Mouse L Cells Expressing Human Prourokinase-type Plasminogen Activator: Effects on Extracellular Matrix Degradation and Invasion

Jean-François Cajot,* Wolf-Dieter Schleuning,† Robert L. Medcalf,‡ Jeanine Bamat,* Joëlle Testuz,* Liora Liebermann,§ and Bernard Sordat*

*Swiss Institute for Experimental Cancer Research, 1066 Epalinges, Switzerland; †Central Hematology Laboratory, University of Lausanne Medical School, 1011 Lausanne, Switzerland; and §BIOGEN Research Corp., Cambridge, Massachusetts 02142

Abstract. A cosmid (cos pUK0322) harboring the complete human urokinase-type plasminogen activator (u-PA) gene and Geneticin resistance as a selectable marker was isolated from a human genomic library and characterized. After transfection of cos pUK0322 into mouse L cells and selection, several plasminogen activator (PA)–expressing clones were obtained and one (L~) was chosen for additional study. The PA expressed was identical to human pro–u-PA in enzymatic, electrophoretic, and antigenic properties. The expression of PA was stable over 50 population doublings. The regulation of the transfected gene was studied by treatment of the cells with various hormones and other effectors. Expression of PA activity was inhibited fivefold by dexamethasone and stimulated two- to threefold by agonists of the adenylate cyclase dependent pathway of signal transduction, such as dibutyryl cyclic AMP and cholera and pertussis toxins. The modulation of PA activity was associated with corresponding changes in mRNA steady-state levels. The phenotypic changes associated with pro–u-PA expression were analyzed in vitro by degradation of 3H-labeled extracellular matrix (ECM), invasion of a matrigel basement membrane analogue, and by light and electron microscopy. L~ cells and reference HT-1080 fibrosarcoma cells, in contrast to control L~o cells transfected with the neomycin resistance gene, degraded the ECM and invaded the matrigel basement membrane. Matrix degradation correlated with the modulation of pro–u-PA gene expression as it was inhibited by dexamethasone and promoted by dibutyryl cyclic AMP. Inhibition of PA or plasmin using anti–u-PA IgG or aprotinin prevented ECM degradation and invasion. These results demonstrate that u-PA expression alone is sufficient to confer to a cell an experimental invasive phenotype.

PLASMINOGEN activators (PA) are highly specific serine proteases that convert plasminogen into the trypsin-like enzyme plasmin (Astrup, 1978). PA-mediated plasmin formation leads to fibrin degradation and to extracellular matrix (ECM) remodeling in the course of morphogenetic events like ontogeny and neoplastic growth (for reviews see Blasi et al., 1987; Dane et al., 1985; Saksela, 1985). There are two genetically distinct PAs: tissue-type PA and urokinase-type PA (u-PA). Of these, u-PA has been implicated in contributing to the mechanism of invasive growth of tumors (for review see Danö et al., 1985). u-PA also appears to play an important role in metastasis formation: antibodies to u-PA inhibited metastasis of Hep3 human epidermoid carcinoma cells in the chick embryo (Ossowski and Reich, 1983) and such antibodies also decreased significantly the number of pulmonary metastases of B16 mouse melanoma cells in mice (Hearing et al., 1988).

Plasmin activates latent collagenase (Mignatti et al., 1986), leading to degradation of collagen constituents of the ECM. Other enzymes such as elastase and various cathepsins are also believed to play a role in ECM degradation (for review see Sträuli et al., 1980). Because of the complexity of these proteolytic events, it is difficult to assess the individual contributions of the various enzymes in wild-type cells. To study the phenotypic change caused by the expression of a single gene, we have transfected a cosmid containing the natural human pro–u-PA gene into mouse L cells, which do not naturally express PA. We demonstrate that the gene remains sensitive to regulatory mechanisms that have previously been shown to be effective at its natural locus. The effects of u-PA expression and regulation in u-PA–transfected L~ cells on the degradation of the ECM and on basement membrane invasion were studied using biochemical and morphological analysis.
Materials and Methods

Materials

Dexamethasone, cholera toxin, dibutyril cyclic AMP, Geneticin, ribonuclease, proteinase K, bovine trypsin (type XI), and salmon testes DNA (type III) were from Sigma Chemical Co. (St. Louis, MO); plasminogen-rich bovine fibrinogen was from Ooporpharma (Zürich, Switzerland); pertussis toxin was from List Biological Laboratories (Campbell, CA); phenol, uranyl acetate, lead citrate, and methylene blue/azure II solution were from Merck (Darmstadt, Federal Republic of Germany); osmium tetroxide was from Elmis (Carouge, Switzerland); in vitro-packaging system from Genoff (Geneva, Switzerland); restriction enzymes were from Boehringer-Mannheim (Rotkreuz, Switzerland); bacterial collagenase (type CLSSPA) was from Worthington Biochemical Corp. (Freehold, NJ); Gene Screen Plus membranes were from New England Nuclear (Boston, MA); cacodylate, Epon 812 and glutaraldehyde were from Fluka AG (Buchs, Switzerland); and tissue culture 100-mm-dish plates and 24-well plates were from Gibco Laboratories (Basingstoke, UK). The pSV2neo plasmid was kindly provided by Dr. P. Cerutti (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). Rabbit anti-human u-PA serum was a gift of Dr. J. Hauert (Hematology Department, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). Mouse L cells were obtained from Dr. J. Maryanski (Ludwig Institute for Cancer Research, Epalinges, Switzerland). Other materials were as previously described (Cajot et al., 1986a,b).

