Cell, Vol. 52, 321–328, February 12, 1988, Copyright © 1988 by Cell Press

Plasminogen Activator Dependent Pathways in the Dissemination of Human Tumor Cells in the Chick Embryo

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Summary

We have previously shown that inhibition of uPA activity of a human tumor—HEp3—results in a drastic reduction of its metastasis in the chick embryo. Using ^{125}IUdR-labeled tumor cells, we have now studied the role of uPA in individual steps of tumor metastasis. We found that, 48 hr after inoculation of tumor cells on the CAM, the organs of the embryos, inoculated with cells in which uPA was inhibited, contained 4-fold less cells than the controls. Neither the initial advance of the tumor mass into the CAM nor the process of extravasation was affected by the inhibition of tumor uPA. However, the infiltration of the CAM mesenchyme by individual tumor cells was blocked when tumor uPA activity or production was inhibited. In addition, indirect evidence implicated uPA as an essential factor in the tumor cell intravasation.

Introduction

Basement membranes of vascular or lymphatic vessels and dense connective tissue pose natural obstacles to the migration of tumor cells from primary sites to distant sites of metastasis. Local proteolytic activity, generated by tumors directly, or indirectly, by a cascade activation of zymogens present in the extracellular milieu, is responsible for partial degradation of these natural barriers and is thought to facilitate tumor cell dissemination (Jones and DeClerck, 1980; Kramer et al., 1982; Salo et al., 1982; Starkey et al., 1984; Sloan et al., 1986; Liotta 1986). Plasminogen activator (PA), a neutral serine protease secreted by most malignant tumors (for a review, see Dano et al., 1985), has the properties required of an initiator of a proteolytic cascade (Reich 1978; Paranjpe et al., 1980; Mignatti et al., 1986).

To determine whether PA plays a role in malignant dissemination, we developed a xenogeneic model of metastasis consisting of a human carcinoma (HEp3), originally described by H. Toolan (Toolan, 1954), growing in a chick embryo. In this model, HEp3 cells, inoculated onto the chorioallantoic membrane (CAM) of a 10 day old embryo, enter the mesenchyme of this highly vascularized membrane and divide, forming a rapidly expanding tumor mass. Within the first 2 to 3 days after inoculation, live tumor cells can be detected in the blood vessels and the organs of the embryo (Ossowski and Reich, 1980). We showed that injection of embryos with rabbit polyclonal antibodies that specifically inhibit tumor uPA activity block metastasis in this system (Ossowski and Reich, 1983b), thus demonstrating that one or more stages of the metastatic process are dependent on PA activity.

To identify the PA dependent stage, metastasis in the above model system was experimentally separated into four steps: entry into the chorioallantoic membrane (CAM); migration of tumor cells away from the primary mass; invasion of the blood vessels; and egress from the circulation.

We found that inhibition of HEp3 uPA activity by anti-uPA IgG interfered with specific events of the metastatic process. These findings were corroborated by experiments in which tumor uPA production was blocked by DMSO (Ossowski and Belin, 1985) or by the use of cell lines with constitutively different uPA levels.

Results

To study the role of uPA in individual stages of HEp3 metastasis, we examined the effect of inhibition of this enzyme on dissemination of radioactively labeled tumor cells in the chick embryo.

The Correlation between uPA Activity of Tumor Cells and Their Accumulation in Organs of Chick Embryos

Before testing the effect of uPA inhibition on tumor cell dissemination, the optimal conditions for these experiments were established. First, using two independent approaches, zymography and "panning" (see Experimental Procedures for details), we determined that radioactivity of the inoculated chick embryos and embryo organs was directly proportional to the number of tumor cells present. Then, three groups, of two chick embryos each, were inoculated onto CAMs with 2, 5, and 10 $\times$ 10^5 radioactive HEp3 tumor cells, and the radioactivity of embryos was measured at 24, 48, and 72 hr after inoculation. The number of radioactive cells in the embryos rose with time with all three inocula used; proportionally, more tumor cells were found in the embryos inoculated with the two lower cell inocula (Figure 1). Therefore, inocula ranging from 2.5 $\times$ 10^5 to 5.0 $\times$ 10^5 cells per embryo were used in all subsequent experiments.

The requirement for uPA activity for tumor cell dissemination from the primary site (CAM) to the embryo was studied with two approaches: radioactive HEp3 cells were used to inoculate embryos pretreated with high levels of anti-uPA IgG, and the dissemination of HEp3 cells was compared to that of three other cell lines that were characterized by widely differing content of uPA.

To examine the effect of uPA inhibition on HEp3 cell accumulation in chick embryos, the embryos were injected intravenously 1 hr prior to inoculation with either rabbit anti-uPA IgG (experimental) or with preimmune IgG (control). For inoculation, tumor cells were resuspended in the appropriate IgG. HEp3 tumor cells were first detected in the hearts and lungs of the control embryos approximately 14 hr after inoculation (Figure 2); thereafter, tumor cell number increased proportionally with time. When

