RECEPTOR-MEDIATED ENDOCYTOSIS OF α_2MACROGLOBULIN–PROTEASE COMPLEXES BY FIBROBLASTS IN CULTURE

Competitive inhibition by bacitracin

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1. Introduction

α_2-Macroglobulin, a major glycoprotein in plasma and body fluids of mammals, is an inhibitor of virtually all endoproteases. Normal human skin fibroblasts in culture are capable of internalizing large amounts of α_2M by receptor-mediated endocytosis [1]. This process was very specific, as not α_2M but only α_2M–protease complexes were recognized by the receptor and selectively internalized [2]. Primary amines interfered at two levels in this system:

(i) Certain small primary amines (e.g., methylamine) inactivate native α_2M to a state very similar to the one obtained with proteases: a complex which is recognized by the cellular receptor [3] and by a monoclonal antibody (F2B2), recognizing a neoantigenic site on α_2M–protease complexes [4].

(ii) Primary amines interfere at the cellular level with intracellular receptor-recycling [5]. Recycling of receptors was inferred from the finding that a rather small, but constant number of receptors at the plasma membrane was capable of massive transport of ligand [5–7] without an apparent large intracellular pool of receptors and without synthesis of new receptors.

Bacitracin, a cyclic peptide antibiotic of bacterial origin, was used as another transglutaminase inhibitor, blocking clustering of fluorescently labeled α_2M [8]. Quantitative data on ^{125}I-labelled α_2M binding to cells and cell plasma membrane preparations was reported in [10]: this binding was inhibited by bacitracin. However, the data showed clear differences between the effect of bacitracin and other suspected transglutaminase inhibitors.

Here we show that bacitracin is a competitive inhibitor of receptor-mediated endocytosis of α_2M–protease complexes inhibiting binding to the cellular receptor. Our data indicate a different mode of action of bacitracin as compared to the primary amine, mono-dansylcadaverine.

2. Materials and methods

Bacitracin and mono-dansylcadaverine were obtained from Sigma. Carrier-free $Na^{125}I$ was from The Radiochemical Centre, Amersham.

2.1. *Cell culture*

Diploid human fibroblast cultures were obtained from skin biopsies of the forearm of healthy adult donors [11]. The cells were grown in Dulbecco's modified Eagle's medium (DME; Grand Island Biological Co., New York), containing 10% (v/v) heat-inactivated newborn calf serum (NCS; Sera-Lab, England), 1 g $NaHCO_3$/l, 15 mM *N*-(tris(hydroxymethyl)methyl-2-amino)ethanesulfonic acid (Tes) and 4-(2-hydroxymethyl)-1-piperazineethane–sulfonic acid (Hepes) buffered to pH 7.4 with 1 N NaOH. All cell lines were karyotyped and monitored regularly for mycoplasma contamination. Between passages 10–15, an equal number of cells of each of 6 donors was pooled. After 2 subcultures the cells were plated in disposo trays (Linbro, Flow Labs., Scotland) at 80 000 cells/well in DME containing 10% (v/v) newborn calf serum and placed in a 37°C incubator in an atmosphere of 5% CO_2, 95% air and 100% humidity. After 48 h the cells were used in the experiments.

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2.2. *Purification of α_2M*

α_2M was purified from human plasma. Only plastic labware was used and the entire procedure was done at 4°C. The plasma was treated with $CaCl_2$ and $BaSO_4$ as in [2]. α_2M was precipitated between 4–8% (w/v) polyethylene glycol 6000. The precipitate was suspended, dialyzed againt PBS and fractionated on a column (5 × 94 cm) of Ultrogel ACA 22 (LKB, Sweden). The final purification step consisted of affinity chromatography on a column (2.6 × 90 cm) of Blue Sepharose (Pharmacia, Sweden). Eluent was a sodium phosphate buffer (25 mM, pH 7.7). Quantitation of α_2M after each step and in column fractions was carried out by rocket immunoelectrophoresis [12]. Protein was determined as in [13], with bovine serum albumin as standard. A typical purification procedure starting with 300 ml plasma resulted in a preparation containing 97% α_2M, in a yield of 58%.