PA Activity Assay

PA activity of tumor cell-conditioned media was assayed using the 125I-fibrin plate method (Unkeless et al., 1973). All activities are expressed in international units by comparison with the international reference preparation of human u-PA (1 IU corresponds to 2 ng two-chain active u-PA enzyme). Zymographic analysis of PA was performed as previously described (Heussen and Dowdle, 1980): 50-μl samples of conditioned media were subjected to SDS–10% PAGE (Laemmli, 1970). Gel slabs, which contained 2.5 mg/ml of copolymerized plasminogen-rich bovine fibrinogen, were incubated for 1 h in 2.5% Triton X-100 to allow protein renaturation and subsequently incubated in 0.1 M glycine/NaOH (pH 8.3). Staining of the gels with amido black revealed white lysis zones against the undigested blue background.

Immunoprecipitation

Conditioned medium was mixed 1:1 (vol/vol) with anti-human u-PA (dilution 1:500) or control nonimmune rabbit serum and incubated for 2 h at 37°C and for 18 h at 4°C. Antigen–antibody complexes were precipitated by addition of 150 μl of cellulose suspensions coated with donkey anti-rabbit IgG to 300 μl of the above mixture followed by centrifugation at 5,000 g for 5 min. Supernatants were recovered and analyzed for the presence of residual PA activity.

Disopropylfluorophosphate (DFP) Sensitivity Assay

DFP sensitivity of zymographic activity was investigated as previously described (Vassalli et al., 1984). Samples were incubated for 1 h at 37°C with 25 mM DFP, and the reaction was terminated by addition of 100 μl of aprotinin. Samples were further incubated for 1 h at 37°C with 27 mM DFP and subjected to zymographic analysis.

Cell Culture

Cells were seeded at a density of 4 × 10^4 into 100-mm-dish tissue culture dishes and grown for 36 h in 15 ml DME supplemented with 10% FCS. Subsequently, the medium was removed and cells washed twice with DME. Cells were then maintained under serum-free conditions in 8 ml DME containing 0.1% BSA. To modulate gene expression of u-PA the following compounds were added to the culture medium: dexamethasone (500 nM), pertussis toxin (5 nM), cholera toxin (50 nM), and dibutyril cyclic AMP (1 mM). Dexamethasone was prepared in 50% ethanol stock solution. All other drugs were prepared in DME containing 0.1% BSA and stored at −80°C until further use. After 24 h, the conditioned media were harvested, centrifuged for 5 min at 3,000 g to remove cellular debris, and stored at −80°C until used. PA activities measured in conditioned media were expressed in IU per 10^6 cells. Cells extracts were prepared by pelleting cells as described above and growing for 36 h. Confluent cultures were then washed twice with serum-free DME followed by incubation in 10 ml lysis buffer (1% SDS, 50 mM imidazole–HCl (pH 7.55), 140 mM NaCl) per 100-mm-diam dish for 15 min at 20°C.

Molecular Cloning and Characterization of a u-PA cDNA Clone and the u-PA Gene

UX0321 was isolated from a cDNA library generated in Agt10 by the method of Guebler and Hoffman (1983) from mRNA isolated and enriched by sucrose gradient centrifugation from human HT-1080 fibrosarcoma cells. Mixed oligonucleotide probes corresponding to a u-PA cDNA sequence (Gänzler et al., 1982) were synthesized and used for screening (Maniatis et al., 1982). The insert of UX0321 was subcloned into pUC8 (Vieira and Messing, 1982) giving rise to pUX0321, sequenced (Maxam and Gilbert, 1977), and found to harbor a 1,023-bp fragment of human u-PA cDNA, reaching from the bp 727 to 1,750, described by Verde et al. (1985). Fragments were separated by gel inversion electrophoresis as described (Frederick et al., 1986).

Isolation of Plasmid, Cosmid, and Genomic DNA

High molecular weight genomic DNA was prepared from cultured cells as described (Reymond, 1987) with the following modifications: the extracted DNA was subjected to an additional step of RNase and proteinase K digestion, phenol–chloroform extraction, ethanol precipitated, and resuspended in NaCl/Tris/EDTA (100:10:1) according to standard procedures (Maniatis et al., 1982). Plasmid DNA was prepared using the alkaline lysis method followed by CsCl/ethidium bromide equilibrium centrifugation (Birnboim and Dol, 1979). Cosmid DNA was packaged in vitro using a commercial kit (Genofit) following the manufacturer's instructions. Escherichia coli HB101 cells were infected with cosmid DNA–containing phages as described by Becker and Gold (1975) and used for cosmid DNA preparation as described above for the preparation of plasmid DNA.

