cancer Res.* 44:1657-1663.
Leighton, J. 1964. Invasion and metastasis of heterologous tumors in the chick embryo. *Prog. Exp. Tumor Res.* 4:98-125.
Liotta, L. A., K. Tryggvason, S. Garbisa, I. Hart, C. H. Foltz, and S. Shafie. 1980. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature (Lond.).* 284:67-68.
McCarthy, J. B., S. T. Hagen, and L. T. Furcht. 1986. Human fibronectin contain distinct adhesion- and motility-promoting domains for metastatic melanoma cells. *J. Cell Biol.* 102:179-188.
Mareel, M., L. De Ridder, M. De Brabander, and L. Vakaet. 1975. Characterization of spontaneous, chemical, and viral transformants of a C3H/3T3-type mouse cell line by transplantation into young chick blastoderm.
Mignatti, P., E. Robbins, and D. B. Rifkin. 1986. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. *Cell.* 47:487-498.
Mira-y-Lopez, R., and L. Ossowski. 1987. Hormonal modulation of plasminogen activator: an approach to prediction of human breast tumor responsiveness. *Cancer Res.* 47:3558-3564.
Morioka, S., G. S. Lazarus, J. L. Baird, and P. J. Jensen. 1987. Migrating keratinocytes express urokinase-type plasminogen activator. *J. Invest. Dermatol.* 88:418-423.
Nakajima, M., T. Irimura, N. DiFerrante, and G. L. Nicolson. 1984. Metastatic melanoma cell heparanase. *J. Biol. Chem.* 259:2283-2290.
Noguchi, P. D., J. B. Johnson, R. O'Donnell, and J. C. Petricciani. 1978. Chick embryonic skin as a rapid organ culture assay for cellular neoplasia. *Science (Wash. DC).* 199:980-983.
Ossowski, L. 1988. Plasminogen activator dependent pathways in the dissemination of human tumor cells in the chick embryo. *Cell.* 52:321-328.
Ossowski, L., and D. Belin. 1985. Effect of dimethyl sulfoxide on human carcinoma cells, inhibition of plasminogen activator synthesis, change in cell morphology, and alteration of response to cholera toxin. *Mol. Cell. Biol.* 5:3552-3559.
Ossowski, L., and E. Reich. 1980. Experimental model for quantitative study of metastasis. *Cancer Res.* 40:2300-2309.
Ossowski, L., and E. Reich. 1983. Antibodies to plasminogen activator inhibit human tumor metastasis. *Cell.* 35:611-619.
Ossowski, L., J. P. Quigley, and E. Reich. 1975. Plasminogen, a necessary factor for cell migration in vitro. *Cold Spring Harbor Conf. Cell Proliferation.* 2:901-913.
Ossowski, L., H. Russo, M. Gartner, and E. L. Wilson. 1987. Growth of a human carcinoma (HEp3) in nude mice: rapid and efficient metastasis. *J. Cell. Physiol.* 133:288-296.
Plow, E. F., D. E. Freany, J. Plescia, and L. A. Miles. 1986. The plasminogen system and the cell surfaces: evidence for plasminogen and urokinase receptors on the same cell type. *J. Cell Biol.* 103:2411-2420.
Pöllanen, J., K. Hedman, L. S. Nielsen, K. Danø, and A. Vaheri, 1988. Ultrastructural localization of plasma membrane-associated urokinase-type plasminogen activator at focal contact sites. *J. Cell Biol.* 106:87-95.
Poste, G., and M. K. Flood. 1979. Cells transformed by temperature-sensitive mutants of avian sarcoma virus cause tumors in vivo at permissive and non-permissive temperatures. *Cell.* 17:789-800.
Poste, G., J. Doll, I. H. Hart, and I. J. Fidler. 1980. In vitro selection of murine B16 melanoma variants with enhanced tissue-invasive properties. *Cancer Res.* 40:1636-1644.
Salo, T., L. A. Liotta, J. Keski-Oja, and T. Turpeenniemi-Hujanen. 1982. Secretion of basement membrane collagen degrading enzyme and plasminogen activator by transformed cells: role in metastasis. *Int. J. Cancer.* 30: 669-673.
Sas, D. F., J. B. McCarthy, and L. T. Furcht. 1986. Clearing and release of basement membrane proteins from substrates by metastatic tumor cell variants. *Cancer Res.* 46:3082-3089.
Scher, C. D., C. Haudenschild, and M. Klagsbrun. 1976. The chick chorioallantoic membrane as a model system for the study of tissue invasion by viral transformed cells. *Cell.* 8:373-382.
Sloane, B. F., J. Rozhin, K. Johnson, H. Taylor, J. D. Crissman, and K. V. Honn. 1986. Cathepsin B: association with plasma membrane in metastatic tumors. *Proc. Natl. Acad. Sci. USA.* 83:2483-2487.
Starkey, J. R., H. L. Hosick, D. R. Stanford, and H. D. Liggitt. 1984. Interaction of metastatic tumor cells with bovine lens capsule basement membrane. *Cancer Res.* 44:1585-1594.
Stoppelli, M. P., C. Tacchetti, M. V. Cubellis, A. Corti, V. J. Hearing, G. Cassani, E. Appella, and F. Blasi. 1986. Autocrine saturation of pro-urokinase receptors on human A431 cells. *Cell.* 45:675-684.
Strickland, S., and W. H. Beers. 1976. Studies of the role of plasminogen activator in ovulation. In vitro response of granulosa cells to gonadotropins, cyclic nucleotides and prostaglandins. *J. Biol. Chem.* 251:5694-5702.
Terranova, V. P., E. S. Hujanen, D. M. Loeb, G. R. Martin, L. Thornburg, and V. Glushko. 1986. Use of reconstituted basement membrane to measure cell invasiveness and select for highly invasive tumor cells. *Proc. Natl. Acad. Sci. USA.* 83:465-469.
Toolan, H. W. 1954. Transplantable human neoplasms maintained in cortisone-treated laboratory animals: HS#1; H. Ep.#1; H. Ep.#2; H. Ep.#3; and H. Emb.#1. *Cancer Res.* 14:660-666.
Valinsky, J. E., E. Reich, and N. M. Le Douarin. 1981. Plasminogen activator in the bursa of Fabricius: correlations with morphogenic remodeling and cell migration. *Cell.* 25:471-476.
Vassalli, J.-D., D. Baccino, and D. Belin. 1985. A cellular binding site for the M_r 55,000 form of the humaan plasminogen activator, urokinase. *J. Cell Biol.* 100:86-92.
Verheijen, J. H., E. Mullaart, G. T. G. Chang, C. Kluft, and G. Wijngaards. 1982. A simple and sensitive spectrophotometric assay for extrinsic (tissue-type) plasminogen activator applicable to measurements in plasma. *Thromb. Haemostasis.* 48:266-269.
Wun, T.-C., L. Ossowski, and E. Reich. 1982. A proenzyme form of human urokinase. *J. Biol. Chem.* 257:7262-7268.