Receptors for Plasminogen Activator, Urokinase, in Normal and Rous Sarcoma Virus-transformed Mouse Fibroblasts

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ABSTRACT

We have prepared a conjugate of the plasminogen activator urokinase (UK) and ferritin, which maintains fibrinolytic activity. Monolayers of BALB/c-3T3 cells and of Rous sarcoma virus-transformed highly malignant line AA12-3T3, subcultured in plasminogen-free serum, were incubated with UK-ferritin at 0° and processed for transmission electron microscopy. Under these conditions, both of the lines showed specific receptors on the cell surface that were distributed in singlets, in small or large clusters. In the presence of excess native UK, the binding of ferritin was reduced by 99%, indicating the interaction of UK:ferritin with a specific receptor. The ligand-receptor interaction involves the catalytic site of UK, since the binding was completely impaired by preincubation of UK:ferritin with p-aminobenzamidine, a competitive inhibitor of the catalytic site of UK. The number and density of receptors decreased about one order of magnitude on the membrane of AA12 cells when compared with normal 3T3 cells. Saturation kinetics, using 125I-labeled UK, indicate the presence of 4 x 10⁴ and 2.5 x 10³ receptors on the membrane of 3T3 and AA12 cells, respectively. At 37°, UK:ferritin redistributed on the plane of the membrane, in a process which was faster in malignant than in normal cells. Ferritin particles clustered in large groups on coated areas of the surface and were internalized by adsorptive pinocytosis. After 10 min at 37°, the vesicles showed a progressively deeper internalization and a fusion with lysosomes, and some were observed in the Golgi complex area. Since the experiments were planned in order to exclude the presence of protease-nexin in the incubation medium, these data suggest the existence of a plasminogen-independent novel receptor for the catalytic site of plasminogen activators, the number on the cell surface of which decreases in Rous sarcoma virus-transformed mouse fibroblasts.

INTRODUCTION

PAs are a family of serine proteases that catalyze the conversion of serum zymogen, plasminogen, to the active fibrinolytic protease, plasmin. Although the role of such enzymes in the fibrinolytic process is well understood (2), little is known of their functions in the regulation of cellular processes. There are many examples of increased PA activity in cell transformation or tissue cancers when compared with their normal counterpart (4, 7, 17, 20), but there are also indications to the contrary, both in surgical specimens of human colorectal tumors (21) and in some cell lines transformed by oncogenic viruses (22). Various serine proteases can stimulate the growth of cells in culture (6, 18). In the presence of plasminogen, PA acts via plasmin on a broad range of substrata, thus regulating proteolysis of extracellular matrix and critical cell functions, such as movement and invasiveness (20), as do many other proteases.

A plasmin-independent role of PA in cultured cells has been described by Quigley (16) using specific inhibitors of PA upon stimulation of cultured fibroblasts with tumor promoters. Quigley concludes that PA is the serine protease responsible for both the altered morphology and changes in cell-to-cell interactions induced by phorbol esters. Keksi-Oja and Vaheri (12) have described a M₄₆₆,000 protein of the cellular matrix which seems to be a plasmin-independent cellular target for urokinase. Recently, Gross et al. (10) have shown that down regulation of receptors for epidermal growth factor correlates with PA activity in human A431 epidermoid carcinoma cells. That epidermal growth factor receptor down regulation is independent of plasmin suggests a direct action of PA in the initiation of the rapid inactivation of receptors that occurs in down regulation. Several reports (3, 13) describe PN, a component released in the medium of normal foreskin cells, that covalently binds and inactivates serine proteases, including thrombin and urokinase. PN resembles antithrombin III, a serum inhibitor of thrombin. The rate of serine protease binding and inactivation, as well as the binding of the nexin moiety of the complex at the cell surface, is heparin dependent.

