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Research Article

Receptor-recognized forms of alpha 2-macroglobulin (alpha 2M*) bind to two macrophage receptors: an endocytic receptor, the low density lipoprotein receptor-related protein/alpha 2M receptor (LRP/alpha 2MR), and a G protein-coupled receptor, the alpha 2M signaling receptor (alpha 2MSR). Binding of alpha 2M* to LRP/alpha 2MR but not alpha 2MSR is inhibited by receptor-associated protein. We now present binding characteristics of alpha 2MSR (kD approximately 50 pm; 1,530 sites/cell) using Scatchard analysis. We also demonstrate that chemical modification of alpha 2M* with cis-dichlorodiammineplatinum (cis-DDP) does not significantly alter binding to either receptor or signaling characteristics as compared with unmodified alpha 2M*. However, internalization by LRP/alpha 2MR is greatly affected. Cis-DDP-modified alpha 2M* (cis-DDP-alpha 2M*) and alpha 2M* show comparable internalization during a single round of endocytosis; however, cis-DDP modification of alpha 2M* results in a > or = 82% reduction in internalization involving receptor recycling and multiple rounds of endocytosis. Results from pH 5.0 dissociation and receptor recycling experiments suggest that the mechanism of decreased internalization of cis-DDP-alpha 2M* involves poor dissociation from the receptor in endosomes and a decrease in available surface receptors over the time of exposure to the ligand.

Altered Interaction of *Cis*-dichlorodiammineplatinum(II)–modified α_2-Macroglobulin (α_2M) with the Low Density Lipoprotein Receptor-related Protein/α_2M Receptor but Not the α_2M Signaling Receptor

Evidence for Interference with Receptor Dissociation and Recycling

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Abstract

Receptor-recognized forms of α_2-macroglobulin (α_2M*) bind to two macrophage receptors: an endocytic receptor, the low density lipoprotein receptor–related protein/α_2M receptor (LRP/α_2MR), and a G protein–coupled receptor, the α_2M signaling receptor (α_2MSR). Binding of α_2M* to LRP/α_2MR but not α_2MSR is inhibited by receptor-associated protein. We now present binding characteristics of α_2MSR ($K_d \sim$ 50 pM; 1,530 sites/cell) using Scatchard analysis. We also demonstrate that chemical modification of α_2M* with *cis*-dichlorodiammineplatinum (*cis*-DDP) does not significantly alter binding to either receptor or signaling characteristics as compared with unmodified α_2M*. However, internalization by LRP/α_2MR is greatly affected. *Cis*-DDP–modified α_2M* (*cis-DDP*-α_2M*) and α_2M* show comparable internalization during a single round of endocytosis; however, *cis*-DDP modification of α_2M* results in a $\geq$ 82% reduction in internalization involving receptor recycling and multiple rounds of endocytosis. Results from pH 5.0 dissociation and receptor recycling experiments suggest that the mechanism of decreased internalization of *cis*-DDP-α_2M* involves poor dissociation from the receptor in endosomes and a decrease in available surface receptors over the time of exposure to the ligand. (*J. Clin. Invest.* 1996. 97:1193–1203.) Key words: cisplatin • macrophage • endocytosis • signaling • proteinase inhibitors

Introduction

Human α_2-macroglobulin (α_2M)[1] ($\sim$ 720,000 M_r) is a homotetramer in the serum in micromolar quantities which has the unique ability to inhibit proteinases from all four mechanistic classes (1–5). Proteinases interact with and cleave native α_2M in a region termed the "bait region," causing a conformational change in the α_2M protein which physically traps the proteinase and sterically hinders it from access to larger substrates and substrate-specific antibodies (3, 6). This conformational change also involves cleavage of internal reactive thiolesters in α_2M, and small nucleophiles, such as methylamine, can activate α_2M by reacting directly with these thiolesters and simulating the conformational change seen upon interactions with proteinases (7, 8).

During the conformational change seen with either proteinase interaction or reaction with methylamine, previously buried receptor recognition sites on α_2M are exposed (9, 10). Since α_2M-proteinase and methylamine derivatives have similar conformations and are recognized equally well by cellular receptors, α_2M* is often used to designate either receptor recognized form. Exposure of these sites on α_2M leads to rapid clearance in vivo as demonstrated in mouse, human, and dog circulation and in vitro by cells expressing a cellular receptor for α_2M* (9, 11–14). These receptor recognition sites have been localized to a carboxyterminal receptor binding fragment of α_2M (RBF) ($\sim$ 20,000 M_r) which can be proteolytically derived (15–17) and has now been cloned and expressed from rat α_1-macroglobulin (18).

α_2M* has been shown to have a variety of biological effects in addition to proteinase inhibition. In macrophages, α_2M* regulates the ability to kill tumor cells, the respiratory burst, proteinase secretion, and prostaglandin production (for review see reference 19). More recent work has shown that α_2M* enhances antigen presentation by macrophages to T cells and stimulates proliferation of smooth muscle cells synergistically with transforming growth factor-β (20, 21).

The low density lipoprotein receptor–related protein/α_2M receptor (LRP/α_2MR) is a high molecular weight cell surface receptor which mediates binding of α_2M* or RBF (22–24). Many cell types have been shown to express this endocytic receptor for α_2M* besides macrophages, including fibroblasts, hepatocytes, adipocytes, and dermal dendritic cells (25–30). LRP/α_2MR is a classical scavenger receptor which binds and internalizes multiple ligands besides α_2M*, including *Pseudomonas* exotoxin A, lipoprotein lipase, apolipoprotein E–enriched lipoproteins, urokinase- and tissue-type plasminogen activators alone or in complexes with plasminogen activator inhibitor-1, tissue factor pathway inhibitor, lactoferrin, lipoprotein(a), and receptor-associated protein (RAP) (31–38). RAP

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1. *Abbreviations used in this paper:* α_2M, α_2-macroglobulin; α_2M*, the receptor recognized form of α_2M, either α_2M-methylamine or α_2M-protease; α_2MSR, the α_2M signaling receptor; $[Ca^{2+}]_i$, intracellular free Ca^{2+}; *cis*-DDP, *cis*-dichlorodiammineplatinum(II); IP_3, inositol 1,4,5-triphosphate; LRP/α_2MR, the low density lipoprotein receptor–related protein/α_2M receptor; RAP, receptor-associated protein; RBF, the 20-kD carboxy-terminal receptor binding fragment of rat α_1-macroglobulin.

inhibits the binding and endocytosis of all known LRP/α_2MR ligands (38).

Recently, studies in this laboratory have shown that α_2M* elicits an increase in intracellular calcium ($[Ca^{2+}]_i$) and inositol triphosphates (IP_3) in murine macrophages via a pertussis toxin–insensitive G protein–coupled receptor termed the α_2M signaling receptor (α_2MSR) (39, 40). Binding to this receptor is calcium dependent (39, 40). The signaling elicited by α_2M* is not blocked by a large molar excess of RAP, the protein which inhibits α_2M* binding to LRP/α_2MR (41). Similar results were obtained with a cloned and expressed RBF from rat α_1-macroglobulin, ruling out any signaling due to possible contamination of α_2M with growth factors and localizing the binding site(s) for this second α_2M receptor to RBF (41). Other than the inability of RAP to block signaling of α_2M* via α_2MSR, no binding characteristics, such as a K_d value or number of receptor sites per cell, have been established for this newly described α_2M* receptor.

Previous studies have used chemical modification techniques to investigate which amino acids are important for α_2M*–receptor interactions. Chemical modification of α_2M* with *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) was shown to dramatically affect clearance in vivo. Structurally, *cis*-DDP is a bidentate cross-linker which forms intramolecular cross-links in α_2M*, involving at least several methionine residues and a few histidine residues (42). Previous studies showed that *cis*-DDP treatment of α_2M* altered the clearance half-life in the murine circulation, lengthening it to that of the native, non–receptor-recognized α_2M (42, 43). However, in vitro binding studies with murine macrophages demonstrated that *cis*-DDP-α_2M* is still an effective competitor for the binding of unmodified α_2M* (42, 43). The mechanism of the decreased clearance has not been investigated. Additionally, the effect of this chemical modification on the ability of the ligand to elicit the newly described signaling cascades has not been studied.