Southern Blot Analysis

For Southern blot analysis (Southern, 1975) 10 μg of genomic DNA and 1 μg of cos pUK0322 DNA were digested with restriction enzymes, electrophoresed through a 0.8% agarose gel slab, and transferred to nitrocellulose filter paper. The filters were prehybridized at 65°C for 2 h in 4× SSC, 20 mM phosphate buffer, 0.1% SDS, 0.005% pyrophosphate and 0.0125% carrier DNA and subsequently hybridized for 20 h at 65°C in the presence of 10^6 cpm of a nick-translated Eco RI-Hind III (607-bp) fragment of pUK0321 (human urokinase cDNA clone). The filter was washed twice for 30 min at 65°C in 500 ml each wash, of successively decreasing salt solutions: 2× SSC, 0.05% SDS, 0.01% pyrophosphate; 1× SSC, 0.005% SDS, 0.001% pyrophosphate; 0.1× SSC, 0.005% SDS, 0.0001% pyrophosphate.

Transfections

Mouse L cells (Sanford et al., 1984) were transfected with cosmid pUK0322 using the calcium phosphate coprecipitation method as previously described (Graham and Van der Eb, 1983) as modified by Wigler et al. (1979). 5 μg of circular cosmid DNA was coprecipitated with 30 μg of carrier salmon testes DNA and added to a 25-cm² tissue culture flask seeded 24 h earlier with 10^6 cells. Control transfections were performed in parallel with either 5 μg of circular pSV2neo DNA plus carrier DNA or carrier DNA alone. DNA-containing media were removed after an 18-h incubation at 37°C, and transfected cells were maintained for a 48-h culture in fresh medium. After trypsinization, 10^6 cells were transferred to 100-mm-dish dishes and cultured in 15 ml of DME, 10% FCS containing 1 mg/ml of Geneticin. The selection medium was changed after 8 d, and drug-resistant clones were isolated with the aid of cloning cylinders after 15 d.

Determination of Gene Copy Number

To quantitate the copy number of the transfected gene, 10 μg of L cells DNA was digested with Sma I and subjected to Southern blot analysis using human u-PA cDNA (pUK0321) as a probe. Serial dilutions of cos pUK0322
containing known copy numbers of the u-PA gene were digested and analyzed under identical conditions. The u-PA gene copy number of transfected cells was calibrated by comparison of the L_{ux} DNA hybridization signal with that of pUK0322 dilutions.

**Northern Blot Analysis**

RNA was isolated as previously described (Medcalf et al., 1986) and subjected to Northern analysis using the specifications of Thomas (1980). Agarose gel electrophoresis of mRNA was performed in the presence of 20% formaldehyde and 300 ng/ml ethidium bromide. After electrophoresis, gels were photographed using a UV transilluminator (260 nm) to localize ribosomal RNA bands and to assess lane variations in RNA loading. Northern blot transfer was performed as described by Thomas (1980), except the Gene Screen Plus membrane was used. Hybridization conditions and processing of filters were as previously described (Medcalf et al., 1988). Densitometric analysis of mRNA signals on autoradiograms was performed using a densitometer (Ultroscan XL model 2222; LKB-Pharmacia, Dübendorf, Switzerland). All calculations were expressed relative to an arbitrary value of one assigned to the signal obtained with untreated L_{ux} cell extract.

**ECM Degradation Assay**

Preparation of [H]proline biosynthetically labeled ECM from R22 rat smooth muscle cells was performed as described (Jones and DeClerck, 1980). Matrix degradation was initiated by plating (at day 0) 50,000 cells per 13-mm-diam well in 500 µl DME containing 10% FCS. Media were changed daily and supernatants were counted for radioactivity in a scintillation counter. Matrix degradation was modulated by addition of various agents in cell culture medium at day 0 and daily addition of fresh drug up to day 4 of the assay (for morphological characterization the assay was performed up to day 7). Final drug concentrations were: 10 µg/ml protein A-Sepharose-purified IgG (preimmune and anti-u-PA); 200 U/ml aprotinin, 500 nM dexamethasone, and 1 mM cyclic AMP. Enzymatic degradation of residual matrix after tumor cell culture was performed by incubating R22 rat smooth muscle cell matrix in presence or absence of tumor cells for 4 d. R22 cells were then eliminated by incubation in 25 mM NH4OH for 20 min at 20°C and the residual matrix was washed three times with 1 ml DME. Enzymes were then added sequentially (trypsin followed by collagenase) at 100 µg/ml final concentration, and each digestion was performed at 37°C for 48 h.

