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

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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_{uPA}) 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 ^{3}H-labeled extracellular matrix (ECM), invasion of a matrigel basement membrane analogue, and by light and electron microscopy. L_{uPA} cells and reference HT-1080 fibrosarcoma cells, in contrast to control L_{neo} 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)[1] 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 neoplasmic growth (for reviews see Blasi et al., 1987; Danø 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 Danø 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_{uPA} cells on the degradation of the ECM and on basement membrane invasion were studied using biochemical and morphological analysis.

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1. *Abbreviations used in this paper*: DFP, diisopropylfluorophosphate; ECM, extracellular matrix; PA, plasminogen activator; u-PA, urokinase-type plasminogen activator.

Materials and Methods

Materials

Dexamethasone, cholera toxin, dibutyryl 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 Opopharma (Zürich, Switzerland); pertussis toxin was from List Biological Laboratories (Campbell, CA); phenol, uranyl acetate, lead citrate, and methylene blue/azur II solution were from Merck (Darmstadt, Federal Republic of Germany); osmium tetroxide was from Elmis (Carouge, Switzerland); in vitro-packaging system from Genofit (Geneva, Switzerland); restriction enzymes were from Boehringer Mannheim (Rotkreuz, Switzerland); bacterial collagenase (type CLSPA) 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-diam dishes and 24-well plates were from Gibco Laboratories (Basel, Switzerland). 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., 1986*a*,*b*).

PA Activity Assay

PA activity of tumor cell-conditioned media was assayed using the ^{125}I-fibrin plate method (Unkeless et al., 1973). All activities are expressed in international units by comparison with a standard curve of u-PA calibrated against 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.

Diisopropylfluorophosphate (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 mIU/ml of plasmin, and the reaction was terminated by addition of 200 U/ml 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 4×10^6 into 100-mm-diam 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 dibutyryl 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. Cell extracts were prepared by plating 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 7.35), 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

λUK0321 was isolated from a cDNA library generated in λgt10 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 (Günzler et al., 1982) were synthesized and used for screening (Maniatis et al., 1982). The insert of λUK0321 was subcloned into pUC8 (Vieira and Messing, 1982) giving rise to pUK0321, 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. (1984). cos pUK0322 was isolated from a human cosmid library (Grosveld et al., 1982) by colony hybridization (Grunstein and Hogness, 1975) using the nick-translated 1,023-bp Eco RI insert of pUK0321. Restriction mapping was performed by the partial digestion method of Smith and Birnstiel (1976) using the indirect labeling method described by Rackwitz et al. (1985). Fragments were separated by field inversion electrophoresis as described (Carle 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 extracted, 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 Doly, 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 anlaysis (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, 0.017% Denhardt's solution, 0.1% SDS, 0.005% pyrophosphate, and 0.0125% carrier DNA and subsequently hybridized for 20 h at 65°C in the presence of 10^7 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, 0.05% SDS, 0.01% pyrophosphate; 0.1× SSC, 0.05% SDS, 0.01% pyrophosphate.

Transfections

Mouse L cells (Sanford et al., 1948) were transfected with cosmid pUK0322 using the calcium phosphate coprecipitation method as previously described (Graham and Van der Eb, 1973) 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-cm^2 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-diam 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_{uPA} 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_{uPA} 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 (0.9%) 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_{uPA} cell extract.

ECM Degradation Assay

Preparation of [^{3}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. Tumor cells were then eliminated by incubation in 25 mM NH_4OH 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% CO_2. 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 (250×) 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 microscopical 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-azur II solution.

Results

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

A series of ten Geneticin-resistant colonies isolated after transfection with cos pUK0322 or pSV2neo were assayed for the presence of PA activity in culture supernatants using the ^{125}I-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_{uPA} and L_{neo}, respectively) for further characterization.

The PA expressed by L_{uPA} 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 kD, 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., 1986*a*; Levin, 1983; Philips et al., 1984). The 33- and 29-kD 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_{uPA} 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_{uPA} cells is immunologically related to human u-PA (Fig. 2, lanes *1–3*). Treatment of L_{uPA} 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_{uPA} 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 plasmin cleavage, L_{uPA}-secreted PA became accessible to DFP inhibition (lanes *5* and *6*). Hence u-PA was secreted from L_{uPA} cells as a DFP-resistant proenzyme (pro-u-PA), which upon limited plasmin 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_{uPA} 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