In the present report, we describe both plasminogen and PN-like molecule independent binding of the PA urokinase at cell receptors, the number of which decreases in transformed mouse fibroblasts when compared with the normal counterpart.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The AA12-3T3, a highly metastasizing cell line obtained by transforming in vitro BALB/c-3T3 fibroblasts with the Bratislava 77 strain of Rous sarcoma virus (5, 9), were obtained from Dr. P. Comoglio, Department of Histology and Embriology, University of Turin, Turin, Italy. BALB/c-3T3 fibroblasts are commonly used in our laboratory. Cultures were maintained in DMEM supplemented with 5% fetal calf serum and antibiotics and were subcultured every 3 days. The last 2 subcultures before experiments were carried out supplementing DMEM with plasminogen-free serum, obtained by eluting fetal calf serum from a column of lysine-Sepharose (8). The cultures were maintained in serum-free conditions for 24 hr after reaching confluence.

Purification of UK and Radioiodination of the Molecule. The human urinary plasminogen activator UK was a gift of Dr. N. Toccalini of the Istituto di Ricerche Cesare Serono, Rome, Italy. UK was purified to homogeneity by affinity chromatography on Sepharose CH-4B (Pharmacia) substituted with p-aminobenzamidine (11). The UK activity, eluted from the column by changing the buffer from 0.1 M sodium phosphate...
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(pH 7.0)/0.4 w NaCl to 0.1 m acetate (pH 4.0)/0.4 w NaCl, was evaluated with the specific chromogenic substrate S-2444 (Kabi Diagnostica, Sweden). When 100,000 units of UK were applied, about 80% of the activity eluted with the retained material. This material was subjected to radioiodination with Na\(^{125}\)I, using a solid-phase support for lactoperoxidase and glucose oxidase (Enzymobead Radioiodination Reagent; Bio-Rad), following the instructions of the manufacturers. Gel filtration on Sephadex G-25 (0.7- x 35-cm column), in the presence of 0.1% albumin, was used to remove the isotope excess. The resulting specific activity was 1.41 \times 10^6 \text{cpm/µg}. Polyacrylamide gel electrophoresis of this material, performed in the absence of β-mercaptoethanol, showed the presence of 2 bands with a mobility corresponding to a molecular weight of 54,000 (70% of the densitometric estimate) and 32,000 (30%), respectively (Fig. 1).

Preparation of UK:Ferritin. The electron microscope-grade ferritin (Polysciences Ltd., United Kingdom) was cross-linked to UK with metaxylyene diisocyanate, using the procedure of Andres and Seegal (1). In order to establish the amount of bound UK, an aliquot of the material was applied onto a fibrin film (15), and the diameter of lysis area was referred to the lysis areas of standard UK. The final activity of the UK:ferritin solution was about 150 units/ml (Fig. 7).

Localization of Cell Bound UK:Ferritin by Electron Microscopy. To label cells at 0° with UK:ferritin for electron microscopy, monolayer cultures of 3T3 and AA12 cells in 30-mm tissue culture dishes were washed 3 times at 0° with 5 ml HBSS; 1 ml HBSS at 0°, containing 50 µl of UK:ferritin complex (corresponding to 7.5 units in terms of fibrinolytic activity), was incubated with cell monolayers for 60 min at 0°. Dishes were then washed 3 times with cold HBSS. The cells were either fixed at this time or further incubated at 37° in the same UK:ferritin-containing HBSS for 20 min. The cultures were then rinsed with HBSS and fixed. Fixation was achieved by adding cold 2.5% glutaraldehyde in 0.1 w cacodylate buffer (pH 7.2) and postfixed with 1.33% osmium tetroxide in the same buffer for 1 hr, then stained en bloc with 2% aqueous uranyl acetate in ethanol, and embedded in situ in Epon 812. Thin sections were cut with a diamond knife, mounted on 300 mesh grids, and examined in a Philips EM 300.

For experimental controls, cells were incubated with 50 µl of unconjugated ferritin. Other cells were incubated with 50 µl of UK:ferritin in the presence of a 500-fold excess of native UK. To study the involvement of the catalytic site of the enzyme in the interaction with the receptor, the PA was incubated with a molar excess of p-aminoenazidine before binding to cell monolayers.

As reported previously, UK binding to foreskin cells can be attributed to the formation of complexes with PN released into the culture medium (3, 13). Using the present conditions of binding, after 60 min of incubation at 0° of 10^6-UK with cell monolayers in the binding medium (HBSS), we did not observe any PN:UK complex (Fig. 1). This suggests that, in our experiments, UK:ferritin binding is not mediated by PN released into the medium.

