SYNTHESIS AND SECRETION OF $\alpha_2$-MACROGLOBULIN BY CULTURED HUMAN FIBROBLASTS

BY DEANE F. MOSHER* AND DAVID A. WING

(From the U. S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701)

Alpha-$\alpha_2$-macroglobulin is a major plasma protein and functions as an inhibitor of a wide variety of proteolytic enzymes (1 and references therein). In the present paper, experiments are described indicating that cultured human fibroblasts synthesize and secrete $\alpha_2$-macroglobulin. This finding may be important for studies of the role of proteases in fibroblast growth and function, both in vitro and in vivo.

Materials and Methods

Reagents were purchased from the following suppliers: goat antisera to human $\alpha_2$-macroglobulin and whole serum and rabbit antiserum to human fibrinogen from Hyland Lab, Costa Mesa, Calif.; TAMe from Schwarz/Mann, Orangeburg, N. Y.; trypsin (TPCK treated) from Worthington Biochemical Corp., Freehold, N. J.; soybean trypsin inhibitor from Calbiochem, San Diego, Cal.; and $[^{35}S]$-methionine (650 mCi/mmol) from Amersham/Searle Corp., Arlington Heights, Ill. Partially (75%) purified human $\alpha_2$-macroglobulin was a gift from the American Red Cross National Fractionation Center, Bethesda, Md. Rabbit antiserum to human cold-insoluble globulin was prepared as previously described (2).

Human WI-38 fibroblasts were obtained from the American Type Culture Collection, Rockville, Md and cultured in Eagle's basal medium (BME) supplemented with 10% fetal calf serum (3). 5 days after subcultivation in this medium protected with 1 U penicillin and 1 $\mu$g streptomycin per ml, protein synthesis was studied in confluent cultures containing approximately $5 \times 10^6$ cells per 75 cm$^2$ flask. In studies of $[^{35}S]$-methionine incorporation, the cultures were washed three times with methionine-free BME and incubated for 18 h in 10 ml of the same medium containing labeled methionine, 6 $\mu$Ci/ml. In studies of synthesis of unlabeled proteins, the cultures were washed three times and incubated with 10 ml of BME supplemented with 0.1% bovine serum albumin. After incubation with $[^{35}S]$-methionine, the medium was centrifuged at 1,850 g for 10 min and mixed with 1/25 vol of citrated human plasma. The proteins in the mixture were precipitated with an equal volume of either saturated ammonium sulfate or 20% trichloroacetic acid. In experiments not utilizing radioisotope, the medium was centrifuged and concentrated by ultrafiltration through a PM-10 membrane (Amicon Corp., Lexington, Mass.).

Immunodiffusion in 1% agarose was performed as described by Ouchterlony and Nilsson (4). The plates were washed in four changes of physiological saline over a 3-day period, dried, stained, and analyzed by autoradiography with Plus X-Pan film (Kodak, Rochester, N. Y.). The concentrations of $\alpha$-macroglobulin and cold-insoluble globulin were determined by Laurell's electroimmunoassay (5). Purified cold-insoluble globulin (2) and partially purified $\alpha_2$-macroglobulin (75% as determined electrophoretically) served as standards. The sensitivity of both assays was 5 $\mu$g/ml; no reactions were seen with fetal calf serum.

$\alpha_2$-Macroglobulin was also assayed by its functional capacity of preserving the enzymic activity of trypsin towards tosyl arginine methyl ester (TAME) from inhibition by soybean trypsin inhibi-
tor (6). Bovine trypsin, 1.6 µg in 200 µl, was incubated for 5 min at 25°C with 200 µl of medium containing α₂-macroglobulin and 200 µl of 100 mM CaCl₂ in 50 mM Tris-chloride, pH 8.1. Soybean trypsin inhibitor, 20 µg in 200 µl, was added; after 5 min, 400 µl of the mixture was transferred to a cuvette containing 600 µl of 1.66 mM TAMe in Tris-chloride buffer. Hydrolysis of TAMe was determined by change in absorbance at 247 nm (7). In the absence of α₂-macroglobulin, there was negligible hydrolysis of TAMe. The rate of change in absorbance versus amount of added α₂-macroglobulin was linear for initial α₂-macroglobulin concentrations ranging from 5–100 µg/ml.

Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate (SDS) was performed as described previously (2). Cylindrical gels containing the labeled proteins were sliced into 2-mm sections, and the amount of radiolabel in each section was estimated by scintillation counting after dissolution of the gel in 200 µl of 30% hydrogen peroxide (8).

Results and Discussion

In studies of [³⁵S]L-methionine incorporation into proteins secreted by fibroblasts into the medium, the medium was mixed with 1/25 vol of human plasma, and the globulins were precipitated from the mixture by 50% saturated ammonium sulfate. The plasma proteins served as carriers, both during the salt precipitation and during subsequent immunodiffusion. After immunodiffusion, radiolabel was detected in the precipitin lines formed by the reactions of the 0–50% saturated ammonium sulfate fraction with antiserum directed against α₂-macroglobulin and cold-insoluble globulin (Fig. 1). There was no suggestion of interference (4) between the radiolabeled precipitin lines, and the immunoprecipitate formed by the reaction of the mixture with antiserum directed against fibrinogen contained no radiolabel. Thus, this experiment suggests that cultured fibroblasts synthesize two plasma proteins: cold-insoluble globulin, as previously described (9), and α₂-macroglobulin. In a similar kind of experiment, Williams et al. (10) found that tissue slices from a variety of mouse organs incorporated labeled amino acids into protein precipitated by anti-α₂-macroglobulin antibody, but did not think this finding was conclusive evidence for α₂-macroglobulin synthesis because of the possibility that other proteins were being labeled and binding to unlabeled α₂-macroglobulin. Our experiment is subject to the same interpretation since the α₂-macroglobulin immunoprecipitate contained unlabeled α₂-macroglobulin.