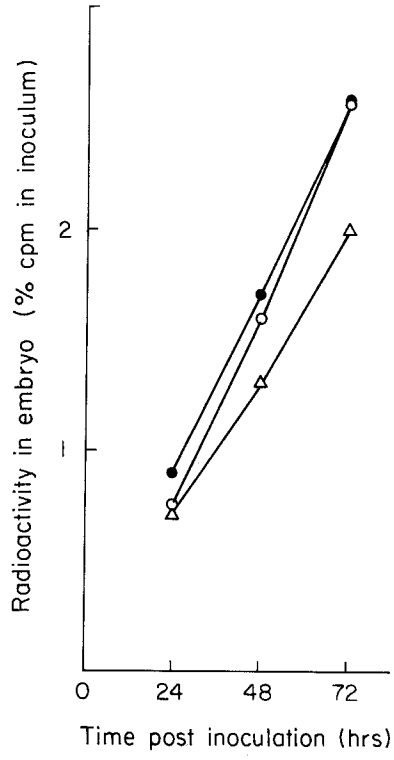

Figure 1. Accumulation of Radioactive Tumor Cells in Chick Embryo

Radioactive HEp3 cells (2.5, 5.0, or 10.0 × 10^5/CAM, 0.15 cpm/cell) were inoculated on CAMs of 10 day old chick embryos. Two embryos of each group were sacrificed at 24, 48, and 72 hr after inoculation, and radioactivity present in the embryos was measured in a gamma counter. Each experimental point is the average of two determinations that differed by less than 10%. Filled circles: 2.5 × 10^5. Open circles: 5.0 × 10^5. Open triangles: 10.0 × 10^5.

tumor cell inoculation on CAMs was preceded by an intravenous anti-uPA IgG treatment (see Experimental Procedures), a pronounced depression in the rate of accumulation of tumor cells was obtained; this effect was most striking between 24 and 48 hr after inoculation. The number of tumor cells present at 48 hr after inoculation in the hearts, the lungs, or the whole bodies of the control embryos was 3- to 4-fold greater than the number present in anti-uPA treated embryos (Figure 2 and Figure 2, bar).

In another set of experiments, four tumor cell lines, chosen for their widely differing content of uPA, were radioactively labeled and inoculated onto CAMs. The proportion of tumor cells found in the embryos at the end of a 24 hr incubation was well correlated with the uPA content of these cells (Table 1).

While in eggs inoculated with A431 cells (the line with highest uPA activity), the embryos contained as much as 14% of the cells present in the CAM, in eggs inoculated with cells of clone 1 (the line with the lowest uPA activity), the embryos contained only 1.5% of the cells. The two lines with intermediate uPA content showed an intermediate ability to disseminate into the embryos.

Is Catalytically Active uPA Required for CAM Invasion?

The inhibition of uPA can be affected at the level of its production or at the level of its catalytic activity. We have shown previously that treatment with DMSO elicits a strong, but reversible, inhibition of the steady state level of uPA-mRNA in HEp3 cells. This inhibition is translated into an overall >90% drop in uPA activity (Ossowski and Belin, 1985). The concentration of DMSO used (1.3%) is not toxic, as evidenced by the undiminished potential of HEp3 cells to divide (Ossowski and Belin, 1985). We have also shown (Ossowski and Reich, 1983b) that anti-human uPA antibody specifically inhibits the catalytic activity of HEp3 uPA without affecting the catalytic activity of PA produced by chicken cells. Both agents were used to assess the role of uPA in the entry of tumor cells into the CAM. The experiments were done using radioactive HEp3 cells, which were either pretreated with DMSO or mixed with anti-uPA IgG before inoculation. The latter cells were inoculated on CAMs of embryos injected 1–2 hr prior to inocu-

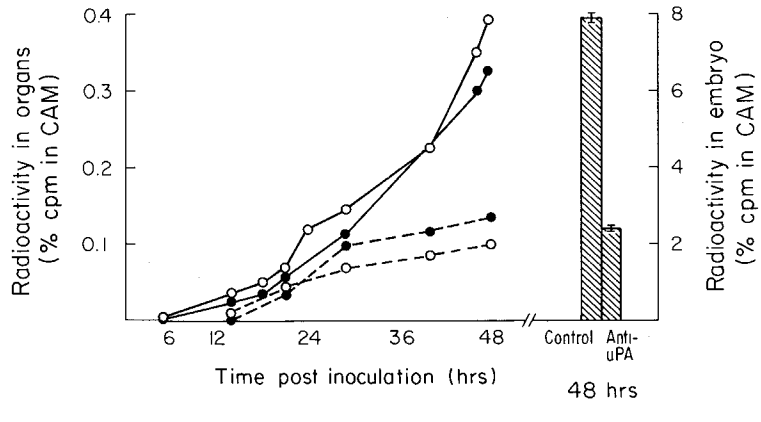

Figure 2. The Effect of uPA Inhibition with Anti-uPA IgG on the Accumulation of Radioactive HEp3 Cells in the Organs of Chick Embryo

Ten to eleven day old chick embryos were injected intravenously, 1–3 hr before inoculation, with 1.5 mg of rabbit preimmune or anti-uPA IgG diluted in 50 μl of PBS. All eggs were inoculated on CAM with radioactive (0.10 to 0.23 cpm/cell) HEp3 cells (3.5–5 × 10^5 per 50 μl of PBS containing 1.5 mg preimmune or anti-uPA IgG). Eggs incubated for 48 hr received an additional intravenous injection of 3 mg of the appropriate IgG. The eggs were incubated at 37°C and, at different times after inoculation, 2–4 embryos per experimental point were dissected. The lungs, heart, and bodies were washed and soaked in 70% ethanol (with three changes) for 24 hr and then counted in a gamma counter. The results of cell dissemination at 0 to 29 hr are from a single experiment (3–4 embryos per experimental point); the results of 40 to 48 hr are an average of three individual experiments. The radioactivity of the individual organs and the rest of the embryos is expressed as percent of the radioactivity of the CAM at the time of dissection. Student t-test of 48 hr values indicated a significant difference ($p < 0.01$) between the mean of control and test samples for isolated organs and the embryo bodies. Open circles: hearts. Closed circles: lungs. Solid line: control. Dashed line: anti-uPA treated embryos. Bars: embryo bodies.

