Brief Definitive Report

SYNTHESIS AND SECRETION OF α_2-MACROGLOBULIN BY CULTURED HUMAN FIBROBLASTS

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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 α_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 α_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]L-methionine (650 mCi/mmol) from Amersham/Searle Corp., Arlington Heights, Ill. Partially (75%) purified human α_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 μg streptomycin per ml, protein synthesis was studied in confluent cultures containing approximately 5×10^6 cells per 75 cm^2 flask. In studies of [^{35}S]L-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 μCi/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]L-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 α-macroglobulin and cold-insoluble globulin were determined by Laurell's electroimmunoassay (5). Purified cold-insoluble globulin (2) and partially purified α_2-macroglobulin (75% as determined electrophoretically) served as standards. The sensitivity of both assays was 5 μg/ml; no reactions were seen with fetal calf serum.

α_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-

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tor (6). Bovine trypsin, 1.6 μg in 200 μl, was incubated for 5 min at 25°C with 200 μl of medium containing α_2-macroglobulin and 200 μl of 100 mM $CaCl_2$ 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 α_2-macroglobulin, there was negligible hydrolysis of TAMe. The rate of change in absorbance versus amount of added α_2-macroglobulin was linear for initial α_2-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 [^{35}S]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 antisera directed against α_2-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 α_2-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-α_2-macroglobulin antibody, but did not think this finding was conclusive evidence for α_2-macroglobulin synthesis because of the possibility that other proteins were being labeled and binding to unlabeled α_2-macroglobulin. Our experiment is subject to the same interpretation since the α_2-macroglobulin immunoprecipitate contained unlabeled α_2-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 α_2-macroglobulin with mole wt[1] of 1.6×10^5 (Fig. 2, middle panel). A minor band of radioactivity migrated at the position of the subunit of cold-insoluble globulin, mol wt = 2.0×10^5. In samples not reduced before electrophoresis, the 1.6×10^5 mol wt band was replaced by a band of higher apparent mol wt (Fig. 2, top panel). This band co-migrated with nonreduced intact α_2-macroglobulin. The observed migration of the major band of radiolabel is compatible with the structure of α_2-macroglobulin which has not bound a proteolytic enzyme, as deduced by Harpel (11) and Jones et al. (12). These investigators concluded that α_2-macroglobulin was composed of four large

[1] Mosher, D. F. Action of fibrin-stabilizing factor on cold-insoluble globulin and α_2-macroglobulin in clotting plasma. In press. Our estimate of the subunit size of α_2-macroglobulin is somewhat lower than Harpel's estimate (11).

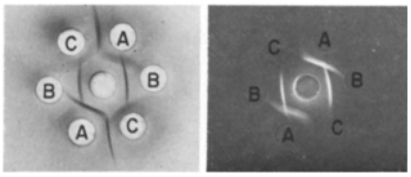

FIG. 1. Immunodiffusion of proteins precipitated from the mixture of labeled medium and unlabeled plasma by 50% saturated ammonium sulfate. The precipitated proteins were dissolved in 1/35 of the volume of the medium, and 5 μl were placed in the center well. Antisera (5 μl) directed against α_2-macroglobulin (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 α_2-macroglobulin in human serum used to absorb the antifibrinogen.

