Monoclonal Antibody Preparation and Purification of a Tumor Cell Collagenase-stimulatory Factor

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ABSTRACT

Tumor cells from several sources produce a factor(s) which stimulates fibroblast collagenase production. Monoclonal antibodies have been raised against the tumor cell collagenase-stimulatory factor from LX-1 human lung carcinoma cells and have been used for purification of the factor from LX-1 cell membranes. These purified preparations stimulated fibroblast collagenase production, and 80% of these preparations contained a single M, ~58,000 protein detectable by immunoblotting; the other 20% contained an additional minor component with a molecular weight of 35,000. A single protein with a molecular weight of ~58,000 was also detected in radiolabeled preparations of the purified factor by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Conditioned media from LX-1 cells contain several species with molecular weights lower than 58,000 which are immunologically cross-reactive with the membrane-derived factor. Immunofluorescence analysis indicates that the tumor cell collagenase-stimulatory factor is distributed on the outer surface of LX-1 cells and is absent from the cell surface of fibroblasts. These and previous results indicate that the factor is present on the tumor cell surface, is released into conditioned media possibly after proteolytic cleavage, and appears to have an important role in inducing collagenolysis of host stroma during tumor invasion.

INTRODUCTION

Previously we have reported that increased collagenase production in cocultures of fibroblasts and tumor cells is influenced by a factor produced by tumor cells (1, 2). The factor activity can be detected in the conditioned medium from tumor cell cultures such that addition of this medium to the fibroblasts stimulates their collagenase production but not vice versa. Furthermore, we have demonstrated that the level of the factor in the conditioned medium depends on particular culture conditions, e.g., use of fibroblast matrix-coated dishes (3). Subsequently we have shown that the factor is associated with tumor cell membranes, can be extracted from the membranes by detergent, and is a hydrophobic protein (4). These observations led us to isolate and characterize the TCSF from the tumor cell membranes.

In this study we report the preparation of monoclonal antibodies against the TCSF and its purification by using immunoaffinity chromatography. Addition of the purified factor to fibroblasts stimulated collagenase production by these cells. Using immunohistology we have observed that the factor is present at the outer surface of the tumor cells.

MATERIALS AND METHODS

Chemicals

Reagents include: pancreatic trypsin (type I) and OG from Sigma (St. Louis, MO); 1-3tosylamido-2-phenylethyl chloromethyl ketone-

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1 This investigation was supported by NIH Grant CA38817.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: TCSF, tumor cell collagenase-stimulatory factor; OG, N-acetyl-D-glucosamine; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate.

trypsin and soybean trypsin inhibitor from Worthington Biochemical Corp. (Freehold, NJ); and [3H]thymidine (20 mCi/mmol) and [35S]-methionine (1134 Ci/mmol) from New England Nuclear (Boston, MA). Type I collagen was isolated and purified from rat tail tendon and labeled with [3H]acetic anhydride as described previously (1). Reagents for purification of IgG (MAPS-II kit) and Affi-Gel-Protein A were from Bio-Rad (Richmond, CA). Rabbit anti-mouse antiserum and goat anti-mouse rhodamine conjugate were from Cooper Biomedical, (Malvern, PA).

Cell Cultures

The sources of human fibroblasts and LX-1 cells and their maintenance have been described previously (2). The nonsecretor variant of mouse myeloma cell P3X63Ag8 (NS-1) was obtained from American Type Culture Collection (Rockville, MD). These cells were maintained in DMEM that contained 4.5 g glucose/liter, 10% fetal calf serum, β-mercaptoethanol (5 × 10⁻⁴ M), and gentamicin (0.5 μg/ml).

Implantation of LX-1 Cells in Nude Mice

LX-1 cells were harvested from monolayer cultures by trypsinization and the cell pellet was washed with sterile PBS and suspended in the same medium. Approximately 4 × 10⁶ cells were injected s.c. into 6-week-old female nude mice (nu/nu; Taconic, Germantown, NY). After 4–6 weeks the animals were killed, tumors were removed, and tissues were homogenized in a buffer that contained 0.15 M NaCl, 0.01 M Tris-HCl (pH 8.2), 2 mM PMSF, 0.5% Nonidet P-40, and 1 mg/ml BSA. The extract was centrifuged at 500 × g for 15 min to remove unextracted tissue fragments and the extract was then centrifuged at 100,000 × g for 30 min. The supernatant was used for polyacrylamide gel electrophoresis and transblotting.