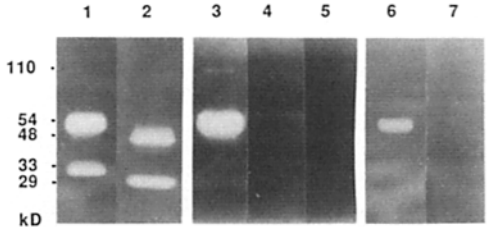

Figure 1. Electrophoretic mobility characterization of human u-PA expressed by transfected mouse L cells. Samples were electrophoretically separated in 10% SDS-polyacrylamide gels containing 2.5 mg/ml copolymerized plasminogen-rich fibrinogen, and PA-related fibrinolytic activity was detected by zymographic analysis. (Lane *1*) Human urine; (lane *2*) murine urine; (lanes *3–5*) conditioned media of L_{uPA}, L_{neo}, and parental L cells, respectively; and (lanes *6* and *7*) cell extracts of L_{uPA} and L_{neo}, respectively.

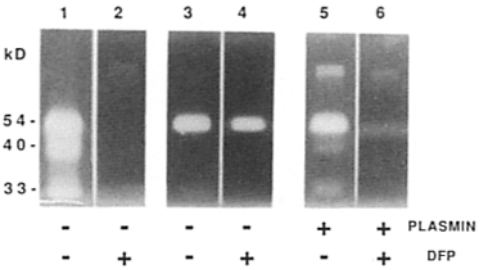

Figure 3. DFP treatment of pro-u-PA. Samples were subjected to sequential treatment with plasmin and DFP as indicated in the figure: (+) treated; (−) untreated. (Lanes *1* and *2*) Human urine-derived u-PA; (lanes *3–6*) L_{uPA}-derived u-PA.

−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 L_{uPA} cells is demonstrated as a 6.8-kb Sma I fragment (lane *6*); no hybridization signal is observed in L_{neo} 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 L_{uPA} 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

L_{uPA} cells were treated with various drugs known to modulate 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 pathway (dibutyryl cyclic AMP, cholera toxin, and pertussis toxin) induced, an increase of PA activity. Zymographic analysis of 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 autoradiograms (Fig. 7 *b*) indicated that dexamethasone decreased human uPA mRNA steady state levels by 50%, whereas cyclic AMP, cholera toxin, and pertussis toxin induced a two- to threefold increase.

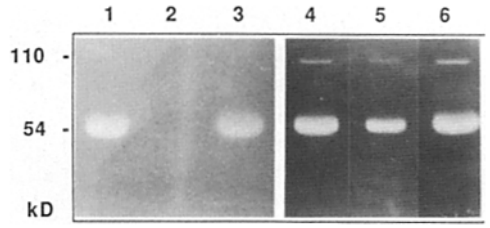

Figure 2. Immunological characterization (lanes *1–3*) and regulation (lanes *4–6*) of u-PA expressed by L_{uPA} cells. L_{uPA}-conditioned media were analyzed by zymography. Immunosupernatants of L_{uPA} conditioned medium obtained after incubation with preimmune serum (lane *1*); anti-human u-PA serum (lane *2*); or buffer alone (lane *3*) followed by immunoprecipitation of antibody–antigen complexes. L_{uPA} cells treated with culture medium alone (lane *4*); 500 nM Dex (lane *5*); or 1 mM dibutyryl cyclic AMP (lane *6*). 50-μl 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 corresponding to 5×10^5 cells/ml.

ECM Degradation by L_{uPA} Cells

L_{uPA} cells degraded [^{3}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 fibrosarcoma HT-1080 cells. L_{neo} control cells showed no effect. Matrix degradation assays were also performed over a 7-d period using L_{uPA} as well as control L_{neo} and parental L cells. These experiments confirmed the ability of L_{uPA} cells to degrade ECM, whereas both L_{neo} or parental L cells exhibited only weak effects (<5% of L_{uPA} degradation). The measurement of fibrinolytic activity in cell culture supernatants (Fig. 8 *b*) demonstrated that the secretion of u-PA by L_{uPA} and HT-1080 cells correlated with matrix degradation. The cell counts obtained for HT-1080, L_{uPA}, and L_{neo} cells during the assay period are shown in Fig. 8 *c*. Hence the difference in the degradation potential observed between L_{uPA} and L_{neo} 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. Modulation 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 L_{neo} (*a* and *b*) or L_{uPA} (*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 transfectants 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