Quantification of Cell-bound UK:Ferritin. The amount of UK:ferritin associated with the cell surface of the various cells examined was determined by counting ferritin particles in randomly selected unstained sections. In each experiment, a minimum of 25 cells for each cell line were evaluated. Each cell perimeter was scanned under established reference points of the electron microscope fluorescent screen. Ferritin particles were counted at the same magnification (x33,000), evaluating singlets, and, arbitrarily, small clusters (less than 10 particles) and large clusters (more than 10 particles) present on the whole-cell perimeter. Values were compared using the multiple confidence interval procedures for the Kruskal-Wallis test (14).

125I-UK-receptor Assay. The UK-receptor assay was performed at 0°.

Monolayers of cells (BALB/c-3T3 and AA12-3T3) were grown to confluence in Nunc 15-mm multidish-24 plates (Nunc-InterMed, Denmark) in DMEM supplemented with 5% plasminogen-free fetal calf serum, as described above. Once at confluence, cells were maintained for 24 hr in serum-free DMEM. Monolayers were washed 5 times with ice-cold HBSS, and 1 ml of ice-cold HBSS containing 2, 5, 10, 20, 30, 40, 50, 60, 80, 100, or 150 ng of 125I-UK was added in triplicate. The plates were incubated on ice for 60 min. Unbound radioactivity was removed by washing the plates 4 times with ice-cold HBSS (total, 8 ml). The washed monolayers were solubilized with 0.3 ml of 1.0 w NaOH for 1 hr at room temperature and counted in a LKB-Minigamma counter. Specific binding was determined by measuring the difference in cell bound radioactivity in the presence and absence of 2.0 µg of unlabeled UK.

Fig. 7. Fibrinolytic activity of the UK:ferritin conjugate. A, area of lysis induced by 5 µl of standard UK (150 units/ml) on a fibrin film. B, area of lysis induced by 5 µl of the final solution of the UK:ferritin conjugate.
RESULTS

Binding of UK:Ferritin to Cellular Receptors. The BALB/c-3T3 and AA12-3T3 cell lines have specific UK-binding sites on the cell surface. Binding of UK at the cell receptors was visualized directly with the electron microscope using a ferritin conjugate of UK. Monolayers of the cell lines were incubated with UK:ferritin (50 μl) at 0° for 60 min and at 37° for 10 min.

Incubation of cells with UK:ferritin at 0° showed no ferritin particles were associated with the plasma membrane of all the cells examined and that the density and distribution of ferritin particles varied in the 2 subclones observed.

In both of the cell lines, UK:ferritin appeared irregularly distributed on the cell surface and was frequently located in small (less than 10 particles) or large groups (more than 10 particles) (Fig. 2).

The degree of grouping was not reduced upon light fixation of cells with formaldehyde before binding the complex, thereby suggesting that the pattern seen in Fig. 2, B and C, was not due to clustering after binding at 0°.

Averaged over the entire cell perimeter, the ferritin particles showed the differential distribution in the 2 cell lines summarized in Table 1.

Aspecific binding occurring when unconjugated ferritin cores (50 μl) were added to cell monolayers was less than 1% when compared to the binding of the UK:ferritin conjugate. To establish whether UK:ferritin binding was specific and occurred on UK-saturable receptors, a 500-fold excess of UK was added together with UK:ferritin. In these conditions, the nonspecific binding was calculated to be less than 1% (Fig. 6, A and B).

In order to observe whether the binding of the UK:ferritin conjugate involved the interaction of the UK catalytic site with the cell receptor, some experiments were carried out adding 50 μl of UK:ferritin preincubated with a molar excess of p-aminobenzamidine, a well-known competitive inhibitor of the catalytic site. Under these conditions, no cell binding was observed (Fig. 6C).

Internalization of UK:Ferritin. When cells were maintained at 0°, no ferritin cores were seen in endocytic vesicles.