Preparation of Monoclonal Antibodies

Antigens Preparation. LX-1 cells were harvested from confluent cultures in 100-mm dishes by mechanical scraping and were then suspended and sonicated in 50 mM Tris (pH 7.4) that contained 0.24 M sucrose, as described previously (4). The sonicated cell suspension was centrifuged at 100,000 × g for 1 h and the membrane pellet was extracted with 50 mM N,N,N′,N′-tetraacetylchloroethylidipropargylamine-N,N,N′,N′-2-ethanesulfonic acid (pH 7.4), containing 30 mM Mg, 0.1 mM DTT, and 0.1 mM PMSF, at 4°C overnight. The extract was centrifuged at 100,000 × g in a Beckman ultracentrifuge for 1 h and the supernatant was dialyzed extensively against PBS followed by DMEM that contained 0.1 mM DTT. The final preparation was used for immunization of mice after its ability to stimulate fibroblast collagenase production in cell culture was tested, as described in "Assay of TCSF Activity" below.

Hybridoma Methodology. The protocol for immunization and fusion is the same as described by Linsenmayer and Hendrix (5). Eight-week-old SJL/J mice were immunized with the antigen preparation (200 μg protein) by s.c. injection. After 2 weeks they were given booster i.p. injections of antigen in incomplete adjuvant. Three months later, just prior to fusion, mice were given i.p. injections of the antigen containing no adjuvant. The splenic lymphocytes were fused with murine myeloma cells (NS-1) by using polyethylene glycol 1000. The hybridomas were selected in medium containing hypoxanthine, thymidine, and aminopterin, and the media were screened with an ELISA as described below. The media from positive wells were further tested by a specific assay procedure utilizing their ability to inhibit stimulation of collagenase production in cocultures of fibroblasts and LX-1 cells, as described below ("Bioassy"). The cells from positive wells were cloned by a limiting dilution technique, where cells were diluted such that each well
in a 96-well flat-bottomed plate received one or no cell. The media from the wells with cell growth were tested by using the ELISA and bioassay (see below). Positive wells were cloned once more and two clones were chosen. They were designated as IIG4F6 and IEnF4, producing IgG1 and IgG2a, respectively. These clones were grown in DMEM with 10% fetal calf serum, in the presence of β-mercaptoethanol and gentamicin, in 150-mm plates and the media were used as a source of antibody.

Antibody Screening Procedures

ELISA. Cultured LX-1 cells were mechanically harvested and, after washing with PBS, pH 7.2, they were plated at 5 × 10^6 cells/well in 96-well polystyrene plates (Immunonol 2; Dynatech, Cambridge, MA). The plates were then incubated to dryness in a 37°C oven and were stored at 4°C until use (6). Before use the plates were washed with PBS that contained 0.2% Tween 20 and 0.02% sodium azide and were then blocked by PBS that contained 1% BSA, for 1 h. Hybridoma culture supernatant (100 µl) was then added to the wells and they were incubated for 1 h at room temperature on an orbital shaker. After incubation, the wells were washed 4 times with PBS and incubated with β-galactosidase-conjugated goat anti-mouse IgG (BRL hybridoma screening kit) for 1 h, with shaking. The color was developed with the substrate p-nitrophenyl-d-galactoside and was read at 405 nm using an ELISA plate reader (Model EL307; BioTek, Burlington, VT).

Bioassay. Hybridoma culture supernatants from positive wells of ELISA were tested for their ability to inhibit TCSF activity of LX-1 cells or membrane extract derived from LX-1 cells, as described below.

Whole LX-1 cells were incubated with hybridoma culture media before coculturing with fibroblasts. Typically 0.2–0.5 × 10^6 LX-1 cells were incubated at 37°C for 45 min with 100 µl of hybridoma media, after which the whole mixture was added to the fibroblasts (1 × 10^6) and cultured as described in "Assay of TCSF Activity" below.

Detergent extracts of LX-1 membranes were dialyzed and incubated either with hybridoma media or with purified IgG preparation for 30 min at 37°C, followed by overnight at 4°C. After incubation, Affi-Gel-protein A-Sepharose suspension (Bio-Rad, Richmond, CA) was added to these samples (10 µl/300-µl sample) and the mixture was agitated at room temperature for 20 min. The Sepharose beads were centrifuged in a Beckman microfuge at 9000 × g for 5 min and the supernatants were collected. These fractions were UV sterilized for 5 min on ice and were added to the fibroblast cultures for assay of TCSF activity.