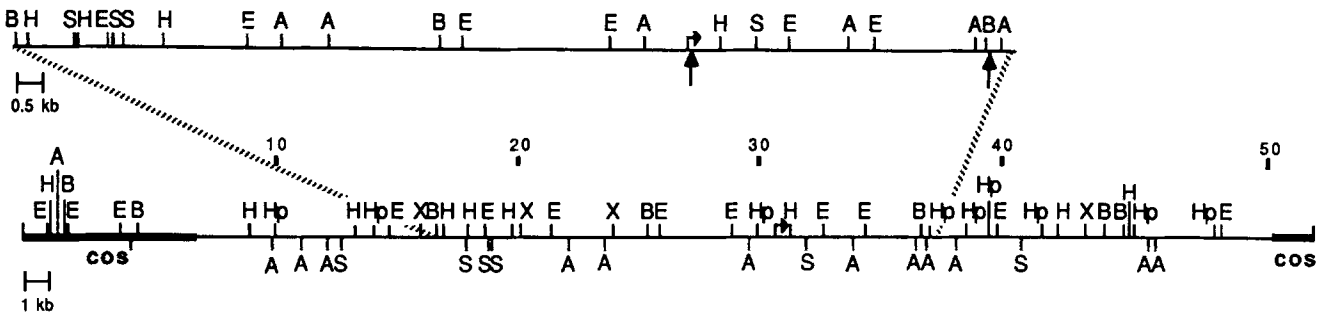

Figure 4. Restriction map of cos pUK0322. The u-PA gene coding region is shown in position 32–38 kb (vertical arrows represent positions of exon I and exon XI, restrictively, according to Riccio et al. [1985]). *Horizontal arrow*, transcription initiation site; *bold line*, cosmid vector DNA (*cos*). The different restriction sites of the cosmid are indicated as follows: *A*, Acc I; *B*, Bam HI; *E*, Eco RI; *H*, Hind III; *Hp*, Hpa I; *S*, Sca I; and *X*, Xho I.

in matrix density occur over the coincubation period and that alterations, especially a loss of density, are more pronounced with L_{uPA}- than with L_{neo}-transfected cells.

Fig. 11 shows at the ultrastructural level a detailed sequence analysis of L_{neo}, L_{uPA}, 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_{uPA} cells (Fig. 11, *g–i*) whereas, for L_{neo} 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.

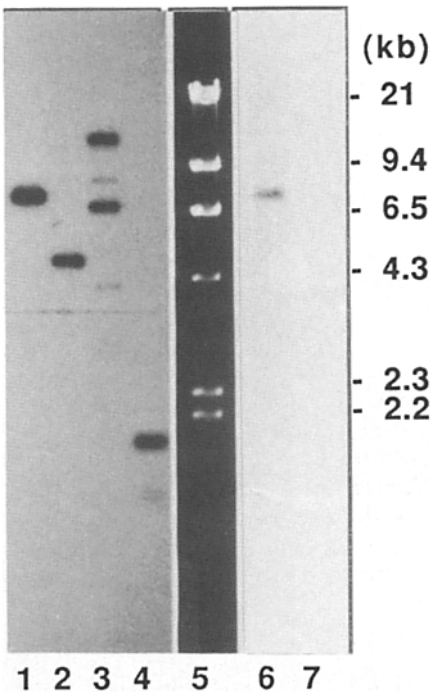

Figure 5. Southern blot analysis of L_{neo} and L_{uPA} genomic DNA as well as cos pUK0322 DNA. 1 μg of cosmid DNA or 10 μg of genomic DNA was digested with the restriction enzymes indicated below, electrophoresed through 0.8% agarose, transferred to nitrocellulose, and hybridized with the nick-translated 0.6-kb Eco RI–Hind III fragment of pUK0321. (Lanes *1–4*) cos pUK0322, digested with Sma I, Eco RI, Bam HI, and Pst I, respectively; (lane *5*) DNA size standard; (lanes *6* and *7*) Sma I–digested genomic DNA derived from pUK0322- or pSVneo-transfected L cells, respectively.

Biochemical Analysis of Residual ECM after Tumor Cell Coculture

To demonstrate which matrix constituents were degraded by L_{uPA} cells, R22 smooth muscle cell matrix which had been incubated 4 d in absence of cells or in presence of L_{uPA} and L_{neo} cells were used for enzyme digestion experiments as shown in Fig. 12. Trypsin digestion of the residual matrix obtained after L_{uPA} cell culture demonstrates a 25% loss of trypsin-sensitive material compared with the control 100% degradation obtained with the intact ECM. No alteration in

