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Limited proteolysis of tumor cells increases their plasmin-binding ability

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Mild proteolytic treatment of SW1116 tumor cells with trypsin or plasmin increases their plasmin-binding ability considerably by increasing the number of binding sites without altering their affinity. This mechanism may be operative for increasing the concentration of active plasmin at the surface of tumor cells. C-terminal lysine residues are involved in plasmin binding to cells, since treatment of cells with carboxypeptidase B decreases this binding by 50%.

Plasmin; Lysine; Lysine-binding site; (Tumor cell)

1. INTRODUCTION

We have previously demonstrated the existence on human carcinoma cells of receptors which bind plasmin and, with lower affinity, plasminogen [1]. These receptors may play an important biological role in concentrating plasmin at the surface of tumor cells and allowing this enzyme to degrade components of the extracellular matrix, thus facilitating the progression of tumor cells.

In the course of these in vitro experiments, we generally used aprotinin in order to prevent autodegradation of plasmin incubated with the tumor cells. Aprotinin did not inhibit plasmin binding, showing that the active site of the enzyme is not involved in cell binding. In some experiments, we studied the influence of aprotinin on the inhibition of radiolabelled plasmin binding by unlabelled plasmin. Surprisingly, in the absence of aprotinin, unlabelled plasmin did not inhibit, but, rather, increased the binding of radiolabelled plasmin. This led us to investigate how mild proteolytic treatment of the tumor cells could modify their ability to bind radiolabelled plasmin.

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2. MATERIALS AND METHODS

The tumor cell line SW1116 (originating from a human colonic carcinoma) was used in all experiments. Cells were grown in MacCoy's medium supplemented with 10% fetal calf serum, then adapted to a defined medium, i.e. MacCoy's medium enriched with insulin (4 μg/ml), transferrin (3 μg/ml) and sodium selenite (10 ng/ml), all purchased from Sigma (St. Louis, MO) and EGF (5 ng/ml) from Collaborative Research (Lexington, MA). After trypsinization, tumor cells were seeded in individual plastic microwells (Remova wells, Bioblock, Strasbourg) at a rate of 10^5 cells per well. They were able to attach and to grow in the same defined medium and reached their initial number at day 5.

Plasminogen and plasmin were purchased from Kabi (Stockholm), trypsin (type IX) from Sigma and DFP-treated carboxypeptidase B from porcine pancreas from Boehringer (Mannheim). Plasminogen was labelled with ^{125}I (Amersham, Les Ulis, France), using the iodogen technique [2]. A specific activity of 6–8 μCi/μg (222–296 kBq) was achieved. 10 μg radiolabelled plasminogen were transformed into plasmin by the addition of 1000 IU urokinase (Choay, Paris). Completion of this activation was checked by polyacrylamide gel electrophoresis followed by autoradiography.

Plasmin (or plasminogen) binding by tumor cells was performed as in [1]. Briefly, tumor cells in each well were incubated with 100 μl of 20 nM labelled plasmin for 30 min at 4°C, then washed, and the binding determined in a gamma spectrometer (Wallac, Finland). In parallel experiments, 20 nM labelled plasmin was mixed with various concentrations of unlabelled plasmin (20 nM–1 μM) in order to yield an inhibition curve. Binding and inhibition experiments were performed in the presence (100 KIU) or absence (in some cases) of aprotinin (Trasylol, Sigma).

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In other experiments, tumor cells were pretreated with an enzyme under various conditions, then washed and incubated with radiolabelled plasmin. The various conditions included: pretreatment with 20 nM unlabelled plasmin for 30 min at 4°C; pretreatment with various concentrations of plasmin (0.66–66 nM) or trypsin (1–100 U/ml) for 10 min at 37°C; pretreatment with DFP-treated carboxypeptidase (0.2–20 U/ml) for 10 min at 37°C; pretreatment with fixed concentrations of enzyme for various times (5–60 min) at 37°C.

In all these experiments aprotinin was omitted during pretreatment and added to radiolabelled plasmin.

Scatchard plots were established from inhibition curves, using a program that allowed calculation of the binding parameters, dissociation constant and number of binding sites. This allowed the comparison of inhibition curves obtained with or without proteolytic pretreatment.

Gel electrophoresis was performed in 10% acrylamide in the presence of 0.1% SDS and 0.2 mM mercaptoethanol. Gels were dried and covered with Hyper films MP (Amersham), the exposure time varying from 6 to 24 h.

3. RESULTS

3.1. *Comparison of inhibition curves of plasmin binding*

In the presence of aprotinin, a typical inhibition curve was obtained using increasing concentrations of unlabelled plasmin. In contrast, when aprotinin was omitted, low doses of unlabelled plasmin increased the binding of radiolabelled plasmin considerably (fig.1). This effect decreased at higher doses of unlabelled plasmin and was followed by a typical inhibition. A similar effect was observed when plasminogen was used as ligand, and plasmin as competitor.