Purification of Immunoglobulin from Hybridoma Media

Immunoglobulins from hybridoma media were purified on an Affi-Gel-protein A column. The hybridoma culture supernatant was brought to 50% saturation with (NH₄)₂SO₄ and the precipitate was suspended in PBS. All buffers contained 0.02% sodium azide.

Purification of TCSF

For purification of TCSF we used an immunoaffinity matrix (7), where Affi-Gel-Protein A was first reacted with rabbit anti-mouse serum (50 mg protein) in order to increase the efficiency of binding of antibody. Diallyzed, (NH₄)₂SO₄-precipitated, rabbit anti-mouse whole serum (50 mg protein) in binding buffer (MAPS-II kit) was passed through a 5-ml column of Affi-gel-protein A that was equilibrated with the same buffer. After extensive washing with the binding buffer, the column was washed and equilibrated with borate buffer, pH 8.2. The hybridoma supernatant was brought to 50% saturation with (NH₄)₂SO₄. The precipitate was suspended in PBS, dialyzed against borate buffer (pH 8.2), and added to the affinity column. The mixture was then cross-linked with 20 mM dimethylpimelimidate dihydrochloride (Sigma), as described by Schneider et al. (7).

LX-1 membranes, prepared as described previously (4), were extracted with 10 mM Tris-HCl (pH 8.2), containing 1 mM EDTA, 0.15 M NaCl, 2 mM PMSF, and 0.5% Nonidet P-40, at 4°C overnight. The supernatant of the centrifuged extract was preabsorbed with a 10% suspension of Staphylococcus aureus Cowan I strain (Enzyme Center, Boston, MA), then added to a 5-ml column of the cross-linked beads, and recirculated through the column for 12 h at 4°C. The column was washed with buffers in the following sequence: (a) 0.5 M NaCl, 50 mM Tris-HCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.1 mM PMSF, pH 8.2; (b) 0.15 M NaCl, 50 mM Tris-HCl, 0.5% Nonidet P-40, 0.1 mM PMSF, 0.1% SDS, pH 8.2; and (c) 0.15 M NaCl, 50 mM Tris-HCl, 0.1 mM PMSF, 30 mM OG, pH 8.2 (two washes). Antibodies bound to the matrix were then eluted from the column with an equal volume of 50 mM diethylamine, pH 11.5, containing 30 mM OG. The eluted material was immediately neutralized by the addition of 0.1 volume of 0.5 M NaH₂PO₄, dialyzed against 0.1 M acetic acid, concentrated approximately 100 times in a Minicon concentrator B-15 (Amicon, Danvers, MA), and stored at 4°C.

Identical results were obtained by using either IE11F4 or IIG4F6 hybridomas for the preparation of the immunoaffinity column.

Preparation of [35S]TCSF

For labeling of LX-1 cells with [35S]methionine, the cells were plated at a density of 1 × 10⁶ cells in 100-mm plates and were incubated under tissue culture conditions until they reached confluence. The media were removed and the dishes were washed with methionine-free DMEM and incubated in the same media for 4 h in the presence of [35S]methionine (30 µCi/ml) under tissue culture conditions. The labeled culture media were saved for analysis of secreted forms of TCSF (see below) and the cells were used for purification of membrane-bound TCSF as described above. Purified [35S]TCSF was electrophoresed in SDS gels (10%) and then autoradiographed (8) using Kodak XAR film.

The TCSF cross-reactive material in the culture media was immunoprecipitated with the IE11F4 monoclonal antibody (100 µl/ml media). The antigen-antibody complex was incubated with Affi-Gel-protein A and the Sepharose beads were collected by centrifugation in a Beckman microfuge at 9000 × g for 5 min. The beads were treated with Laemmli’s sample buffer (9), electrophoresed in a SDS-polyacrylamide gel (10%), and autoradiographed.

Assay of TCSF Activity

Two types of assays were used. In the first assay, the stimulation of collagenase resulting from coculturing of LX-1 cells with fibroblasts was measured. In the second, the effect on collagenase production of addition of putative TCSF-containing samples, e.g., LX-1 membrane extracts, to fibroblast cultures was measured.

Cultures containing human fibroblasts (1 × 10⁶/well) or LX-1 cells (0.2–1 × 10⁶/well) either alone or together, were established in 16-mm wells (Linbro; Flow Laboratories, McLean, VA) in 1 ml of DMEM that was supplemented with 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml), unless otherwise mentioned. The plates were then incubated to dryness in a 37°C oven and were replaced with 0.5 ml of DMEM containing 0.2% lactalbumin hydrolysate and antibiotics (10). For the second type of assay, samples containing TCSF diluted with the same media as above in a total volume of 0.5 ml were added to fibroblast cultures. All cultures were incubated for 2–3 days and the culture fluids were harvested and used for collagenase assay.

