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Membrane Association of Collagenase Stimulatory Factor(s) From B-16 Melanoma Cells

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Past studies have shown that contact between tumor cells and fibroblasts results in stimulation of collagenase production by the fibroblasts. Membrane fractions prepared by differential centrifugation of sonicated B-16 melanoma cells were shown here to contain a collagenase stimulatory factor(s) (CSF). Trypsin treatment of intact B-16 cells prior to membrane fractionation led to loss of 90% of the total activity, indicating that CSF is localized on the outer surface of the cells. Stimulation of fibroblast collegenase production was also observed with dialyzed octylglucoside extracts of the B-16 membranes. Additional of exogeneous lipid, ie, a mixture of phosphatidylcholine and phosphatidylserine, to the detergent extract of the membranes followed by dialysis and centrifugation at 100,000g resulted in 80% recovery of the factor activity in the pellet containing reconstituted lipid vesicles. Fractionation of tritium-labeled, reconstituted lipid vesicles on a Sephacryl S-300 column revealed that the collagenase stimulatory factor coeluted with the radioactive lipid vesicles. The fractionated lipid vesicles lost stimulatory activity completely after trypsin treatment or heating at 65°C, indicating that the factor is a protein.

Key words: cell-cell interactions, tumor invasion, collagenase stimulation

Increased collagenase activity against type I collagen is associated with several neoplasms [1–7] and appears to be important in tumor cell invasion [5,8,9]. We have shown previously that cocultures of several types of tumor cells with fibroblasts produce as much as 50 times more collagenase activity against type I collagen than individual cultures of each cell type. The stimulatory effect was obtained with conditioned media from tumor cell cultures added to the fibroblasts. However, media

Abbreviations used: CSF, collagenase stimulatory factor; OG, octyl-D-glucopyranoside; TPCK, 1-tosylamido-2-chlormethyl ketone; SBTI, soybean trypsin inhibitor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution—calcium and magnesium free; PBS, phosphate-buffered saline—calcium and magnesium free.

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from the fibroblasts added to the tumor cells had no effect, indicating that the source of collagenase in the cocultures was the fibroblasts [10,11]. Further attempts to characterize the nature of the stimulatory factor(s) from B-16 melanoma cells have revealed that its level in conditioned media depends on the particular culture condition and that cell contact between the tumor cells and fibroblasts was often required to elicit stimulation of collagenase production [12]. However, exposure of the B-16 cells to fibroblast matrix constantly gave rise to increased levels of the factor activity in the culture medium [12]. These observations led us to explore the possible association of the factor with the tumor cell plasma membrane. In this study we present data which indicate that a collagenase stimulatory factor(s) (CSF) is associated with tumor cell membranes and is present at the outer surface of the tumor cells. Furthermore, the CSF can be extracted with detergent from the isolated membranes and can be reintercalated into lipid vesicles.

MATERIALS AND METHODS

Materials

Reagents included pancreatic trypsin (type I) and octyl glucoside (OG) from Sigma (St. Louis, MO); 1-tosylamido-2-phenyl ethyl chloromethyl ketone trypsin (TPCK-trypsin) and soybean trypsin inhibitor (SBTI) from Worthington Biochemical Corp. (Freehold, NJ); ^{3}H-dipalmitoyl phosphatidylcholine (51 mCi/mmol) and ^{3}H-dThd (deoxythymidine) (20 mCi/mmol) from New England Nuclear Corp (Boston, MA); and phosphatidylcholine and phosphatidylserine from Avanti Chemicals (Birmingham, AL). Type I collagen was isolated and purified from rat tail tendon and labeled with ^{14}C-acetic anhydride as described previously [13].

Cells and Culture Conditions

The sources of rabbit synovial fibroblasts and mouse B-16 melanoma cells have been described previously [10]. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml) and used for experiments between the 10th and 25th passages.

