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Functional inhibition of endogenously produced urokinase decreases cell proliferation in a human melanoma cell line

(plasminogen activators/tumor growth/proteolysis)

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ABSTRACT **Binding of urokinase-type plasminogen activator (u-PA) to its receptor has been shown not only to focus proteolytic activity to the cell surface but also to exert a mitogenic effect on the human epidermal tumor cell line CCL 20.2. This report shows that u-PA is an autocrine mitogen in the human melanoma cell line GUBSB and that inhibition of receptor-bound u-PA by specific anti-u-PA antibodies causes a significant suppression of cell proliferation in this system. The GUBSB cell line secretes 70–80% of the u-PA in its active form and expresses high-affinity u-PA receptors with a K_d of 5.2×10^{-10} M and 2.8×10^4 binding sites per cell. Approximately 70% of the u-PA receptors on these cells are occupied by endogenously secreted u-PA. Addition of the monoclonal anti-u-PA antibody MPW5UK (10 nM), directed against the active site of u-PA, twice daily to the cell cultures resulted in a significant decrease of [^{3}H]thymidine incorporation by the tumor cells, whereas a 10 times higher concentration of the monoclonal antibody MPW4UK, which does not inhibit plasminogen activator activity of u-PA, was necessary to achieve the same effect. In addition, diisopropyl fluorophosphate-inactivated u-PA, in a concentration 50-fold higher than the concentration necessary to saturate the u-PA receptor (250 pM), decreased [^{3}H]thymidine incorporation similarly to the specific antibody, proving that active u-PA is required for the mitogenic effect. Inhibition of endogenous u-PA production by cycloheximide reduced [^{3}H]thymidine incorporation significantly; after addition of exogenous u-PA, [^{3}H]thymidine incorporation increased again in the cycloheximide-treated cells. Therefore, inhibition of receptor-bound u-PA might represent a tool not only to inactivate cell-bound proteolytic activity, necessary for invasion, but also to exert a specific antiproliferative effect on certain tumor cells.**

Malignant cells contain and secrete plasminogen activators (PAs; EC 3.4.21.31), trypsin-like serine proteases, to a much higher extent than their respective normal counterparts (1–3). Immunological characterization has shown that most malignant cells produce urokinase-type PA (u-PA) rather than tissue-type PA (t-PA) (2). u-PA contains not only an active-site domain consistent with its serine protease character and involved in the activation of plasminogen but also a "kringle" domain, found in several other serine proteases, and a growth-factor domain homologous to the epidermal growth factor (4).

Specific receptors for u-PA have been found in a number of human tumor cell lines: U-937 (histiocytic lymphoma), A-431 (epidermoid carcinoma), and CCL 20.2 (5–9). The distribution of u-PA receptors on normal cells has not been analyzed in detail. However, unstimulated normal monocytes and normal human fibroblasts have also been shown to express specific u-PA receptors (5, 10, 11). Occupation of these receptors by u-PA leads to the focusing of proteolytic activity onto the cell surface. An additional function of the u-PA receptors was shown in the human epidermal tumor cell line CCL 20.2 (9). Binding of intact active high molecular weight u-PA to the receptor resulted in a mitogenic effect that could not be induced by u-PA fragments regardless of whether they lacked the active site or the binding region (9).

Furthermore, correlation of fibrinolytic activity of R3230 rat mammary carcinoma cells and Lewis lung carcinoma cells with invasion and formation of metastases was observed in several animal models (12, 13), and antibodies against u-PA were shown to inhibit the formation of metastases in an animal model employing a human tumor cell line (14). It was the aim of the present study to analyze whether u-PA can act as an autocrine mitogen and whether inhibition of endogenously secreted u-PA by a specific antibody would also reduce cell proliferation. Therefore, we selected a u-PA-secreting melanoma cell line, GUBSB, that possesses high-affinity u-PA receptors and determined the effect of addition of monoclonal antibodies directed against u-PA on cell proliferation *in vitro*.