Collagenase Assay

Fibril Lysis Assay. Routinely, collagenase activity against type I collagen was assayed in culture media, with or without trypsin activation, by a film lysis procedure (2) in which samples are applied to thermally reconstituted [14C]-acetylated rat tail collagen fibrils dried to a film in the microwells of 96-well plates (Linbro; Flow Laboratories). For each assay, 200 µl of culture medium were incubated with or without 20 µl of 1-tosylamide-2-phenylthyl chloromethyl ketone-tryp-
The effect of TCSF on DNA synthesis. Human fibroblasts were plated at a
density of 1 × 10^4 and 1 × 10^5 cells/2 cm² and incubated overnight under tissue culture conditions. After the cell layers were washed three times with DMEM containing no serum, the cells were incubated for 2 days with TCSF and then incubated with [³H]thymidine (0.1 µCi/ml) in DMEM containing 0.2% lactalbumin hydrolysate for 6 h. After incubation, the cell layers were processed for measurement of incorporated radioactivity (4).

BALB/c 3T3 cells (1 × 10⁴) were plated into a 96-well microtiter plate in DMEM and 10% fetal calf serum. After a period of 7 days, the cells were incubated with different concentrations of TCSF, with 4 µCi of [³H]thymidine/ml, and then processed as described elsewhere (16). In both assays epidermal growth factor (Collaborative Research, Lexington, MA) was used as a standard mitogen at concentrations of 5–20 ng/ml.

RESULTS

Identification of Monoclonals. Mice were given injections of detergent extract of LX-1 membranes (see "Materials and Methods"). Before fusion we tested the serum from injected mice for its ability to inhibit stimulation of collagenase production in cocultures of fibroblasts and LX-1 cells, as described in "Assay of TCSF Activity" of "Materials and Methods." There was 70% inhibition of collagenase-stimulatory activity, as compared to preimmune serum (data not shown). After fusion we plated cells into 800 microtiter wells, at approximately 5 × 10⁴ cells/well, and satisfactory growth was obtained in 143 wells. The media from these mixed hybridomas were tested in the ELISA using dried LX-1 cells, as described in "Materials and Methods." This assay was selective for outer cell surface proteins, since no reactivity was obtained using an antibody to Mr 21,000 protein (a gift from Dr. Channing J. Der, LaJolla Cancer Research Foundation, LaJolla, CA), an inner plasma membrane protein shown to be present in LX-1 cells (17). This Mr 21,000 antibody gave a strong reaction in the ELISA if cell extracts were used instead of dried whole cells. Positive reaction in the ELISA, using whole LX-1 cells, was obtained with media from 32 of the mixed hybridoma wells. The media from the 13 wells with the highest ELISA readings were then tested in the bioassay to investigate their ability to inhibit stimulation of collagenase production in cocultures. The cocultures were set up at two cellular ratios of fibroblasts to LX-1 cells, i.e., 1:0.3 and 1:1. Mouse immune serum was used as a positive control in this assay. Four of the mixed hybridoma media showed significant inhibition, compared to controls. These hybridomas were used for cloning. Two of the clones (IE11 and IIG4) showed significant inhibition of TCSF activity (Table 1). They were subcloned and shown to retain positive responses in both

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of hybridoma-conditioned media on collagenase production in cocultures</th>
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<td>Addition</td>
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<tr>
<td>III1</td>
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sin (50 µg/ml) for 5 min at 37°C, after which 20 µl of soybean trypsin inhibitor (250 µg/ml) were added and the mixture was used in the assay.

ELISA. The amount of collagenase in culture medium samples was also measured in an ELISA (11, 12), using an antibody raised against human fibroblast collagenase prepared according to the procedure of Stricklin et al. (13). The wells of a microtiter plate were coated with 50 µl of sample, diluted to 200 µl with ELISA coating buffer (0.17% NaHCO₃, 0.21% Na₂CO₃, pH 8.6, containing 0.02% sodium azide), at 4°C overnight. Excess protein binding sites were blocked by incubation with 1% BSA in PBS for 1 h at room temperature, followed by three washes with PBS containing 0.05% Tween 20. The wells were then incubated for 1 h at room temperature with the rabbit anti-human collagenase antisera (100 µl of 1:500 dilution in ELISA buffer containing 0.2% BSA). The color was developed by using a peroxidase-conjugated goat anti-rabbit immunoglobulin and was measured in an ELISA plate reader. Preimmune rabbit serum was used as a control.