2.3. *^{125}I-α_2M and ^{125}I-α_2M–trypsin complexes*

α_2M was iodinated using $Na^{125}I$ and chloramine-T as in [2]. After labeling, α_2M was reacted with a 3-fold excess crystalline trypsin (on a molar basis). Complexes were separated from free trypsin as in [2]. Labeled α_2M was stored at 4°C.

2.4. *Uptake and degradation of α_2M at 37°C*

The cell layers grown in DME-newborn calf serum were washed 3 times with 1 ml serum-free medium without HCO_3^-, followed by a 15 min incubation at 37°C in this medium. The medium was then replaced by 0.5 ml prewarmed DME containing ^{125}I-α_2M–trypsin at the appropriate concentration. The trays were kept at 37°C for the desired time period, the medium was removed, the cell layers were washed 4 times with ice-cold PBS, and 0.5 ml crystalline trypsin (0.5 mg/ml) was added for 5 min at 37°C. The trypsinized cells were removed and each well was washed with 0.5 ml cold PBS and this wash-fluid was combined with the cell suspension. The cell suspension was centrifuged (800 × *g*, 10 min) and radioactivity in cell pellet and supernatant was counted in an automatic scintillation counter (PRIAS, Packard). Intracellular degradation of ^{125}I-α_2M was followed over a 4 h period. The cell layers were pulsed for 30 min with ^{125}I-α_2M–trypsin, washed 3 times and further incubated in complete medium containing 10% (v/v) serum. At the time periods indicated, the cell layers were trypsinized as described above and counted.

2.5. *Binding of ^{125}I-α_2M at 4°C*

Binding studies were carried out at 4°C with the trays placed on crushed ice in a 4°C refrigerator. The incubation media used in binding studies contained albumin (2 mg/ml) to decrease non-specific binding of α_2M. The cell layers were washed 3 times with cold DME containing bovine serum albumin (0.2%, w/v) and left in this medium for 15 min. The medium was removed and 0.5 ml DME containing ^{125}I-α_2M–trypsin (2 μg/ml) was added. Specific binding was calculated from data with excess unlabeled α_2M–trypsin as in [2].

3. Results

Bacitracin proved a very effective inhibitor of receptor-mediated endocytosis of ^{125}I-α_2M–trypsin complexes by fibroblasts in culture (fig.1). In the presence of 0.25 mg bacitracin/ml endocytosis was

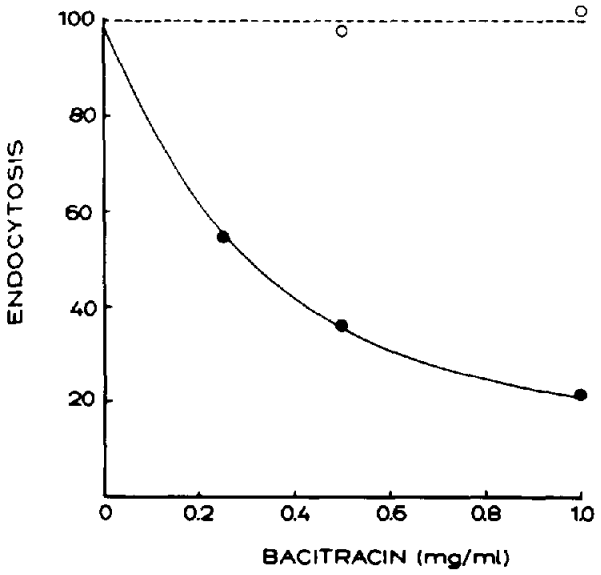

Fig.1. Effect of concentration of bacitracin on receptor-mediated endocytosis of ^{125}I-labeled α_2M–trypsin complexes. Normal human fibroblast cell layers were preincubated with bacitracin in medium or with medium only for 30 min. After washing with medium, ^{125}I-labeled α_2M–trypsin was added (50 μg/ml in medium). After 30 min at 37°C cells were washed and intracellular content of label was determined. Bacitracin was present during the assay (•) at the concentrations indicated or cell layers were pretreated with bacitracin (30 min, 37°C) while the drug was not present during the assay (○). Data are the mean of duplicate determinations and are expressed relative to untreated cell layers.