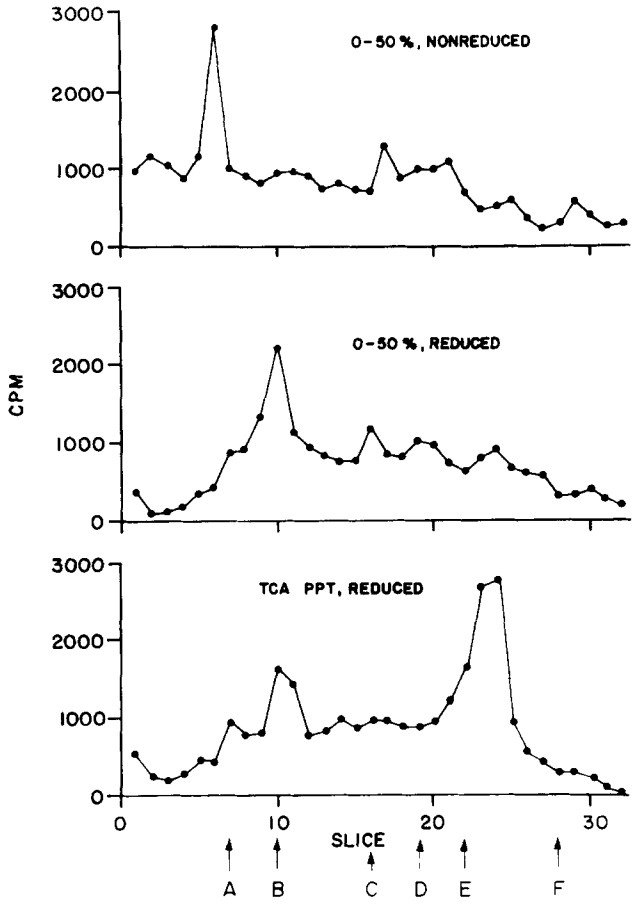

FIG. 2. Polyacrylamide gel electrophoresis in SDS of mixtures of cell culture medium and plasma. The proteins, insoluble in 50% saturated ammonium sulfate, were analyzed with (middle panel) and without (top panel) prior reduction. The bottom panel is of the trichloroacetic acid precipitate (TCA PPT) 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), α_2-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 α_2-macroglobulin was cleaved into fragments exhibiting mol wts approximately half that of the intact subunit (11).

The proteins in the mixture of [^{35}S]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 $\times$ 10^5 mol wt and minor 2.0 $\times$ 10^5 mol wt bands of radioactivity were present along with radiolabel in the 3.0–4.0 $\times$ 10^4 region of the gels.

α_2-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 α_2-macroglobulin increased through 48 h. Medium of the flask sampled at 48 h contained approximately 65 μg of α_2-macroglobulin, corresponding to 10–15 μg per 10^6 cells. Medium from the same flask contained 16 μg of cold-insoluble globulin. Cold-insoluble globulin and α_2-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-

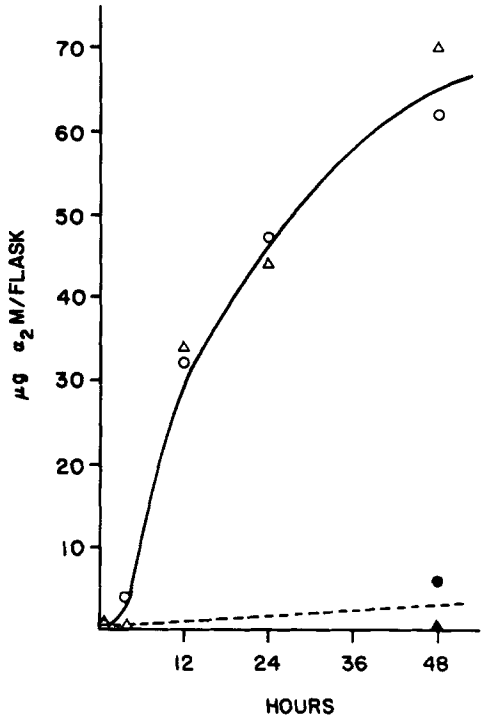

FIG. 3. Quantitative measurements of α_2-macroglobulin (α_2M) production by WI-38 fibroblasts in tissue culture. Confluent cultures of 5 $\times$ 10^6 WI-38 fibroblasts were incubated in BME containing bovine serum albumin, 1 mg/ml, with (closed symbols) or without (open symbols) 0.2 mM puromycin. α_2-Macroglobulin present in the medium at the times shown was measured by Laurell's electroimmunoassay (△) and the TAMe esterase assay (○). The medium was concentrated fivefold before analysis.

ence between the immunologic and functional assays of α_2-macroglobulin suggests, as do the results of electrophoresis in SDS, that there was little binding of proteases to secreted α_2-macroglobulin (1, 11).

The importance of α_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 α_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), α_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 α_2-macroglobulin may be achieved.

Summary

The following observations indicate that cultured human WI-38 fibroblasts synthesize and secrete α_2-macroglobulin into serum-free medium: (*a*) after incubation of cultures with [^{35}S]L-methionine, a labeled protein appeared in the medium which was precipitated by antiserum directed against α_2-macroglobulin; (*b*) after incubation of cultures with [^{35}S]L-methionine, a major band of radioactivity detected by polyacrylamide gel electrophoresis of the proteins in medium co-migrated with α_2-macroglobulin; and (*c*) the amount of α_2-macroglobulin in the medium, estimated both functionally and immunologically, increased with time in normal but not puromycin-treated cultures.

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