Wachtel, U., Eds.) pp 18–33, Thieme, Stuttgart and New York.
Hohn, B. (1979) *Methods Enzymol. 68*, 299–309.
Iwaki, M., Phillips, R. S., & Kaufman, S. (1986) *J. Biol. Chem. 261*, 2051–2056.
Karn, J., Matthes, H. W. D., Gait, M. J., & Brenner, S. (1984) *Gene 32*, 217–224.
Kaufman, S. (1957) *J. Biol. Chem. 226*, 511–524.
Kaufman, S. (1976) *Adv. Neurochem. 2*, 1–132.
Kwok, S. C. M., Ledley, F. D., DiLella, A. G., Robson, K. J. H., & Woo, S. L. C. (1985) *Biochemistry 24*, 556–561.
Ledley, F. D., Grenett, H. E., DiLella, A. G., Kwok, S. C. M., & Woo, S. L. C. (1985) *Science (Washington, D.C.) 228*, 77–79.
Ledley, F. D., Grenett, H. E., & Woo, S. L. C. (1987) *J. Biol. Chem. 262*, 2228–2233.
Lichter, U., Trefz, F. K., Schmidt, H., & Woo, S. L. C. (1985) *Pediatr. Res. 19*, 1074.
Lidsky, A. S., Ledley, F. D., DiLella, A. G., Kwok, S. C. M., Daiger, S. P., Robson, K. J. H., & Woo, S. L. C. (1985) *Am. J. Hum. Genet. 37*, 619–634.
Marvit, J., DeLella, A. G., Brayton, K., Ledley, F. D., Robson, K. J. H., & Woo, S. L. C. (1987) *Nucleic Acids Res. 15*, 5613–5628.
Messing, J. (1983) *Methods Enzymol. 101*, 21–78.
O'Flynn, M. E., Holtzman, N. A., Blaskovics, M., Azen, C., & Williamson, M. L. (1980) *Am. J. Dis. Child. 134*, 769–774.
Orkin, S. H., Kazazian, H. H., Jr., & Antanorakis, S. E. (1982) *Nature (London) 296*, 627–631.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schimmel, P. R., & Flory, P. J. (1968) *J. Mol. Biol. 34*, 105–120.
Schuck, K. D., & Eggert, D. (1975) in *Hannover-Wechsler-Intelligenztest fuer das Vorschulalter (HAWIVA)* (Eggert, D., Ed.) Huber, Bern, Stuttgart, and Wien.
Trefz, F. K., Erlenmaier, T., Hunneman, D., Bartholome, K., & Lutz, P. (1979) *Clin. Chim. Acta 99*, 211–979.
Trefz, F. K., Schmidt, H., Bartholome, K., Mahle, M., Matthes, P., & Pecht, G. (1985) in *Inherited Disease of Amino Acid Metabolism* (Bickel, H., & Wachtel, U., Eds.) pp 86–100, Thieme, Stuttgart and New York.
Wong, G. G., Witek, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenberg, D. P., Jones, S. S., & Brown, E. L. (1985) *Science (Washington, D.C.) 228*, 810–815.
Woo, S. L. C. (1979) *Methods Enzymol. 68*, 389–395.
Woo, S. L. C., Lidsky, A. S., Güttler, F., Chandra, T., & Robson, K. J. H. (1983) *Nature (London) 306*, 151–155.
Zoller, M. J., & Smith, M. (1984) *DNA 3*, 479–488.

Binding of α_2-Macroglobulin–Thrombin Complexes and Methylamine-Treated α_2-Macroglobulin to Human Blood Monocytes†

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ABSTRACT: The binding of α_2-macroglobulin (α_2M) to human peripheral blood monocytes was investigated. Monocytes, the precursors of tissue macrophages, were isolated from fresh blood by centrifugal elutriation or density gradient centrifugation. Binding studies were performed using ^{125}I-labeled α_2M. Cells and bound ligand were separated from free ligand by rapid vacuum filtration. Nonlinear least-squares analysis of data obtained in direct binding studies at 0 °C showed that monocytes bound the α_2M–thrombin complex with a K_d of 3.0 ± 0.9 nM and the monocyte had 1545 ± 153 sites/cell. Thrombin alone did not compete for the site. Binding was divalent cation dependent. Direct binding studies also demonstrated that monocytes bound methylamine-treated α_2M in a manner similar to α_2M–thrombin. Competitive binding studies showed that α_2M–thrombin and methylamine-treated α_2M bound to the same sites on the monocyte. In contrast, native α_2M did not compete with α_2M–thrombin for the site. Studies done at 37 °C suggested that after binding, the monocyte internalized and degraded α_2M–thrombin and excreted the degradation products. Receptor turnover and degradation of α_2M–thrombin complexes were blocked in monocytes treated with chloroquine, an inhibitor of lysosomal function. Our results indicate that human monocytes have a divalent cation dependent, high-affinity binding site for α_2M–thrombin and methylamine-treated α_2M which may function to clear α_2M–proteinase complexes from the circulation.