In this study, we examined the signaling and in vitro binding and internalization by murine macrophages of α_2M* chemically modified with *cis*-DDP. We demonstrate via direct and competition binding studies that *cis*-DDP-α_2M*, RBF, and unmodified α_2M* bind to two distinct receptors on these cells. One binding site is a high affinity ($K_d \sim 50$ pM), RAP-insensitive binding site and the other is a lower affinity ($K_d \sim 4$ nM), RAP-sensitive binding site. *Cis*-DDP modification of α_2M does not significantly alter binding to either α_2MSR or LRP/α_2MR. *Cis*-DDP-α_2M* exhibits greatly decreased internalization by LRP/α_2MR, however, presumably due to decreased dissociation from LRP/α_2MR in endosomes and an interference with recycling of the receptor to the cell surface. The signaling interaction of α_2M* with the second α_2M receptor, α_2MSR, appears to be unaffected by *cis*-DDP modification since macrophages exposed to *cis*-DDP-α_2M* demonstrate an increase in IP_3 synthesis and $[Ca^{2+}]_i$ comparable with the effect induced by unmodified α_2M*.

Methods

Reagents and proteins. Culture medium was purchased from Life Technologies (Grand Island, NY). Bovine serum albumin and Hepes were purchased from Sigma Immunochemicals (St. Louis, MO). Fura-2/AM was purchased from Molecular Probes, Inc. (Eugene, OR). *Cis*-DDP, octylamine, and Freon were purchased from Aldrich Chemical Co. (Milwaukee, WI). 2-[^{3}H]*myo*-inositol (specific activity, 10–20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Gel filtration materials and glutathione-Sepharose were purchased from Pharmacia Biotech Inc. (Piscataway, NJ). ^{125}I for protein iodination was obtained from New England Nuclear (Boston, MA), and Iodobeads were purchased from Pierce (Rockford, IL). Isopropylthio-β-D-galactoside was obtained from 5 Prime–3 Prime, Inc. (Boulder, CO). Pefabloc and DNA modification enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). Hen egg white lysozyme and Sequenase 7-Deaza-dGTP DNA sequencing kits were obtained from United States Biochemical Corp. (Cleveland, OH). All other reagents were of the best commercial grade available.

Preparation of α_2M and cis-DDP-α_2M*.* Human α_2M was purified as previously described (19). α_2M was activated with 200 mM methylamine for 16–18 h at room temperature in the dark and dialyzed extensively against 20 mM Hepes, 150 mM NaCl, pH 7.4, to remove unreacted methylamine. The following method for *cis*-DDP treatment of α_2M* is a modification of that described by Gonias and Pizzo (43). Crystalline *cis*-DDP was dissolved in 20 mM Tris-HCl, 150 mM NaCl, pH 8.0, at $\sim$ 60°C and allowed to cool to 37°C. α_2M* was then incubated with 0.05 or 1.7 mM *cis*-DDP in the dark for 4 h at 37°C. The reaction mixture was then dialyzed extensively against 20 mM Hepes, 150 mM NaCl, pH 7.4, to remove unreacted *cis*-DDP. α_2M* and *cis*-DDP-α_2M* were radiolabeled with ^{125}I using the Iodobead method and applied to a gel filtration PD-10 column for separation from free ^{125}I. The term *cis*-DDP-α_2M* in this manuscript represents 1.7 mM *cis*-DDP–treated α_2M* unless otherwise stated.

Preparation of RBF. Base pairs representing the last 141 amino acid residues of rat α_1M (44) were inserted into the histidine tag expression vector using a PCR cloning strategy. Amplification was performed from the plasmid ATCC 63108 (1) using the following two oligonucleotides: 5′-GCCGGATCCGGCGG-TGAAGGAGAAG-CACCC-3′ and 5′-GGCCAAGCTTTTAGGCATTTCCTTG-3′. PCR amplification was carried out in an MJ research Minicycler using the GeneAmp kit from Perkin Elmer/Cetus (Emeryville, CA). The amplified insert was digested with BamHI and HindIII and ligated into the vector pQE30 obtained from QIAGEN Inc. (Chatsworth, CA).

DH5αF′IQ bacteria harboring the pQE30-6His RBF expression construct were grown at 37°C in 2XTY medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. When the optical density reached 0.8–1.0 at 600 nm, isopropylthio-β-D-galactoside was added to give a final concentration of 1 mM. The bacteria were grown for another 5 h and harvested by centrifugation for 10 min at 15,000 *g*. The pellet was resuspended in 1% of the original culture volume in phosphate buffer (50 mM Na_2HPO_4, pH 7.8, 300 mM NaCl). Lysozyme was added (1 mg/ml) and the bacterial suspension was placed on ice for 30 min. Next, 2% of the original volume in H_2O containing 0.2% (vol/vol) Triton X-100 and 0.5 mM Pefabloc was added forcibly to the lysate. DNase and $MgSO_4$ were added to give final concentrations of 20 mg/liter and 5 mM, respectively. The lysate was mixed well and placed on ice for another 30 min, after which it was centrifuged at 15,000 *g* for 40 min. The supernatant was mixed with 2 ml Ni-NTA agarose/liter of culture and stirred on ice for 1 h. After binding, the resin was packed into a small column and washed with phosphate buffer at 0.5 ml/min until the optical density at 280 nm was ≤ 0.01. The column was eluted with a gradient of 50–250 mM imidazole (total volume 80 ml) and 1-ml fractions were collected. 6His-RBF–containing fractions were identified by running 5 μl on a 12% SDS-PAGE gel and staining with Coomassie brilliant blue. Protein-containing fractions were pooled and dialyzed extensively against 20 mM Hepes, pH 7.3, containing 150 mM NaCl. If necessary, pools were concentrated first by placing in a dialysis bag at 4°C and coating with Sephadex G-50. Protein samples were sterile-filtered and stored in aliquots at −80°C. The amino-terminal sequence was confirmed by analysis on a sequencer (477A; Applied Biosystems, Foster City, CA). The first 20 amino acid residues are MRGSHHHHHHGSGGEGEAPF, with the first glutamate corresponding to rat α_1M residue 1336. The correct nucleotide sequence of the cloned RBF was confirmed by DNA sequencing.

Preparation of RAP. The pGEX 39-kD expression construct was the kind gift of Dr. Joachim Herz (University of Texas, Southwestern, Dallas, TX). The protein was expressed and purified as described in Herz et al. (45) with the following modifications. Before passing the bacterial lysate through needles, DNase and $MgCl_2$ were added to a final concentration of 20 mg/liter and 10 mM, respectively. After a 30-min incubation on ice, the cell debris were removed by centrifugation at 14,000 *g* for 40 min. During the affinity chromatography step, glutathione-Sepharose 4B (Pharmacia Biotech Inc.) was substituted for glutathione-agarose. Protein-containing fractions were pooled and dialyzed extensively against 20 mM Hepes, pH 7.3, containing 150 mM NaCl. Protein samples were sterile-filtered and stored in aliquots at −80°C. The GST component of the RAP-GST protein was cleaved and separated from RAP during the RAP purification using the affinity chromatography step described previously.

Macrophage harvesting. These procedures are given in detail elsewhere and will be described here only briefly (39). C57B1/6 mice were obtained from Charles River Laboratories (Raleigh, NC). Thioglycollate-elicited macrophages were obtained via peritoneal lavage with a solution of 20 mM Hepes, 150 mM NaCl, pH 7.4. The cells were pelleted by centrifugation at ~ 800 *g* for 5 min and resuspended in RPMI 1640 medium containing 12.5 U/ml penicillin, 6.5 μg/ml streptomycin, and 10% fetal bovine serum.

IP_3 measurements. Macrophages were plated on 6-well plates at a density of 4×10^6 cells/well in RPMI 1640 medium containing 12.5 U/ml penicillin, 6.5 μg/ml streptomycin, and 10% fetal bovine serum. The cells were allowed to adhere at 37°C for 3 h in a humidified 5% CO_2 incubator. Medium was aspirated from the monolayers and inositol-free RPMI 1640 medium containing 0.25% BSA was added, followed by addition of 2-[^{3}H]*myo*-inositol (8 μCi/ml) to each well. The cells were incubated at 37°C for an additional 16–18 h. Monolayers were rinsed three times with Hank's balanced salt solution containing 25 mM Hepes (HHBSS) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM LiCl, pH 7.4. A volume of 0.5 ml of this solution was added to each well and the cells were preincubated for 3 min at 37°C before stimulation with ligand for various time periods. The reaction was stopped by aspirating the medium containing the ligand and adding 6.25% perchloric acid solution. The cells were scraped and transferred to tubes containing 1 ml of octylamine/Freon (1:1, vol/vol) and 5 mM EDTA. The tubes were centrifuged at 5,600 *g* for 20 min at 4°C. The upper phase solution was applied to a 1-ml Dowex resin column (AG1-X8 formate; Bio Rad Laboratories, Richmond, CA) and eluted sequentially in a batch process with H_2O, and 50, 200, 400, 800, 1,200, and 2,000 mM ammonium formate containing 0.1 M formic acid (39). Aliquots were evaluated by a liquid scintillation counter to determine radioactivity.