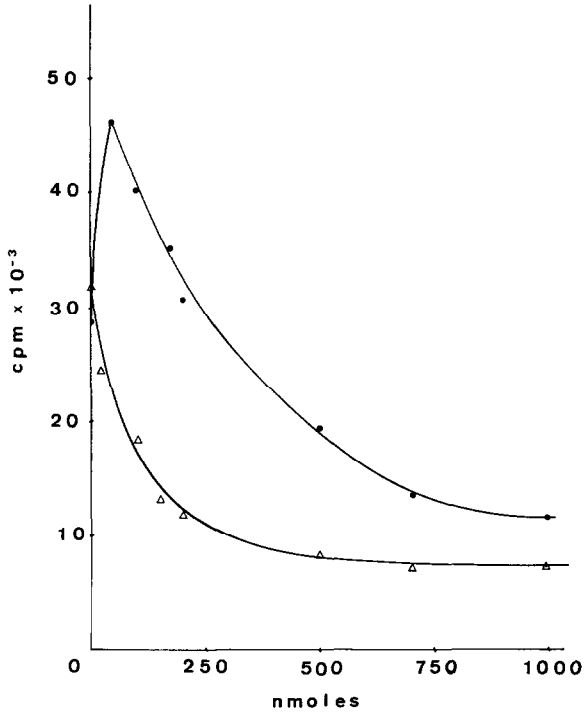

Fig.1. Inhibition of radiolabelled plasmin binding to human SW1116 cells by cold plasmin (2×10^{-8} M to 10^{-6} M) in the presence (Δ—Δ) or in the absence (●—●) of aprotinin (100 KIU).

3.2. *Pretreatment of tumor cells with an enzyme*

When tumor cells were incubated with 20 nM unlabelled plasmin, a 2–5-fold increase in plasmin binding was always observed in comparison to experiments where cells were preincubated with PBS. This increased plasmin binding was specific, as shown by inhibition experiments. For instance, the binding of 20 nM radiolabelled plasmin was inhibited to an extent of 70–80% by 1 μM unlabelled plasmin, whereas 2 μM unlabelled egg albumin gave rise to inhibition of only 0–10%.

When tumor cells were preincubated for 10 min at 37°C with various concentrations of plasmin or trypsin, the same effect as before was observed even for low doses of protease (fig.2); binding increased progressively until a maximum was attained (0.25 nM for plasmin, 20 U/ml trypsin). Much higher concentrations of proteases still produced the same effect. The stimulating effect on plasmin binding was seen after 5 min preincubation time and increased until a maximum after 30 min. This remained the same after 1 h preincubation with 0.1 μM plasmin, but decreased with 0.4 μM plasmin.

3.3. *Pretreatment with carboxypeptidase B*

When carboxypeptidase B was used for pretreatment of cells, a clear-cut decrease in plasmin binding was observed. This amounted to about 30% when the concentration of carboxypeptidase B was 0.2 U/ml and 50% for higher levels (2–20 U/ml). A similar effect was noted when carboxypeptidase B was used after pretreatment of cells with plasmin.

3.4. *Comparison of Scatchard plots*

By comparing Scatchard plots derived from inhibition curves of plasmin binding (fig.3) obtained after preincubation of tumor cells with plasmin or

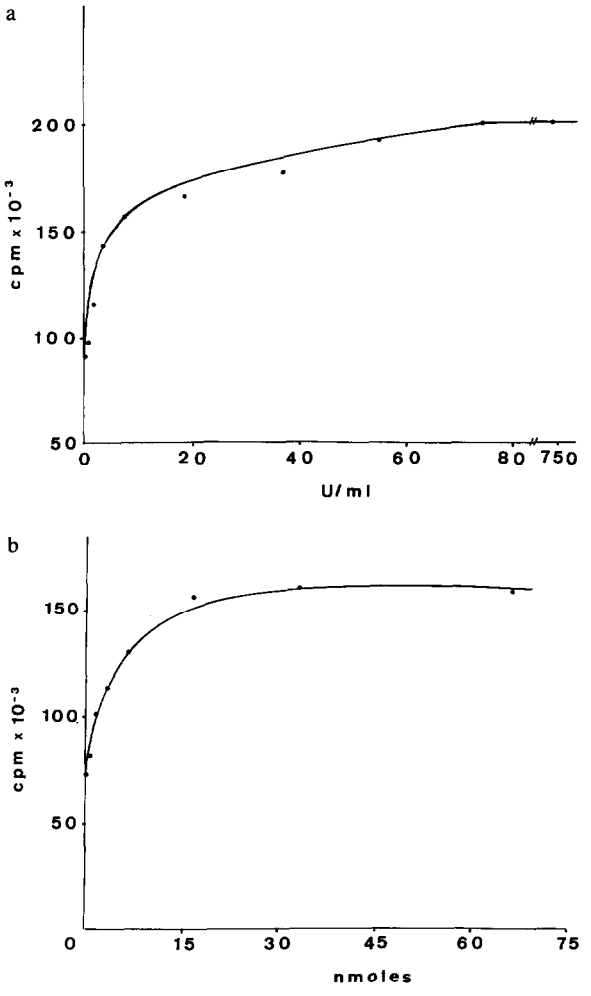

Fig.2. Increase of plasmin binding after cell pretreatment by various concentrations of trypsin (a) or plasmin (b).