α_2-Macroglobulin (α_2M)[1] is a plasma proteinase inhibitor that binds proteolytic enzymes of all four classes (Barrett & Starkey, 1973). α_2M–proteinase complexes are bound, internalized, and degraded by tissue macrophages of the mouse and rabbit (Debanne et al., 1976; Kaplan & Nielsen, 1979a,b; Imer & Pizzo, 1981). The goal of this study was to determine whether the human peripheral blood monocyte, the precursor of the tissue macrophage (Ebert & Florey, 1939), has a

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[1] Abbreviations: α_2M, α_2-macroglobulin; α_2M-Ma, methylamine-treated α_2M; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HBSS, Hank's balanced salt solution supplemented with $NaHCO_3$ (4 mM) and HEPES (10 mM), pH 7.1; HBSS–BSA, HBSS with 0.5% bovine serum albumin; EDTA, disodium ethylenediaminetetraacetate; TCA, trichloroacetic acid.

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binding site for α_2M–proteinase complexes and, if so, to characterize this binding site.

Structurally, the α_2M molecule consists of four identical subunits, each with a molecular weight of 185 000, and covalently bound to one other subunit by two disulfide bonds (Jensen & Sottrup-Jensen, 1986). Two of these dimers are associated noncovalently to form the tetrameric molecule (Sottrup-Jensen et al., 1984). α_2M is locked in its native conformation by an intrachain thiol ester bond on each of its four subunits (Swensen & Howard, 1979; Sottrup-Jensen et al., 1980). When a proteinase cleaves α_2M, the thiol ester bonds are broken (not proteolytically), and the α_2M molecule undergoes a conformational change (Barrett et al., 1979; Bjork & Fish, 1982; Gonias et al., 1982; Straight & McKee, 1982). Reaction of small nucleophiles, such as methylamine, with the thiol esters causes a similar conformational change. It is this conformationally altered α_2M that binds to rabbit and mouse macrophages (Debanne et al., 1975; Kaplan & Nielsen, 1979a; Imber & Pizzo, 1981). α_2M Binding to macrophages has been shown to modulate certain functions such as production of O_2^- metabolites and proteinase secretion, suggesting that α_2M may play a role in controlling the inflammatory response (Johnson et al., 1982; Hoffman et al., 1983). In addition α_2M–proteinase complexes have been reported to cause chemotaxis of human blood monocytes (Forrester et al., 1983). Given the relationship between monocytes and macrophages, and the suggestion of an interaction between α_2M and monocytes, we felt it likely that human monocytes would have a binding site for α_2M–proteinase complexes and such a site might prove important in controlling monocyte function. During completion of our work, a study was published that showed cultured monocytes do have a binding site for α_2M–trypsin complexes (Petersen et al., 1987).

In this study, we have determined that human monocytes have a high-affinity, divalent cation dependent binding site for α_2M–proteinase complexes, and for the conformationally similar methylamine-treated α_2M, but not for native α_2M. In addition, our studies suggest that at 37 °C α_2M–thrombin complexes are internalized and subsequently degraded in the lysosome.

Experimental Procedures

Materials. Human thrombin was a gift from Dr. David Aronson, Bureau of Biologics, Federal Drug Administration, Bethesda, MD. Lymphocyte separation medium was from Bionetic Laboratory Products, Percoll and Dextran T500 were from Pharmacia, and RPMI 1640 was from Hazleton Research Products. Glass fiber filters (GF/C) were obtained from Whatman, and the vacuum filtration apparatus used in the cell binding studies was from Hoefer Scientific. All binding studies were performed in Hank's balanced salt solution (Gibco) supplemented with $NaHCO_3$ (4 mM), HEPES (10 mM), and bovine serum albumin (0.5%) at pH 7.1 and at 0 °C unless stated otherwise.

α_2M Purification. Human α_2M was purified from fresh frozen plasma as previously described (Arnaud & Gianazza, 1982). α_2M concentrations were determined by using an extinction coefficient ($E^{1\%}_{280\ nm}$) of 8.9 (Hall & Roberts, 1978) and a molecular weight of 725 000 (Jones et al., 1972).

Radiolabeling of α_2M. α_2M was radioiodinated using carrier-free $Na^{125}I$ (Amersham) as described by David and Reisfeld (1974). The specific radioactivity of the resulting ^{125}I-α_2M was usually 250 cpm/fmol.

Preparation of α_2M–Thrombin Complexes and Methylamine-Treated α_2M. α_2M or radiolabeled α_2M was treated with a 1.5-fold molar excess of human thrombin (0.05 M triethanolamine–0.1 M NaCl, pH 8.0, room temperature overnight). Prior to binding studies, the α_2M–thrombin was dialyzed against HBSS (4 °C).