EXPERIMENTAL PROCEDURES

Materials. The following were obtained as indicated: tissue culture cluster plates (24 flat-bottom wells; Costar); Tween 20, 1,3,4,6,-tetrachloro-3α,6α-diphenylglycouril, diisopropyl fluorophosphate (DFP), and cycloheximide (Sigma); bovine plasminogen-containing and plasminogen-free fibrinogen (Poviet, Amsterdam); thrombin (Topostasin, 3000 units per vial; Hoffmann–La Roche); RPMI 1640 medium and fetal bovine serum (Flow Laboratories); L-glutamine (200 mM; Seromed, Darmstadt, F.R.G.); penicillin/streptomycin (GIBCO); Sephadex G-25 and G-200 (Pharmacia); acrylamide, *N,N'*-methylenebisacrylamide, sodium dodecyl sulfate, and low molecular weight protein standards for SDS/PAGE (Bio-Rad); AquaSil (Pierce); Na^{125}I and [*methyl*-^{3}H]thymidine (17.4 Ci/mg and 6.7 Ci/mmol, respectively; 1 Ci = 37 GBq; New England Nuclear); GFR 25/3 filters (Whatman); Liquifluor (Beckman).

Analytical Methods. Protein was determined by the method of Lowry *et al.* (15). PA activity was monitored on plasminogen-rich fibrin/agarose plates (16) and were expressed in international units (IU) by comparison with the First International Reference Preparation for Urokinase (coded 68/48; Institute for Biological Standards and Control, London).

SDS/7.5% PAGE was performed according to Laemmli (17) to estimate molecular weights of the purified reduced or unreduced enzymes. Gels were either silver-stained (18) or, in the case of radiolabeled samples, subjected to autoradiography using Agfa-Gevaert x-ray films. PA activity or PA

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Abbreviations: PA, plasminogen activator; u-PA, urokinase-type PA; t-PA, tissue-type PA; DFP, diisopropyl fluorophosphate; IU, international unit(s).

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inhibitor activity was determined by fibrin autography (19) or reverse fibrin autography (20), respectively.

Cell Line and Culture Conditions. The human melanoma cell line GUBSB was selected in a previous study (21) and maintained in Hepes-buffered RPMI 1640 medium with Hanks' salts, supplemented with 10% fetal bovine serum and antibiotics at 37°C in a 5% CO_2 atmosphere. For experiments, cells grown in large flasks were washed three times and seeded in the same medium but without serum in tissue culture cluster plates (24 flat-bottom wells) at a density of 0.6×10^6 per well. The cell line was screened for production of PA and PA inhibitor by SDS/PAGE followed by fibrin autography and reverse fibrin autography, respectively.

Preparation and Radiolabeling of u-PA. u-PA (double chain; M_r 54,000) was isolated from human urine by affinity chromatography on anti-u-PA monoclonal antibody (MPW5UK)-Sepharose followed by affinity chromatography on agmatine-Sepharose (22). SDS/PAGE followed by silver staining showed a single band in the unreduced samples, corresponding to M_r 54,000. u-PA (50 μg) was inactivated by incubation with 0.05 M DFP in 0.01 M potassium phosphate buffer (pH 7.4) for 2 hr at room temperature followed by excessive dialysis to remove free DFP; inactivated u-PA was found to be <0.5% as active as the native enzyme on plasminogen-rich fibrin/agarose plates (16). u-PA was labeled with ^{125}I by the iodogen method (23). u-PA (25 μg in 50 μl) was added to a vial coated with 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodo-Gen) together with 1 mCi of $Na^{125}I$ for 20 min at room temperature; the reaction was terminated by addition of 350 μl of 0.05 M sodium phosphate buffer (pH 7.4). Free iodine was separated on a column (30 × 1.4 cm) of Sephadex G-25 in 0.05 M Tris buffer (pH 8.0) containing 0.1 M NaCl. Specific radioactivity of the ^{125}I-labeled u-PA was 5.4×10^6 cpm/μg of protein. No alterations in molecular weight as judged by SDS/PAGE occurred during the labeling procedure.

