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In Vitro Binding and In Vivo Clearance of Human α_2-Macroglobulin after Reaction with Endoproteases from Four Different Classes

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Summary: The binding of human α_2-macroglobulin complexed with trypsin, papain, thermolysin and cathepsin-D to murine macrophages was studied at 4°C. Similar dissociation constants (0.4 nM) were determined for all of the complexes except α_2- macroglobulin-cathepsin-D (0.7 nM). Radioiodinated α_2-macroglobulin-protease complexes were injected into mice, and the clearance studied. Native α_2-macroglobulin cleared slowly, as previously reported, while greater than 50% of the complexes formed with trypsin, papain and thermolysin cleared in less than 5 min. The clearance of α_2-macroglobulin-cathepsin-D was biphasic, suggesting that only about half the α_2-macroglobulin was present in a reacted complex.

Human α_2-macroglobulin (α_2M) is a high molecular weight plasma protein that inhibits endoproteases from all four major classes (1). The molecule, M_r about 718,000, is composed of four identical subunits and is formed by the noncovalent association of disulfide bonded pairs (2). α_2M-serine protease complexes specifically bind to macrophages that do not recognize native α_2M (3). The cell binding and clearance of α_2M-methylamine and α_2M-trypsin are similar (4,5). Thus, it has been suggested that receptor recognition of α_2M-protease complex depends on the conformational change induced in the α_2M and not on the reactant that induced that change.

Macrophages undergo significant functional changes under the influence of various stimuli (6-8). The binding of α_2M-protease complexes, in particular, regulates macrophage function (9). α_2M can scavenge exogenous as well as endogenous proteases in vivo (10,11). Thus, α_2M complexed to proteases of diverse origin may be of significance in regulating macrophage function.

The purpose of this study was to further define the specificity of the binding of α_2M-protease complexes to macrophages. While it has been suggested that the binding of α_2M complexes is independent of the protease, this has not been tested

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rigorously. In this study, the binding of α_2M complexed with a representative of each of the four classes of protease is compared.

Experimental Procedures

Materials: Murine peritoneal macrophages were obtained from C57B1/6 mice 3 days after an intraperitoneal injection of Brewer's thioglycollate broth and plated at a density of 500,000 cells per well as previously described (12). CD-1 female mice were obtained from Charles River laboratories. Trypsin was obtained from Worthington. All other enzymes were from Sigma. ^{125}I was obtained from New England Nuclear; and lactoperoxidase, coupled to Sepharose beads, was obtained from P-L Biochemicals. All other reagents were of the highest quality available.

Methods: α_2M was purified by the method of Kurecki (13) as previously modified (4). Trypsin was active site titrated as described by Chase and Shaw (14). α_2M-trypsin complexes were prepared by incubating α_2M with a five fold molar excess of active trypsin for five min at room temperature in 0.02 M sodium phosphate, 0.15 M NaCl,pH 7.4. Soybean trypsin inhibitor was added to stop the reaction, and complex was separated from uncomplexed protease on a Sephadex G-150 column.

Thermolysin complexes were prepared by incubating a ten fold molar excess of thermolysin with α_2M in 0.01 M $CaCl_2$, 0.01 M Tris, pH 8.0, for 30 min at room temperature. Complex was purified on a Sephadex G-150 column. Papain complexes were prepared by first incubating papain in 0.05 M sodium phosphate, 5 mM cysteine, 2 mM EDTA, pH 7.3 for 5 min at room temperature. A 20-fold molar excess of this protease was added to α_2M in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4 and allowed to react for 30 min at 4°C. Complex was resolved by Sephadex G-150 chromatography and concentrated by dialysis against a solution of 30% polyethylene glycol (average molecular weight 20,000).

Cathepsin-D complexes were prepared by incubating a ten-fold molar excess of the protease with α_2M at pH 6.0, and ambient temperature for nine days. An additional ten-fold molar excess of protease was then added; and, after four more days, the mixture was chromatographed on a Sephadex G-150 column. Native α_2M after incubation for a comparable period of time in the same buffer showed no change in electrophoretic mobility. The stoichiometry of the α_2M-cathepsin-D reaction was determined with gel filtration chromatography as previously described (15).

α_2M-methylamine was prepared as previously reported (15). The concentration of inhibitor in solutions containing unreacted α_2M or α_2M-methylamine was determined spectrophotometrically assuming an absorption coefficient of 8.93 (15,16). The concentration of α_2M-protease complexes was determined by Lowry protein assay (17) using unreacted α_2M as a standard. Nondenaturing gel electrophoresis was conducted on a Tris-borate polyacrylamide gel system (4,18).

Iodination of complexes was performed by the solid phase lactoperoxidase method (19). Macrophage binding and in vivo clearance studies in mice were performed as previously reported (4,20).