inhibited by ~50%. When the cell layers were pretreated with bacitracin and subsequently examined for endocytosis in the absence of the drug, no inhibition was observed (fig.1). As this latter finding was completely different from our observations on the effect of primary amines on receptor-mediated endocytosis [5], we examined in the same set of experiments the effect of bacitracin on endocytosis at 37°C and on receptor binding at 4°C, in comparison to mono-dansylcadaverine (m-DNSC) (table 1).

It is clear from these data that bacitracin was an effective inhibitor of the endocytosis and the binding of ^{125}I-α_2M–trypsin but only when the drug was present during the assay. Pretreatment of the cells did not affect endocytosis or binding. This was very much unlike m-DNSC which was only maximally effective when cells were pretreated for at least 30 min [5]. Moreover, the mere presence of m-DNSC at 4°C did not affect binding of α_2M–trypsin while bacitracin inhibited binding 60% under these conditions (table 1).

Table 1
Effect of bacitracin and mono-dansylcadaverine on internalization and binding of α_2M–trypsin complexes

	Bacitracin (1 mg/ml)	m-DNSC (0.2 mM)
Endocytosis (37°C)		
pre-30 min	104	43
present	22	70
pre-30 min + present	24	38
Binding (4°C)		
pre-30 min	97	48
present	41	99
pre-30 min + present	40	37

Normal human fibroblast monolayers were examined for receptor-mediated endocytosis at 37°C or for receptor-binding at 4°C with ^{125}I-labeled α_2M–trypsin complexes. Endocytosis at 37°C was determined for 30 min with 100 μg ligand/ml medium. Binding was measured after 4 h at 4°C with 1 μg ^{125}I-α_2M–trypsin/ml medium. Non-specific binding was determined by addition of 100 μg α_2M–trypsin/ml. Results are expressed relative to controls receiving no drugs, but under otherwise identical conditions. Pretreatment of cell layers was at 37°C for 30 min for both endocytosis and for binding. For measurements of endocytosis cell layers were subsequently washed with warm medium, containing the drug or not when indicated. For measurements of binding, cell layers were washed with ice cold medium while trays were placed on crushed ice. After 15 min the assay was started by addition of labeled α_2M–trypsin

The type of inhibition of both drugs on receptor-mediated endocytosis was examined (fig.2). Whereas m-DNSC clearly decreased the maximal rate of endocytosis of α_2M–trypsin, bacitracin did not. From the double-reciprocal plot it would appear that bacitracin is a purely competitive inhibitor of endocytosis.

As these data point to inhibition of binding to the receptor, we examined whether bacitracin could inhibit the binding of the monoclonal antibody F2B2. This antibody binds to the receptor-recognition site on α_2M–protease complexes, thus preventing the latter from being internalized by fibroblasts [4]. The results indicated that bacitracin even at concentrations much higher than those used in the cellular