Alternatively, α_2M or radiolabeled α_2M was reacted with 0.1 M CH_3NH_2 overnight at room temperature and then dialyzed against two changes (500 mL) of HBSS at 4 °C.

Purification of Human Monocytes by Centrifugal Elutriation. Blood (450 mL) from a healthy donor was brought to 10 units/mL heparin and mixed with an equal volume of 3% Dextran T500 in 0.9% NaCl. After 25 min at room temperature, the supernatant was fractionated using lymphocyte separation medium (Boyum, 1968). The mononuclear fraction containing monocytes (20–30%) and lymphocytes (70–80%) was removed and washed 3 times with HBSS–BSA. The monocytes were separated from the lymphocytes by countercurrent elutriation employing a Beckman J-6M centrifuge equipped with a JE-6B elutriation rotor based on the principles described previously (Lindahl, 1947; Sanderson et al., 1976). The monocytes were concentrated, suspended, and counted to determine the final yield and purity.

Purification of Human Monocytes by Percoll Gradient Centrifugation. Blood and cells were treated as described in the previous section to the step prior to elutriation. The mononuclear cells were suspended in RPMI 1640, layered on 5 mL of 43% Percoll (diluted with RPMI and phosphate-buffered saline, pH 7.1), and centrifuged at 550*g* at room temperature for 30 min. The monocytes, appearing in a band above the pellet, were collected and washed twice in HBSS–BSA.

Cell viability was assessed by using trypan blue. The cell populations were characterized by differential staining (Diff-Quik, American Scientific Products) and/or myeloperoxidase staining (Kaplow, 1965). Cell populations used in binding studies were 80–95% monocytes with some contamination by lymphocytes and platelets. Control experiments described below indicate the contaminating cells did not interfere with the results obtained. Results of binding studies described below were similar using either method of purification.

Binding Studies of α_2M–Thrombin, Methylamine-Treated α_2M, and Native α_2M to Human Monocytes. Total ^{125}I-α_2M–thrombin and ^{125}I-α_2M-Ma binding to monocytes was measured by incubating various concentrations of the radiolabeled ligand with (1.5–4.0) × 10^6 monocytes in 200-μL total volume on ice. Nonspecific binding of radiolabeled ligand was determined in the presence of a 100-fold molar excess of unlabeled α_2M–thrombin. The unbound ligand was separated from the cells and cell-bound ligand by rapid vacuum filtration on glass fiber filters as previously described (Lefkowitz, 1976; Williams et al., 1977). Studies were performed on ice to avoid potential complications due to endocytosis. Binding constants and the number of sites per cell were determined by the method of Scatchard (1949) and by nonlinear curve fitting as previously described (Fletcher & Sharger, 1973; Hancock et al., 1979).

In competitive binding experiments, a constant amount of ^{125}I-α_2M–thrombin or ^{125}I-α_2M-Ma (3.0 nM) and various concentrations of the unlabeled competing ligand (0–1.0 μM) were incubated as above, and the reaction was stopped by vacuum filtration.

Characterization of α_2M–Thrombin Binding to Monocytes at 37 °C. The time course of EDTA-resistant (irreversible) ^{125}I-α_2M–thrombin binding was determined as follows: ligand (10.0 nM) was incubated with monocytes (7 × 10^6) in 200-μL total volume at 37 °C. At various times, samples were diluted

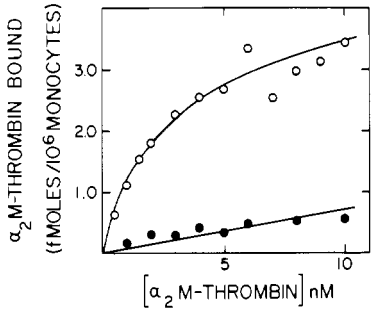

FIGURE 1: Binding of ^{125}I-α_2M–thrombin to human peripheral blood monocytes. Various concentrations of radiolabeled α_2M–thrombin complexes (0–10 nM) were incubated at 0 °C with human monocytes (2×10^6 in 200 μL of HBSS–BSA). After 60 min, the monocytes and bound ligand were separated from free ligand by vacuum filtration on glass fiber filters. Total binding (O) was determined by γ counting of the filters. Nonspecific binding (●) was determined in a similar manner, but in the presence of a 100-fold molar excess of unlabeled α_2M–thrombin. The curve shown was generated by nonlinear least-squares analysis of the data and describes a single class of sites with a K_d of 3.0 ± 0.9 nM and 1545 ± 153 sites/cell.