**Invasion Assay with Reconstituted Basement Membrane**

Invasion assay using matrigel-coated nuclepore filters was performed as described (Albini et al., 1987). Nuclepore filters (13 mm filter diameter; 8 µm pore diameter) were coated with 100 µl of various dilutions of basement membrane matrigel at 10 mg/ml and dried at 37°C for 2 h. Blind well Boyden chambers were filled with 200 µl DME, 10% FCS in the lower compartment and coated filters mounted in the chamber. The upper compartment was filled with 800 µl of cell suspension at 300,000 cells/ml in DME, 10% FCS. Where stated, aprotinin was added to the cell suspension at 200 U/ml. The wells were incubated for 12 h at 37°C in 5% CO2. The filters were then removed, fixed 10 min in 30% methanol, 7% acetic acid solution, and stained for 20 min in 20% Giemsa solution. All material from the upper surface of the filter was carefully removed by scraping with a cotton tip, and invasive cells adhering to the lower surface of the filter were counted by light microscopy (250x) at randomly chosen areas (each sample was assayed using quadruplicate filters and filters were counted at four areas). Results are expressed as cell number per visual field (one microscopic field represents 1/200 of the filter surface).

**Preparation for Light and Electron Microscopy**

Cells cultured onto the R22 radiolabeled matrix in 24-well tissue culture plates were prefixed for 1 h at room temperature and then fixed at 4°C with 2.5% cacodylate-buffered (0.1 M, pH 7.35) glutaraldehyde at days 1, 4, and 7. After extensive washing with 0.1 M cacodylate, cultures were fixed for 1 h at room temperature with 2% osmium tetroxide in 0.1 M cacodylate, dehydrated, and embedded in Epon 812. Thin sections were contrasted with uranyl acetate and lead citrate. Control, thick thin sections for light microscopy were stained with methylene blue-azure II solution.

Results

**Expression of Human Pro-u-PA by Mouse L Cells**

A series of ten Genetically-resistant colonies isolated after transfection with cos pUK0322 or pSV2neo were assayed for the presence of PA activity in culture supernatants using the ^125I-fibrin method. All cos pUK0322 transfectants expressed PA activity (ten clones assayed; 0.8-4.5 IU/ml range), whereas no activity was detected in the conditioned media of control pSV2neo transfected cells (five clones). From each of these transfectants (pUK0322 and pSVneo), representative clones were selected and established as cell lines (L_{ux} and L_{neo}, respectively) for further characterization.

The PA expressed by L_{ux} cells was shown by zymography to comigrate with human but not with mouse u-PA (Fig. 1, lanes 1-3). A feeble band, observed at a position corresponding to 110 kDa, represents a complex between u-PA and a specific PA inhibitor that is found in the conditioned medium of several cells producing PA and PA inhibitor (Cajot et al., 1986a; Levin, 1983; Philips et al., 1984). The 33- and 29-kDa species represent degradation products of human and mouse high relative molecular mass u-PA, respectively. PA activity was not detected in conditioned medium derived from either L_{neo} or parental L cells (Fig. 1, lanes 4 and 5). Cell extracts were also analyzed for PA activity. A 54-kD band was associated with L_{ux} cells, whereas no PA-related activity was observed in L_{neo} cell extract (Fig. 1, lanes 6 and 7). To establish the immunological identity of the PA expressed, conditioned medium was immunoprecipitated with anti-human u-PA or control rabbit nonimmune serum, and the supernatants were subjected to zymographic analysis. The results demonstrate that the 54-kD band released by L_{ux} cells is immunologically related to human u-PA (Fig. 2, lanes 1-3). Treatment of L_{ux} cells with dexamethasone or dibutyryl cyclic AMP resulted in modulation of the 54-kD band. As shown in lanes 4-6, dexamethasone inhibited, whereas dibutyryl cyclic AMP stimulated, u-PA expression compared with untreated cells. These results also demonstrate that essentially the 54-kD PA species is affected by these drugs, while the 110-kD PA-PA inhibitor complex appears unaltered. DFP sensitivity of PA expressed by L_{ux} cells is shown in Fig. 3. Conditioned medium-derived u-PA was largely resistant to DFP (lanes 3 and 4). Under identical experimental conditions, urinary u-PA activity was totally quenched (lanes 1 and 2). After activation by limited plasmid cleavage, L_{ux}-secreted PA became accessible to DFP inhibition (lanes 5 and 6). Hence u-PA was secreted from L_{ux} cells as a DFP-resistant proenzyme (pro-u-PA), which upon limited plasmid treatment has been shown to be converted to the active two-chain form (Nielson et al., 1982; Wun et al., 1982).