Determination of u-PA Antigen. u-PA antigen levels in the cell culture supernatants were determined by a competitive radioimmunoassay for high molecular weight u-PA. All determinations were done in triplicate. The lower detection limit was 50 pg/ml, the intraassay variation was ≈4%, and the interassay variation was ≈6% (24).

Binding Studies with the Iodinated Ligand. Twenty-four hours after seeding of the cells, the adherent cultures were washed three times with Dulbecco's phosphate-buffered saline (PBS, without Ca^{2+} or Mg^{2+}), and the cells were treated with 0.05 M glycine hydrochloride at pH 3.0 for 5 min (acid treatment) to remove bound endogenous u-PA (8). Binding studies were performed by adding 500 pM labeled u-PA in 1 ml of PBS to the acid-treated cells for 5–60 min at 37°C in the absence or, to account for nonspecific binding, in the presence 100-fold excess (50 nM) unlabeled u-PA. No appreciable internalization of the occupied receptor or dissociation of u-PA from the receptor occurred as determined according to the literature (7, 8). For determination of cell-bound radioactivity, the cells were washed three times with PBS and lysed by addition of 1 ml of 0.1 M NaOH/0.1 M EDTA/1% (vol/vol) Tween 20. Radioactivity was measured in a Beckman Gamma 8000 γ counter (Beckman) and expressed in fmol per 0.6×10^6 cells. Binding parameters were calculated by the method of Scatchard (25).

Characterization of the Antibodies. The monoclonal antibodies MPW4UK and MPW5UK (22), both belonging to the IgG1 subclass and directed against u-PA, reacted with high as well as with low molecular weight u-PA. MPW5UK additionally interfered with plasminogen activation by u-PA. Radiolabeling of MPW5UK was performed as described for u-PA, resulting in a specific activity of 3.79×10^7 cpm/μg. The monoclonal antibody T14, directed against a surface epitope on human lymphocytes and used as a control antibody, was kindly provided by W. Knapp (University of Vienna, Austria).

Differentiation Between Endogenously Occupied and Total u-PA Receptors. To determine the total number of u-PA receptors the cells were incubated with 5 IU of u-PA in 1 ml of PBS (equivalent to a concentration of 1 nM, which is sufficient to saturate receptors) for 40 min at 37°C. The cells were washed three times and incubated for 30 min at 37°C with 250 pM ^{125}I-labeled anti-u-PA antibody (MPW5UK) in the absence or presence of a 20-fold molar excess of unlabeled antibody to account for nonspecific binding. The cells were washed three times with PBS and lysed in 2 ml of 0.1 M NaOH/0.1 M EDTA/1% (vol/vol) Tween 20. Cell-bound radioactivity was quantified with a Beckman γ counter. To quantify endogenously occupied u-PA receptors, exogenous u-PA was omitted from the first incubation.

Determination of [^3H]Thymidine Incorporation. At fixed intervals ranging up to 5 days, [^3H]thymidine was added (1 μCi/ml) to the culture medium; after 6 hr the medium was removed and the cultures were washed three times with ice-cold PBS. Then the cells were solubilized by addition of 1 ml of ice-cold 10% (wt/vol) trichloroacetic acid and kept at 4°C for 30 min. The precipitate was collected on a Whatman GFR filter under light suction and washed with 30 ml of 10% trichloroacetic acid. The filter was dried before scintillation counting in Liquifluor/toluene in a Beckman liquid scintillation spectrophotometer (LS 6500).