Table 1. Relationship between Cell uPA Levels, Invasion of Injured CAM, and Dissemination of Tumor Cells into Chick Embryos

Cell Line	uPA Content (mU/mg protein)	Tumor cells in CAM (% inoculum) Mean	(SD)	Tumor cells in Embryo (% cells in CAM) Mean	(SD)
A431	400	28	(±13)	14	(±4)
Clone 5 (IH4)	340	29	(±3)	7	(±2)
HEp3	140	45	(±14)	4	(±2)
Clone 1 (IC8)	50	46	(±15)	1.5	(±0.3)

Tumor cell lines were labeled for 24 hr in culture with 0.2 μCi/mo ^{125}IUdR. In individual experiments, the specific activity varied from 0.13 to 0.47 cpm per cell. Cells (3×10^5 to 5×10^5) were inoculated onto CAMs and, following 24 hr of incubation, the number of tumor cells in the CAM and in the embryo were measured (see Experimental Procedures). Each value represents an average of two to three experiments and a total of 8 to 19 eggs for each experimental point. Tumor cell uPA content was measured as described in Experimental Procedures. Each uPA value is the average of two determinations (difference <10%) from a single, representative experiment. SD: standard deviation.

Table 2. Lack of Correlation between Inhibition of Tumor uPA and Invasion of Injured CAM

Treatment	HEp 3 Cells in the CAM (% inoculum)	
	Mean	*(SD)*
None	35.4	(±16.8)
DMSO	32.4	(±21.2)
Preimmune IgG	40.3	(±12.3)
Anti-uPA IgG	35.4	(±12.8)

HEp3 cells grown in culture for 2 days, without or with 1.3% DMSO (to reduce uPA production [Ossowski and Belin, 1985]), were labeled with 0.2 μCi/ml ^{125}IUdR for the last 24 hr in culture. In individual experiments, the specific activity varied from 0.14 to 0.28 cpm per cell. The cells (2.5 to 4.0×10^5) were inoculated onto freshly prepared (see Experimental Procedures) CAMs in 50 μl of PBS. For anti-uPA treatment, the eggs received 1.5 mg of antibody 1 hr prior to cell inoculation, and 1.5 mg was mixed with cells. Controls were treated similarly with preimmune IgG. Following 24 hr of incubation, the CAMs were excised, and the proportion of invading tumor cells was determined (described in Experimental Procedures). The results with DMSO are the average of five experiments; each experimental group consists of four to five eggs. The results with anti-uPA IgG are the average of two experiments in which a total of 20 eggs was used and divided equally between control and anti-uPA. Student t-test did not reveal a significant difference between the groups. SD: standard deviation.

lation with the anti-uPA IgG. Following a 24 hr incubation, the CAMs were excised, and the proportion of tumor cells in the CAMs was determined using the procedure described in Experimental Procedures. The results in Table 2 show that inhibition of uPA production or uPA activity did not result in a significant drop in the proportion of tumor cells that were found inside the CAMs. Injection of IgG intravenously, rather than its delivery on the CAM, did not change the outcome of these experiments (data not shown).

A similar conclusion was reached when cell lines with different uPA content were examined for their ability to penetrate the CAM. As shown in Table 1, no correlation was noticed between the level of uPA and the proportion of tumor cells that were found inside the CAMs.

A striking qualitative difference in tumor cell distribution was noticed, however, when histological sections of CAMs were examined. When uPA activity was left uninhibited, the advance of the tumor cell mass from the site of inoculation appeared to be more rapid. More importantly, many individual cells were found migrating ahead of the main tumor mass (Figures 3A and 3D). In contrast, in CAMs of embryos pretreated with anti-uPA IgG, or inoculated with DMSO-treated HEp3 cells, the tumor–host interface appeared even, and infiltration by individually migrating cells was extremely rare (Figures 3B, 3C, and 3E). Analysis, based on counting of tumor cells located in the CAM mesenchyme at a distance equal to, or greater than, 1 cell diameter from the main tumor mass (Table 3), provided a quantitative confirmation of the above findings.

Lack of Effect of uPA Inhibition on Tumor Cell Extravasation

To examine the egress of cells from the circulation, radioactive tumor cells were injected intravenously, and their distribution in the organs of chick embryos was determined at different times after injection. Figure 4 shows that the level of tumor cells in organs was the highest immediately following injection, and that it declined with time until it reached a plateau. Although the kinetics of tumor cell loss in the different organs was not identical in all cases (Figure 4), no further drop in radioactivity was noted between 24 to 48 hr after injection, suggesting that, in all likelihood, the cells no longer resided in the intravascular compartment. This notion is supported by the finding that extensive intravenous perfusion of embryos with proteolytic enzymes prior to sacrifice did not further reduce the organ associated radioactivity (data not shown).

To determine whether tumor uPA inhibition affects the number of extravasated cells, embryos were injected intravenously with 3 mg of anti-uPA antibody followed, 2 hr later, by intravenous injection of HEp3 cells. We have shown previously (Ossowski and Reich, 1983b) that more than 30% of the injected IgG remains in the embryo circulation 24 hr after injection. The experiment was terminated after 28 hr. As seen in Table 4, no significant difference was found in the proportion of tumor cells in the organs of chick embryos treated with anti-uPA IgG compared with the preimmune IgG. A similar result was obtained in one experiment in which the tumor cells were preincubated with the anti-uPA IgG for 1 hr in vitro and then injected intravenously (not shown).