The distribution of UK:ferritin in singlets or clusters varied between normal and transformed cells, when the monolayers were examined after warming to 37° for 20 min. As shown in Table 1, the singlets decreased in both subclones, while the percentage of clustered ferritin cores resulting increased. In a comparison of the 2 cell lines, it is evident that the extent of clustering is increased in the malignant cells. The pattern of binding after 20 min at 37° is shown in Fig. 3. Ferritin clusters can clearly be seen located in areas of the membrane with no particular features (Fig. 3A) and on coated pits (Fig. 3, B and C).

After 20 min at 37°, ferritin particles could be observed inside cytoplasmic vesicles. These vesicles were formed by absorptive pinocytosis (Fig. 3C). A series of progressively deeper internalizations can be observed. A number of the internalized ferritin cores was located throughout the cytoplasm in endosomes (Figs. 4 and 5A) or in multivesicular bodies (Fig. 5B). Some of these vesicles were observed in the Golgi complex area (Fig. 5A).

Binding of 125I-UK at 0°. In order to clarify whether the presence of PN in the incubation medium could account for the binding observed, monolayers were washed 3 times at 0° with 5 ml of HBSS and then incubated for 60 min at 0° with 1 ml of cold HBSS containing 200 ng of 125I-UK. The electrophoretic pattern of this sample did not show any difference when compared with the pattern of unincubated 125I-UK (Fig. 1).

This suggests that, under our conditions, release of PN into the incubation medium and the eventual binding of PN:125I-UK complexes to the cell surface do not account for our results.

Charts 1 and 2 show that the specific binding of 125I-UK to fibroblast monolayers is saturable with respect to the concentration of 125I-UK, for both subclones.

At saturation of binding, approximately 4 x 10⁵ sites are occupied on the surface of normal mouse 3T3 fibroblasts, and 2.5 x 10⁵ are on that of the transformed counterpart.

Charts 1 and 2 also show that the addition of 125I-UK preincubated with a molar excess of p-aminobenzamidine inhibits the specific binding of the plasminogen activator.

Thus, it is evident that (a) both normal and transformed 3T3 cells, cultured in plasminogen-free conditions, have specific receptors for a sequence of plasminogen activator involving the catalytic site of the molecule, and (b) the number of UK receptors on RSV-transformed 3T3 fibroblasts is markedly less than on their nontransformed counterpart.

DISCUSSION

A ferritin conjugate of UK has been prepared that retains biological activity in fibrinolytic tests.

The BALB/c-3T3 and AA12-3T3 cell lines have specific binding sites for the UK:ferritin conjugate on the cell surface. The degree of aspecific binding of unconjugated ferritin cores was less than 1% when compared to the binding of UK:ferritin.

Table 1

<table>
<thead>
<tr>
<th>Binding of UK: ferritin at 0° and 37°: quantitation of ferritin particles/10 μm of cell surface</th>
<th>Internalized vesicles with ferritin cores</th>
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</thead>
<tbody>
<tr>
<td><strong>Singlets</strong></td>
<td><strong>Small clusters</strong></td>
</tr>
<tr>
<td>0° BALB/c-3T3 (n = 27)</td>
<td>18.6 ± 5.2c</td>
</tr>
<tr>
<td>AA12-3T3 (n = 26)</td>
<td>3.3 ± 1.9c</td>
</tr>
<tr>
<td>37° BALB/c-3T3 (n = 25)</td>
<td>5.4 ± 2.3</td>
</tr>
<tr>
<td>AA12-3T3 (n = 25)</td>
<td>1.9 ± 1.1</td>
</tr>
</tbody>
</table>

a Small clusters and large clusters have been arbitrarily evaluated as groups of ferritin particles containing less or more than 10 particles of ferritin, respectively. Each group was considered as a single entity.

b Mean ± S.D. for a number of determinations (n).

c Numbers in parentheses, percentage number of each arbitrary entity within a single cell line.

d Significantly different from the value of BALB/c-3T3 at p < 0.01.

e Usually, an internalized vesicle showed a number of ferritin cores between 5 and 80.

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p-aminobenzamidine for each concentration used.