Neutral Protease Activity

Neutral protease activity was measured by using a SDS substrate gel according to the procedure described by Heusson and Dowdle (14). Gelatin was added to the standard Laemmli acrylamide polymerization mixture, at a final concentration of 1 mg/ml. Samples were mixed with the sample buffer with or without DTT, incubated for 1 h at room temperature, electrophoresed, and stained with amido black.

Dot-Immunoblot Analysis of TCSF

The presence of TCSF in different preparations was assayed by dot-blot analysis. Using a Bio-Dot apparatus (Bio-Rad) and following the manufacturer's procedure, sample was dotted on a nitrocellulose filter. The nonspecific sites were blocked with 3% horse serum in Tris-buffered saline (50 mM Tris and 200 mM NaCl, pH 7.4) and then the dots were incubated with the hybridoma supernatant for 1 h, followed by goat anti-mouse IgG. The color was developed with 4-chloro-l-naphthol in methanol, and 15 µl of H2O2 in Tris-buffered saline, in a total volume of 30 ml. Positive reactions are indicated by blue spots.

Polyacrylamide Gel Electrophoresis and Transblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli's procedure (9). After electrophoresis, the proteins were transferred electrophoretically to Zetaprobe paper (Bio-Rad) (15). After the nonspecific sites were blocked with 10% BSA in Tris-buffered saline at 45°C for 12 h, the paper was incubated with hybridoma supernatant overnight at 4°C. The sheet was washed 3 or 4 times with PBS and incubated for 1 h with peroxidase-conjugated anti-mouse IgG. The color was developed with 4-chloro-1-naphthol prepared as described above.

Immunofluorescence Histology

Sterile glass coverslips (22 x 22 mm) were laid down on a 35-mm tissue culture dish and seeded with cells. Cells were allowed to grow for 2 days under standard culture conditions (see above). The cell monolayers on the coverslips were washed with PBS and fixed in 1% paraformaldehyde in PBS for 45 min at room temperature. The fixed cells were washed again in PBS and quenched with 0.1 M Tris, pH 7.4, for 1 h at room temperature. Nonspecific binding sites were blocked with a solution containing 1% BSA, 0.01% Triton X-100, 1% normal goat serum, and 2% nonfat milk in PBS that contained 0.02% sodium azide. After washing with PBS, the coverslips were incubated at 37°C for 1 h with hybridoma supernatant followed by rhodamine-conjugated goat anti-mouse IgG (Cappel Worthington Biochemicals, Malvern, PA). The coverslips were washed and mounted on slides and fluorescence microscopy was performed on a Zeiss (IR-35) microscope. The images were photographed on Kodak Ektachrome film.

[³H]Thymidine Incorporation

Both human fibroblasts and 3T3 cells were used to investigate the effect of TCSF on DNA synthesis. Human fibroblasts were plated at a
the ELISA and bioassay procedures. Two subclones derived from IE11 and IIG4 (IE11F4 and IIG4F6) were cloned again to confirm their purity and stability. Ninety% of these subclones were positive in the ELISA, assuring us of their clonal origin. Ouchterlony diffusion assay showed that clone IE11F4 produced IgG2a and IIG4F6 produced IgG1, thus indicating that they were not identical clones. These two subclones were used in all subsequent experiments. In most of the experiments presented in this study IE11F4 was used but virtually identical results were also obtained with IIG4F6.

To confirm the effectiveness of the clones, dialyzed LX-1 membrane extract was preincubated with the immunoglobulin fraction isolated from these cultures, the antigen-antibody complex was absorbed on protein A-Sepharose beads, and the unabsorbed fractions were tested for TCSF activity (see “Bioassay”). Table 2 shows the results from three such experiments. With increasing antigen-antibody ratios there was more inhibition of TCSF activity as compared to controls.

Western blot analysis of the membrane extract with either antibody usually revealed a single band with an approximate molecular weight of 58,000 (Fig. 1, Lane 4). In 1 of 10 samples, a minor band with an approximate molecular weight of 35,000 was obtained. For comparison, a Coomassie blue-stained gel of the same extract is shown in Fig. 1, Lane 2; this serves to emphasize the specificity of the monoclonal antibodies, in that they do not react with the vast majority of the proteins present in the extract. Extract prepared from nude mouse-grown LX-1 tumor also showed a M, 58,000 band (Fig. 1, Lane 3) and, occasionally, other bands of approximate molecular weights of 35,000–42,000.