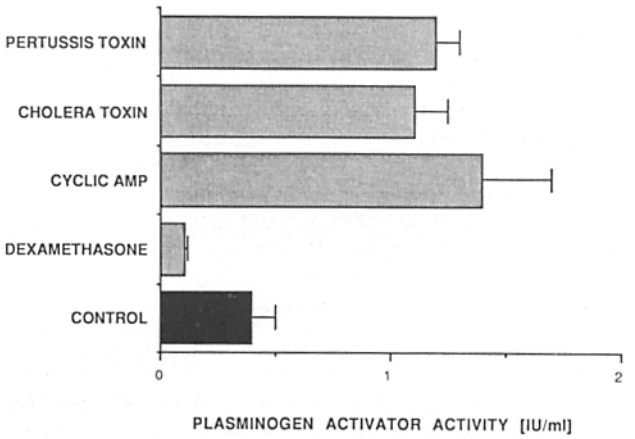

Figure 6. Modulation of human urokinase gene expression in mouse L cells. L_{uPA} cells were treated with various drugs, and the resulting conditioned media were assayed for PA activity using ^{125}I-fibrin plates. Drug concentrations: dexamethasone (500 nM); dibutyryl cyclic AMP (1 mM); cholera toxin (50 nM); and pertussis toxin (5 nM).

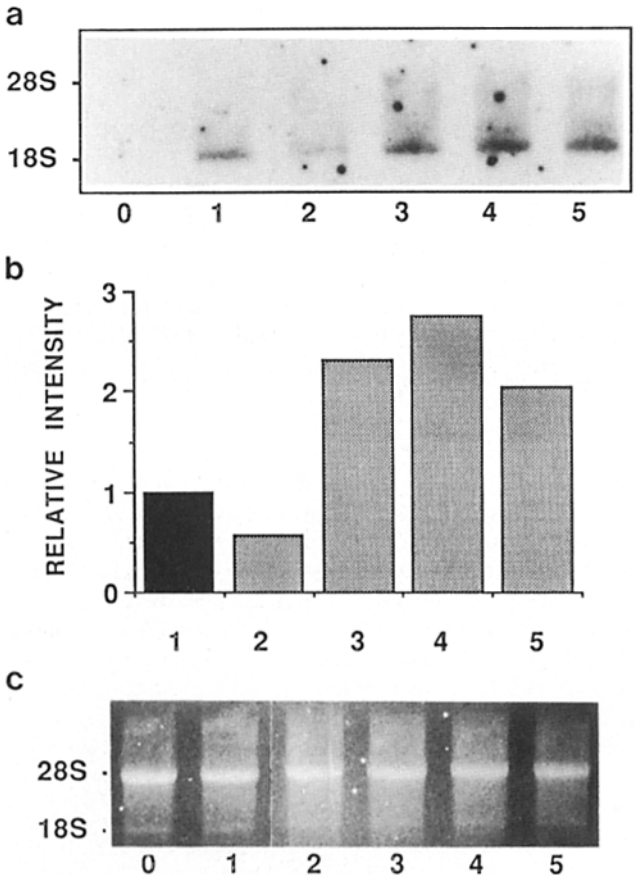

Figure 7. Regulation of u-PA mRNA in transfected mouse L cells. mRNA prepared from 24-h cultures of nontreated or treated transfected mouse L cells was assessed by Northern blot analysis. Relative changes of u-PA mRNA were determined by hybridization to a random primer-labeled 600-bp Eco RI insert of 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 L_{uPA} 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 L_{neo} cells; (lane *1*) nontreated, cos pUK0322-transfected L_{uPA}cells; (lane *2*) L_{uPA} cells treated with dexamethasone (500 nM); (lane *3*) L_{uPA} cells treated with cAMP (1 mM); (lane *4*) L_{uPA} cells treated with pertussis toxin (5 nM); and (lane *5*) L_{uPA} cells treated with cholera toxin (50 nM).

the amount of trypsin-sensitive material was observed when analyzing ECM cultured with control L_{neo} cells. Collagenase digestion performed under similar conditions demonstrates a comparable degradation of the collagen substrate in all three conditions. These results demonstrate that L_{uPA}-mediated matrix degradation leads to solubilization of the trypsin-sensitive components, while the collagen components are left unaltered.

Basement Membrane Matrigel Invasion

Fig. 13 *a* demonstrates the ability of L_{uPA} cells to migrate through various dilutions of basement membrane matrigel. When compared with L_{neo} control cells, L_{uPA}-transfected 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 (threefold higher for L_{uPA} cells compared with L_{neo}). 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. L_{uPA} invasion through a 25-μg matrigel-coated filter was inhibited by >90% using aprotinin at 200 U/ml, indicating that