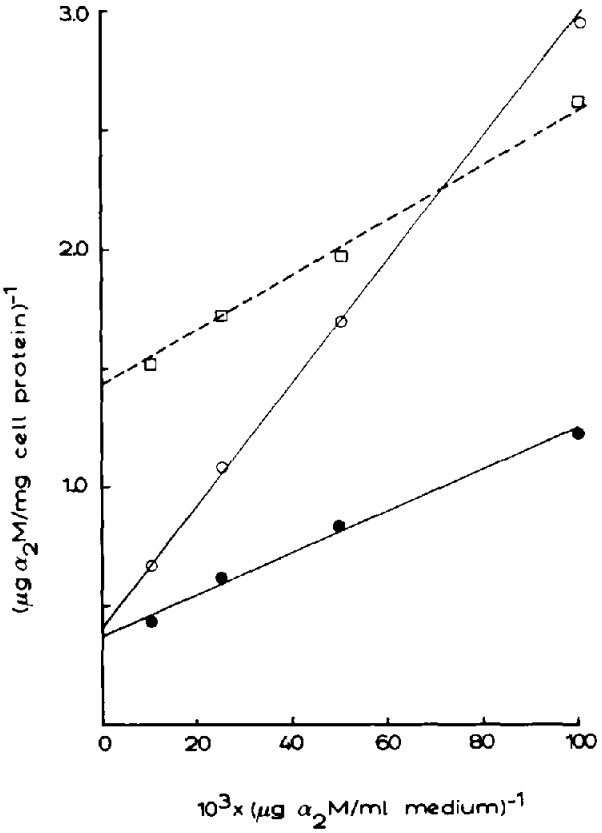

Fig.2. Kinetic parameters of receptor-mediated endocytosis, in the presence of bacitracin or mono-dansylcadaverine. Double reciprocal plot of receptor-mediated endocytosis with concentration of ^{125}I-labeled α_2M–trypsin as indicated, under control conditions (•), in the presence of 0.25 mg bacitracin/ml (○) or in the presence of 0.2 mM mono-dansylcadaverine (□). For m-DNSC only, cells were also pretreated with the same concentration for 30 min. Data are the mean of duplicate determinations on separate cell layers.

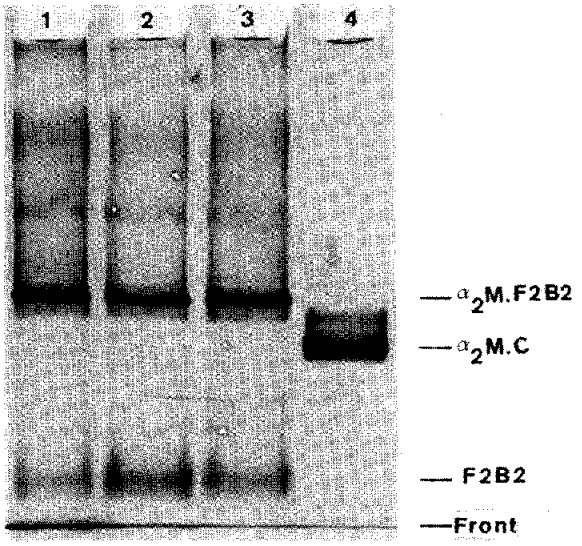

Fig.3. Effect of bacitracin on binding of the monoclonal antibody F2B2, to α_2M–complexes. α_2M was reacted with methylamine (25 mM, Tris–HCl (pH 8.0)). Rate electrophoresis on 5% polyacrylamide gel as in [3] indicated α_2M–MA complexes to be formed with a small amount of slow form α_2M still present (4). When α_2M–complexes are reacted with excess F2B2, subsequent rate electrophoresis shows the pattern as in (3): fast form α_2M is shifted to a slower mobility (see also [4]). When antibody is added to α_2M–complexes in the presence of bacitracin no differences in the pattern are observed: (1) 5 mg bacitracin/ml; (2) 1 mg bacitracin/ml. Similar patterns were observed with α_2M–trypsin complexes. *Abbreviations:* α_2M . C, α_2M–complex; F2B2, monoclonal antibody (see text); α_2M . F2B2, immune complex of α_2M–complex and monoclonal antibody. Bacitracin migrates at the dye front.

experiments, did not inhibit binding of the monoclonal antibody to α_2M–complexes (fig.3).

Finally, we examined the effect of bacitracin on intracellular degradation of ^{125}I-labeled α_2M–trypsin complexes, internalized by receptor-mediated endocytosis. Under control conditions intracellular half-life of the complexes was 2.1 h confirming [2,5]. In the continuous presence of 0.25 mg bacitracin/ml intracellular half-life was 2.5–2.7 h.