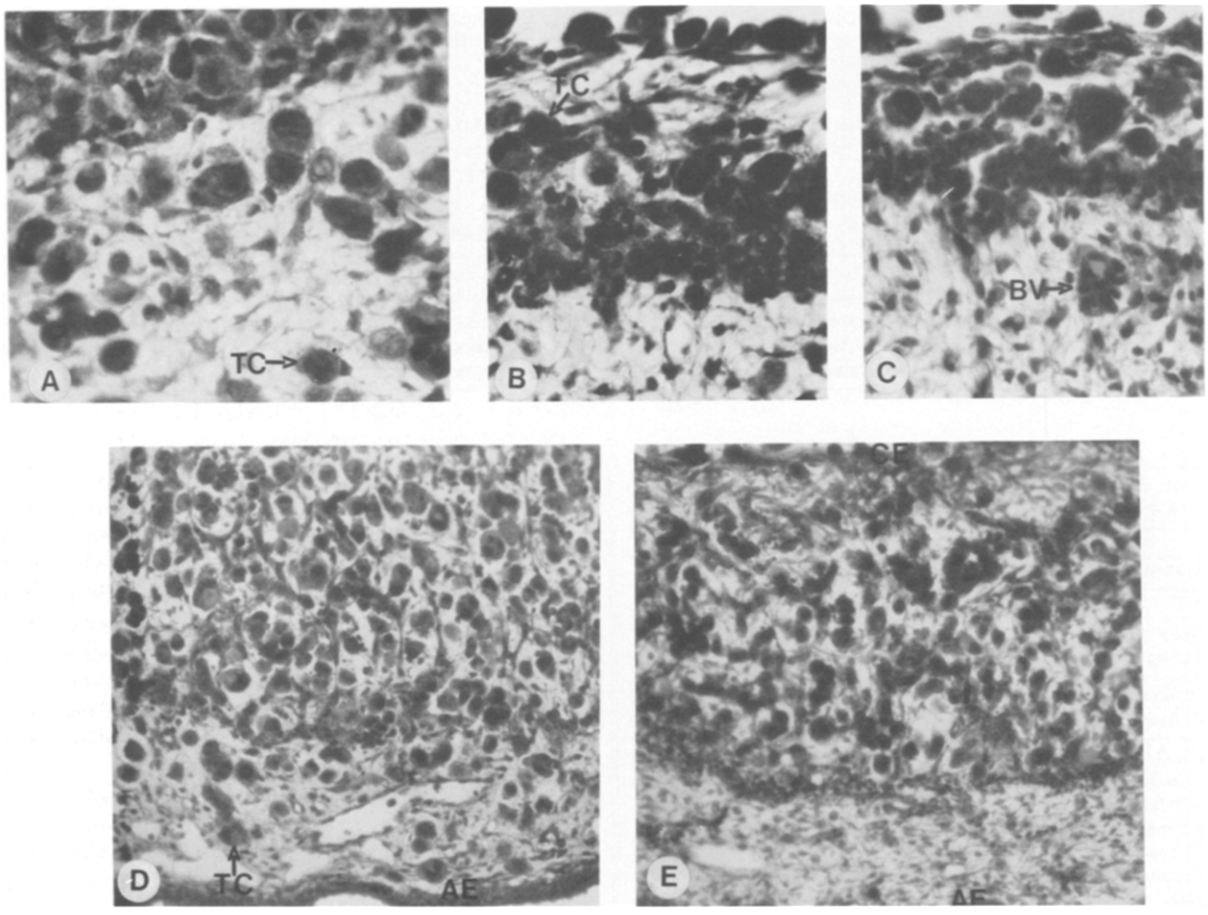

Figure 3. The Distribution of HEp3 Cells in the CAM: The Effect of Inhibition of Tumor uPA

HEp3 tumor cells (4×10^5/CAM) were resuspended in 1.5 mg anti-uPA IgG and inoculated onto CAMs that were pretreated with the same amount of IgG 1 hr prior to cell inoculation. Embryos treated with preimmune IgG served as controls. Eggs incubated for 48 hr received an intravenous injection of 3 mg of the appropriate IgG 24 hr after the initial inoculation. The inoculation with DMSO-treated cells (4×10^5/CAM) was exactly as described in the legend to Table 2. To remove unattached tumor cells, the CAMs were washed with PBS before excision. They were then excised, fixed in formalin, and histological sections were prepared and stained with H&E. A, B, C: 24 hr after inoculation; magnification 400×. D, E: 48 hr after inoculatoin; magnification 100×. A, D: controls. B: DMSO-treated. C, E: anti-uPA IgG. TC: tumor cells. AE: allantoic epithelium. CE: chorionic epithelium. BV: blood vessel. Note individually migrating tumor cells, away from the tumor mass, in A and D, and a relatively even interface between the tumor mass and the host mesenchyme in B, C, and E.

Table 3. The Effect of uPA Inhibition on the Infiltration of CAM Mesenchyme by Tumor Cells

Incubation (hr)	Number of HEp3 Cells/Unit Area[a]					
	Control			Treated[b]		
	Mean	(SD)	Range	Mean	(SD)	Range
24	3.6	(±5.2)	0–21	0.5	(±0.9)	0–3
48	4.9	(±3.9)	0–15	0.3	(±1.4)	0–7

Hep3 tumor cells (4×10^5/CAM) were inoculated on CAMs, and 24 or 48 hr later the CAMs were washed, excised, fixed in formalin, sectioned, and stained with H&E. Infiltrating tumor cells (see Figures 3A and 3D), distinguished by their very large size, were counted.

[a] One microscopical field (400× magnification) represents 1 unit area. Each experimental point represents the mean tumor cell number calculated by counting 20 fields (10 each of 2 adjacent sections) of four individual CAMs. SD: standard deviation.