$[Ca^{2+}]_i$ measurements. The methods used for measuring $[Ca^{2+}]_i$ have been published earlier by Misra et al. (39, 40). Briefly, macrophages were plated on glass coverslips sitting in 35-mm Petri dishes at a density of 1.5×10^5 cells/cm^2 and incubated for 16–18 h in a humidified 5% CO_2 incubator at 37°C. Cells were removed from the incubator and 4 μM Fura-2/AM was added and allowed to incubate with the cells for 30 min in the dark. $[Ca^{2+}]_i$ measurements were obtained using a digital imaging microscope as described earlier (39, 40). After obtaining baseline measurements for 5 min, ligands were added and multiple $[Ca^{2+}]_i$ measurements were taken.

Direct binding assays and Scatchard analysis. Macrophages were plated in 48-well plates (1×10^6 cells/well) and incubated for 3 h at 37°C in a humidified CO_2 incubator. The plates were moved to 4°C, and the cells were allowed to equilibrate for ~ 30 min. The monolayers were rinsed three times with ice-cold buffer A (HHBSS containing 12.5 U/ml penicillin, 6.5 μg/ml streptomycin, 5% bovine serum albumin). To assess nonspecific binding, some wells were rinsed three times with ice-cold buffer B (HHBSS without Ca^{2+} and Mg^{2+} containing 12.5 U/ml penicillin, 6.5 μg/ml streptomycin, 5% bovine serum albumin, 5 mM EDTA). Increasing concentrations of ^{125}I-ligands were added to each well and allowed to incubate at 4°C for 16–18 h. Radioligand solutions were removed from the wells, and the wells were rinsed two times in ice-cold buffer A or B. Solubilization solution was added to the wells (1.0 M NaOH, 0.1% SDS) and allowed to incubate at room temperature for ~ 5 h before transferring the solution to tubes to be counted in a γ-counter. Nonspecific binding was determined by using buffer B in some of the wells containing radioligands and averaged 10–30% of total binding over several assays. Specific binding is reported as total binding minus nonspecific binding. The competitor RAP was included in some wells along with ^{125}I-α_2M*, ^{125}I-*cis*-DDP-α_2M*, or ^{125}I-RBF to determine which class of binding sites represents LRP/α_2MR. Scatchard analysis was used to determine the best fit to either one- or two-site binding models, number of binding sites per cell, and an apparent K_d for each class of sites determined. Three or more independent experiments were used for the determination of the SEM for each ligand tested.

Multiple round uptake assay. Macrophage monolayers containing 250,000 cells/well were plated in 48-well plates and prepared as described in *Direct binding assays.* Increasing concentrations of ^{125}I-ligands were added to each well and allowed to incubate at 37°C for 2 h. Wells were rinsed two times in ice-cold buffer A or B, and well contents were solubilized as described in *Direct binding assays.* Nonspecific uptake was determined by using buffer B in some of the wells containing radioligand and averaged ≤ 10–20% of total uptake over several assays. Specific uptake is reported as total uptake minus nonspecific uptake.

Binding competition assays at 4°C. Binding competition assays were performed to determine K_d values for α_2M* and *cis*-DDP-α_2M* for use in some of the following assays. Briefly, macrophages were plated in 24-well (2×10^6 cells/well) or 48-well plates (1×10^6 cells/well) and prepared as described in *Direct binding assays.* ^{125}I-α_2M (1.0 nM) or ^{125}I-*cis*-DDP-α_2M* (15.0 nM) was added to each well along with various concentrations of unlabeled α_2M* or *cis*-DDP-α_2M*, respectively, and allowed to incubate at 4°C for 16–18 h. Radioligand solutions were removed from the wells, and the wells were rinsed two times in ice-cold buffer A or B. Well contents were solubilized as described in *Direct binding assays.* Nonspecific binding was determined by using buffer B in some of the wells containing radioligand and averaged ≤10% of total binding over several assays. Specific binding is reported as total binding minus nonspecific binding. Using the SYSTAT program from SYSTAT, Inc. (Evanston, IL), K_d values were obtained from the data as follows: α_2M* K_d = 0.6 nM r^2 = 0.98 and *cis*-DDP-α_2M* K_d = 7.0 nM r^2 = 0.98. The SEM in the region of the curve used to calculate the K_d was ≤ 10% for either ligand. These K_d values agree with previous studies of α_2M* (0.5–1.25 nM) and *cis*-DDP-α_2M* (11.0 nM) (42, 46).

Binding competition assays at 37°C. Binding competition assays with α_2M* and *cis*-DDP-α_2M* were also performed at 37°C in the presence of 75 μM chloroquine, which is known to inhibit internalization of α_2M* ligands, in order to determine K_d values at 37°C for use in some of the following assays. To ensure that the binding characteristics of α_2M* and *cis*-DDP-α_2M* at both 4 and 37°C were not affected by the presence of chloroquine, direct binding studies were first performed with ^{125}I-α_2M* and ^{125}I-*cis*-DDP-α_2M* in the absence and presence of 75 μM chloroquine. Then, binding competition studies were performed at 37°C in the presence of 75 μM chloroquine as described previously in *Binding competition assays at 4°C.* Using the SYSTAT program, K_d values were obtained from the data as follows: α_2M* K_d = 1.2 nM r^2 = 0.98 and *cis*-DDP-α_2M* K_d = 16.0 nM r^2 = 0.97. The SEM in the region of the curve used to calculate the K_d was ≤ 10% for either ligand. These values are not significantly different from those published previously for studies at 4°C or for the 4°C K_d values determined above in *Binding competition assays at 4°C.*

Single-round endocytosis assay. Macrophage monolayers were plated in 48-well plates and prepared as described in *Direct binding assays.* ^{125}I-α_2M (1.0 nM) or ^{125}I-*cis*-DDP-α_2M* (7.0 nM) was added to the monolayers at 4°C and allowed to bind for 4 h after which unbound ligand was rinsed away with buffer A or B. The monolayers were then quickly brought to 37°C. After different time intervals at 37°C, an aliquot of the medium was removed to determine degrada-

tion. The monolayers were rinsed two times with buffer A or B and then a pH 3.0 solution (50 mM glycine, 150 mM NaCl) was added to the cells and incubated at 4°C for 2 min before collecting for γ-counting to determine surface bound ligand. Well contents were solubilized as described in *Direct binding assays.* Degradation was determined by adding TCA to the aliquots of medium collected to a final concentration of 15%, centrifuging the samples, and incubating them on ice for several hours before collection of an aliquot for γ-counting. Nonspecific degradation, surface binding, and uptake were determined by using buffer B in some of the wells containing radioligand and averaged ≤ 10–20% of total binding or uptake and ≤ 30% of total degradation over several assays. Specific values are reported as total minus nonspecific.

pH 5.0 dissociation studies. The endocytic vesicles which α_2M* encounters during receptor-mediated endocytosis have been shown to have a pH of ~ 5.0±0.2, and several other ligands have been demonstrated to rapidly dissociate from their receptors at this pH, including epidermal growth factor, insulin, and asialoglycoproteins (47–50). Macrophages monolayers were plated in 48-well plates and prepared as described in *Direct binding assays.* ^{125}I-ligands were added to each well and allowed to incubate at 4°C for 16–18 h. Wells were rinsed two times in ice-cold buffer A or B, and either 0.25 ml of buffer A, adjusted to pH 5.0, or trypsin (50 μg/ml) in buffer A not containing BSA was added to the wells. After 30 min at 4°C, the solutions were collected and one rinse with buffer A or B was added to the collected solutions and the amount of released radioligand was counted on a γ-counter. Wells treated with the pH 5.0 solution were subsequently treated with the trypsin solution to determine if any additional radioligand could be removed from the cell surface. Remaining cell-associated ligand was solubilized as described in *Direct binding assays.* Nonspecific release and binding was determined by using buffer B in some of the wells containing radioligand and averaged ≤ 10% of total over several assays. Specific release or binding is reported as total minus nonspecific.