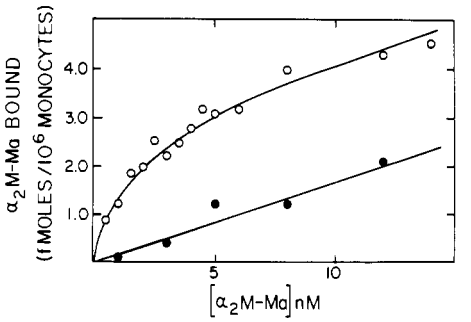

FIGURE 2: Binding of ^{125}I-methylamine-treated α_2M to monocytes. Radiolabeled methylamine-treated α_2M (0–14 nM) was incubated with human monocytes (3×10^6 in 200 μL of HBSS–BSA, 0 °C). Total binding (O) was determined as described in the legend to Figure 1. Nonspecific binding (●) was determined in the presence of a 50-fold molar excess of α_2M-Ma. The curve shown was generated as in Figure 1 and represents a single class of binding sites with a K_d of 2.5 ± 0.7 nM and 1607 ± 176 sites/cell.

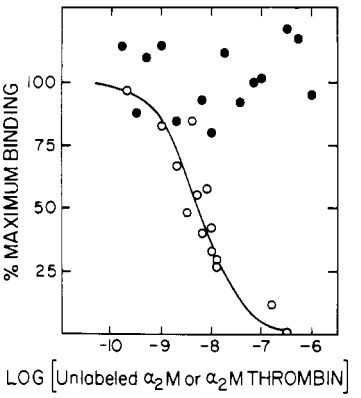

FIGURE 3: Competitive binding of α_2M, α_2M–thrombin, and methylamine-treated α_2M to human monocytes. ^{125}I-Methylamine-treated α_2M (3.0 nM) was incubated for 60 min (0 °C) with unlabeled α_2M–thrombin (0–0.3 μM) in the presence of monocytes (3×10^6). Binding (O) was determined as described in the legend to Figure 1. Alternatively, ^{125}I-α_2M–thrombin (3.0 nM) was incubated with various concentrations (0–1.0 μM) of native α_2M, and binding (●) was determined as above.

with 4 mL of HBSS–BSA (no Ca^{2+} or Mg^{2+} and 0.01 M EDTA, 0 °C) and incubated for 10 min. The reaction was stopped by rapid vacuum filtration. In a parallel experiment, after various times at 37 °C, samples were centrifuged and portions of the supernatant treated with cold trichloroacetic acid (10%). After 10 min at 0 °C, the TCA-treated samples were centrifuged, and the radioactivity in the supernatant was determined.

Alternatively, monocytes (15×10^6/mL) were incubated with ^{125}I-α_2M–thrombin (5.0 nM) at 0 °C for 60 min and centrifuged, and the excess unbound ligand was removed. The cells were suspended in HBSS–BSA at 37 °C; samples were removed at various times and treated as before to determine EDTA-resistant binding.

To determine the effect of the lysosomal inhibitor chloroquine on internalization and degradation, monocytes were incubated with 0.5 mM chloroquine at 37 °C (30 min). The cells were then incubated (37 °C) with radiolabeled α_2M–thrombin and treated as above to determine the amount of EDTA-resistant binding and TCA-soluble products.

Results

Binding of α_2M–Thrombin Complexes to Monocytes. Direct binding studies indicate the monocyte has a high-affinity binding site for the α_2M–thrombin complex (Figure 1). Analysis of the results of five such experiments employing Scatchard plots suggested a single class of sites with a K_d of 2.5 ± 0.5 nM, and 1684 ± 390 sites/monocyte. Nonlinear least-squares analysis of the data (Figure 1) gave a K_d of 3.0 ± 0.9 nM and 1545 ± 153 sites/cell. In the absence of Ca^{2+} or Mg^{2+} (≈1 mM each), α_2M–thrombin binding was similar to nonspecific binding (data not shown).

Binding studies performed using a cell population that was 90% lymphocytes did not show any specific saturable binding (not shown). In addition, in studies performed using monocyte populations contaminated by large numbers of lymphocytes and platelets, all the binding could be accounted for by the monocytes present (not shown) and was similar to that exhibited by highly purified monocytes. These results indicate that lymphocytes and platelets do not contribute to or interfere with the observed binding. Results reported by other investigators also indicate that lymphocytes do not bind α_2M (Debanne et al., 1975).

Specificity of the Monocyte Binding Site for the Various Forms of α_2M. The α_2M molecule can exist in its native circulating form or in a conformationally altered form. Reaction with proteinases or small primary amines, such as methylamine, produces the conformationally altered form. Our results (Figure 2) demonstrate that ^{125}I-α_2M-Ma is bound in a manner similar to α_2M–thrombin. Nonlinear least-squares analysis suggested a K_d of 2.5 ± 0.7 nM and 1607 ± 176 sites/cell. Competitive binding studies were performed to determine whether the α_2M-Ma and α_2M–thrombin complexes bind to the same site. An experiment employing ^{125}I-α_2M-Ma and α_2M–thrombin (Figure 3) shows that the two forms of α_2M do compete for the same site. The line drawn through the points is the theoretical curve for a single class of sites with a K_d of 2.5 nM and 1500 sites/cell. In contrast, native α_2M (1.0 μM) did not compete with ^{125}I-α_2M–thrombin for binding sites on the monocyte (Figure 3). In another experiment (results not shown), thrombin alone (1.0 μM) also did not compete with α_2M–thrombin for the monocyte binding site.