Cultures containing either fibroblasts, tumor cells, or both were established in 20-mm-diameter wells (Linbro) at a density of 10^5 cells of each cell type per well containing DMEM and 5% FBS. The cells were allowed to attach for 24 hr at 37°C in a humid atmosphere of 5% CO_2 and 95% air, after which their media were replaced with fresh media prior to beginning the experiment. Total volume in each well was 0.5 ml. Each experimental condition was in triplicate wells. Culture fluids were harvested and used for the collagenase assay.

Preparation and Extraction of Membranes

Cells were allowed to reach confluence using standard culture conditions. The media were removed and the cultures were rinsed four times with Hanks' balanced salt solution (HBSS). The cells were then scraped from the dishes with a rubber policeman and suspended in 50 mM Tris (pH 7.4) containing 0.24 M sucrose (2 ml per 100-mm dish containing 3–5 $\times$ 10^6 cells). The suspension was sonicated in a Branson sonicator using two bursts of 30 sec each at 4°C. The sonicate was centrifuged at 500g for 20 min at 4°C, then the supernatant was collected and centrifuged

at 100,000g for 1 hr in a Spinco type 40 rotor at 4°C. In some experiments the 500g supernatant was first centrifuged at 20,000g in a Sorvall SS-34 for 20 min at 4°C, and then the supernatant was further centrifuged at 100,000g for 1 hr.

The supernatant fractions were dialyzed exhaustively against HBSS overnight followed by DMEM and then used for the CSF assay as described below. The membrane fractions were assayed for CSF activity before and after detergent treatment. For the latter, membranes suspended in HBSS were extracted with an equal volume of 60 mM OG containing 10 mM HEPES (pH 7.4) and 0.2 mM dithiothreitol at 4°C for 30 min with occasional mixing in a Vortex mixer [14]. The suspension was centrifuged at 100,000g for 1 hr in a Beckman rotor type 40. The supernatant was removed, dialyzed extensively against HBSS and DMEM as mentioned above and used in the CSF assay. The protein concentration of the preparations was determined by Lowry's procedure [15]. Protein concentration in the extracted samples varied between 250 and 500 μg/ml for different preparations.

Reconstitution of Stimulatory Factor Into Membrane Vesicles

To the extract described above, phenylmethyl sulfonyl fluoride and ovalbumin were added to 0.1 mM and 1 mg/ml, respectively [14]; and then the solution was dialyzed extensively at 4°C against phosphate buffered saline—Ca^{++}/Mg^{++} free (PBS)—containing 0.1 mM dithiothreitol. A final dialysis was done against DMEM containing 0.1 mM dithiothreitol. During this entire procedure tissue culture glasswares were used, the dialysis tubings were soaked in PBS and UV-sterilized for 10 min at 4°C prior to use, and all reagents contained penicillin (100 units/ml) and streptomycin (100 μg/ml). The association of the stimulatory factor activity with membrane vesicles was tested by centrifugation of the dialyzed detergent extract at 100,000g for 1 hr followed by assaying the stimulatory activity in the supernatant and the pellet containing the reconstituted vesicles. The effect of addition of exogenous lipid on vesicle reconstitution was tested by adding a mixture of soybean phosphatidylcholine and phosphatidylserine at a ratio of 4:1 (w/w) [16] to the detergent extract of the membrane before dialysis. The lipid mixture was dissolved in chloroform, after which the chloroform was removed by evaporation under N_2; the dried residue was taken up in HBSS, sonicated in a Branson sonicator for 1.5 min, and added to the detergent extract of the membrane. For vesicle-labeling studies, ^{3}H-dipalmitoyl phosphatidylcholine was added to the mixture of phosphatidylcholine and phosphatidylserine in chloroform and processed as above. The amount of ^{3}H-radioactivity used was approximately 2×10^7 cpm/ml of extract. Vesicle preparations were suspended in DMEM prior to further experimentation.

Isolation of Vesicle-Membrane Factor Complex

The reconstituted ^{3}H-labeled vesicle-membrane factor complex was fractionated on a Sephacryl S-300 column. The column (1.8 × 20 cm) was equilibrated with PBS containing 0.1 mM dithiothreitol, penicillin (100 units/ml), and streptomycin (100 μg/ml). The sample was dialyzed against the same buffer before use. The column fractions were screened for absorbance at 280 nm, for radioactivity, and for CSF activity.