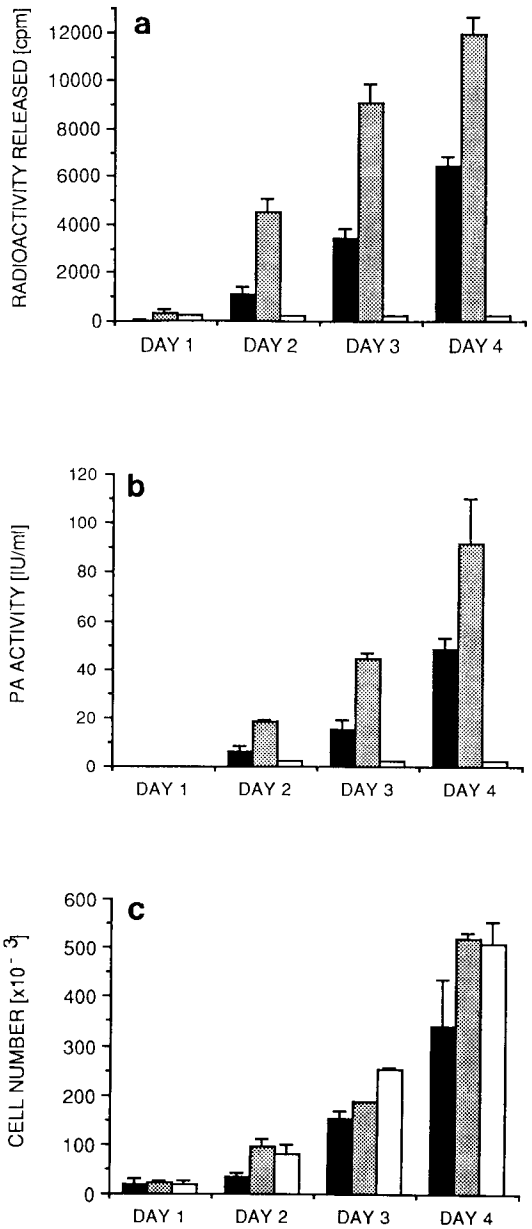

Figure 8. ECM degradation by HT-1080, L_{uPA}, and L_{neo} cells. (*a*) Cumulative plot of the total radioactivity solubilized from R22 smooth muscle cell ECM by HT-1080 (*black boxes*), L_{uPA} (*grey boxes*), and L_{neo} cells (*white boxes*). (*b*) Cumulative plot of PA activity released in supernatants by tumor cells in the course of matrix degradation. (*c*) Corresponding cell counts at days 1–4 of tumor cells.

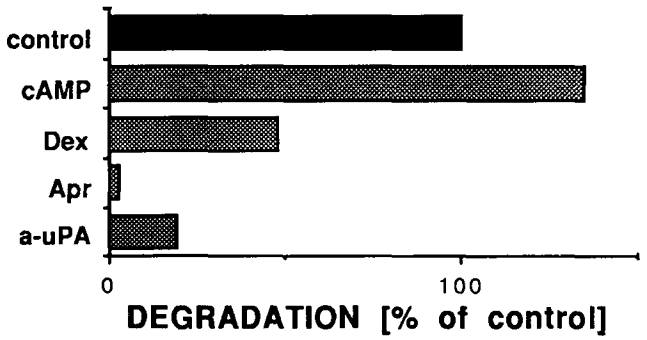

Figure 9. Modulation of ECM degradation by L_{uPA} cells with anti-u-PA IgG (*a-uPA*; 10 μg/ml), aprotinin (*Apr*; 200 U/ml), dexamethasone (*Dex*; 500 nM), and dibutyryl cyclic AMP (*cAMP*; 1 mM). *Grey boxes*, treated cells; *black box*, control untreated cells (control for anti-u-PA IgGs were nonimmune 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 structral 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 L_{uPA} 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 L_{uPA} 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 L_{uPA} 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 L_{uPA} 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-