Therefore, the 0–50% saturated ammonium sulfate fraction was further analyzed by electrophoresis in 4% polyacrylamide gels containing SDS. In reduced samples a major band of radioactivity was present which migrated at the same position as the subunit of intact α₂-macroglobulin with mol wt¹ of 1.6 × 10⁶ (Fig. 2, middle panel). A minor band of radioactivity migrated at the position of the subunit of cold-insoluble globulin, mol wt = 2.0 × 10⁶. In samples not reduced before electrophoresis, the 1.6 × 10⁶ mol wt band was replaced by a band of higher apparent mol wt (Fig. 2, top panel). This band co-migrated with nonreduced intact α₂-macroglobulin. The observed migration of the major band of radiolabel is compatible with the structure of α₂-macroglobulin which has not bound a proteolytic enzyme, as deduced by Harpel (11) and Jones et al. (12). These investigators concluded that α₂-macroglobulin was composed of four large

¹ Mosher, D. F. Action of fibrin-stabilizing factor on cold-insoluble globulin and α₂-macroglobulin in clotting plasma. In press. Our estimate of the subunit size of α₂-macroglobulin is somewhat lower than Harpel's estimate (11).
Fig. 1. Immunodiffusion of proteins precipitated from the mixture of labeled medium and unlabeled plasma by 50% saturated ammonium sulfate. The precipitated proteins were dissolved in 1/35 of the volume of the medium, and 5 μl were placed in the center well. Antisera (5 μl) directed against a2-macroglobulin (A), cold-insoluble globulin (B), and fibrinogen (C) were placed in the outer wells. The plate was stained for protein (left) and analyzed by autoradiography (right). The precipitin line between wells A and C is thought to represent α2-macroglobulin in human serum used to absorb the antifibrinogen.

![Immunodiffusion Image](image)

Fig. 2. Polyacrylamide gel electrophoresis in SDS of mixtures of cell culture medium and plasma. The proteins, insoluble in 50% saturated ammonium sulfate, were analyzed with (middle panel) and without (top panel) prior reduction. The bottom panel is of the trichloroacetic acid precipitate (TCA PPT) analyzed after reduction. During sample preparation, proteins in the medium were concentrated approximately 17-fold (ammonium sulfate precipitate) or 9-fold (trichloroacetic acid precipitate); 20-μl samples were analyzed. The gels were sliced and solubilized, and the radioactivity of each slice was determined as counts per minute (CPM). Arrows indicate that positions of migration of reduced cold-insoluble globulin (A), α2-macroglobulin (B), phosphorylase (C), Aα chain of fibrinogen (D), heavy chain of IgG (E), and bromophenol blue (F).
subunits arranged as two dimers, and that subunits within each dimer were held together by disulfide bonds. Harpel found that upon binding a protease the subunit of α₂-macroglobulin was cleaved into fragments exhibiting mol wts approximately half that of the intact subunit (11).

The proteins in the mixture of [35S]L-methionine-labeled medium and unlabeled plasma were also precipitated by 10% trichloroacetic acid and analyzed by gel electrophoresis after reduction (Fig. 2, bottom panel). The prominent 1.6 × 10⁵ mol wt and minor 2.0 × 10⁴ mol wt bands of radioactivity were present along with radiolabel in the 3.0–4.0 × 10⁴ region of the gels.

α₂-Macroglobulin was assayed both immunologically and functionally in medium concentrated by ultrafiltration in the absence of carrier plasma proteins (Fig. 3). It was not detectable immediately after placing the cells in serum-free medium; it was first detected after 12 h incubation. The concentration of α₂-macroglobulin increased through 48 h. Medium of the flask sampled at 48 h contained approximately 65 μg of α₂-macroglobulin, corresponding to 10–15 μg per 10⁶ cells. Medium from the same flask contained 16 μg of cold-insoluble globulin. Cold-insoluble globulin and α₂-macroglobulin were not detected in the media of flasks incubated with 0.2 mM puromycin, indicating that appearance of these proteins in the medium was due to synthesis by the cells. The correspond-

![Graph](image-url)
ence between the immunologic and functional assays of $\alpha_2$-macroglobulin suggests, as do the results of electrophoresis in SDS, that there was little binding of proteases to secreted $\alpha_2$-macroglobulin (1, 11).

The importance of $\alpha_2$-macroglobulin to fibroblast function will require further investigation. Fibroblasts in tissue culture have been shown to synthesize and secrete a variety of proteolytic enzymes, including a collagenase (13, 14), a neutral protease (14), and, in transformed variants, an activator of plasminogen (15, 16). Secretion of $\alpha_2$-macroglobulin would afford the cells a means of modifying the activities of these enzymes. Because of its large size, mol wt = 6.5–8.0 $\times$ $10^5$ (11, 12), $\alpha_2$-macroglobulin would be expected to diffuse slowly away from the cells, especially in the ground substance of connective tissue (17), and high local concentrations of $\alpha_2$-macroglobulin may be achieved.

Summary

The following observations indicate that cultured human WI-38 fibroblasts synthesize and secrete $\alpha_2$-macroglobulin into serum-free medium: (a) after incubation of cultures with $[35S]$-l-methionine, a labeled protein appeared in the medium which was precipitated by antiserum directed against $\alpha_2$-macroglobulin; (b) after incubation of cultures with $[35S]$-l-methionine, a major band of radioactivity detected by polyacrylamide gel electrophoresis of the proteins in medium co-migrated with $\alpha_2$-macroglobulin; and (c) the amount of $\alpha_2$-macroglobulin in the medium, estimated both functionally and immunologically, increased with time in normal but not puromycin-treated cultures.

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