Results

Reaction with methylamine, trypsin, papain or thermolysin, under the conditions described, resulted in a nearly complete transition in the electrophoretic mobility of the α_2M (from "slow" to "fast") (Fig. 1). This transition is correlated with conformational change in the inhibitor and loss of further protease binding

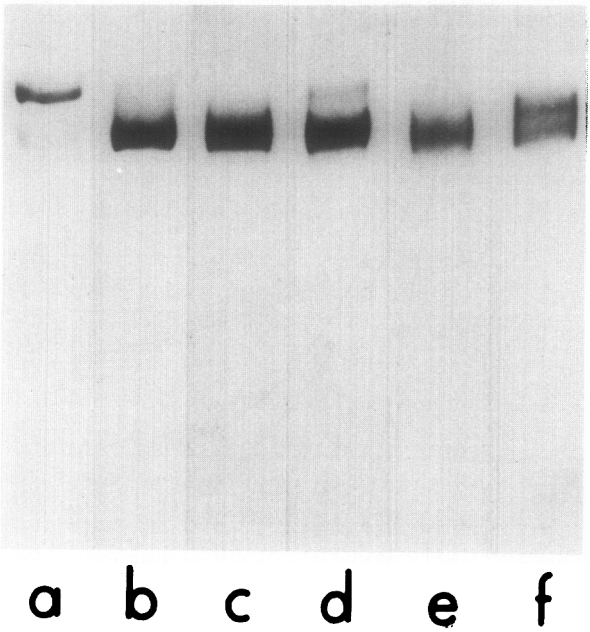

Figure 1. Polyacrylamide gel electrophoresis of α_2M and its complexes. Lane a, native α_2M; lane b, α_2M-methylamine; lane c, α_2M-trypsin; lane d, α_2M-papain; lane e, α_2M-thermolysin; lane f, α_2M-cathepsin-D.

capacity (15,18). Only part of the α_2M showed increased mobility after reaction with cathepsin-D. This result, suggesting heterogeneity in the reacted population of inhibitor molecules, was confirmed with stoichiometry experiments that demonstrated no more than 0.5 mol of cathepsin-D bound per mol of α_2M.

Results are presented in Table 1 for the binding of α_2M-protease complexes to macrophages at 4°C. Experiments were performed in the presence of calcium

Table I

Dissociation constants of α_2M-protease complexes

Complex	K_d(nM)
α_2M-trypsin	0.5
α_2M-papain	0.4
α_2M-thermolysin	0.3
α_2M-cathepsin-D	0.7
α_2M-methylamine	0.4
Native α_2M	17.0

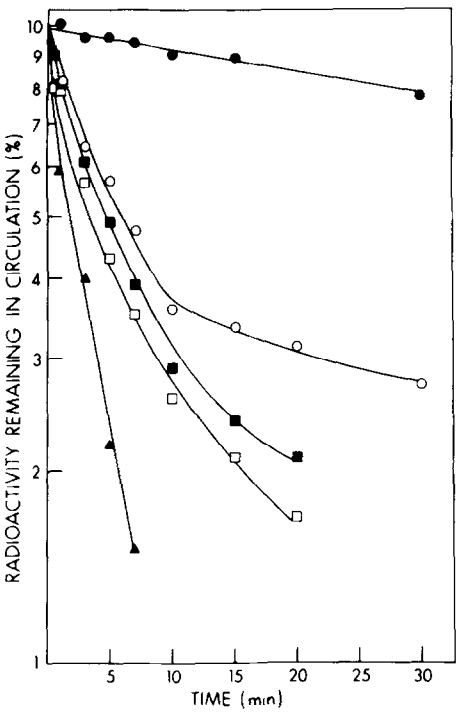

Figure 2. Clearance of α_2M and α_2M-protease complexes in mice. Native α_2M (●), α_2M-thermolysin (□), α_2M-papain (■), α_2M-trypsin (▲), and α_2M-cathepsin-D (○).

to determine total binding and in the presence of EDTA to determine nonspecific binding. Specific binding is the difference between total and nonspecific binding. Dissociation constants (K_d) were determined from Scatchard analysis (21) of the binding data (Table 1). The K_d determined for α_2M-cathepsin-D was somewhat greater than that of the other protease complexes, while the constant for native α_2M was about 30 times greater.

Human α_2M-protease complex and mouse α_2M-protease complex are bound and cleared by the same receptor system when injected into the circulation of mice (22). Fig. 2 shows the clearance data for complexes of ^{125}I-human α_2M and the four representative proteases. Native α_2M cleared only slowly, while each of the α_2M-protease complexes cleared rapidly. The clearance of α_2M-cathepsin-D complex was biphasic with approximately half clearing quickly and half slowly.

Discussion

α_2M-trypsin, α_2M-plasmin and α_2M-methylamine bind to macrophages and clear in vivo similarly (4,20, and unpublished data from this laboratory). In this

report it is shown that complexes of α_2M and proteases from each of the four classes, also bind to macrophages and clear from the circulation similarly. The K_d determined for α_2M-cathepsin-D was slightly greater than that of the other complexes. This difference is best explained by incomplete reaction of the α_2M with the cathepsin-D. The heterogeneity observed in electrophoresis and stoichiometry experiments performed with α_2M-cathepsin-D support this hypothesis. Incomplete reaction of this acid protease with α_2M is not surprising since even at pH 6 one would expect an acid protease to react only slowly (23). Native α_2M bound to macrophages with a K_d about 30 times greater than that of complex. This is consistent with the presence of less than 5% contaminant of complex in the native α_2M preparation.

Clearance experiments confirmed the results of the macrophage binding studies in an in vivo system. The clearance of the α_2M-cathepsin-D was biphasic, as might be expected. It is concluded that the interaction of reacted forms of α_2M with receptors occurs independently of the protease bound for all four major classes of proteases.

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