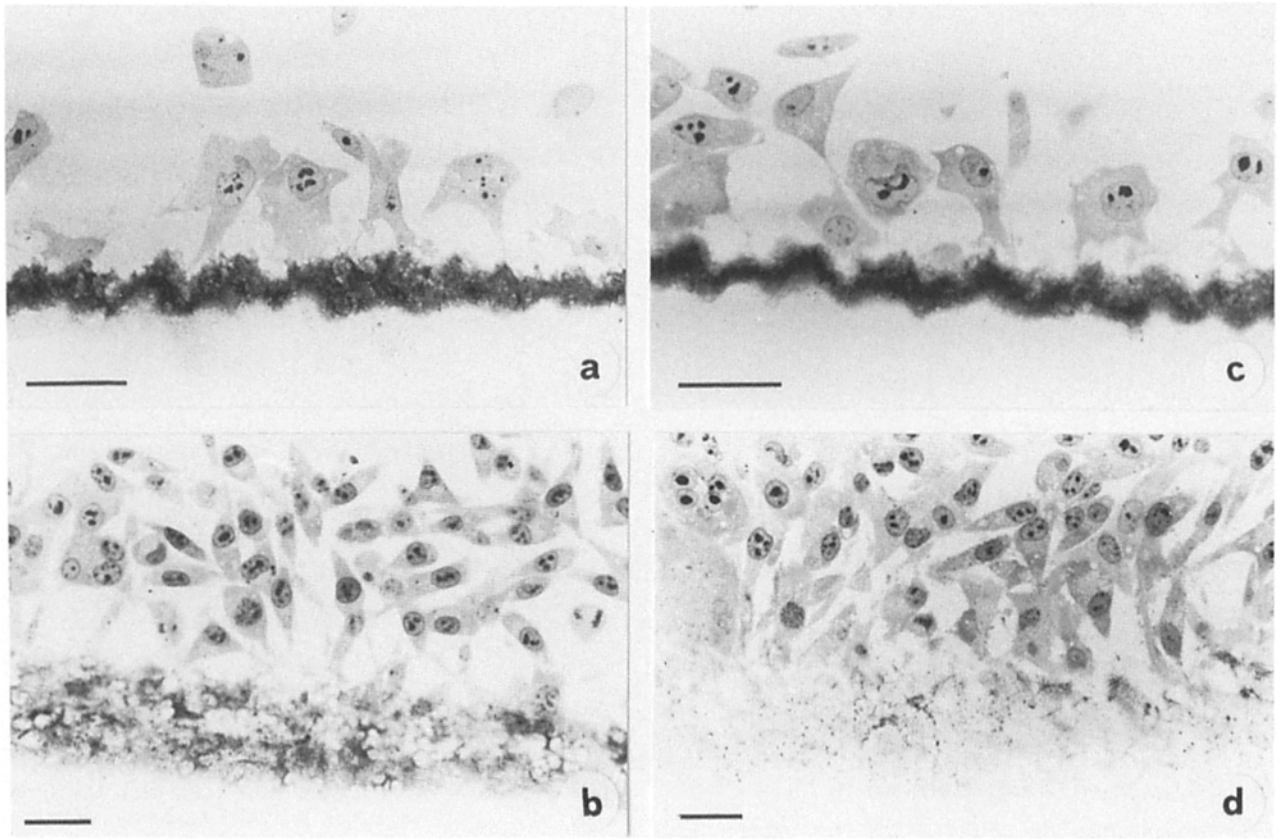

Figure 10. Thick thin sections from Epon-embedded material taken at day 1 (*a* and *c*) and 7 (*b* and *d*) from cultures of L_{neo} (*a* and *b*) and L_{uPA} (*c* and *d*) cells. The sections were cut at right angles to the plane of the culture dish. Bars, 10 μm.