[b] At 24 hr tumor uPA was inhibited by DMSO, at 48 hr by anti-uPA IgG.

Discussion

The studies described here were made possible by the use of ^{125}IUdR labeled tumor cells, which allowed for direct quantitation of as few as 100 metastatic tumor cells in the chick embryo organs. The highly sensitive "indirect" assay of metastasis, used previously to show the blocking effect of anti-uPA IgG on metastasis (Ossowski and Reich, 1983b), could not be used to test organs from 11 day old embryos as required by the current experiments. In contrast with cells from older embryos, which divide slowly when transplanted on CAMs (Ossowski and Reich, 1980), cells from young embryos divide rapidly, causing a drop in the relative number of tumor cells in the cell mixtures.

Although ^{125}IUdR was shown to be a reliable label for quantitation of tumor cells in rodents (Fidler, 1970), its usefulness in the chick embryo was not previously established.

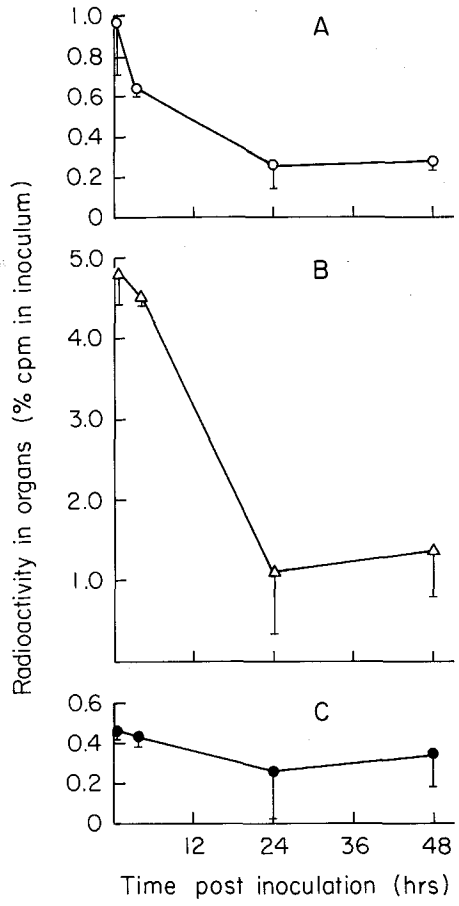

Figure 4. Accumulation of Radioactive HEp3 Cells in Hearts (A), Livers (B), and Lungs (C) of Chick Embryos Inoculated Intravenously

Chick embryos (11 days old), received an intravenous injection of 2.5 × 10^5 radioactive (0.15 cpm/cell) HEp3 cells. At different times after inoculation, the embryos were sacrificed, their organs dissected, and the number of radioactive cells measured in a gamma counter. The results shown are the mean of four embryos per experimental point; the bars indicate standard deviation.

Two approaches were used to validate ^{125}IUdR radioactivity as a measure of tumor cell number: in one, human uPA in chick embryo organs was quantitated by zymography; in another, tumor cells were separated from mixtures of chicken cells by the "panning" technique. Both methods confirmed that the measure of radioactivity was a reliable way of quantitating tumor cells (see Experimental Procedures for details).

The initial steps of tumor cell spread in this system involve the dissemination of the inoculum from the surface of the CAM into the CAM mesenchyme. It has been shown that, while tumor cells cannot penetrate an undamaged CAM (Armstrong et al., 1982), the epithelium of CAM prepared for tumor cell inoculation, by standard methods, is so extensively damaged that it poses no resistance to tumor cell penetration (Ossowski and Reich, 1983a). We determined that a reduction in tumor uPA activity or content did not affect the number of tumor cells present in the CAM mesenchyme. A difference was observed, however, when sections of the CAMs were examined microscopically: individual tumor cells, migrating far ahead of the tumor mass, were seen almost exclusively in those tumors in which uPA was not inhibited. (Figures 3A and 3D). This observation was confirmed by a quantitative analysis, which revealed that 7- to 16-fold more individually migrating cells were found in the mesenchyme of CAMs inoculated with tumor cells in which uPA was not inhibited (Table 3). These results are not unexpected in view of previous findings made in a number of studies (Ossowski et al., 1975; Strickland et al., 1976; Vassalli et al., 1976, Valinski et al., 1981; Morioka et al., 1987), all of which indicate a requirement for active uPA for cell migration or invasion.

Extravasation was studied by injecting radioactive tumor cells intravenously into control embryos or embryos that received anti-uPA IgG. After an initial decline, because of intravascular tumor cell death (Liotta and De Lisa, 1977), the radioactivity in the organs remained constant for at least 24 to 48 hr. We assume that this radioactivity represents a fraction of the tumor cell population which has already left the vascular bed and entered the parenchyma of the embryo organs. This conclusion is supported by findings (Leighton, 1964; Easty et al., 1969) that show that in chick embryos, a majority of the detectable tumor cells are found outside the vascular compartment as early as 18 to 48 hr after intravenous injection (Leighton, 1964; Easty et al., 1969). As shown in Table 4, the anti-uPA IgG did not have an effect on the size of this population. A similar result was obtained in mice inoculated intravenously with B16 melanoma and treated with a uPA inhibitor (Ostrowski et al., 1986). Although the most likely explanation of these findings would be the lack of re-

Table 4. Lack of Effect of Anti-uPA IgG on Extravasation of Tumor Cells

	Radioactive Tumor Cells in Organs (% inoculum)					
	Heart		Liver		Lungs	
IgG	Mean	(SD)	Mean	(SD)	Mean	(SD)
Preimmune	0.19	(± 0.07)	1.27	(± 0.44)	0.22	(± 0.03)
Anti-uPA	0.22	(± 0.09)	1.15	(± 0.3)	(± 0.27	(± 0.1)