Membrane extracts obtained from fibroblasts in the same way as described for the tumor cells were also analyzed for cross-reactivity with the monoclonal antibodies to TCSF. Neither dot-blot analyses (Fig. 2) nor Western blots (data not shown) showed the presence of any cross-reactive material in the fibroblast preparation.

**Purification of TCSF from LX-1 Membranes.** Using immunoaffinity column chromatography, we attempted purification of TCSF from the detergent extract of LX-1 membranes. The hybridoma IE11F4 was used for the preparation of the column. Seventeen mg of protein from 2.4 x 10^9 cells were loaded on a 5-ml column (1.5 x 1.8 cm), and the column was washed and eluted as described in “Materials and Methods.” The eluted fractions were tested by dot-blot analysis using the starting material as a control. The fractions with positive cross-reactivities were pooled, dialyzed against 0.1 M acetic acid, concentrated, and stored at 4°C. By Western blot analysis the purified TCSF showed a single band with a molecular weight of ~58,000, in 24 of 30 preparations; in the other 6 preparations, a minor band (M, ~35,000) was also observed (Fig. 1, Lane 3). Variable amounts of high molecular weight aggregates were also observed in overloaded gels, such as that shown in Fig. 1, Lane 3. Similar data were obtained using the hybridoma IIG4F6. The amount of protein in the final preparation of TCSF was very low, thus leading to difficulty in obtaining an accurate measurement in the protein assay. However, from several different experiments it appears that approximately 500-fold purification was obtained by the immunoaffinity chromatography. The low protein content of the final preparation also led to difficulty in obtaining staining in SDS-polyacrylamide gels. Thus the purity of the preparation was tested by autoradiography of purified 35S-labeled TCSF. Radiolabeled 35S-TCSF, isolated and purified in the same manner as described above from membranes of LX-1 cells metabolically labeled with [35S]methionine, showed one band with an approximate molecular weight of 58,000 after sodium dodecyl sulfate-gel electrophoresis and autoradiography (Fig. 3).

Storage of purified TCSF at 4°C for 6–8 weeks at acid or neutral pH resulted in the loss of biological activity. Appropriate conditions for stabilization of the pure protein have still not been determined.

The purified TCSF was also assayed directly for collagenase-stimulatory activity by addition to fibroblast cultures. Table 3 shows that with increasing concentration of TCSF there is increased collagenase production by the fibroblasts. This has been confirmed in four separate experiments. A similar result

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**Table 2 Effect of IgG isolated from conditioned media of clone IIG4F6 on fibroblast collagenase production**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>No antibody</th>
<th>0.3:1*</th>
<th>1.3:1*</th>
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<tr>
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<td>7</td>
</tr>
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<td>3</td>
<td>16</td>
<td>4.4</td>
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* Antibody:antigen ratio.
° ND, not determined.

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**Fig. 1. Western blot of various preparations that contain purified TCSF or TCSF-like material, using hybridoma IE11F4. Lanes 1 and 2, SDS-polyacrylamide gel; Lane 1, molecular weight markers; Lane 2, OG extract of LX-1 membranes. Lanes 3–5, Western blots; Lane 3, purified TCSF (this lane is deliberately overloaded to reveal minor components); Lane 4, OG extract of LX-1 membranes; Lane 5, extract prepared from nude mouse-grown LX-1 tumor. For details see “Materials and Methods.” The same results were obtained with the hybridoma, IIG4F6.
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Fig. 2. Dot-blot analysis of various preparations for the presence of TCSF using media from two hybridoma clones. Rows A and B, duplicate dots with clone IG12F6; rows C and D, duplicate dots with clone IE11F4. Column 1, blank; Column 2, purified TCSF; Column 3, whole membrane extract from LX-1 cells; Column 4, whole membrane extract from human fibroblasts; Column 5, DMEM with 5% fetal calf serum as control; Column 6, concentrated conditioned media from LX-1 incubated with DMEM containing 0.2% lactalbumin hydrolase; Column 7, concentrated conditioned media from LX-1 incubated with DMEM that contained 5% fetal calf serum.

has also been obtained using an ELISA for measurement of the amount rather than activity of collagenase produced on stimulation (Fig. 4).