Data are expressed as the average of triplicate determinations, as 125I-UK preincubated with a molar excess of p-aminobenzamidine for each concentration used.

Chart 1. Effect of 125I-UK concentration on binding to BALB/c-3T3 cells. Indicated concentrations of 125I-UK were added to confluent culture plates (15-mm multidish), as described in the text. Specific binding was determined after 60 min at 0°. Data are expressed as the average of triplicate determinations as 125I-UK preincubated with a molar excess of p-aminobenzamidine for each concentration used.

At 0°, UK:ferritin is initially localized exclusively on the plasma membrane. Most of the binding appears irregularly distributed on the cell surface, and it often occurs at the basis of membrane digitations, where a clustered distribution of ferritin particles is observed.

When unfixed cells were warmed to 37°, the conjugate rapidly redistributed in the plane of the plasma membrane, showing a distribution in small and large clusters, which was particularly marked in transformed cells. The binding of the PA to its receptor probably predisposes the receptor to either associate with other UK:receptor complexes or to move toward a zone of the cell membrane where clustering is somehow facilitated. It is also possible that an increased fluidity of the cell membrane upon binding of the PA can favor the clustering of enzyme:receptor complexes (19).

In both the normal and transformed cells, a large part of clusters appeared localized exclusively on coated pits and internalized in cytoplasm vesicles.

We observed a significant difference in the number and distribution of receptors for PA between normal and RSV-transformed cells. The density distribution of receptors decreases in RSV-transformed cells. The Scatchard analysis of affinity binding studies with 125I-UK has shown that, at saturation of binding, about 4 x 10^4 sites are located on the plasma membrane on normal 3T3 cells, while only 2.5 x 10^3 are on that of the RSV-transformed fibroblasts.

In the present model system, the electron microscopic data obtained at 37° also show a transformation-dependent increase in the rate of internalization of PA:receptor complexes. What these differences connote we cannot say at present.

The catalytic site of PA is clearly implicated in the interaction with the receptor; in fact, the addition of p-aminobenzamidine to the UK:ferritin and to 125I-UK impairs the cellular binding of the ligand.

Recent reports (3, 13) indicate that binding, internalization, and degradation of 125I-UK is mediated by PN. In our experimental conditions, PN was not present in the incubation medium, as shown in Fig. 1. On the basis of these data, one is tempted to tentatively suggest that the presence of PN:UK complexes did not complicate our results, which probably identify a novel receptor for the catalytic site of plasminogen activators.

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Fig. 2. Binding of UK-ferritin at 0°C. The conjugate bound to the plasma membrane (PM) can be observed as single ferritin cores (A) (BALB/c-3T3, x 133,000) or large (arrow) and small clusters: (B) AA12, x 270,000; or (C) BALB/c-3T3, x 245,000).

Fig. 3. Binding of UK-ferritin at 37°C. A, redistribution of UK-ferritin at 37°C on the plasma membrane of a BALB/c-3T3 cell (x 315,000). B, a large cluster of UK-ferritin located on a coated area of the plasma membrane (BALB/c-3T3, x 93,500). C, a large cluster is seen apparently in the process of being internalized into a coated pit (arrow) (AA12, x 84,000). up, uncoated pit.
Fig. 4. Binding and internalization of UK:ferritin at 37°. UK:ferritin is seen on the plasma membrane as a large cluster (arrow) and inside endocytic vesicles (double arrow). AA12, × 110,000.

Fig. 5. Accumulation of ferritin inside the cell. In A, the arrows indicate an endosome located close to the Golgi complex (BALB/c-3T3, × 79,000). B, presence of ferritin in multivesicular bodies. × 84,000.

Fig. 6. In A, no ferritin cores are present on the plasma membrane of cells incubated with unconjugated ferritin. BALB/c-3T3, × 84,000. In B, ferritin cores are not observed on the plasma membrane of cells incubated in the presence of a large excess of native urokinase, added together with UK:ferritin. BALB/c-3T3, × 63,000. In C, BALB/c-3T3 monolayers incubated with UK:ferritin preincubated with a molar excess of p-aminobenzamidine. No binding is present on the plasma membrane. × 63,000.

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