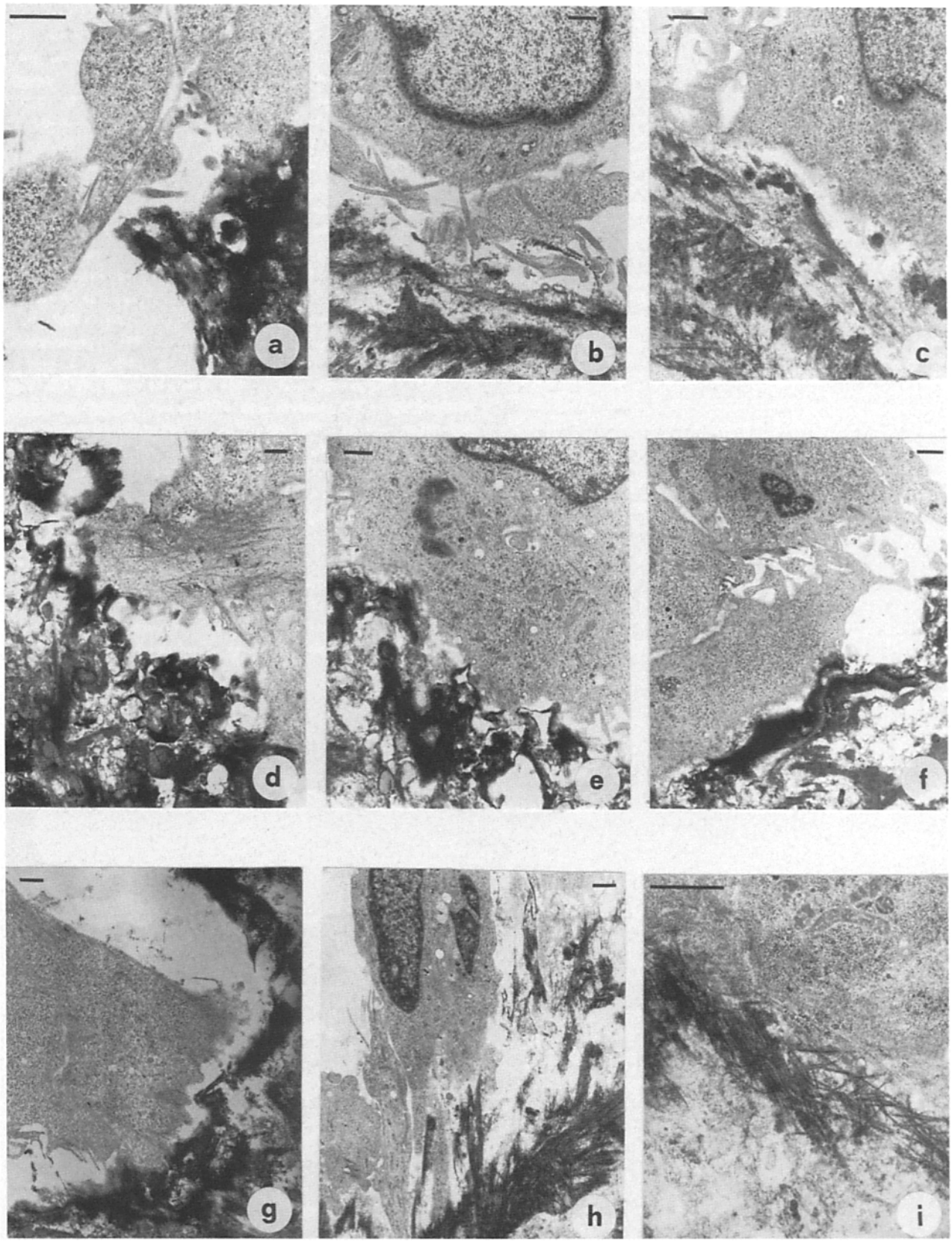

Figure 11. Ultrastructural characterization by transmission electron microscopy of tumor cell-mediated R22 matrix degradation. (*a–c*) HT-1080, (*d–f*) L_{neo}, and (*g–i*) L_{uPA} cells all at days 1, 4, and 7, respectively. Bars, 1 μm.

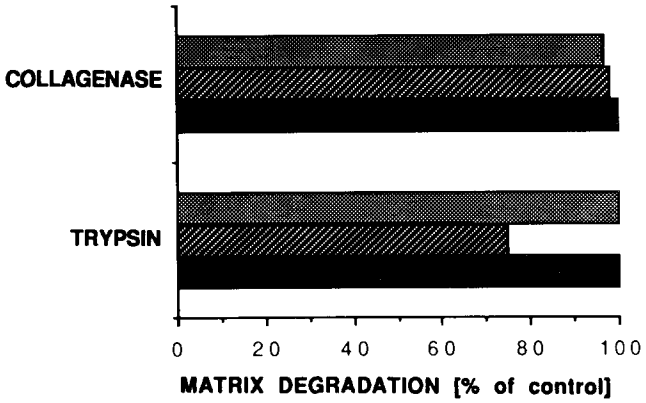

Figure 12. Enzymatic digestion of residual R22 smooth muscle cell ECM after tumor cell coculture. ECM was incubated 4 d in the presence of L_{uPA} (*hatched bars*) and L_{neo} cells (*grey bars*) or in absence of cells (*black bars*). The tumor cells were then lysed by incubation with 25 mM NH_4OH, and 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.

tion of matrix components; and (*c*) migration of cells within this matrix. u-PA has been reported to play an important role in protease-mediated matrix degradation (Danø et al., 1985; Saksela, 1985). It generates plasmin which in turn degrades glycoproteins such as fibronectin and laminin or it activates latent collagenases (Liotta et al., 1981).

L cells are particularly suited to the study of the effects of u-PA expression on the cell–matrix interaction because: (*a*) they do not express u-PA constitutively; (*b*) unlike HT-1080 cells, they express only very little or no PA inhibitor (Pannekoek et al., 1986; Rehemtulla et al., 1987); and (*c*) murine u-PA receptor does not bind human u-PA (Appella et al., 1987; Huarte et al., 1987). Hence the protease effect per se can be studied without having to account for the modulating effect of u-PA receptor or PA inhibitor metabolism. We have demonstrated that L_{uPA} cells break down the ECM and, by quantitation of the release of radiolabeled products, shown that the degree of degradation is comparable with the one affected by human HT-1080 fibrosarcoma cells. Control L_{neo} cells, which do not express endogenous PA, do not degrade such a model matrix.