The ^{3}H-labeled vesicle membrane factor complex was further examined by discontinuous sucrose gradient centrifugation. Sephacryl S-300 fractionated, reconstituted vesicles were layered on top of the sucrose gradient made with 5 ml each of

60%, 30%, and 17% sucrose in DMEM layered sequentially, and then centrifuged at 4°C for 16 hr at 26,000 rpm in a Beckman L8-80 ultracentrifuge using a SW 28 rotor. Fractions of 0.5 ml were collected from the bottom and analyzed for ^{3}H-radioactivity.

Assay for CSF Activity

Aliquots of the crude membrane fractions, dialyzed detergent-extracted membranes or column fractions derived therefrom were tested for stimulation of rabbit fibroblast collagenase production. The fractions were UV-sterilized for 10 min at 4°C and then added to rabbit fibroblasts plated at a density of 1×10^5 cells/2 cm^2 well as described in Cells and Culture Conditions. The media collected after 3 days were analyzed for collagenase activity. In these assays fractions containing CSF activity were routinely tested over a range of protein concentrations (50–100 μg) in a final volume of 0.5 ml/culture well.

Collagenase Assay

Measurement of type I collagen-degrading activity was performed using ^{14}C-labeled rat tail tendon collagen in a gel film lysis assay as decribed previously [13]. Both active and trypsin-activable collagenase activities were assayed. Typically 200 μl of culture medium was incubated with or without 20 μl or TPCK-trypsin (1 mg/ml) for 5 min at 37°C after which 20 μl of SBTI (5 mg/ml) was added, the mixture was left at room temperature for 2 min, and then used in the collagenase assay. The amount of trypsin giving optimal activation was determined by using a range of trypsin concentrations. Each assay included a trypsin blank and a buffer blank as controls and total incubation time was 4 hr at 37°C.

A unit of collagenase activity is defined as micrograms of collagen degraded per minute at 37°C. Positive collagenase activity was confirmed by SDS-polyacrylamide gel electrophoretic [17] analysis of the characteristic TC_A and TC_B fragments obtained on digestion of collagen by animal collagenase [13].

DNA Synthesis

To investigate the effect of membrane factor on DNA synthesis, cells were first incubated with the factor for different time periods under tissue culture conditions and then pulsed for 6 hr with ^{3}H-dThd (0.1 μCi/ml). After washing the cell layers with PBS two times they were incubated at 37°C for 5 min with 0.25% trypsin, 0.02% EDTA in PBS. The cell suspension was precipitated by the addition of 50% trichloroacetic acid to a final concentration of 10%. After standing for 60 min in ice, the pellets were collected by centrifugation in a Beckman Microfuge B for 3 min. The pellet was dissolved in 100 μl of 0.2 N NaOH then acidified with 20 μl of 1.0 N acetic acid. The radioactivity in the final sample was then measured.

RESULTS

Membrane Association of the Collagenase Stimulatory Factor Activity

Various membrane fractions prepared from mechanically harvested B-16 cells were tested for CSF activity by addition to cultures of fibroblasts and measurement of collagenase production (see Materials and Methods). Both active and trypsin-activable collagenase activity was measured in the assay samples. However, only

trypsin-activable enzyme activity was detected in all the samples. The conditions for optimal trypsinization were determined by measurement of activity subsequent to treatment with a wide range of trypsin concentrations [13]. Most of the CSF activity (78%) was associated with the 20,000g and 100,000g pellets, two-thirds of this CSF activity being present in the 20,000g pellet (Table I). This then suggests that the CSF is membrane bound in nature and is preferentially enriched in large membrane particles. Since 81% of the total factor activity can be recovered in the combined 20,000g and 100,000g pellets, compared to 54% in the 20,000g pellet alone, the combined membrane fractions obtained by centrifugation at 100,000g, after centrifugation at 500g, were used for subsequent experiments. The CSF activity of this combined membrane fraction was equivalent to approximately 25% of that of the number of cells from which it was derived (Table II). When the combined membrane fraction was assayed directly for collagenase it did not have any endogenous enzyme activity (Table II).