Receptor recycling assays using direct binding. Macrophage monolayers were plated in 48-well plates as described in *Direct binding assays.* Unlabeled α_2M (100 nM), RBF (300 nM), or 0.05 or 1.7 mM *cis*-DDP-α_2M (300 nM) was added to the wells and incubated for 90 min at 37°C. After 90 min, the monolayers were rinsed three times with ice-cold buffer A or B and rapidly cooled to 0–4°C on ice. Increasing concentrations of ^{125}I-α_2M were then added to the wells in buffer A or B and allowed to incubate for 6 h at 4°C. The radioligand solution was removed from the wells, and the wells were rinsed two times in ice-cold buffer A or B. Well contents were solubilized as described in *Direct binding assays.* Nonspecific binding was determined by using buffer B in some of the wells containing radioligands and averaged ≤ 10% of total binding over several assays. Specific binding is reported as total binding minus nonspecific binding.

Receptor recycling assays using antibodies to LRP/α_2MR. The polyclonal rabbit anti-LRP/α_2MR 777 antibody and the monoclonal mouse anti-LRP/α_2MR 5A6B6 antibody used in these studies were the kind gift of Dr. Dudley Strickland (American Red Cross, Rockville, MD). Macrophage monolayers were plated in 96-well plates at a concentration of 250,000 cells/well and prepared as described in *Direct binding assays.* Unlabeled α_2M (100 nM), RBF (300 nM), or 1.7 mM *cis*-DDP-α_2M (300 nM) was added to the wells in either buffer A or B and incubated for 90 min at 37°C. Some wells received no ligand treatment. After 90 min, the monolayers were rinsed three times with ice-cold buffer A or B and rapidly cooled to 0–4°C on ice. Blocking buffer (buffer A containing 1.0% goat IgG) was then added to the wells and allowed to incubate with the cells at 4°C for 1 h. Primary antibodies were then added to the cells and allowed to incubate at 4°C for 2 h. The cells were then rinsed several times with buffer A solution and the appropriate goat secondary antibody conjugated to horseradish peroxidase was added to the wells and allowed to incubate at 4°C for 1 h. The cells were again rinsed several times with buffer A solution and *o*-phenylenediamine dihydrochloride (OPD) substrate was added to the wells. After development of color, H_2SO_4 was added to the well contents and the absorbance at a wavelength of 490 nm was determined using a plate reader. Controls included primary antibodies only, secondary antibodies only, OPD substrate only, no cells, and pooled mouse IgG or rabbit IgG with appropriate secondary antibodies.

Results

The effect of α_2M and cis-DDP-α_2M* on IP$_3$ synthesis and [Ca^{2+}]$_i$ by macrophages.* We studied the ability of α_2M* and *cis*-DDP-α_2M* to induce IP$_3$ synthesis by macrophages. Fig. 1

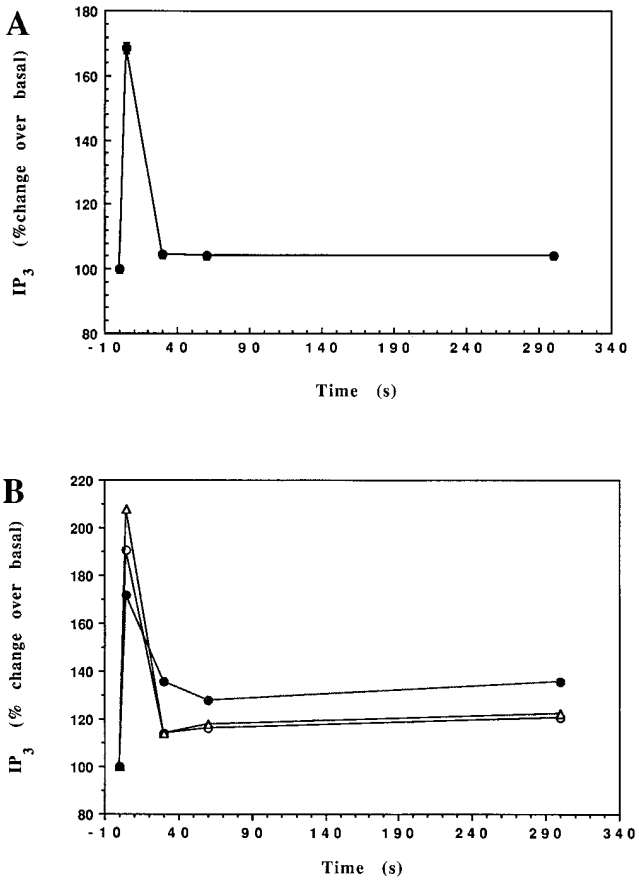

Figure 1. IP$_3$ formation in ligand-stimulated macrophages. (*A*) 2-[^{3}H]*myo*-inositol–labeled macrophages were exposed to 40 nM α_2M* (●) for various time periods and processed for quantitation of IP$_3$ radioactivity. Values represent the average of three independent experiments performed in triplicate. Values are expressed as the percent change in radioactivity over basal value from 0 to 300 s. (*B*) 2-[^{3}H]*myo*-inositol–labeled macrophages were exposed to 40 nM *cis*-DDP-α_2M* (○) for various time periods and processed for quantitation of IP$_3$ radioactivity. Values represent the average of three independent experiments performed in triplicate. Values are expressed as the percent change in radioactivity over basal value from 0 to 300 s. Experiments with *cis*-DDP-α_2M* using macrophages pretreated with pertussis toxin (△) or in the presence of excess RAP (●) are also represented. SEM values for each point are not shown in *B* to simplify presentation. SEM values for the spikes were 14.3, 14.7, and 7.4% for experiments with *cis*-DDP-α_2M* alone, with pertussis toxin–pretreated cells, and in the presence of excess RAP, respectively. Incubation of macrophages with buffer or free *cis*-DDP (100 μM) did not statistically alter IP$_3$ synthesis (data not shown) consistent with previous observations (39–41).

demonstrates that the generation of IP_3 upon ligand binding is similar for α_2M* and *cis*-DDP-α_2M*. Ligation with either α_2M* (Fig. 1 *A*) or *cis*-DDP-α_2M* (Fig. 1 *B*) resulted in a rapid but transient increase in IP_3 levels. The maximum increases in IP_3 levels within 5 s of stimulation with α_2M* and *cis*-DDP-α_2M* were 169 and 190% over basal levels, respectively. These values are comparable with the increase seen upon stimulation with α_2M* as previously reported (39). The increase in IP_3 stimulated by *cis*-DDP-α_2M* is not blocked by RAP in excess (172%) or pretreatment of the cells with pertussis toxin (208%) in agreement with the known characteristics of signaling by α_2M* ligation of the α_2MSR.

Concomitant with a rise in IP_3 levels, ligation of *cis*-DDP-α_2M* by macrophages resulted in a significant rise in $[Ca^{2+}]_i$. The cell shown in Fig. 2 demonstrated a twofold increase in $[Ca^{2+}]_i$ from a resting level of 169±6 nM. Multiple cells were examined for this study and analyzed by digital imaging microscopy using Fura-2/AM–loaded cells. Approximately 85–90% of the cells responded to *cis*-DDP-α_2M* exposure in a manner comparable with that shown in Fig. 2. This is similar to the rate of response seen in macrophages to α_2M* exposure (39). Exposure of cells to buffer, *cis*-DDP alone, or boiled *cis*-DDP-α_2M* did not result in any significant change in $[Ca^{2+}]_i$ (3, 5, and 6% maximum increase, respectively). The averages of the maximum percentage increase in $[Ca^{2+}]_i$ over basal values seen upon stimulation of macrophages with either α_2M* or *cis*-DDP-α_2M* were 185±14 and 212±28%, respectively.

α_2M, cis-DDP-α_2M*, and RBF binding to macrophages at 4°C.* Direct binding of ^{125}I-α_2M* to macrophages is shown in Fig. 3 *A*. The data represent the mean values obtained from two experiments performed in triplicate. The inset shows Scatchard analysis of the data which yields a two-site fit of $r^2 = 0.92$ for site 1 and $r^2 = 0.95$ for site 2. Analysis of binding of ^{125}I-α_2M* to site 1 yields an apparent K_d of 30±11 pM with 1,660±150 sites/cell. Analysis of binding of ^{125}I-α_2M* to site 2 yields an apparent K_d of 3.7±0.2 nM with 21,500±300 sites/cell. Direct binding of α_2M* in the presence of 100-fold molar excess of RAP is given in Fig. 3 *B* with an inset of the Scatchard analysis. The data represent the mean values obtained from two experiments performed in triplicate. Scatchard analysis of the binding to site 1 in the presence of RAP yields an apparent K_d of 60±15 pM with 1,860±200 sites/cell. Analysis of binding to site 2 yields an apparent K_d of 2.4±0.1 nM with 7,200±100 sites/cell. Direct binding of ^{125}I-*cis*-DDP-α_2M* to macrophages is shown in Fig. 4 *A*. The data represent the mean values obtained from two experiments performed in triplicate. The inset shows Scatchard analysis of the data which yields a two-site fit of $r^2 = 0.98$ for site 1 and $r^2 = 0.99$ for site 2.