Morphologically, tumor cells contact the matrix surface by multiple pseudopodial extensions. In u-PA–expressing cells (HT-1080 and L_{uPA}), the electron density of amorphous material of the matrix is markedly decreased. Interestingly, fibrillar material appears largely resistant to degradation, suggesting a lack of collagenolytic and/or elastolytic activities in our experimental system. Jones et al. (1979) have shown that R22 cells produce connective tissue proteins, in a cross-linked insoluble form, that remain anchored to the surface of the culture dish. By performing a sequential enzyme digestion of the residual ECM obtained after culture with L_{uPA} tumor cells, we demonstrated that partial digestion (25%) of the ECM had occurred and that the degradation induced by L_{uPA} cells affected essentially trypsin-sensitive constituents, whereas collagen components remained unaltered. These results are consistent with the observation that L cells do not express collagenase (Murphy, G., personal communication). Recently, fine cytoplasmic extensions have been shown to express u-PA antigen in HT-1080 cells (Pöllanen et al., 1988). Focal and/or extended areas of matrix–cell contacts are likely regions where degradative processes take place.

Modulation of u-PA gene expression by dexamethasone or dibutyryl cyclic AMP demonstrated that the effect was correlated with the potential of stimulated cells to degrade the ECM. Specific antibodies directed against human u-PA, as well as the serine protease inhibitor aprotinin, inhibited matrix degradation. This militates against a fibronectin-degrading activity of u-PA, unaffected by aprotinin (Quigley et al., 1987). Our results also bear on questions associated with the function of the u-PA receptor. Human u-PA does not bind to the mouse receptor (Appella et al., 1987; Huarte et al., 1987), hence such binding was not required for the increased potential of L_{uPA} cells, described in the present study, to degrade and/or invade the matrix. These results are in contrast with the recent demonstration by Ossowski (1988) of a role for the u-PA receptor in the invasion of the chick chorioallan-

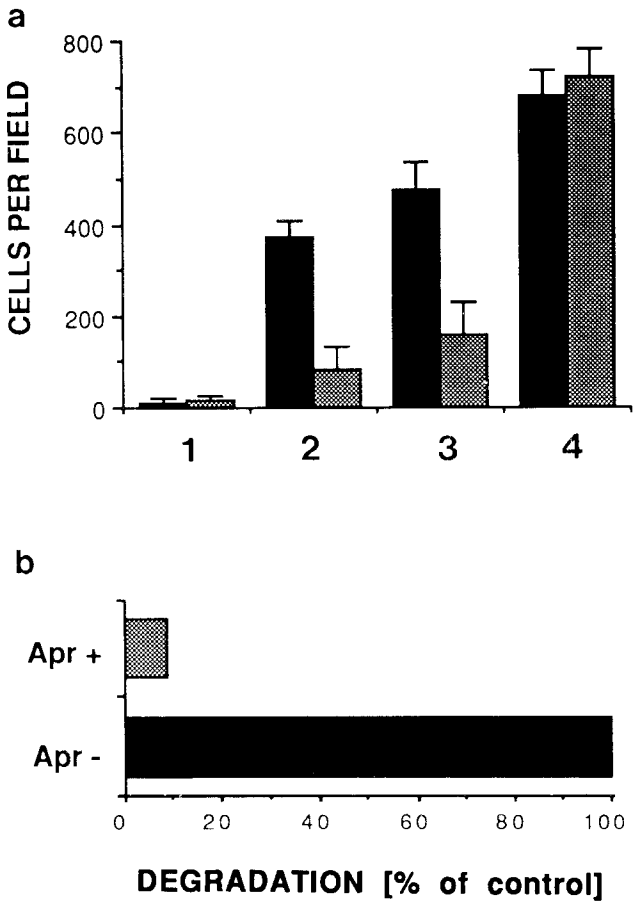

Figure 13. Basement membrane invasion assay. (*a*) Invasion of the artificial basement membrane by L_{uPA} (*black boxes*) or L_{neo} cells (*grey boxes*). The polycarbonate filters were coated with various matrigel concentrations ([*1–3*] 100, 25, or 12.5 μg/filter, respectively) or with collagen IV ([*4*] 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_{uPA} cells in the presence of 200 kIU/ml of aprotinin (*Apr*) using filters coated with 25 μg of matrigel.

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_{uPA} cells would allow an analysis of its role in invasion and metastasis.

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