Eleven day old chick embryos were injected intravenously with 3 mg of preimmune or anti-uPA IgG. Two hours later, all embryos were injected intravenously with 2.5 × 10^5 radioactive HEp3 cells (0.1 cpm/per cell). Following 28 hr of incubation, the embryos were sacrificed, their organs dissected, and counted in a gamma counter (see Experimental Procedures). Each experimental point is the average of four to seven embryos. SD: standard deviation. Student t-tests showed no significant difference between the control and the anti-uPA treated group.

quirement of active uPA for extravasation, other possibilities must be also considered. First, intravenous injections result in a sudden introduction of a mass of circulating tumor cells into the vascular compartment. Their presence may cause many effects different from the ones initiated by a spontaneous process. It is also possible that the large inocula required to ensure experimentally measurable levels of radioactivity in the embryo organs exceed the neutralizing capacity of the injected anti-uPA IgG. This does not seem to be the case, however, since preincubation of the HEp3 cells with an excess of anti-uPA IgG prior to injection (data not shown) did not change the outcome of this experiment. Other possibilities must also be considered. Since extravasation is such a complex process, it is easy to envision a scenario in which uPA would play multiple but contrasting roles, resulting in an apparent lack of effect and misinterpretation.

We have shown here that, under the conditions used, the CAM did not pose a barrier to initial tumor cell entry and that active uPA was not required for this stage of dissemination. Similarly, the process of extravasation did not appear to require active uPA. However, the magnitude of the overall inhibitory effect on tumor cell accumulation in the chick embryo, achieved by suppression of tumor uPA activity, indicates an important role for uPA in this process. What then may the uPA dependent step be? Although a direct involvement of proteases in intravasation in vivo has not been documented, histological studies show that degradation of the basal lamina of the CAM blood vessels by tumor cells precedes tumor cell entry into the circulation (Vlaeminck et al., 1972). We have a clear indication (see Figure 3) that individual tumor cells in the CAM display a highly invasive behavior, characterized by enhanced migration, only when uPA is fully active. It is possible that this leads to an increase in the number of encounters between tumor cells and the vascular walls of the CAM blood vessels. It is more likely, however, that these cells represent a population which both migrates and intravasates more efficiently because of its higher uPA content. The following facts support this last possibility: 48 hr after inoculation, when the main tumor cell mass has advanced almost through the entire thickness of the CAM, all cells, regardless of their uPA status, should have the same chance to come in contact with blood vessel walls. And yet, in comparison with control cells, the number of uPA inhibited tumor cells, which migrate from the CAM to the embryo, is reduced 4-fold (Figure 2). Similarly, in four tumor cell lines examined, the ability of cells to disseminate from the CAM to the embryo, but not their ability to enter the CAM mesenchyme, was found to be strictly correlated with their uPA content (Table 1). All of the above facts suggest that proteolytic activity is required for tumor cell intravasation and that the participation of uPA is essential for generating this activity. The fact that in this model system tumor cell intravasation appears to be dependent on uPA, while the enzyme does not appear to be required for extravasation, may not be too surprising. The environment encountered by the circulating tumor cells inside the blood vessels, determined by factors such as plasma proteins, blood cells, shearing forces, endothelium etc., is unlike that existing in the stroma. Such differences may limit the population of cells able to complete a particular process to those cells that possess the required capabilities. An example of a modifying influence of endothelium on the invasive properties of a fibrosarcoma cell line has been published (Jones et al., 1981). The notion that different properties may be crucial for different stages of metastasis is also supported by the findings (Price et al., 1984) that show that in primary mammary tumors, a link between the ability of tumor cells to colonize lungs upon intravenous injection (extravasation) and the ability to form spontaneous metastasis does not exist in all tumors. Distinct lung colonizing and lung metastasizing populations have also been isolated from a B16 mouse melanoma (Stackpole, 1981). Also, the possibility that different components of the basement membrane need to be degraded in order to facilitate the crossing of tumor cells from opposite directions of a blood vessel wall cannot be excluded.

Experimental Procedures

Materials

COFAL-negative embryonated eggs were obtained from SPAFAS (Norwich, CT); Dulbecco's modified Eagle's medium from Grand Island Biological Co. (Grand Island, NY); fetal bovine serum from Hazleton Dutchland Inc. (Denver, PA); Triton X-100, collagenase type I and DMSO from Sigma Chemical Co. (St. Louis, MO); ^{125}IUdR (specific activity 2200 Ci/mmol) from New England Nuclear Corp. (Boston, MA); human urokinase standard from Leo Pharmaceuticals, (Ballerup, Denmark). Human plasminogen was purified from fresh human plasma as previously described (Deutsch and Mertz, 1970; Strickland and Beers, 1976), bovine fibrinogen (Calbiochem/Behring, San Diego, CA) and rabbit preimmune and anti-uPA IgG were prepared as described (Strickland and Beers, 1976; Ossowski and Reich, 1983b; Wun et al., 1982). Goat anti-rabbit IgG (specific for Fc portion of the immunoglobulin) were from Cappel, Cooper Biomedicals Inc. (Malvern, PA); rabbit anti-HEp3 IgG were as described (Becker at al., 1981).