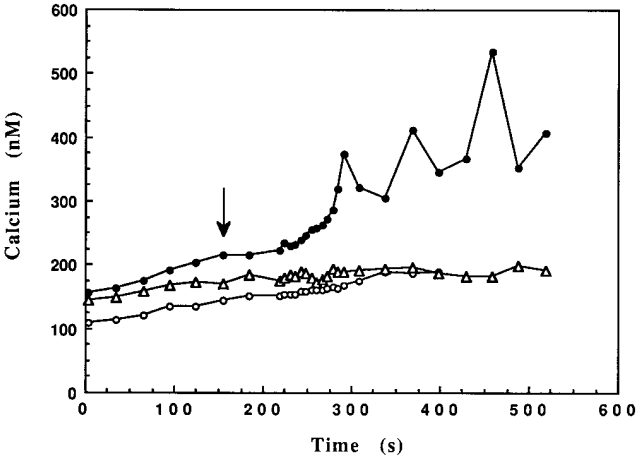

Figure 2. Representative $[Ca^{2+}]_i$ signal upon *cis*-DDP-α_2M* stimulation. Macrophages were preloaded with 4 μM Fura-2/AM for 30 min at 37°C and changes in $[Ca^{2+}]_i$ after stimulation with α_2M* or *cis*-DDP-α_2M* (40 nM) were measured according to published methods (39, 40). Typical cell responses to stimulation with *cis*-DDP-α_2M* (●), *cis*-DDP alone (○), and boiled *cis*-DDP-α_2M* (△) are shown. The changes in $[Ca^{2+}]_i$ are representative of three independent experiments using 55–60 individual cells in each experiment. The arrow indicates the time of addition of ligand. The average increases in $[Ca^{2+}]_i$±SEM for stimulation with α_2M* or *cis*-DDP-α_2M* are given in the text.

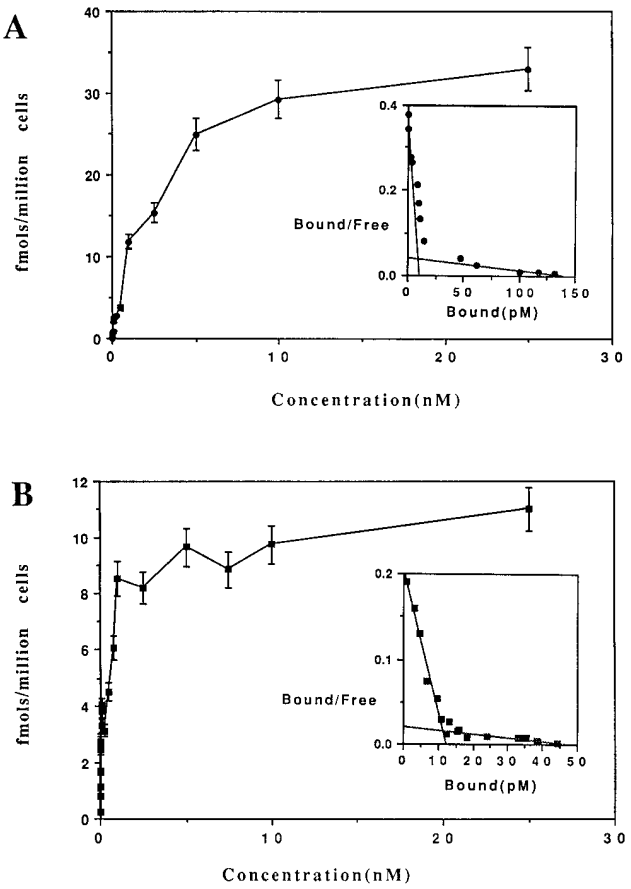

Figure 3. Direct binding of ^{125}I-α_2M* by macrophages at 4°C in the absence or presence of 100-fold molar excess RAP. (*A*) Femtomoles of bound ^{125}I-α_2M* at increasing concentrations of ^{125}I-α_2M*. Increasing concentrations of ^{125}I-α_2M* were added to macrophage monolayers at 4°C for 16–18 h. Cell-associated activity was collected and counted in a γ-counter. The inset shows the lines derived by Scatchard analysis of the data. (*B*) Femtomoles of ^{125}I-α_2M* bound in the presence of 100-fold molar excess of RAP. Increasing concentrations of ^{125}I-α_2M* were added to macrophage monolayers at 4°C for 16–18 h in the presence of 100-fold molar excess of RAP. Cell-associated activity was collected and counted in a γ-counter. The inset shows the lines derived from Scatchard analysis of the data. Nonspecific binding was determined in the presence of 5 mM EDTA and subtracted from the total binding at each concentration tested. The details of the Scatchard analysis are given in the text.

Analysis of binding of ^{125}I-*cis*-DDP-α_2M* to site 1 yields an apparent K_d of 50±10 pM with 1,250±250 sites/cell. Analysis of binding of ^{125}I-*cis*-DDP-α_2M* to site 2 yields an apparent K_d of 25±2 nM with 19,000±200 sites/cell. Direct binding of *cis*-DDP-α_2M* in the presence of 100-fold molar excess of RAP is given in Fig. 4 *B* with an inset of the Scatchard analysis. The data represent the mean values obtained from two experiments performed in triplicate. Scatchard analysis of the binding to site 1 in the presence of RAP yields an apparent K_d of 120±45 pM with 1,860±200 sites/cell. Analysis of binding to site 2 yields an apparent K_d of 10±0.5 nM with 6,800±100 sites/cell. The data concerning direct binding of the *cis*-DDP-α_2M* shown in Fig. 4 demonstrate that the binding characteristics of the chemically modified α_2M* are similar to unmodified α_2M* for the high affinity site and the K_d value for the lower affinity site is slightly higher than for unmodified α_2M* as expected from previous studies (42, 43). *Cis*-DDP-α_2M* is able to occupy the same number of high and low affinity receptor sites (1,250±250 sites/cell and 19,000±200 sites/cell, respectively) as unmodified α_2M*. The ability of RAP to inhibit ~67–90% of α_2M* or *cis*-DDP-α_2M* binding is consistent with observations of Warshawsky et al. (51) and Williams et al. (38) studying RAP competition for α_2M* binding to rat hepatoma cells and purified LRP/α_2MR, respectively. Determinations of K_d values for RBF to the two binding sites were also obtained using direct binding techniques. Analysis of the data yields a two-site fit of $r^2 = 0.91$ for site 1 with an apparent K_d of 140±30 pM and $r^2 = 0.99$ for site 2 with an apparent K_d of 38±5 nM.