The membrane fraction was also prepared from B-16 cells after incubation of the intact cells with trypsin. The trypsin treatment of the intact cells would destroy CSF exposed on the external side of the plasma membrane but would not destroy

TABLE I. Membrane Association of Collagenase Stimulatory Factor Activity of B-16 Cells*

Fraction	Collagenase stimulatory activity	
	Total units	% of total
500g pellet	0.6	4.2
20,000g pellet	7.6	54.2
100,000g pellet	3.8	27.0
100,000g supernatant	2.0	14.3

*B-16 melanoma cells were harvested, sonicated, and fractionated by differential centrifugation into three pellet fractions and one supernatant fraction. The pellet fractions were suspended in 1 ml of HBSS per pellet obtained from 10 × 100-mm culture dishes. The suspected pellets and supernatant were dialyzed against HBSS followed by DMEM then assayed for CSF activity as (see Materials and Methods). The amount of each fraction used in the assay was normalized to the original cell number. Values are average of duplicate measurements.

TABLE II. Effect of Trypsinization of B-16 Cells on CSF Activity of Isolated Membranes*

Experimental condition	Collagenase (m units/ml ± SEM)
Fibroblasts alone	< 10
Fibroblasts plus B-16 cells	840 ± 90
Fibroblasts plus B-16 membrane	210 ± 10
Fibroblasts plus B-16 membrane from trypsinized cells	18 ± 5
B-16 cells alone	< 10
B-16 membrane alone	< 10

*A high-speed membrane (100,000g) fraction was prepared from sonicated B-16 cells as described in Materials and Methods. For trypsinization, the B-16 cell pellet obtained after mechanical scraping from monolayer cell cultures was incubated with 0.01% TPCK-trypsin in HBSS for 30 min at 37°C (10 ml per cell pellet obtained from 10 dishes) followed by the addition of an equal volume of 0.05% SBTI in HBSS. The cell pellet collected after centrifugation at 1,000g was washed once with 0.05% SBTI in HBSS and used for fractionation of membranes. The CSF activities of the membrane fractions were compared with that of an equivalent number of intact B-16 cells. The levels of collagenase produced by the cells or membranes alone were also measured after incubation for an equivalent period, ie, 3 days at 37°C. The values given are means of three measurements.

CSF associated with internal membranes since they are not accessible to the trypsin. This pretreatment with trypsin resulted in 90% loss of CSF activity in the membranes (Table II), suggesting that most of the activity is attached to the outer surface of B-16 cells.

Detergent Extraction of CSF and Reconstitution Into Lipid Vesicles

The membrane preparation was extracted with 30 mM OG and the dialyzed detergent extract was tested for CSF activity. Results shown in Table III indicate that the dialyzed detergent extract of the membrane stimulated fibroblast collagenase production. The extract had more activity than the whole membrane, possibly because of more uniform dispersion of the factor. The activity of the extract was calculated to be similar to that of the number of cells from which it was derived. The extracts did not show any intrinsic collagenase activity when tested after 3 days of incubation at 37°C under tissue culture conditions or when assayed directly.

Reconstitution of CSF into artificial lipid vesicle membranes was explored by addition of exogenous lipid to the detergent-containing extract followed by dialysis and centrifugation at 100,000g. A mixture of phosphatidylcholine and phosphatidylserine in a ratio of 4:1 was added to the OG extract of membrane at several phospholipid-to-protein ratios, and the mixture was then dialyzed extensively against DMEM. The dialyzed samples were centrifuged at 100,000g, and the supernatants and pellets were assayed. The total CSF activity recovered in the combined supernatant and pellet fractions did not change significantly upon addition of increasing amounts of exogenous phospholipid at ratios of lipid to protein ranging from 0:1 to 1:1. However, without the addition of exogenous phosholipid, the amount of CSF activity that became associated with the pellet fraction was only 5% of the total activity recovered. When lipid was added at the ratio of 0.1:1, the proportion in the pellet was 40%, and between 0.2:1 and 1:1 the proportion remained constant at approximately 80%. Thus in further experiments, exogenous phospholipid was added at a ratio of 0.2:1. In these experiments, where 70–80% of CSF activity was recovered in reconstituted vesicles, only 38% of the total protein was obtained in this fraction. This indicates that the CSF was preferentially associated with the vesicles as compared to most of the other proteins in the detergent extract.