Dissociation of the α_2M–Thrombin Complex from Monocytes in the Presence of EDTA. Our analysis of binding studies is based on the assumption that the α_2M–thrombin complexes remain on the surface of the monocyte when bound and are not internalized under the conditions of the assay. To confirm this assumption, we measured the extent of dissociability after the system had reached apparent equilibrium. When EDTA was added to a mixture of monocytes and ^{125}I-α_2M–thrombin

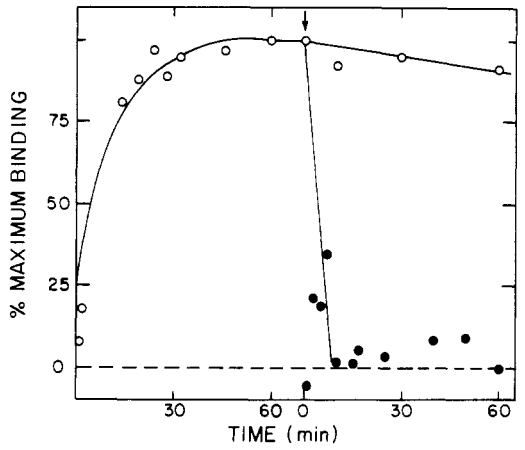

FIGURE 4: Association of ^{125}I-α_2M–thrombin with monocytes, and dissociation from monocytes after removal of divalent cations with EDTA. ^{125}I-α_2M–thrombin (5.0 nM) was incubated for various times with monocytes (4×10^6/200 μL). Binding (○) was determined as described in the legend to Figure 1. To determine the reversibility of binding in the presence of EDTA, ^{125}I-α_2M–thrombin (10 nM) was incubated for 60 min (0 °C) with monocytes (3.5×10^6/200 μL). Unbound ^{125}I-α_2M–thrombin was removed by aspiration after rapid centrifugation. Cells were suspended in HBSS–BSA (+Ca^{2+}/Mg^{2+}) (○) or HBSS–BSA (–Ca^{2+}/Mg^{2+} + 0.01 M EDTA) (●), and binding was determined at various times as described in the legend to Figure 1.

Table I: Effect of Chloroquine on the Internalization and Degradation of ^{125}I-α_2M–Thrombin Complexes by Monocytes[a]

	EDTA-resistant binding[b]	TCA-soluble radioactivity[c]
control	8.0 ± 1.9	5.1 ± 0.3
+chloroquine	2.6 ± 1.3	1.8 ± 1.6

[a] Results expressed as femtomoles per 10^6 monocytes. [b] Monocytes were incubated 30 min at 37 °C ± chloroquine, and then ^{125}I-α_2M–thrombin was added to each sample. After 30 min, samples were treated with cold HBSS–BSA + EDTA for 10 min and subjected to vacuum filtration, and the filter-bound radioactivity was determined (n = 2). [c] Portions of samples incubated with ^{125}I-α_2M–thrombin in the presence or absence of chloroquine (37 °C, 60 min) were centrifuged, and the supernatant was treated with TCA (10%, 0 °C). The acid-soluble radioactivity was then determined (n = 3).

at equilibrium, the ligand immediately dissociated from the binding site to the level of nonspecific binding (Figure 4). Dissociation was not due to damage to the cell or the binding site, since cells treated with EDTA and then washed with buffer containing calcium and magnesium bound the α_2M–thrombin complex as well as cells that had not been treated with EDTA (results not shown).

Internalization and Degradation of α_2M–Thrombin Complexes by Monocytes. Ligand binding to a cell is frequently followed by internalization and subsequent degradation of the ligand. One measurement of internalization is EDTA-resistant binding (Kaplan & Nielsen, 1979b). As described above (Figure 4), at 0 °C, binding of α_2M–thrombin to monocytes was reversible in the presence of EDTA. In contrast, at 37 °C, binding of α_2M–thrombin rapidly became resistant to EDTA dissociation (Table I). In a similar experiment, where excess unbound ligand was removed from the system prior to incubation at 37 °C, results were obtained which were characteristic of a system in which the ligand is bound, internalized, degraded, and excreted (Figure 5). To test this hypothesis, we measured the appearance of trichloroacetic acid soluble (degraded) radioactivity in the incubation medium. Results shown in Table I demonstrate a 3-fold increase in acid-soluble