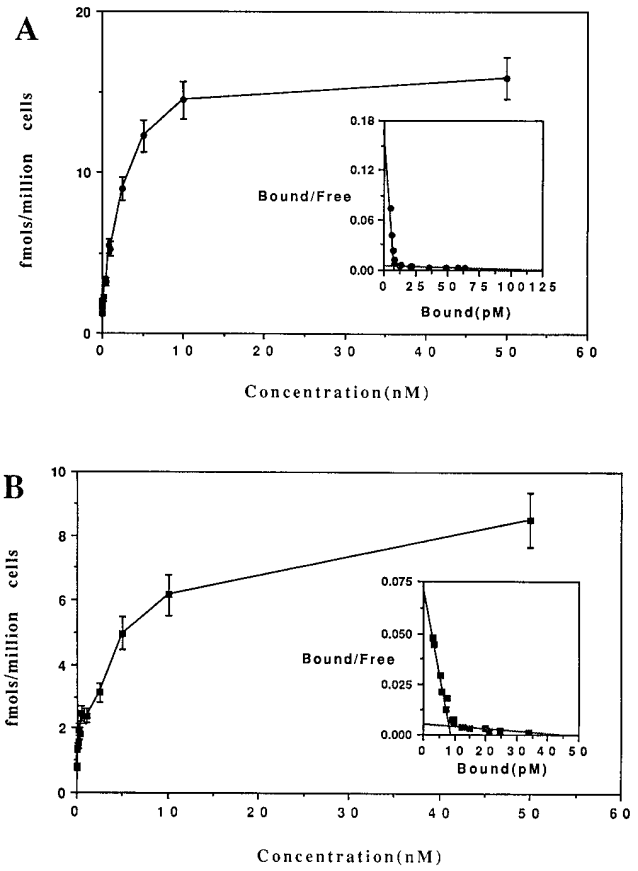

Figure 4. Direct binding of ^{125}I-*cis*-DDP-α_2M* by macrophages at 4°C in the absence or presence of 100-fold molar excess RAP. (*A*) Femtomoles of bound ^{125}I-*cis*-DDP-α_2M* at increasing concentrations of ^{125}I-*cis*-DDP-α_2M*. Increasing concentrations of ^{125}I-*cis*-DDP-α_2M* were added to macrophage monolayers at 4°C for 16–18 h. Cell-associated activity was collected and counted in a γ-counter. The inset shows the lines derived by Scatchard analysis of the data. (*B*) Femtomoles of ^{125}I-*cis*-DDP-α_2M* bound in the presence of 100-fold molar excess of RAP. Increasing concentrations of ^{125}I-*cis*-DDP-α_2M* were added to macrophage monolayers at 4°C for 16–18 h in the presence of 100-fold molar excess of RAP. Cell-associated activity was collected and counted in a γ-counter. The inset shows the lines derived from Scatchard analysis of the data. Nonspecific binding was determined in the presence of 5 mM EDTA and subtracted from the total binding at each concentration tested. The details of the Scatchard analysis are given in the text.

α_2M, RBF, and cis-DDP-α_2M* internalization by macrophages at 37°C.* Internalization involving multiple rounds of endocytosis at increasing concentrations of ^{125}I-α_2M* and ^{125}I-*cis*-DDP-α_2M* is compared in Fig. 5. At each concentration, the internalization of the modified form of α_2M* is ~ 18% or less than that of the unmodified α_2M*. Incubation of cells with *cis*-DDP alone before incubation with ^{125}I-α_2M* was not found to affect internalization of the radioligand. The data represent the mean values obtained from three experiments performed in duplicate.

Single-round endocytosis studies of ^{125}I-α_2M* and ^{125}I-*cis*-DDP-α_2M* are shown in Fig. 6, *A* and *B*, respectively. In either case, all of the surface bound ligand was rapidly internalized within 15 min of exposure to 37°C and the amount of ligand found inside the cells began to decrease by 45–60 min. Although internalization of ligands was comparable, degradation differed in that degradation products of α_2M* could be identified after a lag of ~ 20 min whereas degradation of *cis*-DDP-α_2M* appeared to have a longer lag time of ~ 50 min. The data represent the mean values obtained from two experiments performed in triplicate.

pH 5.0 dissociation experiments. Studies of the dissociation of 4°C bound ^{125}I-α_2M* and ^{125}I-*cis*-DDP-α_2M* in a pH 5.0 or trypsin solution are given in Fig. 7, *A* and *B*, respectively. Trypsin treatment alone released 53±7% of bound ^{125}I-α_2M*,

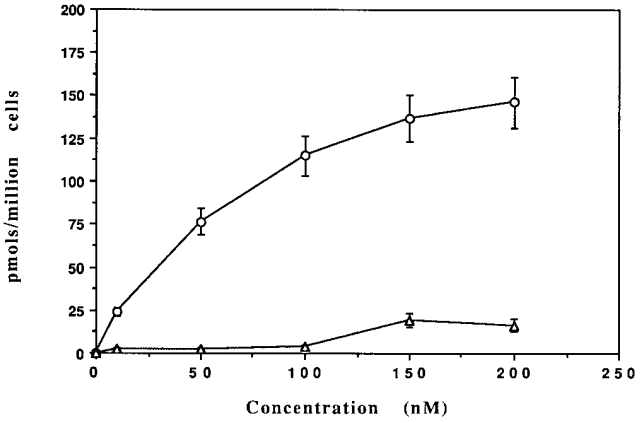

Figure 5. Multiple round internalization of ^{125}I-ligands by macrophages at 37°C. Increasing concentrations of α_2M* (○) or *cis*-DDP-α_2M* (△) were added to macrophage monolayers for 2 h at 37°C. Cell-associated radioactivity was collected and counted in a γ-counter. Nonspecific internalization was determined in the presence of 5 mM EDTA and subtracted from total internalization.

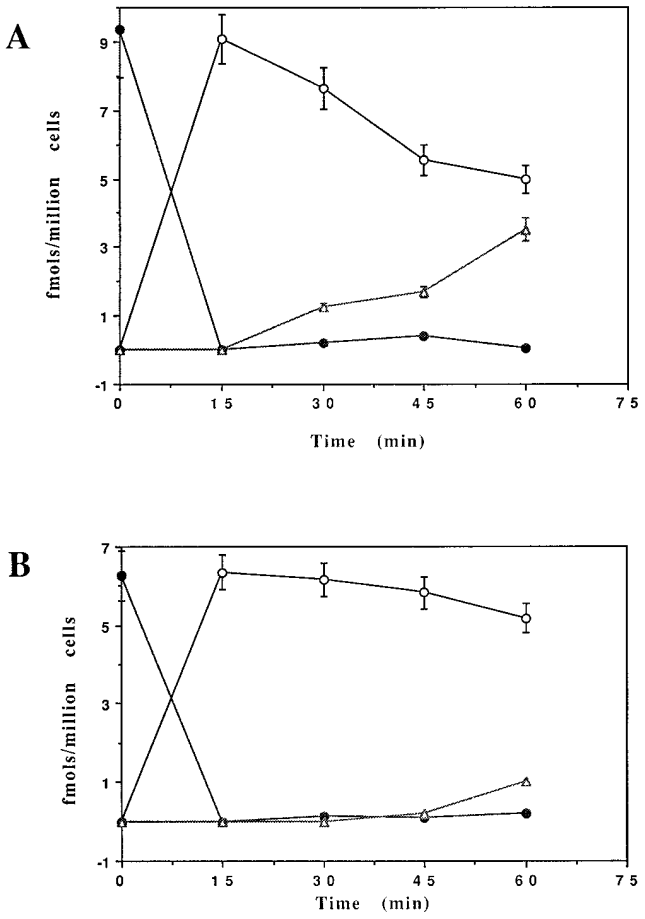

Figure 6. Single-round endocytosis of surface bound ^{125}I-ligands by macrophages. (*A*) Endocytosis of ^{125}I-α_2M*. 1.0 nM of ^{125}I-α_2M* was added to macrophage monolayers and incubated for 6 h at 4°C. Cells were rinsed and the monolayers were warmed quickly to 37°C. At time intervals, aliquots of medium were taken for determination of degradation of ligand by TCA precipitation, and a pH 3.0 solution was added to determine surface bound ligand. Remaining cell-associated radioactivity was collected and counted in a γ-counter. (*B*) Endocytosis of ^{125}I-*cis*-DDP-α_2M*. 7.0 nM of ^{125}I-*cis*-DDP-α_2M* was added to macrophage monolayers and incubated for 6 h at 4°C. Cells were rinsed and the monolayers were warmed quickly to 37°C. At time intervals, aliquots of medium were taken for determination of degradation of ligand by TCA precipitation, and a pH 3.0 solution was added to determine surface bound ligand. Remaining cell-associated radioactivity was collected and counted in a γ-counter. Data are presented for surface bound ligand (●), internalized ligand (○), and degraded ligand (△) with increasing time at 37°C.