Confirmation of reconstitution of CSF into the lipid vesicles was achieved by using ^{3}H-phospholipid in the reconstitution mixture and fractionating the ^{3}H-lipid vesicles on a gel filtration column. Fractionation of the ^{3}H-vesicles on a Sephacryl S-300 column revealed that most of the factor activity and ^{3}H-radioactivity eluted together in the void volume along with the major protein peak (Fig. 1). Approximately

TABLE III. CSF Activity of Octylglucoside Extract of B-16 Membranes*

Experimental condition	Collagenase (m units/ml ± SEM)
Fibroblasts alone	< 10
Fibroblasts plus B-16 membrane	425 ± 30
Fibroblasts plus OG extract of B-16 membrane	1,400 ± 158

*The membrane fraction from sonicated B-16 cells was extracted with OG as described in Materials and Methods. The dialyzed extract was centrifuged at 100,000g for 1 hr, and the supernatant fraction was assayed for CSF activity. The CSF activities of equivalent amounts of membrane and detergent extract were compared. Values are means of three measurements.

85% of the recovered factor activity was eluted in the void volume with the vesicles (Fig. 1). A similar profile of elution of radioactivity was obtained for ^{3}H-lipid vesicles reconstituted in the absence of the factor (data not shown). These results have been reproduced in five separate experiments.

Further demonstration of vesicle formation was assessed by discontinuous sucrose gradient fractionation as shown in Figure 2. Sephacryl S-300 fractionated ^{3}H-vesicles reconstituted from extracts of CSF were layered on top of the sucrose gradient and subjected to centrifugation for 16 hr at 26,000 rpm in a Beckman SW28 rotor. The major peak of radioactivity layered at the 17%–30% interface, indicating the buoyant nature of the sample. In addition the Sephacryl S300 fractionated ^{3}H-vesicles were subjected to high-speed centrifugation before and after detergent treatment. The radioactivity recovered in the 100,000g pellet of untreated ^{3}H-vesicles was 85%. However, after pretreatment of the ^{3}H-vesicles with 1% Triton X-100, only 9% of the total radioactivity sedimented in the pellet. These data support the conclusion that the ^{3}H-labeled lipid which voided on Sephacryl S-300 was present as vesicles. Attempts to remove Triton by treating the samples with Biobeads SM-2 in order to assay CSF activity were unsuccessful, as were attempts to extract the CSF from the reconstituted vesicles with 30 mM OG, even though this detergent was used in the extraction of the original cell membranes. Further experimentation is necessary to clarify this issue.

Properties of Reconstituted CSF

Treatment of the reconstituted vesicles with trypsin completely destroyed the factor activity and 80% of the factor activity was lost upon heating at 65°C for 15