Functional Inhibition of Endogenously Produced u-PA. To determine the effects of monoclonal antibodies directed against u-PA or of DFP-inactivated u-PA, cells (0.6×10^6 per ml per well) were washed three times with PBS and afterwards incubated with medium containing various concentrations of monoclonal anti-u-PA antibodies or DFP-inactivated u-PA (concentrations are given in the figure legends). Addition was performed twice daily to ensure constant levels of the reagents. At various time points [^3H]thymidine incorporation was determined as described above. In additional experiments, cells (10^6 per ml per well) were incubated with cycloheximide (5 μg/ml) for 3 hr. After washing and an additional 6 hr in fresh culture medium, supernatants were tested for PA and PA inhibitor, and endogenously occupied and total u-PA receptors on the cells were determined. Thereafter, 5 IU of u-PA was added twice daily. At various time points [^3H]thymidine incorporation was determined and compared to a control culture without addition of u-PA.

Statistical Analysis. Significant differences in [^3H]thymidine incorporation at different time points during a 5-day period for GUBSB cells treated in different ways were calculated by analysis of variance. The respective *P* values are given in the figure legends.

RESULTS

The human melanoma cell line GUBSB, grown in adherent cell cultures, was tested for the production of PA and PA inhibitor in the absence or presence of fetal bovine serum. Cells grown in serum-free medium secreted u-PA but no inhibitor. When cells were grown with 1% or 10% fetal bovine serum, the presence of PA inhibitor was made evident by the shift of part of the PA activity to a higher molecular weight of about 110,000, representing an activator–inhibitor complex. By comparing data obtained by a radioimmunoassay for u-PA antigen (24) and a functional assay (16) it was found that 70–80% of the enzyme molecules were active (data not shown).

To determine the presence of u-PA receptors on the surface of these cells, binding of enzymatically active ^{125}I-labeled u-PA to GUBSB cells was followed as a function of time (Fig. 1 *Left*). Binding of labeled u-PA was fast, and half-maximal specific binding was achieved in about 5 min at

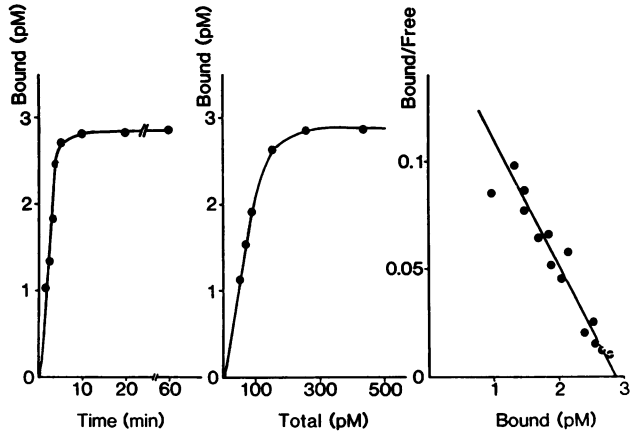

FIG. 1. Binding of ^{125}I-labeled u-PA to GUBSB cells. (*Left*) Time course. Acid-treated cells (0.6×10^6 per ml per well) were cultured at 37°C for various times in the presence of ^{125}I-labeled u-PA (500 pM). After incubation the cells were washed and cell-associated radioactivity was determined. Values represent specific binding of a single experiment. Similar data were obtained in three experiments. Nonspecific binding never exceeded 9% of the total radioactivity bound to the cells. (*Center*) Concentration dependence. Acid-treated cells (0.6×10^6 per ml per well) were incubated for 10 min at 37°C with various concentrations of ^{125}I-labeled u-PA (50–450 pM). After incubation the cells were washed and cell-associated radioactivity was determined. Values represent specific binding of a single experiment. Similar data were obtained in three experiments. Nonspecific binding never exceeded 6% of the total radioactivity bound to the cells. (*Right*) Scatchard analysis of the data presented in *Middle*.