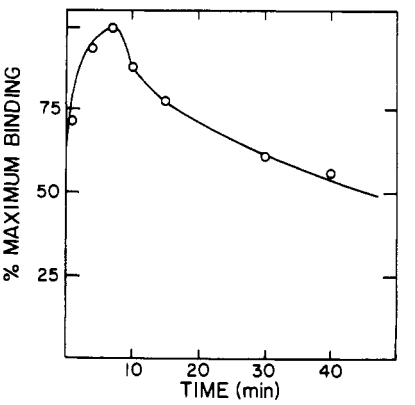

FIGURE 5: Interaction of ^{125}I-α_2M–thrombin with monocytes at 37 °C. ^{125}I-α_2M–thrombin (5 nM, 0 °C) was incubated for 60 min with monocytes (15×10^6/mL). Unbound ligand and cells were separated by centrifugation, then the cells were suspended at 37 °C, samples (200 μL) were removed at various times and treated with EDTA, and the binding was determined as in the legend to Figure 1.

radioactivity when ^{125}I-α_2M–thrombin was incubated with monocytes at 37 °C for 60 min. In a control experiment in the absence of cells, but otherwise identical conditions, there was no increase in soluble radioactivity over the time course of the experiment.

Chloroquine is an inhibitor of lysosomal activity. This amine rasises the pH in the lysosome and thus inhibits protein degradation (Ohkuma & Poole, 1978). When monocytes were pretreated with chloroquine, the production of TCA-soluble products was inhibited (Table I). Internalization was also inhibited (Table I), but we did observe a rapid increase of EDTA-resistant binding to the level indicated after 15 min. This level remained constant through 60 min.

Discussion

Results shown in Figures 1–3 indicate that α_2M–thrombin and α_2M-Ma bind to the same sites on monocytes with a similar affinity. The fact that the monocyte binds both α_2M–thrombin and α_2M-Ma equally well means the monocyte recognizes part of the α_2M molecule, not thrombin. This conclusion is also supported by results of a competitive binding study showing that thrombin did not compete with α_2M–thrombin complexes for monocyte binding sites (results not shown). This is important, because monocytes have a well-characterized thrombin binding site (Goodnough & Saito, 1982).

Our studies also show that the high-affinity binding site on human blood monocytes specifically recognizes α_2M molecules that have undergone a conformational change after reaction with thrombin or methylamine. The monocyte does not appear to recognize native α_2M, as this form does not compete with α_2M–thrombin complexes for binding (Figure 3). Our results agree with those reports indicating tissue macrophages from rabbits and mice bind only the conformationally altered α_2M (Debanne et al., 1975; Imber & Pizzo, 1981). The monocyte's ability to discriminate between the native and conformationally altered protein is important because the plasma concentration of α_2M is 3 μM, and thus would saturate the binding sites in circulation if the monocyte bound native α_2M. In addition, if formation of the α_2M–proteinase complex and its subsequent interaction with the monocyte is a significant physiologic event, then specific recognition of only this form is essential.

Although our results are qualitatively similar to those reported for macrophages, the affinity and the number of sites per cell are lower for the monocyte than for the macrophage (0.2–0.4 nM and 20 000–100 000 sites/cell, respectively, for

macrophages) (Kaplan & Nielsen, 1979a; Feldman et al., 1983). This difference in affinity and binding site number may reflect a species difference. On the other hand, one is tempted to speculate that during its maturation from a monocyte to a tissue macrophage, the cell might develop more α_2M–proteinase binding sites. This would correlate well with the increased phagocytic and lysosomal hydrolase potential of macrophages relative to monocytes. Indeed, a recently published work indicates that monocytes in culture show a 15-fold increase in binding sites over a period of 2–4 weeks (Petersen et al., 1987). In this work, the authors found approximately 2000 binding sites/monocyte in cells subjected to culture conditions for only 1.5 h, thus agreeing reasonably well with our results.

One important question raised by these results is whether the low number of receptors found on monocytes represents a subset of cells that have a large number of sites or a uniform distribution of a small number of sites per cell. The relatively constant number of receptors found among individuals in our study, and between our two methods for isolation of monocytes, suggests a uniform distribution of receptors among cells. However, this question will only be answered by additional studies.