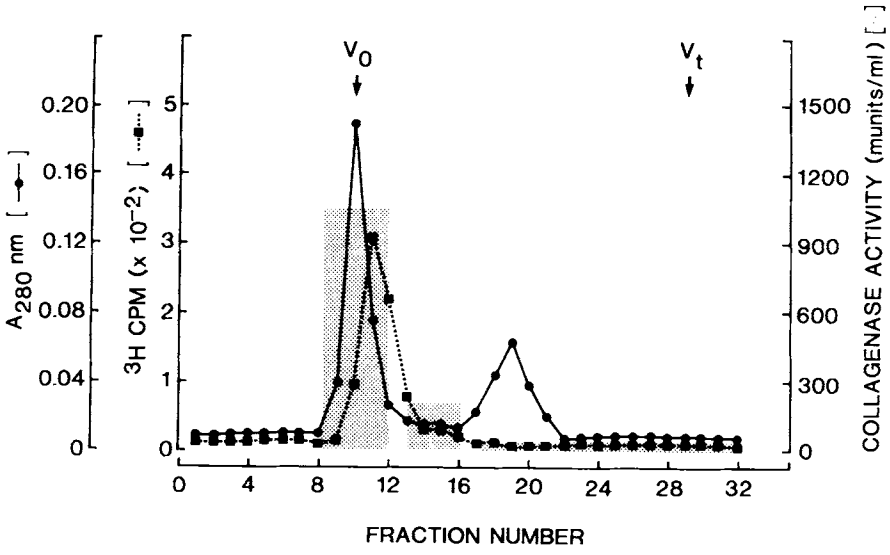

Fig. 1. Fractionation of reconstituted lipid vesicles on a Sephacryl S-300 column. Reconstituted lipid vesicles were prepared under optimal conditions in the presence of ^{3}H-phosphatidylcholine and fractionated on a 1.8×20cm column equilibrated with PBS containing 0.1 mM dithiothreitol. Fractions of 1 ml were collected and analyzed for radioactivity and absorbance at 280 nm. Pooled fractions were dialyzed against DMEM and assayed for CSF activity. Approximately 80% of the radioactivity and 67% of the CSF activity were recovered from the column.

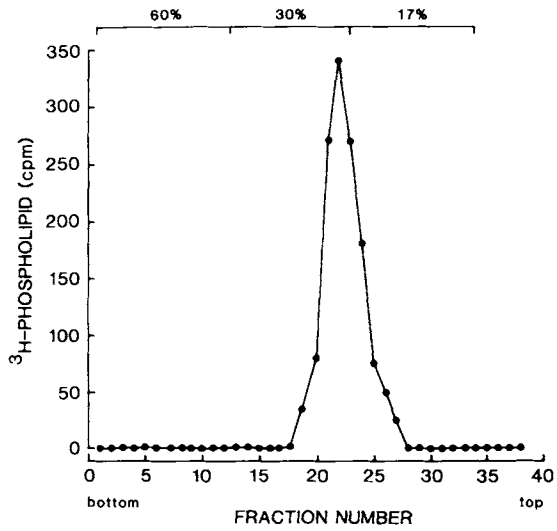

Fig. 2. Fractionation of reconstituted lipid vesicles on a discontinuous sucrose gradient. One milliliter of pooled fractions from Sephacryl S-300 column chromatography containing both CSF activity and ^{3}H radioactivity (Fig. 1) was layered on top of a discontinuous sucrose gradient (60%,30%, and 17% w/v) and centrifuged at 26,000 rpm for 16 hr (see Materials and Methods). Fractions of 0.5 ml were collected and used for radioactivity measurement. The pelleted fraction was also analyzed after suspension in PBS. Total recovery of radioactivity was 80%.

min (data not shown). These data along with its nondialyzable nature indicate that the stimulatory factor(s) is a protein.

To investigate the minimum time period necessary for incubation of fibroblasts with CSF, the fibroblasts were incubated with the fixed concentration of 100 μg/ml of protein for different time periods. The cultures were then extensively washed with DMEM to remove the factor and further incubated for 3 days. A maximal response was obtained after the cells were preincubated with the factor for 24 hr; however, a significant response was obtained after only 8-hr exposure to the factor (Fig. 3). The CSF activity of the reconstituted lipid vesicles was assayed at various protein concentrations. Figure 4 shows that the fibroblasts collagenase activity was linear up to a protein concentration of 200 μg/ml.

The effect of the factor on ^{3}H-dThd incorporation into DNA by fibroblasts was investigated under conditions of culture where collagenase stimulation was obtained. No significant effect was observed on addition of the factor at several different concentrations (Table IV). Addition of cycloheximide at concentrations ranging from 10^{-9} to 10^{-7}M to fibroblast cultures incubated with the reconstituted vesicles inhibited the stimulation by CSF (Table V), suggesting that de novo protein synthesis is necessary for the production of collagenase as is the case in most other systems [13,18,19].