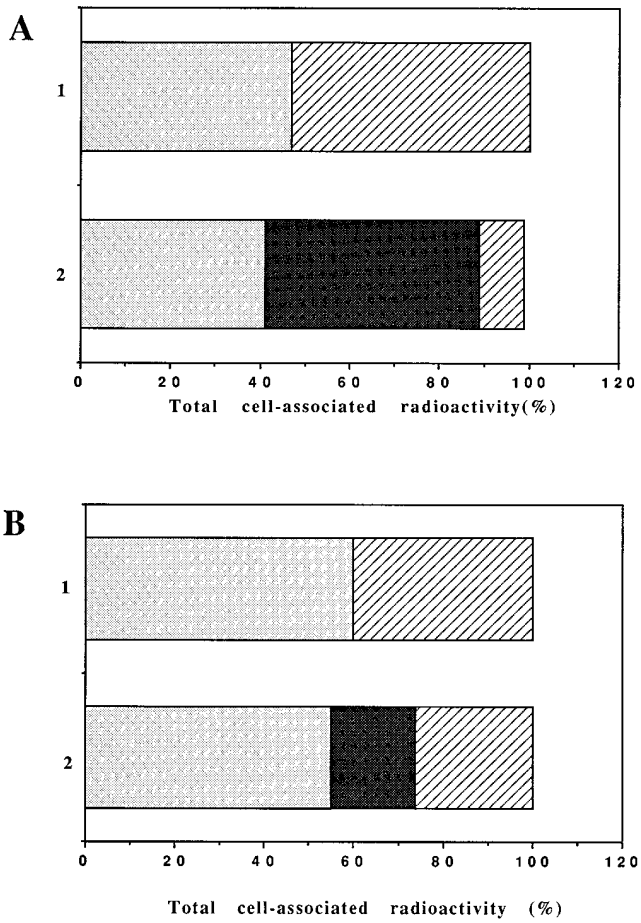

Figure 7. pH 5.0 dissociation of bound ^{125}I-ligands. Either 1.0 nM ^{125}I-α_2M* (*A*) or 7.0 nM ^{125}I-*cis*-DDP-α_2M* (*B*) was added to macrophage monolayers at 4°C for 16–18 h. Cells were rinsed and a trypsin (50 μg/ml) solution was added and incubated with the cells for 30 min at 4°C (*1*) or the cells were rinsed and treated with the pH 5.0 solution for 30 min at 4°C, followed by treatment with the trypsin solution for 30 min at 4°C (*2*). Released ligand and cell-associated radioactivity were collected and counted in a γ-counter. The values are given as a percentage of the total amount of ligand bound after 16–18 h at 4°C. The percentage of ligand which is cell associated is shown as the gray solid compartment. The percentage of ligand released into the medium with the pH 5.0 treatment is shown as the black solid compartment. The percentage of ligand released by trypsin treatment after the pH 5.0 treatment is shown as the diagonal striped compartment. The specific SEM values for the various percent values are given in the text but the values were ≤ 9%.

leaving 47±5% associated with the cell surface. Treatment of cells with the pH 5.0 solution released 48±5% of bound ^{125}I-α_2M*. Subsequent treatment of these cells with trypsin solution released an additional 10±2% of bound ^{125}I-α_2M*, leaving 42±5% associated with the cell surface. The cause of this retained binding of ^{125}I-α_2M* to the cell surface is unclear, but similar results are presented in studies by Ney et al. (13) who also demonstrated that no covalent cross-linking between the receptor and ligand occurs to account for this retained binding. Trypsin treatment alone released 39±8% of bound ^{125}I-*cis*-DDP-α_2M*, leaving 61±9% associated with the cell surface. Treatment of cells with the pH 5.0 solution released only 19±2% of bound ^{125}I-*cis*-DDP-α_2M*. Subsequent treatment of these cells with trypsin solution released an additional 25±1% of bound ^{125}I-*cis*-DDP-α_2M*, leaving 56±7% associated with the cell surface. The release of ^{125}I-*cis*-DDP-α_2M* at pH 5.0 is ~ 40% of the release of ^{125}I-α_2M* at pH 5.0. The data represent the mean values obtained from two experiments performed in quadruplicate.

Receptor recycling studies using direct binding. The direct binding of ^{125}I-α_2M* to macrophages after exposure to either 20 nM α_2M*, 500 nM RBF, or 300 nM of 0.05-mM–treated or 1.7-mM–treated *cis*-DDP-α_2M* is given in Fig. 8. Pretreatment of cells for 90 min at 37°C with either α_2M* or RBF allows for the average binding of 500±50 fmol/million cells when exposed to 50 nM ^{125}I-α_2M* at 4°C. In comparison, pretreatment

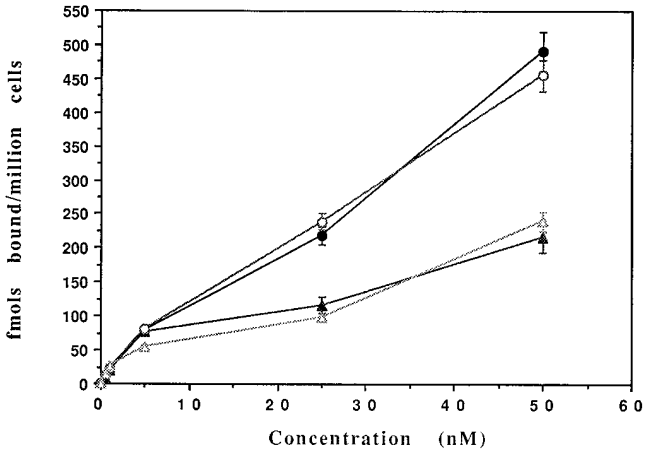

Figure 8. Recycling of α_2M* surface receptors: direct binding assay. Macrophage monolayers were incubated with either 20 nM α_2M* (●), 500 nM RBF (○), 300 nM of 0.05 mM–treated *cis*-DDP-α_2M* (▲), or 300 nM of 1.7 mM–treated *cis*-DDP-α_2M* (△) for 90 min at 37°C. Cells were rinsed and the monolayers were quickly cooled to 4°C. Increasing concentrations of ^{125}I-α_2M* were added to macrophage monolayers for 6 h at 4°C. Cell-associated radioactivity was collected and counted in a γ-counter. Nonspecific binding was determined in the presence of 5 mM EDTA and subtracted from the total binding at each concentration tested.

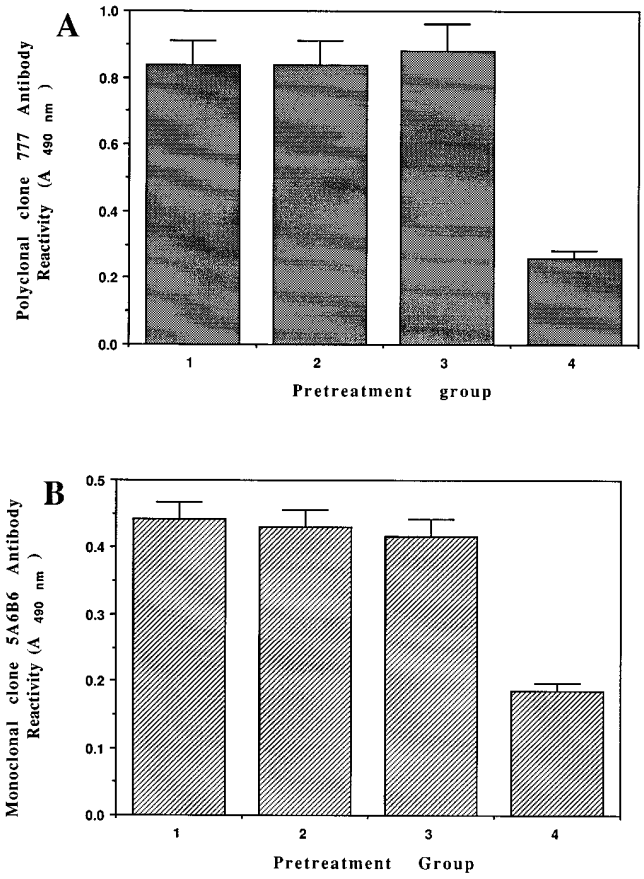

Figure 9. Recycling of α_2M* surface receptors: anti-LRP/α_2MR assay. Macrophage monolayers were incubated with either no ligand (*1*), 20 nM α_2M* (*2*), 500 nM RBF (*3*), or 300 nM of 1.7 mM–treated *cis*-DDP-α_2M* (*4*) for 90 min at 37°C. Cells were rinsed and the monolayers were quickly cooled to 4°C. Primary polyclonal or monoclonal anti-LRP/α_2MR antibodies were added and incubated with the cells at 4°C. Secondary antibodies conjugated to horseradish peroxidase were subsequently added, and *o*-phenylenediamine dihydrochloride substrate was added before analysis of absorbance at a wavelength of 490 nm by a plate reader. Control incubations were performed as described in the text.

with 1.7-mM–treated *cis*-DDP-α_2M* results in a decreased capacity for surface binding of ^{125}I-α_2M* by 50%. Pretreatment with α_2M* modified with more than a 50-fold lower concentration of *cis*-DDP (0.05 mM) yielded the same results. The concentrations chosen for pretreatment are ~ 40-fold the K_d values for the ligands based on 4°C binding competition studies. Incubation of the cells with *cis*-DDP alone was not found to alter the direct binding of ^{125}I-α_2M* from that of no pretreatment. The data represent the mean values obtained from three experiments performed in triplicate.