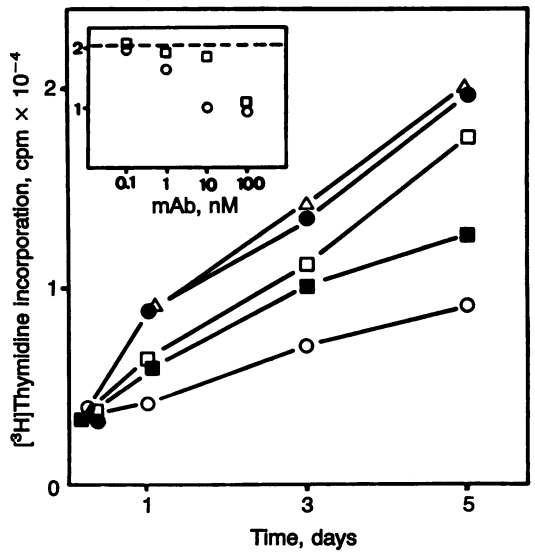

FIG. 2. Effect of antibodies against u-PA on [^{3}H]thymidine incorporation by GUBSB cells. Cells (0.6×10^6 per ml per well) were cultured for 5 days under serum-free conditions in the presence of monoclonal antibodies against u-PA (MPW4UK, 10 nM, □; MPW5UK, 10 nM, ○), control antibody (T14, 10 nM, △), and DFP-inactivated u-PA (15 nM, ■), which were added twice daily. At various times incorporation of [^{3}H]thymidine during a 6-hr period was determined and compared to incorporation by an untreated control culture (●). For clarity, only means of a single experiment are presented. SEM for triplicates averaged 8% of the mean. Similar data were obtained in three experiments. The time-dependent increase in [^{3}H]thymidine incorporation was significantly different ($P < 0.01$; analysis of variance) in MPW5UK-supplemented cultures as compared to untreated controls or to cultures supplemented with the control antibody; there was no significant effect of antibody MPW4UK at 10 nM. The effect of DFP-inactivated u-PA was significant ($P < 0.04$). On day 5, the respective cell counts for the experiment shown in the figure were as follows; control, 4.2×10^6; control antibody, 4.2×10^6; MPW5UK, 1.5×10^6; MPW4UK, 3.8×10^6; DFP u-PA, 2.3×10^6. (*Inset*) Effect of monoclonal antibody (mAb) concentration on [^{3}H]thymidine incorporation by GUBSB cells after 5 days. Cells (0.6×10^6 per ml per well) were incubated with medium containing various concentrations of MPW4UK (□) or MPW5UK (○), and [^{3}H]thymidine incorporation was determined on day 5.

37°C. In all subsequent experiments, binding was therefore determined after 10 min. Nonspecific binding in the presence of a 100-fold molar excess of unlabeled u-PA was in the range of 4.5% to 9.5% of total binding in all experiments. During binding studies, molecular characteristics of labeled u-PA remained unchanged as revealed by SDS/PAGE followed by autoradiography of material obtained by acid treatment (data not shown).

Binding assays using various concentrations of ^{125}I-labeled u-PA showed that saturation occurred at 250 pM (Fig. 1 *Center*). A Scatchard plot of these data (Fig. 1 *Right*) exhibits linearity, suggesting a single class of binding sites. From a series of three binding studies a dissociation constant (K_d) of $5.2 \pm 0.8 \times 10^{-10}$ M and $2.8 \pm 0.6 \times 10^4$ binding sites per cell were calculated by least-squares analysis (mean ± SD).

The specificity of u-PA binding was investigated by examining the ability of various unlabeled proteins at 20- and 200-fold molar excess to compete with ^{125}I-labeled u-PA for binding to GUBSB cells. Unlabeled u-PA as well as DFP-inactivated u-PA caused a concentration-dependent decrease in binding of ^{125}I-labeled u-PA, whereas plasminogen, t-PA, and albumin had no effect even when present in 200-fold molar excess.

We determined the ratio of endogenously occupied to total u-PA receptors by means of an ^{125}I-labeled antibody against u-PA. As calculated from five experiments, about 70% of the u-PA receptors were occupied by endogenously produced u-PA.