**Restriction Mapping of Cosmid pUK 0322 and Southern Blot Analysis of Human u-PA DNA Sequences in L_{ux} Cells**

The restriction map (Fig. 4) demonstrates that pUK0322 embodies the structural gene of pro-u-PA including at least 23 kb of 5' flanking sequences, some of which were previously described (Riccio et al., 1985). The map agrees with the published data within an acceptable limit of error, except for the presence of an additional Eco RI site in map position.
cosmid DNA digestion with Sma I, Eco RI, Bam HI, and
Mouse L Cells
calibration of the hybridization signal obtained for L,A
Modulation of Human Pro-u-PA Gene Expression in
digestion fragments to a nick-translated human urokinase
cDNA probe. The presence of human u-PA-specific DNA
of flanking sequences.
way (dibutyryl cyclic AMP, cholera toxin, and pertussis toxin)
assayed for PA activity. As shown in Fig. 6, dexamethasone
late u-PA gene expression, and the conditioned media were
was shown to contain two copies of the human u-PA gene by
hybridization signal obtained for Lnp, control cells (lane 7). The genome of this clonal line
showed a fragment at -9.3 kb, which may represent the site of a polymorphism.
At the 3' end of the gene, pUK0322 contains at least 12 kb
of flanking sequences.
Southern blot analysis of pUK0322 is shown in Fig. 5 after
cosmid DNA digestion with Sma I, Eco RI, Bam HI, and
Pst I (lanes 1-4, respectively) followed by hybridization of
digestion fragments to a nick-translated human urokinase
cDNA probe. The presence of human u-PA-specific DNA
sequences in Lnp, cells is demonstrated as a 6.8-kb Sma I
fragment (lane 6); no hybridization signal is observed in
Lnr, control cells (lane 7). The genome of this clonal line
was shown to contain two copies of the human u-PA gene by
calibration of the hybridization signal obtained for Lnp,
genomic DNA against serial dilutions of pUK0322 containing
known copy numbers of the u-PA gene.

Modulation of Human Pro-u-PA Gene Expression in
Mouse L Cells
Lnp, cells were treated with various drugs known to modu-
late u-PA gene expression, and the conditioned media were
assayed for PA activity. As shown in Fig. 6, dexamethasone
suppressed, whereas agonists of the adenylate-cyclase path-
way (dibutyryl cyclic AMP, cholera toxin, and pertussis toxin)
induced, an increase of PA activity. Zymographic analysis of

| 110 | 54 |
|-----|-----|
| 29  | 29  |

Figure 2. Immunological characterization (lanes 1-3) and regu-
lation (lanes 4-6) of u-PA expressed by Lnp cells. Lnp-conditioned
media were analyzed by zymography. Immunosupernatants of Lnp
conditioned medium obtained after incubation with preimmune se-
rum (lane 1); anti-human u-PA serum (lane 2); or buffer alone
(lane 3) followed by immunoprecipitation of antibody-antigen com-
plexes. Lnp cells treated with culture medium alone (lane 4); 500
nM Dex (lane 5); or 1 mM dibutyryl cyclic AMP (lane 6). 50-td
aliquots of conditioned media were loaded at a dilution of 1:5 for
lanes 1-3. For semiquantitative estimation of PA activity, samples
were corrected for cell number and represent the conditioned media
containing 5 x 10³ cells/ml.

conditioned medium (Fig. 2) furthermore demonstrated that
only the 54-kD human u-PA but not the 48-kD mouse u-PA
was regulated. These results were corroborated by Northern
blot analysis (Fig. 7 a). Densitometric analysis of the auto-
radiograms (Fig. 7 b) indicated that dexamethasone decreased
human uPA mRNA steady state levels by 50%, whereas cy-
clic AMP, cholera toxin, and pertussis toxin induced a two-
to threefold increase.

ECM Degradation by LnpA Cells
Lnp, cells degraded [H]proline-labeled rat smooth muscle
ECM as monitored by the release of solubilized degradation
products in cell culture supernatants (Fig. 8 A). These cells
mediated matrix degradation as efficiently as human fibro-
sarcoma HT-1080 cells. Lnr, control cells showed no effect.
Matrix degradation assays were also performed over a 7-d
period using Lnp as well as control Lnr, and parental L cells.
These experiments confirmed the ability of Lnp cells to de-
grade ECM, whereas both Lnr or parental L cells exhibited
only weak effects (<5% of Lnp degradation). The measure-
ment of fibrinolytic activity in cell culture supernatants (Fig.
8 b) demonstrated that the secretion of u-PA by Lnp, and
HT-1080 cells correlated with matrix degradation. The cell
counts obtained for HT-1080, Lnp, and Lnr cells during the
assay period are shown in Fig. 8 c. Hence the difference in
the degradation potential observed between Lnp, and Lnr
cells was not due to a difference in cell numbers.

In the presence of anti-u-PA IgG, ECM degradation was
inhibited by 80% when compared with incubation with nonimmune IgG (Fig. 9). Addition of aprotinin, a potent
plasmin inhibitor, likewise inhibited degradation. Modula-
tion of u-PA expression by various agents also influenced the
ECM degradation: dexamethasone inhibited >50%, whereas
cyclic AMP led to a 40% increase in matrix solubilization.

Morphological Analysis of Cell–Matrix Interaction
Fig. 10 illustrates the changes in morphology of R22 matrix
associated with the coincubation of Lnr (a and b) or LnpA (c
and d) transfectants (a and c are taken at day 1; b and d at
day 7). There was evidence of cell proliferation of both cell
types over the 4-d culture period, as already shown by cell
count determinations (see Fig. 8 c). Both types of transfec-
tants appeared to adhere equally to the matrix layer, by direct
contacts or by multiple cytoplasmic extensions. By comparing
Fig. 10, a with b or c with d, it is seen that major changes

![Figure 1](https://example.com/figure1.png)

-9.3 kb cannot be resolved by cloning because of
the length of human genomic DNA.

![Figure 2](https://example.com/figure2.png)

![Figure 3](https://example.com/figure3.png)

>918

The Journal of Cell Biology, Volume 109, 1989
in matrix density occur over the coincubation period and that alterations, especially a loss of density, are more pronounced with L_{un} than with L_{un}-transfected cells.