Other Properties of TCSF. To determine whether TCSF has any mitogenic activity, both human fibroblasts and 3T3 cells were incubated with [3H]thymidine in the presence of TCSF. In both assays, no increase in thymidine incorporation was observed compared to controls (data not shown). In our assay human fibroblasts were nonresponsive to epidermal growth factor, whereas 3T3 fibroblasts were responsive. Neither cell type responded to TCSF, suggesting that TCSF is not closely related to epidermal growth factor. However, more studies are necessary to obtain a definitive answer to this question.

TCSF did not show any intrinsic protease activity, assayed by gelatin-incorporated gel lysis assay (14), and it did not activate latent collagenase (data not shown).

Presence of TCSF in Conditioned Media of LX-1 Cells. In previous studies we reported the presence of collagenase-stimulatory activity in LX-1-conditioned media (2). Therefore, we explored the presence of TCSF-like material in the conditioned media of these cultures. Using either hybridoma, dot-blot analysis revealed positive cross-reactivity with proteins in conditioned media harvested from LX-1 cells cultured with or without fetal calf serum (Fig. 2). However, this reactivity could be detected only after 100-fold concentration, indicating that the

Table 3 Effect of purified TCSF on fibroblast collagenase production

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>% of total cpm released</th>
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<tbody>
<tr>
<td>Fibroblasts</td>
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</tr>
<tr>
<td>Fibroblasts + 0.52 μg TCSF</td>
<td>11.0</td>
</tr>
<tr>
<td>Fibroblasts + 1.3 μg TCSF</td>
<td>23.0</td>
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Fig. 3. Autoradiogram of purified TCSF preparation and conditioned media of LX-1 cells. Lane 1, SDS gel of purified [35S]TCSF (2500 cpm); Lane 2, SDS gel of material from conditioned media of [35S]-labeled LX-1 cells immunoprecipitated with TCSF antibody IE11F4 (2700 cpm). For details see "Materials and Methods."

Fig. 4. ELISA for collagenase in culture media of fibroblasts incubated with purified TCSF. The wells of a microtiter well were coated with media from fibroblasts incubated with different concentrations of pure TCSF and reacted with polyclonal antibody for human collagenase, as described in "Materials and Methods" ("Collagenase assay: ELISA.")
amount of cross-reactive material present in these samples is low or that fewer antigenic sites are present.

Conditioned media from metabolically labeled LX-1 cells showed several bands with molecular weights ranging from 35,000–68,000 after SDS gel electrophoresis of the immuno-precipitated material (with IE11F4) and autoradiography (Fig. 3, Lane 2). The proportions of these bands varied in different preparations but the $M_r$ 58,000 band was always present.

Immunolocalization of TCSF. The localization of TCSF in LX-1 cells was investigated by indirect immunofluorescence staining (Fig. 5). The LX-1 cells showed positive staining with media from the monoclonal antibodies IE$_{11}$F$_4$ and IIG$_4$F$_6$ but not with media from a clone producing antibody to type I collagen (a kind gift from Dr. T. F. Linsenmayer, Tufts University, Boston, MA). The staining was observed at the outer surface of the LX-1 cells but no staining was obtained with human fibroblasts (Fig. 5).

**DISCUSSION**

Studies from several laboratories indicate that interactions between host fibroblasts and tumor cells are responsible for increased collagenase production in neoplastic tissue. Bauer and colleagues (18, 19) have shown that fibroblasts derived from human basal cell carcinoma produce increased amounts of collagenase, compared to normal skin fibroblasts, and that extracts of this tumor stimulate normal fibroblasts to produce elevated levels of collagenase. Baici et al. (20) have shown that explants of rabbit V2 carcinoma stimulate explants of normal tissue in a similar fashion. A related study has been reported by Dabbous et al. (21). Using immunological methods we have previously shown that collagenase present in the rabbit V2 carcinoma implanted in the nude mouse is at least in part produced by cells of the nude mouse host (22). We have also demonstrated that fibroblasts in culture can be stimulated to produce high levels of collagenase by factor(s) of either animal or human tumor cell source but the reverse is not the case (1, 2). The stimulatory factor activity is present in conditioned media of tumor cells but its presence therein is influenced by fibroblast-produced matrix (3). Recently we have shown that the stimulatory factor is associated with the outer cell surface of tumor cells and that it can be extracted with detergent from isolated tumor cell membranes (4). Although there is evidence that some tumor cells themselves are capable of producing collagenase against type I collagen (23), the above studies indicate that interactions of fibroblasts and tumor cells play a significant role in elaboration of collagenase activity associated with tumors.