One likely functional consequence of ligand binding is internalization and subsequent degradation of the ligand. This has been shown to be true when α_2M–proteinase complexes interact with macrophages at 37 °C (Debanne et al., 1976; Kaplan & Nielsen, 1979b). Our results (Table I) show that ^{125}I-α_2M–thrombin becomes irreversibly bound (EDTA resistant) to monocytes at 37 °C, thus suggesting internalization does occur. To investigate what happens to the ligand following internalization, we allowed binding of ^{125}I-α_2M–thrombin to come to apparent equilibrium at 0 °C, removed free ligand, and incubated samples at 37 °C. EDTA-resistant binding increased rapidly (Figure 5) but then began a gradual decrease after 5–7 min. This suggests the ligand was internalized and subsequently excreted in a degraded form or some other form that did not reassociate with monocytes. To determine whether ^{125}I-α_2M–thrombin was degraded by monocytes, we measured the appearance of TCA-soluble radioactivity in monocyte media (Table I). The amount of acid-soluble material increased with time at 37 °C, thus supporting the proposition that ^{125}I-α_2M–thrombin at 37 °C is bound, internalized, degraded, and excreted by monocytes. To further characterize this process, we treated monocytes with the inhibitor of lysosomal activity, chloroquine. Results of these experiments (Table I) show that chloroquine-treated monocytes internalize α_2M–thrombin complexes but only slowly secrete TCA-soluble products. In addition, internalized α_2M–thrombin did not increase after a short time even in the presence of excess ligand. The amount of cell-associated radioactivity present at this time indicates about 1000 α_2M–thrombin complexes were inside the cell. This suggests that each α_2M binding site binds one ligand, is internalized, and is inhibited by chloroquine from returning to the cell surface or is rendered inactive in some way. Thus, the monocyte–α_2M–thrombin system appears to operate like the fibroblast in which it is believed that an α_2M–proteinase complex binds, collects in coated pits, is internalized, goes to the lysosome where the receptor releases the α_2M, and returns to the cell surface (Van Leuven et al., 1978; Willingham et al., 1979). The α_2M is degraded and the products excreted from the cell. These results suggest that a function of monocytes is clearance of α_2M–proteinase complexes from circulation. Studies under way will determine whether, in addition to being cleared by monocytes, α_2M–proteinase complexes modulate monocyte function.

On the basis of our results, we conclude that the human peripheral blood monocyte offers a homologous system for the study of human α_2M–proteinase–cell interactions in suspension, and potentially in culture. The description of a specific binding site for α_2M–thrombin suggests a physiologic role for this interaction at sites where monocytes may accumulate and active proteinases are present, such as in inflammation due to tissue injury, neoplasm, or infection. This report is the first to characterize the binding of α_2M–proteinase complexes to a circulating cell. It will now be possible to study the functional significance of this interaction in suspension and then follow the behavior of the monocyte binding site in culture, under conditions where these cells take on characteristics of the macrophage (Hammerstrom, 1979).

References

Arnaud, P., & Gianazza, R. (1982) *FEBS Lett. 137*, 157–161.

Barrett, A. J., & Starkey, P. M. (1973) *Biochem. J. 133*, 709–723.

Barrett, A. J., Brown, M. A., & Sayers, C. A. (1979) *Biochem. J. 181*, 401–408.

Bjork, I., & Fish, W. W. (1982) *Biochem. J. 207*, 347–356.

Boyum, A. (1968) *Scand. J. Clin. Lab. Invest. 21*, 77–89.

David, G. S., & Reisfeld, R. A. (1974) *Biochemistry 13*, 1014–1021.

Debanne, M. T., Bell, R., & Dolovich, J. (1975) *Biochim. Biophys. Acta 411*, 295–304.

Debanne, M. T., Bell, R., & Dolovich, J. (1976) *Biochim. Biophys. Acta 428*, 466–475.

Ebert, R. H., & Florey, H. W. (1939) *Br. J. Exp. Pathol. 20*, 342–356.

Feldman, S. R., Ney, K. A., Gonias, S. L., & Pizzo, S. V. (1983) *Biochem. Biophys. Res. Commun. 114*, 757–762.

Fletcher, J. E., & Sharger, R. I. (1973) Technical Report No. 1, U.S. Department of Health, Education, and Welfare, Washington, DC.

Forrester, J. V., Wilkinson, P. C., & Lackie, J. M. (1983) *Immunology 50*, 251–259.

Gonias, S. L., Reynolds, J. A., & Pizzo, S. V. (1982) *Biochim. Biophys. Acta 705*, 306–314.

Goodnough, L. T., & Saito, H. (1982) *J. Lab. Clin. Med. 99*, 873–884.

Hall, P. K., & Roberts, R. C. (1978) *Biochem. J. 173*, 27–38.

Hammerstrom, J. (1979) *Acta Pathol., Microbiol. Immunol. Scand., Sect. C. 87C*, 113–120.

Hancock, A. A., DeLean, A. L., & Lefkowitz, R. J. (1979) *Mol. Pharmacol. 16*, 1–9.

Hoffman, M., Feldman, S. R., & Pizzo, S. V. (1983) *Biochim. Biophys. Acta 760*, 421–423.

Imber, M. J., & Pizzo, S. V. (1981) *J. Biol. Chem. 256*, 8134–8139.

Jensen, P. E. H., & Sottrup-Jensen, L. (1986) *J. Biol. Chem. 261*, 15863–15869.

Johnson, W. J., Pizzo, S. V., Imber, M. J., & Adams, D. O. (1982) *Science (Washington, D.C.) 218*, 574–576.

Jones, J. M., Creeth, J. M., & Kekwick, R. A. (1972) *Biochem. J. 127*, 187–197.

Kaplan, J., & Nielsen, M. L. (1979a) *J. Biol. Chem. 254*, 7323–7328.