Receptor recycling assays using antibodies to LRP/α_2MR. The reactivity of the cell surface of macrophages to anti-LRP/α_2MR antibodies with no ligand pretreatment and with pretreatment with either 20 nM α_2M*, 300 nM *cis*-DDP-α_2M*, or 500 nM RBF is given in Fig. 9. Reactivity to the polyclonal anti-LRP/α_2MR antibody is shown in Fig. 9 *A*, and reactivity to the monoclonal anti-LRP/α_2MR antibody is shown in Fig. 9 *B*. Reactivity to either antibody after pretreatment with α_2M* or RBF was not significantly different from that of the reactivity with no pretreatment. Reactivity to the polyclonal and monoclonal antibodies after pretreatment with *cis*-DDP-α_2M*, however, was decreased by 69±6 and 58±6%, respectively. The data represent the mean values obtained from two experiments performed in triplicate. Incubation of cells with *cis*-DDP alone was not found to alter the antibody reactivities as compared with the no pretreatment findings (data not shown).

Discussion

α_2M* is capable of binding to both LRP/α_2MR (24) and α_2MSR (39, 41), but only the latter is coupled to a signaling cascade activated by α_2M* binding. While α_2M* binding to LRP/α_2MR is inhibitable by RAP, binding of α_2M* to α_2MSR is unaffected by RAP (41). Exposure of macrophages to α_2M* results in the rapid generation of IP_3, followed by a rise in $[Ca^{2+}]_i$. Increases are also seen in cAMP levels, activities of several different phospholipases, and the activity of protein kinase C (52). These signaling events do not occur with exposure of macrophages to native α_2M, thus a signaling cascade would only be activated in vivo in situations involving proteolysis.

Direct α_2M* binding data presented in this paper identify two classes of binding sites on macrophages. It is possible that the high affinity site (50 pM) represents the dimerization of receptors by the multivalent ligand α_2M*; however, direct binding experiments using the monomeric RBF also identify two similar classes of binding sites. Therefore, this second class of sites is not due to dimerization of receptors by the multivalent α_2M* but represents a distinct class of cell surface binding sites. We hypothesize that the 50 pM site, which is insensitive to binding competition with RAP, represents α_2MSR and that the 3.7 nM RAP-sensitive site represents LRP/α_2MR. This designation of the lower affinity site as LRP/α_2MR is consistent with the known characteristics of the receptor, including RAP competition and number of sites per cell (24). Additionally, several other authors have described similar K_d values for

α_2M* binding to this receptor on cells ranging from 1.6 to 5.5 nM (28, 53–55).

Certain chemical modifications of α_2M* have been shown to have effects on binding and/or clearance characteristics. One particular modification with the antitumor agent, *cis*-DDP, was of interest due to its dramatic effect on clearance with little apparent effect on binding to macrophages (42). No studies have been performed concerning the effects of *cis*-DDP modification of α_2M* on its ability to elicit cellular signaling since the α_2MSR was only recently discovered, and specific internalization studies with macrophages have not been explored. In this study, we show that *cis*-DDP modification of α_2M* has no effects on its ability to initiate signaling cascades. *Cis*-DDP-α_2M* elicits a rapid increase in IP_3 followed by an increase in $[Ca^{2+}]_i$ similar to α_2M*. Binding studies using Scatchard analysis likewise demonstrate no change in the binding to the high affinity, RAP-insensitive binding site. Additionally, the capacity of binding to the lower affinity, RAP-sensitive site remains unaltered. However, a significant effect of *cis*-DDP modification on the internalization via LRP/α_2MR over a period of 2 h is demonstrated. The internalization of α_2M* is decreased by $> 80\%$ after treatment with *cis*-DDP. Studies were completed to investigate the mechanism of this decreased internalization given the apparently unaltered binding to LRP/α_2MR.

Endocytosis of α_2M* is thought to occur after a clustering of α_2M*-LRP/α_2MR complexes in clathrin-coated pits on the cell surface (22, 56–59). Complexes then enter endosomes and possibly early lysosomes where the pH decreases to 5.0 and the ligand dissociates from the receptor (47). Typically, the ligand continues on to be degraded in lysosomes, and the receptor is recycled to the surface where it is free to bind another ligand and undergo endocytosis again (60). It has been proposed that if a ligand fails to dissociate from its receptor, the ligand may either be recycled with the receptor to the cell surface or result in degradation of the entire ligand–receptor complex (61). Studies of pH 5.0 dissociation of bound α_2M* and *cis*-DDP-α_2M* suggest that the chemical modification with *cis*-DDP interferes with the ability of α_2M* to dissociate from its receptor in endosomes. This endosomal pH is thought to cause a conformational change in the ligand which facilitates dissociation from the receptor. The cross-linking of amino acid residues within α_2M* which occurs with *cis*-DDP modification may result in a decreased ability to change conformation with changes in pH . If the *cis*-DDP-α_2M* does remain associated with the receptor, one possibility is that the entire complex would recycle to the cell surface. Continual exposure to *cis*-DDP-α_2M* during multiple rounds of endocytosis would therefore result in very little accumulation of the ligand intracellularly.

One possibility is that the failure of the ligand to dissociate from the receptor may result in an intracellular localization of the receptor or even accelerated degradation of the receptor. Binding studies show that α_2M* is a very effective competitor of *cis*-DDP-α_2M* for binding to cell surface receptors, and it has been demonstrated previously that no covalent association occurs between α_2M* and its receptor (13). Thus, it would be expected that at high enough concentrations, α_2M* would be able to compete off any *cis*-DDP-α_2M* which recycled with a receptor to the cell surface and saturate the same number of available receptors as if no exposure to *cis*-DDP-α_2M* had occurred. Thus, experiments were performed investigating the direct binding of α_2M* after pretreatment at 37°C with α_2M* or *cis*-DDP-α_2M*. The data suggest that there is a 50% decrease in the number of available receptors on the surface. It is also possible that LRP/α_2MR recycles to the surface after internalization of *cis*-DDP-α_2M* but is unable to bind new α_2M*; however, studies using anti-LRP/α_2MR antibodies also suggest a decrease in the available amount of LRP/α_2MR on the cell surface after internalization of *cis*-DDP-α_2M*. Whether this apparent decrease in surface receptors is due to an intracellular localization of receptors or the accelerated degradation of ligand–receptor complexes cannot be determined from these experiments; however, the single-round endocytosis studies of *cis*-DDP-α_2M* suggest that degradation may actually be delayed instead of accelerated. Finally, these studies cannot exclude the possibility that *cis*-DDP-α_2M* interaction with LRP/α_2MR results in a subtle alteration of the receptor which makes it appear as if the surface receptor concentration is decreased when it is not.

Of interest is the fact that modification of α_2M* with a very low concentration of *cis*-DDP (50 μM) results in a *cis*-DDP-α_2M* ligand which has similar effects on the number of available α_2M* surface receptors as that of the high *cis*-DDP concentration modification. Clinically, levels of 50 μM cisplatin are achieved in the plasma after short-infusion high-dose chemotherapy.[2] Previous studies have demonstrated that the large majority of cisplatin is bound rapidly by plasma proteins, including albumin (62–64). Studies of albumin and α_2M have shown that *cis*-DDP–albumin complexes are capable of chemically modifying α_2M as effectively as *cis*-DDP alone (64). With such a large reservoir of available *cis*-DDP for reaction with α_2M, it is possible that significant amounts of α_2M are modified as in these studies. The effects of decreasing the available LRP/α_2MR on cells which are exposed to this modified α_2M are unknown. Given that the modified α_2M* is still capable of eliciting intracellular signaling, the signaling may be potentiated by a decrease in the clearance of the ligand from the cell surface. The results of potentiating this cellular signaling are unknown. Internalization of α_2M*–antigen complexes is involved in the antigen presentation enhancement seen with α_2M*; thus, it is possible that a decrease in the surface LRP/α_2MR concentration would result in a decrease in this mode of antigen presentation. LRP/α_2MR is an endocytic receptor for many ligands besides α_2M* whose roles vary from lipoprotein metabolism to plasminogen activation. Interference with the LRP/α_2MR receptor system may play a role in the therapeutic and/or toxic effects of *cis*-DDP by affecting these different ligand systems. It is also possible that chemical modification of other serum proteins occurs with effects on pH-dependent ligand–receptor dissociation in other systems.

Acknowledgments

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2. This information was supplied by Bill Petros and represents results from studies in the bone marrow transplant unit at Duke University Medical Center of plasma platinum levels in patients undergoing short-infusion high-dose chemotherapy with cisplatin.

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