To investigate a possible bioregulatory function of the endogenously produced and receptor-bound u-PA on GUBSB cells, various amounts of the monoclonal anti-u-PA antibodies MPW4UK and MPW5UK (of which only MPW5UK interferes with plasminogen activation by u-PA) were added twice daily to GUBSB cells cultured under serum-free conditions over a 5-day period. The effect on cell proliferation was tested by measuring the incorporation of [^{3}H]thymidine into GUBSB cells as well as by counting the cells (Fig. 2). Addition of the antibody MPW5UK at 10 nM decreased [^{3}H]thymidine incorporation significantly. Addition of the antibody MPW4UK, which does not inhibit plasminogen activation by u-PA, at the same concentration did not significantly alter the [^{3}H]thymidine incorporation into GUBSB cells. However, at a 10 times higher concentration of MPW4UK, the same decrease in cell proliferation as with MPW5UK was observed. An unrelated antibody (T14) had no effect on cell proliferation. The suppressive effect of the antibodies on [^{3}H]thymidine incorporation was dose-dependent between 0.1 and 10 nM for antibody MPW5UK and between 0.5 and 100 nM for antibody MPW4UK, respectively (Fig. 2 *Inset*).

To prove that the active site of u-PA is involved in the mitogenic effect, [^{3}H]thymidine incorporation was determined after addition of various amounts of DFP-inactivated u-PA. DFP-inactivated u-PA has been shown by us (9) and others (8, 10) to bind to various cell lines with the same properties as active u-PA. The amount of DFP-inactivated u-PA necessary to elicit a similar decrease in cell proliferation as the antibody MPW5UK was about 15 nM, which is ≈50-fold higher than the concentration necessary for saturation of the u-PA receptors (Fig. 2).

Inhibition of protein synthesis by cycloheximide prior to the experiments completely inhibited the production of u-PA (Fig. 3 *Inset*). Also the percentage of endogenously occupied

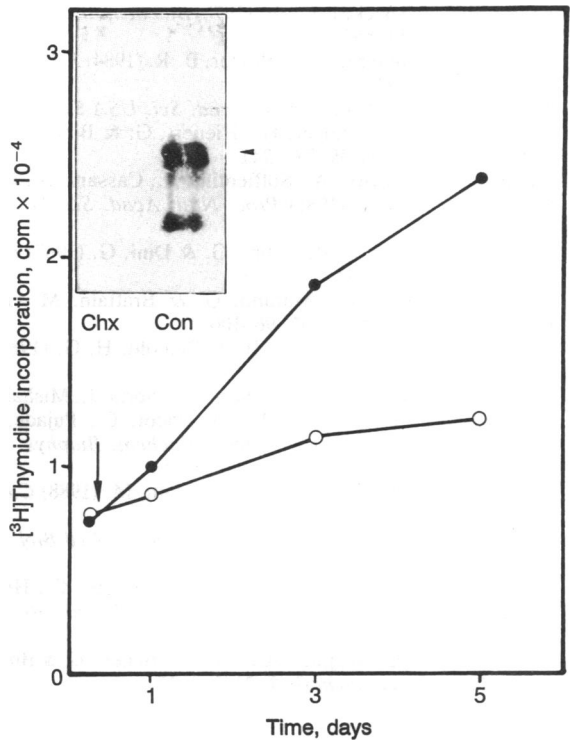

FIG. 3. Addition of u-PA reverses the decrease in [^{3}H]thymidine incorporation by cycloheximide-treated cells. Acid-treated cells (1.0×10^6 per ml per well) were incubated with cycloheximide (5 μg/ml) for 3 hr at 37°C. After three washes, cells were allowed to rest for 6 hr in medium without serum. Thereafter (arrow) 5 IU of u-PA was added twice daily to the medium. At various times, incorporation of [^{3}H]thymidine was determined (●) and compared to incorporation by a control culture lacking u-PA in the culture medium (○). Values represent means from triplicate determinations of a single experiment. Similar data were obtained in two experiments. The effect of added u-PA on cycloheximide-treated cells was significant ($P < 0.01$; analysis of variance) as compared to the control cultures, which were treated with cycloheximide but not with u-PA. (*Inset*) SDS/PAGE followed by fibrin overlay of cell culture supernatants derived from cells cultured in the absence (Con) or presence (Chx) of cycloheximide. Arrowhead, M_r 54,000.

u-PA receptors decreased to 24%, while the total number of u-PA receptors remained almost the same ($2.5 \pm 0.4 \times 10^4$ binding sites per cell). The low rate of incorporation of [^{3}H]thymidine into cycloheximide-treated cells as compared to an untreated control culture was reversible by addition of u-PA to the culture medium twice daily. The incorporation of [^{3}H]thymidine reached levels comparable to those for control cultures not treated with cycloheximide prior to the experiment (Fig. 3).