To facilitate understanding the nature and the mechanism of action of TCSF, we undertook purification of the factor. We raised monoclonal antibodies against TCSF and used these antibodies to purify the factor on an antibody affinity column. We have shown that the antibody produced by two purified clones inhibited the stimulation of fibroblast collagenase production on incubation with tumor cells or with membrane extract from tumor cells. Both antibodies recognized a protein with a molecular weight of ~58,000 in unfractionated membrane extracts of LX-1 cells. Purified TCSF was shown to contain a protein with a molecular weight of ~58,000 by immunoblotting and by SDS-polyacrylamide gel electrophoresis autoradiography of purified radiolabeled material. In 80% of the 30 preparations produced to date, only a single protein was detectable; in the other cases, a small amount of a $M_r$ ~35,000 protein was observed in immunoblots. The latter may have arisen by degradation of the $M_r$ 58,000 protein during preparation or storage. Our data strongly imply that the collagenase-stimulatory activity resides in the $M_r$ 58,000 protein, for the following reasons: (a) the monoclonal antibodies almost always recognize only one protein band at $M_r$ 58,000 in crude membrane extracts or purified preparations (Fig. 1); (b) purified radiolabeled TCSF shows only one band at $M_r$ 58,000 in autoradiograms (Fig. 3); (c) the purified TCSF stimulates fibroblast collagenase production (Table 3; Fig. 4) and the level of mRNA for collagenase (24); (d) the monoclonal antibodies inhibit TCSF activity (Table 2); (e) polyclonal antiserum raised against the $M_r$ 58,000 protein band excised from a SDS-poly-
acrylamide gel inhibits TCSF activity.4

Purified TCSF preparations stimulate fibroblast collagenase production but the extent of fibroblast collagenase stimulation by purified TCSF was lower than that obtained in cocultures of fibroblasts and tumor cells (2). It is conceivable that under the bioassay conditions, TCSF molecules are unstable, undergo aggregation, or stick to the culture plates. An alternative possibility is that another cellular component may be needed to express the full potential of TCSF activity. We are currently exploring these possibilities. The purified TCSF did not have any mitogenic activity towards the human fibroblasts used for measuring TCSF activity or in a conventional 3T3 proliferation assay. Preliminary experiments5 using a number of growth factors and their antibodies, e.g., platelet-derived growth factor, epidermal growth factor, transforming growth factor α, and transforming growth factor β, indicated lack of cross-reactivity with TCSF. However, a more detailed analysis is required to confirm these results. To understand the molecular nature of TCSF and to investigate further whether it bears any structural or functional homology to proteins already described in the literature, we are currently attempting to determine the amino acid sequence of the M, 58,000 protein.

Examination of conditioned media from cultures of LX-1 cells showed the presence of several molecular weight forms cross-reacting with the TCSF antibodies. One of the major forms had a molecular weight of 58,000, suggesting that membrane TCSF may be shed in an intact form into the medium. The TCSF in the medium may be bound to membrane vesicles. Vesiculation of the plasma membrane is an active process in many tumor cells in culture (25). The other major species in conditioned medium had molecular weights less than 58,000 and may have arisen by proteolytic cleavage. In support of this idea, we have obtained data indicating that limited protease digestion of the M, 58,000 membrane-derived TCSF gives rise to a M, 35,000 form which is identical in size to one of the species often found in conditioned media.6 We do not know the origin of the higher molecular weight M, 68,000 form; however, it is a minor component of the media, is not always found, and may represent an aggregate of smaller forms, since aggregation is commonly seen with the membrane-derived species.

Immunofluorescence analysis indicates that TCSF is present on the outer surface of LX-1 cells, and is absent from normal fibroblasts. Further studies7 indicate that it is present at the plasma membrane of several human tumor cell types. The process of tumor invasion and metastasis involves a number of biological steps, one of which is production of type I collagenase and other neutral proteases. These enzymes are thought to have a significant role in the degradation of host connective tissue, thus allowing penetration of the tumor cells (26). Our studies indicate that the production of type I collagen-degrading enzyme by fibroblasts is influenced by a tumor cell surface protein. Thus we propose that, during tumor invasion, this enzyme is secreted upon interactions between host fibroblasts and tumor cells, thereby facilitating movement of the latter through the host tissue.

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Monoclonal Antibody Preparation and Purification of a Tumor Cell Collagenasestimulatory Factor

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