DISCUSSION

Morphological and biochemical evidence from several laboratories indicates that extracellular matrix degradation and increased collagenase activity against type I collagen are associated with several neoplasms [1–9]. However, immunolocalization

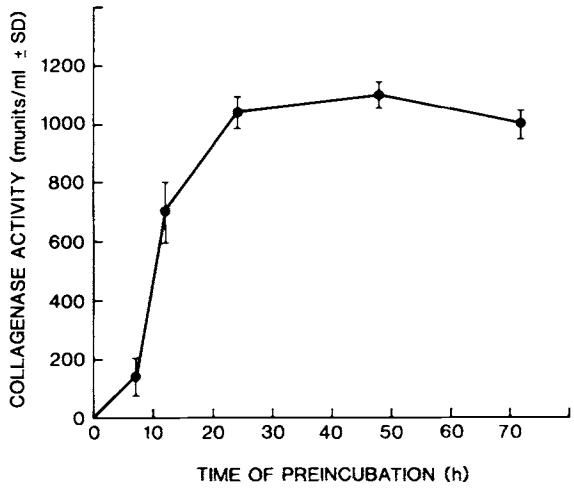

Fig. 3. Time course of fibroblast response to CSF. A fixed amount of reconstituted vesices (protein = 100 μg) was added to fibroblasts for assay of CSF activity as described in Materials and Methods. After different periods of incubation with the vesicles the culture wells were washed three times with DMEM containing 5% FBS and antibiotics and further incubated for three days with 0.5 ml of the same media. The media collected at the end of this 3-day incubation period were used for collagenase assay.

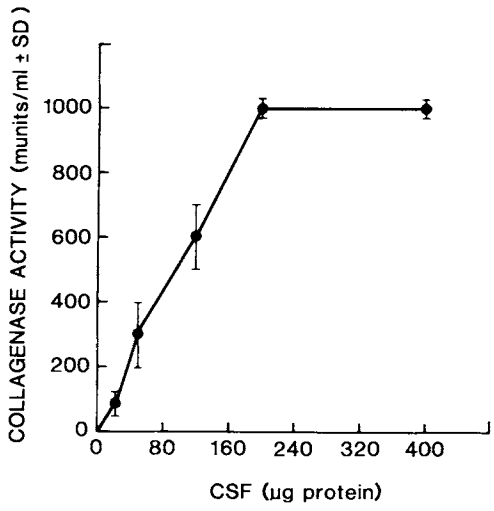

Fig. 4. Dose-response curve for reconstituted lipid vesicles containing CSF. Different amounts of Sephacryl S-300-fractionated, reconstituted lipid vesicles were assayed for CSF activity as decribed in Materials and Methods.

studies of a variety of tumors [5,20] have demonstrated that this collagenase is present in the collagenous stroma surrounding tumor cells rather than asociated with the tumor cells themselves. Bauer and coworkers have reported that fibroblasts derived from human basal cell carcinoma produce increased amounts of collagenase compared to normal skin fibroblasts [21] and that extracts of this tumor stimulate normal fibroblasts to produce elevated levels of collagenase [22]. Baici et al [23] have shown that explants of rabbit V2 carcinoma stimulate explants of normal subcutaneous tissue

TABLE IV. Effect of CSF on DNA Synthesis*

Experimental condition	cpm incorporated (± SEM)	% control
Fibroblasts alone	5,215 ± 860	
Fibroblasts plus 10 μl CSF	4,570 ± 697	92
Fibroblasts plus 24 μl CSF	4,139 ± 69	80
Fibroblasts plus 50 μl CSF	4,877 ± 850	93

*Fibroblasts were incubated for 2 days with different concentrations of S-300 fractionated CSF (500 μg protein/ml), then pulsed with ^{3}H-dThd (total counts = 50,700 cpm) as described in Materials and Methods. The optimal conditions for thymidine incorporation were predetermined in a separate experiment. Collagenase activity obtained in cultures with 50 μl CSF was 750 m units/ml.