Kaplan, J., & Nielsen, M. L. (1979b) *J. Biol. Chem. 254*, 7329–7335.

Kaplow, L. S. (1965) *Blood 26*, 215–219.

Lefkowitz, R. J. (1976) in *Methods in Receptor Research* (Blecher, M., Ed.) pp 53–72, Marcel Dekker, New York.

Lindahl, P. E. (1947) *Nature (London) 161*, 648–649.
Ohkuma, S., & Poole, B. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3327–3331.
Petersen, C. M., Ejlersen, E., Hansen, P. W., & Gliemann, J. (1987) *Scand. J. Clin. Lab. Invest. 47*, 55–61.
Sanderson, R. J., Bird, K. E., Palmer, N. F., & Brenman, J. (1976) *Anal. Biochem. 71*, 615–622.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.
Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Wierzbicki, D. M., Jones, C. M., Londblad, P. B., Magnusson, S., & Petersen, T. E. (1984) *J. Biol. Chem. 259*, 8318–8327.
Straight, D. L., & McKee, P. A. (1982) *Biochemistry 21*, 4550–4556.
Swensen, R. P., & Howard, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4313–4316.
Van Leuven, F., Cassiman, J. J., & Van Den Berghe, H. (1978) *Exp. Cell Res. 117*, 273–282.
Williams, L. T., Snyderman, R., Pike, M. C., & Lefkowitz, R. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1204–1208.
Willingham, M. C., Maxfield, F. R., & Pastan, I. H. (1979) *J. Cell Biol. 82*, 614–625.

Purification and Characterization of Mouse Protamines P1 and P2. Amino Acid Sequence of P2†

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ABSTRACT: Two mouse protamines, denoted as P1 and P2, have been purified directly from mature sperm nuclei and characterized as distinct polypeptide species. The complete primary structure of P2 was determined by peptide sequencing analyses. P1 and P2 were purified by a sequence of cation-exchange chromatography on Bio-Rex 70 and permeation chromatography on Bio-Gel P10, both in the presence of guanidine hydrochloride. Biochemical analyses demonstrate P1 has a molecular weight of 7400 and is characterized by the presence of arginine, cysteine, lysine, and tyrosine. By contrast, P2 is unusual in containing an abundance of arginine, histidine, lysine, and cysteine, but no tyrosine. The primary structure of P2 was determined from the sequencing of overlapping, high-pressure liquid chromatography purified peptides generated by thermolysin and endoproteinase Lys-C digestions and by chemical cleavage at each of four serine residues. Sequence analyses have demonstrated that P2, with a molecular weight of 8841, contains 62 amino acids, in the sequence NH_2-Arg-Gly-His-His-His-His-Arg-His-Arg-Arg-Cys-Ser-Arg-Lys-Arg-Leu-His-Arg-Ile-His-Lys-Arg-Arg-Arg-Ser-Cys-Arg-Arg-Arg-Arg-Arg-His-Ser-Cys-Arg-His-Arg-Arg-Arg-His-Arg-Arg-Gly-Cys-Arg-Arg-Ser-Arg-Arg-Arg-Arg-Arg-Cys-Arg-Cys-Arg-Lys-Cys-Arg-Arg-His-His-COOH. Thus, the primary structure includes six clusters of arginine and histidine, distributed throughout the polypeptide, each ranging from five to eight amino acids in length. Sequence comparisons of mouse and human protamines by the Dayhoff program have revealed greater homology exists between human P2 and mouse P2 than within the P1 family from the two mammalian species.

During mammalian spermiogenesis, the germ cell nucleus undergoes a marked transition as many chromosomal proteins are removed and then replaced by an array of novel polypeptides (Bellvé et al., 1975; O'Brien & Bellvé, 1980a,b). In this process of nuclear transformation, the histones are replaced initially by a set of testis-specific proteins (TSP)[1] (Grimes et al., 1977) and then by the protamines (Bellvé et al., 1975; Balhorn et al., 1984), the predominant proteins comprising the mature sperm nucleus. On the basis of current evidence, the low molecular weight protamines exist in two classes: a ubiquitous P1 family rich in arginine, lysine, cysteine, and tyrosine and a P2 species rich in arginine, histidine, and lysine but lacking tyrosine. Expression of the latter protamine has been detected in the mouse (Bellvé et al., 1975; Balhorn et al., 1977; Bellvé & Carraway, 1978) and human (Kolk & Samuel, 1975). Complete amino acid sequences for members of the P1 family have been determined for bulls (Coelingh et al., 1972; Mazrimas et al., 1986), boars (Tobita et al., 1983), rams (Sautiere et al., 1984), and humans (McKay et al., 1985;

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[1] Abbreviations: M_r, relative molecular weight; HPLC, high-pressure liquid chromatography; Gdn·HCl, ultrapure guanidine hydrochloride; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; TSP, testis-specific protein(s); SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin.

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