PBS, we were able to demonstrate that proteolytic pretreatment did not alter the affinity of plasmin for tumor cells (K_d = 1.5 × 10^{-7} M after proteolytic pretreatment vs 1 × 10^{-7} M in the control experiment) but increased the number of binding sites strongly (60000 vs 15000/cell).

3.5. *Proteolytic pretreatment*

Proteolytic pretreatment did not change the structure of bound radiolabelled plasmin, as evaluated by SDS-polyacrylamide gel electrophoresis of the cell eluates. Typical plasmin heavy and light chains were seen with or without proteolytic pretreatment (not shown).

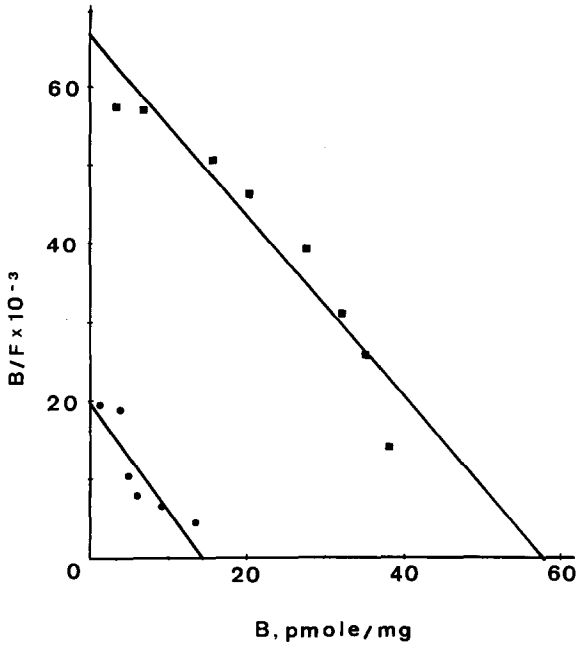

Fig.3. Comparison of Scatchard plots obtained in parallel experiments after (●—●) and without (■—■) proteolytic pretreatment.

3.6. *Effect of fibrinogen*

The possible role of fibrinogen as plasmin-binding protein on the cell surfaces was ruled out in two ways. Anti-fibrinogen serum (Behring) did not stain SW1116 cells as determined using immunofluorescence, nor did it inhibit plasma binding to these cells.

4. DISCUSSION

We have demonstrated an increase in plasmin binding by treatment of tumor cells with a trypsin-like proteinase. It is likely that such pretreatment unmasks lysine residues which react with lysine-binding sites of plasmin. Their involvement is based on two facts: plasmin binding by tumor cells is inhibited strongly by lysine (not shown) and partially by carboxypeptidase B, which is known to cleave off C-terminal lysine and arginine residues [3]. This effect of plasmin may play an important role in tumor invasion and metastasis. It is likely that in vivo tumor cells bind plasminogen and activate it to plasmin through the action of

plasminogen activators, especially urokinase. Once small amounts of plasmin have been formed, they increase the subsequent cell binding of other plasminogen and/or plasmin molecules. The final concentration of enzyme may thus be strongly increased, and hence its possible degrading action on components of the extracellular matrix.

Our data are similar to those of Tran-Thang et al. [4], who studied the binding of plasminogen to fibrin. In their experiments, pretreatment of fibrin with a low concentration of plasmin increased plasminogen binding several fold, by generating high-affinity binding sites reacting with the lysine-binding sites of plasminogen. Furthermore, Christensen [5] showed that a similar effect on fibrinogen molecules due to plasmin treatment was suppressed by the addition of carboxypeptidase B. These results proved that C-terminal lysine residues of proteolytically degraded fibrinogen were essential for binding to plasminogen. In our system, carboxypeptidase B reduced, but did not suppress plasmin binding by tumor cells, showing that not all C-terminal lysine residues of plasmin-binding molecules are susceptible to treatment with this enzyme. Furthermore, plasmin-binding molecules present on tumor cells are different from fibrinogen, as proved by our negative data with anti-fibrinogen serum.

The plasmin receptor studied here seems different from those found by another group on platelets [6] and on cells of the U937 monocytic cell line [7], since these receptors have the same affinity (K_d = 1 μM) for plasminogen and plasmin.

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