Cell Lines

HEp3 cells were obtained by dissociation of HEp3 tumors serially passaged on CAMs of 10 day old chick embryos, using previously described methods (Ossowski and Reich, 1980). Clone 1 (IC8) and clone 5 (IH4) were isolated from an HEp3 tumor and have been described previously (Ossowski and Reich, 1983a). A431 is a human squamous cell carcinoma (Giard et al., 1973) that produces very high levels of uPA. All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Preparation of Cells for uPA Assay

HEp3, clones 1 and 5, and A431 cells were plated at 5×10^5 in 60 mm culture dishes and grown in medium with 10% fetal bovine serum. Following 24 hr of incubation, the cultures were washed repeatedly and incubated with medium without serum. Aliquots of conditioned medium and cells were taken at 24 and 48 hr and assayed for uPA activity exactly as described (Strickland and Beers, 1976).

Radioactive Labeling of Cells

Cells (1.5 to 2.0 $\times$ 10^6) were plated in 100 mm tissue culture dishes in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and allowed to grow for 24 hr, at which time all cultures were refed medium containing 5% serum and 0.2 μC/ml of ^{125}IUdR. Following 24 to 48 hr of incubation, the cultures were washed extensively (six washes with 10 ml medium before detachment, three to four washes, 40 ml each, in suspension) until the cell free counts dropped below 3% of total radioactivity. Depending on the conditions and the cell type used, the specific activity varied in individual experiments from 0.10 to 0.47 cpm/cell; autoradiography showed that the nuclei of most cells were labeled under these conditions.

The Effect of uPA Inhibition on Tumor Cell Accumulation in the Organs of Chick Embryo

The methodology used in these series of experiments is essentially similar to that described (Ossowski and Reich, 1983b) with the exception that here, we radioactively labeled the HEp3 cells and estimated their numbers by determining the radioactivity of the organs early after inoculation. Briefly, an artificial air sac was made on the side of a 10.5 to 11 day old egg. Rabbit preimmune or anti-uPA IgG (1.5 mg in 50 μl) was inoculated into the chorioallantoic vessels and, 1 hr later, radioactively labeled tumor cells (4–5 × 10^5), washed extensively to remove unbound label and resuspended in 1.5 mg of the appropriate IgG, were inoculated onto CAMs. In experiments in which incubation continued for 48 hr, the eggs received an additional intravenous dose of 3 mg of IgG 24 hr after inoculation. Hearts, lungs, and bodies (without internal organs) were dissected at different times after inoculation, washed, soaked for 24 hr (with three changes) in 70% ethanol (Fidler, 1970), and counted in a gamma counter.

The Effect of uPA Inhibition on Invasion of CAM

Two approaches were used: First, the production of HEp3 uPA was inhibited by DMSO treatment in culture prior to inoculation of the HEp3 cells on CAMs. To achieve a 65% inhibition of cell associated uPA and a greater than 95% inhibition of the total uPA production (Ossowski and Belin, 1985), HEp3 cells were incubated with 1.3% DMSO for 48 hr. Second, the catalytic activity of HEp3 uPA was inhibited in vivo by anti-uPA IgG. This method was essentially similar to the method described above for inhibiting the accumulation of tumor cells in the embryos, except that 1.5 mg (in 50 μl of PBS) of preimmune or anti-uPA IgG was delivered onto the CAMs 1 hr prior to the inoculation of cells. Once the IgG was adsorbed, ^{125}IUdR-labeled tumor cells, resuspended in 50 μl of PBS containing 1.5 mg of the appropriate IgG, were inoculated onto the CAMs. Unless specified, the eggs were incubated for 24 hr, at which time the CAMs were washed in situ with PBS (four times, 1 ml), excised, counted in a gamma counter, and placed in 2 ml of trypsin (0.05%) mixed with EDTA (1mM) and incubated for 20 min at 37°C. The CAMs were washed in PBS and again counted in a gamma counter. We have shown (unpublished data) that this treatment releases only the tumor cells that have not entered the CAM and which remain unprotected from the action of trypsin.

To examine whether the radioactivity present in the CAM corresponded to live, ^{125}IUdR-labeled tumor cells, HEp3 cells were labeled in culture (0.16 cpm/cell) and inoculated on 4 CAMs (3.5 × 10^5 cells /CAM). Following 16 hr of incubation, the CAMs were washed with PBS, excised, treated with trypsin-EDTA for 20 min at 37°C, minced finely, and incubated at 37°C for 45 min with 0.15% collagenase. The radioactivity and the cell number, present in each of the 4 CAMs that had been dissociated to a single cell suspension, have been determined. During the 16 hr of incubation, the specific label associated with tumor cells dropped from 0.16 cpm/cell to 0.145 (±0.006) cpm/cell or 9.4%, while at the same time, the number of live (Trypan blue excluding) tumor cells recovered from the CAMs increased from 3.5 to 3.9 × 10^5 (±0.23 × 10^5) cells, or 11.4%. The observed correlation between the drop in specific label of cells and the rate of cell divisions indicates that the radioactivity is a valid measure of viable tumor cells.

The Effect of uPA Inhibition on Extravasation of Tumor Cells

Chick embryos (10.5 to 11 days old) were inoculated intravenously with 3 mg of preimmune or anti-uPA IgG followed by intravenous injection of radioactive tumor cells 1 hr later. At 28 hr after inoculation, the hearts, lungs, and livers of the embryos were dissected, soaked in 70% ethanol, and counted in a gamma counter. To show that tumor cells, which may become opsonized in vivo by rabbit anti-uPA IgG, are not destroyed by chicken complement, the following experiment was performed: HEp3 cells were incubated in vitro with 0.5 mg/ml of the anti-uPA IgG followed by either fresh rabbit serum (as a source of complement) or fresh serum from 12 day old chick embryos. HEp3 cells, preincubated with anti-HEp3 IgG and the two sources of complement, served as a positive control. The percent of dead cells was determined by Trypan blue exclusion. The backgrounds (dead cells in presence of IgG but in absence of complement) were subtracted. The percent of dead cells in the mixtures incubated with anti-uPA IgG and rabbit complement was 3% and with chick embryo serum 0%. Of cells preincubated with anti HEp3 IgG and rabbit complement, 68% were dead while no dead cells were found in mixtures of the IgG and chick serum. These results show that 12 day old chick embryos do not have detectable levels of circulating complement or, alternatively, that complement does not interact with the rabbit IgG.