4. Discussion

These data confirm that bacitracin interferes with receptor-mediated endocytosis of α_2M–trypsin complexes by normal human fibroblasts in culture [10]. The mode of action of bacitracin is however different from m-DNSC:

(i) Bacitracin does not require preincubation with the cells to become inhibitory;
(ii) Bacitracin inhibits binding of α_2M–trypsin to the receptor;
(iii) Bacitracin inhibits endocytosis in a competitive fashion;
(iv) Bacitracin does not appreciably affect intracellular degradation of endocytozed α_2M.

Taken together these findings indicate that bacitracin is only effective extracellularly by inhibiting the binding of α_2M–complexes to the receptor, whereas m-DNSC is only effective intracellularly and does not affect binding of α_2M–complexes to the receptor. Mono-dansylcadaverine and other primary amines inhibit the recycling of α_2M–receptors in fibroblasts [5]. This was refuted on the basis that bacitracin and other transglutaminase inhibitors inhibited internalization of α_2M [10] and binding of α_2M to plasma membranes [9]. These authors explained inhibition by bacitracin [10], as an inhibition of receptor-clustering at 0°C as the result of an enzymatic (transglutaminase-mediated) process, while other transglutaminase inhibitors were not effective under the same conditions. Furthermore, inhibition of binding of α_2M to plasma membranes by m-DNSC was described in [9] while the same drug was not effective on binding to intact cells [10].

It must be clear from these results that both drugs interfere with receptor-mediated endocytosis of α_2M–complexes, but at different steps in the process. Therefore it is unlikely that bacitracin and m-DNSC block receptor-mediated endocytosis by virtue of their being transglutaminase inhibitors, as claimed [9,10]. An explanation of all findings would be that bacitracin interferes directly with binding of α_2M–complexes to the cellular receptor, while primary amines such as m-DNSC act intracellularly on receptor-recycling [5]. The latter process might or might not be mediated by a transglutaminase activity [5]. The difference in mode of action between bacitracin and m-DNSC is stressed further, as preliminary results indicate that bacitracin is not an effective inhibitor of fibroblast transglutaminase activity (unpublished).

Our finding that bacitracin is a competitive inhibitor of α_2M–endocytosis is quite exciting from a biochemical point of view. The question arises whether bacitracin binds to the receptor or to the α_2M–complexes. In this regard it is of some interest that bacitracin is a chelator of bivalent metal ions. Ca^{2+} seems involved in receptor-mediator endocytosis of α_2M–

complexes probably at the binding step [10]. However, it is well known that α_2M itself binds metals, such as Ca^{2+} and Zn^{2+}, and it is not clear whether Ca^{2+} is needed at the cellular receptor or on the ligand, or on both. As bacitracin binds Zn^{2+} more effectively than Ca^{2+} [14], the metal chelating characteristics of bacitracin are worth consideration. Our findings that bacitracin does not affect the recognition of α_2M–complexes by the monoclonal antibody, F2B2, might be explained by such a mechanism. If the metal-chelating properties of bacitracin are not involved, which remains to be determined, other structural features such as the cyclic peptide or the thiazoline ring of bacitracin need further consideration. Preliminary experiments, in which bacitracin was hydrolyzed in acid, known to open the thiazoline ring [15], indicated this structure not to be important in inhibitions. Other derivatives would yield more information on the type of interaction producing inhibition. This kind of reasoning will direct further experiments to understand the exact nature of the α_2M–protease complex interaction with the cellular receptor. This implies of course that bacitracin is somehow specific for α_2M-binding to the cellular receptor. Apparently, receptor-mediated endocytosis of low density lipoprotein is not affected by bacitracin [16]. Other fibroblast surface antigens might however be acted upon, as we found bacitracin to be a very potent inhibitor of aminopeptidase M (unpublished) shown to be present at the fibroblast plasma membrane [17,18].

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