Fig. 11 shows at the ultrastructural level a detailed sequence analysis of L_{un}, L_{un}, as well as HT-1080 cells coincubated on the R22 matrix. Fig. 11, a-c, illustrates at days 1, 4, and 7, respectively, the interactions of human fibrosarcoma HT-1080 cells with the heterogeneous constituents of the matrix. Numerous fine cytoplasmic extensions maintain close contacts (focal or extended) with the matrix, which itself shows heterogeneous electron densities (very dense amorphous as well as fibrillar materials) and irregular borders. With time, the loss of matrix density appears to affect mostly the compact amorphous material (Fig. 11, cf. a–c) making the fibrillar constituents more apparent. This sequence of events is quite comparable with respect to L_{un}, cells (Fig. 11, g–i) whereas, for L_{un}, cells, the dense amorphous material is present throughout the coincubation period (Fig. 11, d–f). Irregular matrix borders, focal or extended areas of adhesion have been observed for the three types of cell populations: the two transfectants and the reference HT-1080 line.

Biochemical Analysis of Residual ECM after Tumor Cell Coculture

To demonstrate which matrix constituents were degraded by L_{un}, cells, R22 smooth muscle cell matrix which had been incubated 4 d in absence of cells or in presence of L_{un}, and L_{un}-cells were used for enzyme digestion experiments as shown in Fig. 12. Trypsin digestion of the residual matrix obtained after L_{un}, cell culture demonstrates a 25% loss of trypsin-sensitive material compared with the control 100% degradation obtained with the intact ECM. No alteration in
cells exhibit a fivefold increase in their ability to invade and migrate through a 25-μg matrigel barrier. At a higher concentration (100 μg/filter), no significant invasion occurred under the experimental conditions used, while at lower concentration (2.5 μg/filter) the difference in invasiveness observed between the two cell lines was less pronounced (threelfold higher for Lnoc cells compared with Lmo). Using collagen IV-coated filters (5 μg/filter), which promotes cell adherence but does not constitute a physical barrier to invasion, both cell lines exhibited a comparable migration potential. Lmo invasion through a 25-μg matrigel-coated filter was inhibited by >90% using aprotinin at 200 U/ml, indicating that the amount of trypsin-sensitive material was observed when analyzing ECM cultured with control Lmoc cells. Collagenase digestion performed under similar conditions demonstrates a comparable degradation of the collagen substrate in all three conditions. These results demonstrate that Lnop-mediated matrix degradation leads to solubilization of the trypsin-sensitive components, while the collagen components are left unaltered.

**Basement Membrane Matrigel Invasion**

Fig. 13a demonstrates the ability of Lnop cells to migrate through various dilutions of basement membrane matrigel. When compared with Lmoc control cells, Lnop-transfected

---

**Figure 7. Regulation of u-PA mRNA in transfected mouse L cells.**

mRNA prepared from 24-h cultures of nontreated or treated transfected mouse L cells was assessed by Northern blot analysis. Relative changes of u-PA mRNA were determined by hybridization to a random primer-labeled 600-bp Eco RI insert of the human u-PA cDNA. 4 μg of polyadenylated mRNA was applied per lane. (a) Northern blot analysis of u-PA mRNA. (b) Changes in the relative intensity of the signals presented were quantified by densitometric analysis of the autoradiograms by assigning an arbitrary score of 1 to the intensity of the mRNA signal obtained for nontreated, cos pUK0322-transfected Lnop cells. Lanes 1–5 of the bar graph (b) correspond to lanes 1–5 of a. (c) Ethidium bromide staining of total mRNA, which indicate similar loadings of mRNA for each sample. (Lane 0) Nontreated, pSVneo-transfected Lmoc cells; (lane 1) nontreated, cos pUK0322-transfected Lnop cells; (lane 2) Lnop cells treated with dexamethasone (500 nM); (lane 3) Lnop cells treated with cAMP (1 mM); (lane 4) Lnop cells treated with pertussis toxin (5 nM); and (lane 5) Lnop cells treated with cholera toxin (50 nM).

**Figure 8. ECM degradation by HT-1080, Lnop, and Lmoc cells.**

(a) Cumulative plot of the total radioactivity solubilized from R22 smooth muscle cell ECM by HT-1080 (black boxes), Lnop (grey boxes), and Lmoc cells (white boxes). (b) Cumulative plot of PA activity released in supernatants by tumor cells in the course of matrix degradation. (c) Corresponding cell counts at days 1–4 of tumor cells.
DEGRADATION [% of control]

Figure 9. Modulation of ECM degradation by LMPo cells with anti-u-PA IgG (a-uPA; 10 μg/ml), aprotinin (Apr; 200 U/ml), dexamethasone (Dex; 500 nM), and dibutyryl cyclic AMP (cAMP; 1 mM). Grey boxes, treated cells; black box, control untreated cells (control for anti-u-PA IgGs were nonimmune IgGs at same concentration and, for all other conditions, culture medium without drug). Results are displayed as the percentage matrix breakdown obtained after various tumor cell treatments relative to that of the control untreated cells. All experiments were repeated twice and the standard deviations obtained were <10%.

serine-proteases, such as plasmin and/or u-PA, played a major role in this invasive process (Fig. 13 b).