DISCUSSION

Malignant cells and tissues contain and secrete higher amounts of u-PA than their normal counterparts (2). In addition, a shift of the ratio of u-PA activity to antigen was observed in human renal cell carcinoma, indicating a possible role of active u-PA for cellular functions in malignancy (26). Besides increased production of u-PA by malignant cells, expression, modulation, and occupation of the u-PA receptor might reflect a response to pathophysiological situations (27–31). *In vivo*, aggressively growing strains of a human colon carcinoma cell line expressed about 10 times more receptors than indolent strains and showed significantly higher levels of u-PA in the conditioned medium (32). These findings indicate that u-PA receptors are involved in focusing extracellular proteolysis to the surface of the cells, because binding of u-PA does not require the catalytic site of the protease (33). Furthermore, interaction of enzymatically active u-PA (M_r 54,000), but not of any u-PA fragments, with its receptor is mitogenic in malignant cells (9, 34, 35). We therefore stated (9) that endogenously produced u-PA, through binding to its receptor, acts as an autocrine mitogen.

If our hypothesis is valid, removal of active enzyme from the receptor as well as inhibition of the active site of receptor-bound u-PA should decrease cell proliferation. To test this assumption we selected the human melanoma cell line GU-BSB, a cell line expressing u-PA receptors, producing u-PA, and exhibiting high receptor occupation, and studied the effect of anti-u-PA antibodies on [^{3}H]thymidine incorporation by these cells. In fact, addition of a monoclonal antibody (MPW5UK) directed against the active site of u-PA gave a dose-dependent decrease of cell proliferation, reaching the half-maximal effect at a concentration of 2 nM. The second monoclonal antibody (MPW4UK), which does not interfere with plasminogen activation by u-PA, showed a similar pattern, but at a 10-fold higher concentration. This fact could be explained by the higher affinity of the antibody MPW5UK for u-PA or by the possibility that inhibition of the active site of bound u-PA leads to a more prominent effect. That in fact active u-PA is primarily involved in eliciting the mitogenic effect was proven by DFP-inactivated u-PA replacement experiments leading to a significant reduction of cell proliferation. This requirement of active u-PA for mitogenesis of GUBSB cells is consistent with the findings in the CCL 20.2 cell line. These data indicate that endogenously secreted active u-PA is able to induce a mitogenic effect in the GUBSB cell line.

To further prove this assumption, experiments were performed with cells treated with the protein synthesis inhibitor cycloheximide. Such cells should still possess u-PA receptors, which have a half-life of 8–12 hr (7), but should exhibit no occupation of receptors by endogenous u-PA because of cycloheximide inhibition of u-PA production. The cells should exhibit slower proliferation than untreated cells, an effect that should be reversible upon addition of u-PA. In fact, cycloheximide-treated cells exhibited a lower rate of incorporation of [^{3}H]thymidine, and addition of exogenous u-PA to the culture medium was able to increase cell proliferation to values seen in untreated cultures.

The data indicate that endogenously produced u-PA interacts with its receptor to elicit a mitogenic effect that is dependent on the active site of u-PA. Therefore, u-PA can be regarded as an autocrine mitogen in the human melanoma cell line GUBSB. Inhibition of u-PA might be a potential means for the modulation of tumor growth *in vivo*, not only by inactivating cell-bound proteolytic activity necessary for invasion, but also by exerting a specific antiproliferative effect on tumor cells.

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