Correlation between Radioactivity and Tumor Cells

Radioactivity was shown to be a reliable method for tumor cell quantitation in embryos on the basis of the following two experiments: Aliquots (50 μl) of 0.5% Triton X-100 lysates of chick embryos (one 11 day old embryo was lysed in 1 ml of Triton X-100) were mixed with 0 to 4000 HEp3 cells. Aliquots (5 μl) of the mixtures were analyzed by gel electrophoresis and zymography (Granelli-Piperno and Reich, 1978); the zymographs were photographed, and the lysis zones corresponding to human uPA were cut out, weighed, and used to prepare a standard curve. The sensitivity of this technique is such that it allows for the detection of lysis zones produced by 100 to 200 tumor cells per 5 μl aliquot. Considering the dilution factor, this represents 20,000 to 40,000 tumor cells per embryo. This method was used to test the correlation between the level of radioactivity of embryos, inoculated on CAMs with radioactive HEp3 cells, and the level of human uPA activity. Twelve embryos (four for each cell concentration), inoculated with 0.5, 1.0, and 2.0 × 10^6 radioactive HEp3 cells, were excised after 24 and 48 hr, minced, and homogenized in Triton X-100 and counted in a gamma counter. The average number of counts per embryo inoculated with 0.5 × 10^6, 1.0 × 10^6, and 2.0 × 10^6 cells and excised after 24 hr was 1300 cpm, 2600 cpm, and 2800 cpm and, after 48 hr, 3700 cpm, 6700 cpm, and 7800 cpm, respectively. Aliquots of homogenates were assayed by gel electrophoresis and zymography. The lysis zones were excised, weighed, and used to calculate the number of HEp3 cells from the previously prepared standard curve. We found that when tumor cell number per aliquot tested exceeded 200, the number of HEp3 cells calculated from zymographic vs. radioactive measurements differed only by 7% or less. A worse correlation (a difference of up to 40%) was found with embryos harboring fewer cells because the weaker lysis zones produced by these aliquots photographed poorly, and it was difficult to determine their exact outlines.

The above test showed that radioactivity and human uPA activity of the embryos were well correlated. To determine if the radioactivity is tumor cell associated, we used a selective adherence technique or "panning," a method often employed in separating immune cells. Petri dishes were coated with goat anti-rabbit Fc portion of the IgG (60 μg/6 ml PBS/100 mm dish). To calibrate, 5,000 to 40,000 radioactive HEp3 cells preincubated with anti-HEp3 IgG were added to the anti-rabbit Fc coated dishes. The ability of HEp3 cells alone and HEp3 cells mixed with 8 × 10^7 chick embryo lung cells to adhere to the IgG coated surface was determined. With HEp3 alone, 94% to 99% of the input radioactivity was recovered as an adherent population, but only 29% to 31% of the radioactivity was recovered from the dishes seeded with the HEp3 mixed with chick embryo lung cells. This reduced efficiency is probably due to the interference by the great excess (2000- to 8000-fold) of chicken cells. The adherence of HEp3 cells to the anti-Fc IgG coated dishes was critically dependent on the preincubation of these cells with anti-HEp3 IgG.

To determine if this method would be useful in identifying metastatic cells in organs of HEp3 inoculated chick embryos, four groups of five lungs each, obtained from embryos that received 1.0 × 10^6 radioactive cells 48 hr earlier, were subjected to "panning." The lungs were dissociated with collagenase (60 min, 37°C) and incubated on ice for 20 min in 2 ml of Dulbecco's modified Eagle's medium with 5% fetal bovine serum containing 800 μg of anti-HEp3 cells IgG. Following incubation, the cells were washed and seeded onto the goat-anti rabbit Fc (experimental) or BSA (60 μg/6 ml PBS, control) coated dishes, incubated for 10 min on ice, centrifuged for 6 min at 300 rpm, washed, and stained with Giemsa. In a parallel set of dishes, cells were solubilized with 1 N NaOH and counted in a gamma counter. A second round of "panning" (performed in two of the four experiments) resulted in only a small improvement of yield. The radioactivity recovered from the dishes in four individual experiments was 26%, 28%, 31%, and 33% of the input. These values are in good agreement with the values obtained for standard curves (see above) in which known numbers of HEp3 cells were directly mixed with chick embryo lung cells, and they suggest that the radioactivity measurements of the chick embryo organs are a good representation of the HEp3 cell number. In addition, estimates of the

HEp3 cells in the Giemsa stained dishes correlated well with the calculated numbers of HEp3 cells based on the radioactivity dissociated by treatment with NaOH from parallel dishes. Similar results were obtained when whole, metastasis-bearing embryos were dissociated with collagenase and examined for the presence of HEp3 cells by "panning."

Acknowledgments

This work was supported by USPSH Research Grant CA-40758. I would like to thank Gloria Bertuzzi for outstanding assistance, Judy Adams for help with the preparation of the photomicrographs, Dr. A. Granelli-Piperno for help with the "panning" technique, and Drs. R. Mira y Lopez and Z. Cohn for help and critical reading of the manuscript.

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Received September 23, 1987; revised November 23, 1987.

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