Discussion

To assess the potential role of pro-u-PA in the degradation and invasion of the ECM, we expressed the respective gene in mouse L cells. Restriction mapping demonstrated that cos pUK0322 contained the structural human pro-u-PA gene and at least 23 kb of 5' and 10 kb of 3' flanking sequence. The identity of the PA expressed by LMPo cells with human pro-u-PA was established by several independent criteria: antigenicity and molecular weight characterization, as well as DFP resistance (Vassalli et al., 1984). The expression of pro-u-PA was stable over 10 passages.

u-PA gene expression is known to be influenced by several hormones, protooncogene and oncogene products, and a variety of other effectors (Blasi et al., 1987). Human u-PA expression by LMPo cells was inhibited by dexamethasone. Such an effect has previously been observed in HT-1080 cells (Andreasen et al., 1986; Medcalf et al., 1986) and various breast carcinoma cell lines (Busso et al., 1987). Agonists of the adenylate cyclase pathway, such as dibutyryl cyclic AMP, cholera toxin, and pertussis toxin, induced u-PA expression in LMPo cells as shown in other cell systems (Mira-y-Lopez et al., 1983; Nagamine et al., 1983; Vassalli et al., 1976). The modulation of PA activity and mRNA was related to human and not to murine u-PA since: (a) zymography demonstrated human (54-kD) u-PA; and (b) murine u-PA mRNA did not hybridize under the conditions applied. Hence the regulation of u-PA gene expression in LMPo cells is in all likelihood mediated by cis-acting regulatory elements present in the insert of cos pUK0322.

Invasion is based on a series of complex interactions between tumor cells and various types of ECM (Liotta et al., 1984). It involves: (a) attachment of cell surface receptors to glycoproteins such as laminin, fibronectin, or thrombospondin; (b) protease activation and pericellular degrada-

Figure 10. Thick thin sections from Epon-embedded material taken at day 1 (a and c) and 7 (b and d) from cultures of Lneo (a and b) and LMPo (c and d) cells. The sections were cut at right angles to the plane of the culture dish. Bars, 10 μm.
Figure 11. Ultrastructural characterization by transmission electron microscopy of tumor cell-mediated R22 matrix degradation. (a–c) HT-1080, (d–f) L_reno, and (g–i) L_ortho cells all at days 1, 4, and 7, respectively. Bars, 1 µm.
COLLAGENASE

TRYPSIN

0 20 40 60 80 100

MATRIX DEGRADATION [% of control]

Figure 12. Enzymatic digestion of residual R22 smooth muscle cell ECM after tumor cell coculture. ECM was incubated 4 d in the presence of L

(hatched bars) and L

cells (grey bars) or in absence of cells (black bars). The tumor cells were then lysed by incubation with 25 mM NH4OH, and the residual matrix was digested by sequential addition of 100 µg/ml trypsin and collagenase. The resulting degradation obtained is expressed as the percentage of released radioactivity using tumor cell-cocultured ECM relative to ECM incubated in the absence of tumor cells.

Figure 13. Basement membrane invasion assay. (a) Invasion of the artificial basement membrane by L

(black boxes) or L

cells (grey boxes). The polycarbonate filters were coated with various matrigel concentrations ([I] 100, 25, or 12.5 µg/filter, respectively) or with collagen IV ([I] 5 µg/filter). The results are expressed as the number of cells per field counted at the end of migration. (b) Inhibition of basement membrane invasion by L

cells in the presence of 200 kIU/ml of aprotinin (Apr) using filters coated with 25 µg of matrigel.

DEGRADATION [% of control]
toic membrane by human tumor cells, suggesting that u-PA binding to its receptor was critical, in this particular invasion assay, for cell migration. Receptor binding may (a) concentrate the activity and (b) localize proteolysis at critical sites of the cell surface. We present a model system suited for further investigation of the u-PA receptor: isolation of the human uPA receptor gene and transfection into L-m cells would allow an analysis of its role in invasion and metastasis.

We thank Dr. Kathryn Hessien and Dr. Harry Mead for the construction and screening of the HT-1080 cDNA library; Dr. Jacqueline Tests for helpful discussions and participation in the early stage of this work; Dr. Peter Grant for careful reading of this manuscript; Mr. Pierre Dubied and Mr. Marco Giuffrida for expert preparation of the figures; and Dr. Max Hausermann (LKB-Pharmacia, Dblendorf, Switzerland) for densitometric analysis of the autoradiograms. We are particularly obliged to Dr. Karl-Dieter Woehn who provided the pUK3022 restriction map. We appreciate the provision of laboratory space and the interest of Prof. F. Bachmann in this work.

This work was supported by grants from the Swiss Science Foundation for Scientific Research (Nr. 3.334.086, Nr. 3.406.83, and Nr. 3.350.82). Received for publication 4 July 1988 and in revised form 3 February 1989.