TABLE V. Effect of Cycloheximide on CSF Activity*

Experimental condition	Collagenase (m units/ml ± SEM)
Fibroblasts plus CSF	750 ± 65
Fibroblasts plus CSF plus cycloheximide (10^{-9}M)	140 ± 20
Fibroblasts plus CSF plus cycloheximide (10^{-8}M)	< 50
Fibroblasts plus CSF plus cycloheximide (10^{-7}M)	< 50

*Fibroblasts were incubated for 3 days with a dialyzed OG extract of B-16 cells (equivalent to 100 μg protein concentration) as described in Table III in the absence and presence of different concentrations of cycloheximide.

in a similar fashion. In past studies we have shown, using immunological methods, 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 [13]. We have also demonstrated that fibroblasts in culture can be stimulated to produce high levels of collagenase by tumor cell factor(s) of either animal or human tumor cell sources whereas the reverse is not the case [10,11]. Although there is evidence that some tumor cells themselves are capable of production of collagenase against type I collagen [24,25], the above studies suggest very strongly that interactions between fibroblasts and tumor cells lead to the observed high levels of collagenase activity associated with tumors in vivo.

Our attempts to characterize the CSF of B-16 melanoma cells led to the finding that the presence of the factor in B-16-cell-conditioned medium depends on the culture conditions. Of particular interest was the observation that in some cases contact between the tumor cells and fibroblasts was necessary for collagenase stimulation [12]. The data presented here indicate that CSF is in fact a membrane-bound protein. The finding that most of the CSF activity of intact cells is accessible to degradation by trypsin indicates that the active site of CSF is external to the plasma membrane. Since the CSF activity is 1) associated with membrane fractions from B-16 cells, 2) is absent in membrane fractions prepared from trypsin-treated B-16 cells, 3) requires detergent for its extraction, and 4) codistributes with lipid vesicles, it is most likely derived from the plasma membrane. Similar results have been obtained in human lung carcinoma LX-1 cells [43]. The lipid vesicle reconstitution experiments suggest that the CSF contains a hydrophobic domain which mediates intercalation into lipid membranes. However, further experiments are necessary to rule out other possibilities such as formation of large protein-protein or protein-lipid aggregates on removal of detergent.

We propose, on the basis of the above evidence and our past studies [12], that in cocultures of tumor cells and fibroblasts, the exposed active site-containing region of the tumor cell CSF interacts with the surface of fibroblasts either directly via cell contact or subsequent to shedding into the culture medium under the influence of fibroblast-produced matrix. The exact molecular nature of CSF, the relationship between the secreted form and membrane bound form, and its possible relation to other collagenase-stimulating factors—eg, from epithelia [26,27] or tumor extracts [22]—remain to be established. Preliminary evidence indicates that CSF does not have any significant mitogenic effect under the assay conditions used here. Whether CSF is related to growth factors such as platelet-derived growth factor [28] or epithelial growth factor [29] which have been shown to stimulate collagenase in other systems, requires further study under a wider range of experimental conditions.

Movement of tumor cells through normal tissue involves penetration of both basement membranes and interstitial stroma. A variety of molecular changes have been associated with these invasive events—eg, degradation of type I collagen [9,13], type IV collagen [30], and heparan sulfate-proteoglycan [31] as well as alterations in amounts of various extracellular macromolecules [32–36]. Degradation of the basement membrane components, type IV collagen, and heparan sulfate-proteoglycan appears to be due to production of degradative enzymes by the tumor cells themselves [31,37,38]. However, the increased production in tumors of type I collagenase [10,11,22], other proteases [23,39], hyaluronate [40,41], and proteoglycan [42] appears to be medited by interactions between the tumor cells and neighboring fibroblasts. The data presented here indicate that the latter events may be due to plasma membrane factors derived from the tumor cells which interact with the surface of the fibroblasts, thereby facilitating changes in the extracellular matrix associated with tumor invasion.

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