References

Albini, A., Y. Iwamoto, H. K. Kleinman, G. R. Martin, S. A. Aaronson, J. M. Kozlowski, and R. N. Ewan. 1987. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res. 47:2339–2345.

Andreasen, P. A., L. S. Nielsen, P. Kristensen, P. Grondhald-Hansen, L. Skriver, and K. Damé. 1986. Plasminogen activator inhibitor from human fibrosarcoma cells binds urokinase-type plasminogen activator, but not its proenzyme. J. Biol. Chem. 261:7644–7651.

Appella, E., E. A. Robinson, S. J. Ullrich, M. P. Stoppelli, A. Corti, G. Casmani, and F. Blasi. 1987. Urokinase-type plasminogen activator in rodent mammary tumors by hormones and other effectors. Cancer Res. 47:6437–6440.

Astrup, T. 1978. Fibrinolysis: an overview. Prog. Chem. Fibrinolysis Thrombosis. 3:1–57.

Becker, A., and M. Gold. 1975. Isolation of the bacteriophage lambda A gene. Proc. Natl. Acad. Sci. USA. 72:581–585.

Birnboim, H. C., and L. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513–1522.

Blasi, F., J. D. Vassalli, and K. Dané. 1987. Urokinase-type plasminogen activator: proenzyme, receptor, and inhibitors. J. Cell Biol. 104:801–804.

Busso, N., M. Collart, J. D. Vassalli, and D. Belin. 1987. Antagonist effect of RU 486 on transcription of glucocorticoid-regulated genes. Exp. Cell Res. 173:425–430.

Cajot, J. F., B. Sordat, E. K. Kruithof, and F. Bachmann. 1986a. Human primary colon carcinoma xenografted into nude mice. I. Characterization of plasminogen activators expressed by primary tumors and their xenografts. J. Natl. Cancer Inst. 77:703–712.

Cajot, J. F., E. K. Kruithof, B. Sordat, and F. Bachmann. 1986b. Plasminogen activators, plasminogen activator inhibitors and procollagen analyzed in twenty human tumor cell lines. Int. J. Cancer. 38:719–727.

Carle, G. F., M. Frank, and M. V. Olson. 1986. Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. Science (Wash. DC). 232:63–68.

Dané, K., P. A. Andreasen, J. Gründahl-Hansen, P. Kristensen, L. S. Nielsen, and L. Skriver. 1985. Plasminogen activators, tissue degradation and cancer. Adv. Cancer Res. 44:129–266.

Graham, F. L., and A. J. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology. 52:456–467.

Grosfeld, F. G., T. Land, E. J. Murray, A. L. Mellor, H. H. M. Dahl, and R. A. Flavell. 1982. The construction of cosmid libraries which can be used for screening recombinant plasmid DNA. Nucleic Acids Res. 10:259–266.

Grunstein, M., and D. Hogness. 1975. Colony hybridization: a method for the provision of laboratory space and the interest of Prof. F. Bachmann in this work.

This work was supported by grants from the Swiss Science Foundation for Scientific Research (Nrs. 3.334.086, 3.406.83, and 3.350.82). Received for publication 4 July 1988 and in revised form 3 February 1989.
Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–517.

Sträuli, P. A., A. J. Barrett, and A. Baici. 1980. Proteinases and tumor invasion. Raven Press, Inc., New York. 215 pp.

Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201–5205.

Unkeless, J. C., L. Ossowski, J. P. Quigley, D. B. Rifkin, and E. Reich. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic I. Chick embryo fibroblast cultures transformed by avian RNA tumor viruses. *J. Exp. Med.* 137:85–111.

Vassalli, J. D., J. Hamilton, and E. Reich. 1976. Macrophage plasminogen activator; modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors, and cyclic nucleotides. *Cell.* 8:271–281.

Unkeless, J. C., L. Ossowski, J. P. Quigley, D. B. Rifkin, and E. Reich. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic I. Chick embryo fibroblast cultures transformed by avian RNA tumor viruses. *J. Exp. Med.* 137:85–111.

Vassalli, J. D., J. Hamilton, and E. Reich. 1976. Macrophage plasminogen activator; modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors, and cyclic nucleotides. *Cell.* 8:271–281.

Vassalli, J. D., J. M. Dayer, A. Wohlwend, and D. Belin. 1984. Concomitant secretion of prourokinase and of a plasminogen activator–specific inhibitor by cultured human monocytes macrophages. *J. Exp. Med.* 159:1653–1668.

Vere, P., M. P. Stopelli, P. Galeffi, P. Di Nocera, and F. Blasi. 1984. Identification and primary sequence of an unspliced human urokinase poly(A)++ RNA. *Proc. Natl. Acad. Sci. USA.* 81:4727–4731.

Vieira, J., and J. Messing. 1982. The pUC plasmids, and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene (Amst.)* 19:259–268.

Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin. 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA.* 76:1373–1376.

Wun, T. C., L. Ossowski, and E. Reich. 1982. A proenzyme form of human urokinase. *J. Biol. Chem.* 257:7262–7268.