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Matrix Influence on the Tumor Cell Stimulation of Fibroblast Collagenase Production

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Cocultures of rabbit fibroblasts and mouse B-16 melanoma cells produce increased levels of collagenase against type I collagen. This stimulatory effect was also found when fibroblasts were cultured in conditioned media from tumor cells. However, the level of the stimulatory factor in conditioned media was influenced by matrix deposited by fibroblasts. Thus, conditioned media collected from monolayers of B-16 plated on fibroblast matrix consistently showed high levels of the factor activity. The influence of the matrix on the level of the factor was not removed by treating the fibroblast matrix with collagenase or chondroitinase ABC and was not reproduced by collagen-coated dishes.

Key words: matrix, collagenase, tumor cells, cell-matrix interactions

In previous studies, I have demonstrated that cocultures of rabbit fibroblasts and mouse tumor cells [1] or human fibroblasts and human tumor cells [2] produce increased levels of collagenase against type I collagen compared to cultures of the individual cell types. This stimulatory effect was also found when fibroblasts were cultured in conditioned media from the tumor cells. Further attempts to characterize the stimulatory factor from B-16 melanoma cells have revealed that its level in the conditioned media of these cells depends on the particular culture conditions. In this study, I report that the presence of the factor in culture medium from B-16 cells is influenced by plating the cells on matrix deposited by fibroblasts.

MATERIALS AND METHODS

Reagents

Pancreatic trypsin (type I) and chondroitinase ABC were from Sigma (St. Louis, MO); 1-tosylamido-2 phenyl ethyl chloromethyl ketone-trypsin, soybean trypsin inhibitor, and bacterial collagenase (CLSPA) were from Worthington Diagnostic Systems (Freehold, NJ). Type I collagen was isolated and purified from rat tail tendon and labeled with ^{14}C-acetic anhydride as described before [1,2].

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Cells

Rabbit synovial fibroblasts were grown from tissue explants as described before [1]. The mouse B-16 melanoma cell line used in previous studies [1] was routinely passaged through C-57/Bl6 mice. All cells were maintained in Dulbecco's modified Eagle's medium containing 5% fetal calf serum (FCS).

Culture Conditions

Cultures containing either fibroblasts, tumor cells, or both were established in 16-mm diameter wells (Linbro) at a density of 10^5 cells of each cell type per well containing 1.0 ml Dulbecco's modified Eagle's medium (DMEM) and 5% fetal calf serum. 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 medium was replaced with fresh complete medium (0.5 ml/well). Each experimental condition was done in triplicate wells. Culture fluids were harvested and replaced with fresh medium at day 3. The harvested media were used for collagenase assay after activation with TPCK-trypsin, followed by the addition of soybean trypsin inhibitor as described before [1,2].

Preparation of Fibroblast Matrix

Fibroblast matrix, ie, substratum-attached material (SAM), from rabbit synovial fibroblast cultures was prepared according to the procedure described by Murray and Culp [3]. Briefly, 0.5×10^5 cells were plated in 16-mm diameter wells containing 1 ml DMEM and 5% fetal calf serum. After the cells were allowed to attach for 24 hr at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, the medium was replaced with fresh complete medium (0.5 ml/well). After 24 hr, the cell layers were washed twice with Ca^{2+}, Mg^{2+} free PBS (PBS-A) and then incubated at 37°C with shaking in 0.5 mM EGTA in PBS-A. The cell layers were lifted off the wells by gently pipetting up and down. The total time of incubation required for removing the cells from the wells varied from 60–90 min between experiments. The cells were removed by gentle suction and the wells were washed three times with PBS-A and used as the substratum (SAM) for B-16 cells. In some experiments, SAM was pretreated with enzymes, ie, bacterial collagenase or chondroitinase ABC, followed by 4× washing with PBS-A.

Preparation of B-16 Conditioned Medium

B-16 cells were plated on fibroblast-SAM at a density of 1×10^5 cells/16-mm well in a total volume of 1 ml of DMEM and 5% FCS. The cells were allowed to attach for 24 hr, after which the medium was replaced with 0.5 ml fresh culture medium. The cells were incubated at 37°C for 3 days in a humid atmosphere of 5% CO_2 and 95% air. Media from these cultures were collected and centrifuged at 200g to remove floating cells. Fresh fetal calf serum was added to bring the concentration to 5% [1] and these media were used for assay of collagenase stimulatory activity using fibroblast cultures as described below. Control conditioned media were prepared from B-16 cells plated in tissue culture wells without SAM. In some experiments, collagen-coated dishes were used [4,5], in which case bacteriological plastic dishes (60 mm) were coated with purified rat tail collagen (2 mg/ml in 0.5 N acetic acid) and air dried at room temperature. After drying, the dishes were washed three times with PBS-A containing serum albumin (1 mg/ml) to block nonspecific binding sites. The dishes were washed three times with PBS-A, UV sterilized for 15 min, and used for B-16 plating.

Assay of Collagenase Stimulatory Factor

B-16–conditioned medium was added to rabbit synovial fibroblasts plated in culture wells (1×10^5 cells/16 mm), then the cultures incubated at 37°C for 3 days. The final concentration of FCS in both control and experimental cultures was 5%. The media from these wells were assayed for collagenase activity.

Collagenase Activity

Collagenase activity against type I collagen was assayed in the culture media, after trypsin activation, by a film lysis procedure as described previously [1,6]. In this assay activity is proportional to the release of labeled degradation products from ^{14}C-labeled collagen films polymerized in multiwell plates. Assays were performed at 37°C for 4 hr and included a buffer blank and a trypsin blank as controls. One unit of collagenase activity is defined as the amount of enzyme degrading 1 μg of collagen per minute at 37°C. The specificity of the assay was confirmed by identification of collagenase reaction products (α_1A, α_2A, α_1B, α_2B) by polyacrylamide gel electrophoresis [7] as described previously [1,2].

RESULTS

Cocultures of B-16 melanoma cells and rabbit fibroblasts consistently yield increased levels of collagenase activity compared to separate cultures of the two different types of cells [1]. Conditioned medium harvested from B-16 cell cultures plated in a conventional manner on plastic tissue culture dishes often stimulated the fibroblasts to produce similar levels of collagenase activity to the cocultures (Table I, Experiment A). However, in some cases B-16–conditioned medium did not have this effect (Table I, Experiment B). B-16–conditioned medium prepared in this manner was effective in 12 out of 20 such experiments performed to date, but in all 20 experiments cocultures gave high levels of collagenase activity. Using a preparation

TABLE I. Variable Effect of Conditioned Medium From B-16 Cells on Collagenase Production by Rabbit Fibroblasts*

Experiment	Cell type	Agent added	Collagenase (m units/ml)
A	Rabbit synovial fibroblasts	None	<20
	B-16	None	<20
	Rabbit synovial fibroblasts + B-16	None	1,240
	Rabbit synovial fibroblasts	B-16 CM (40%)	470
	Rabbit synovial fibroblasts	B-16 CM (80%)	1,000
B	Rabbit synovial fibroblasts	None	<20
	B-16	None	<20
	Rabbit synovial fibroblasts + B-16	None	1,140
	Rabbit synovial fibroblasts	B-16 CM (80%)	<20

*Rabbit synovial fibroblasts were incubated alone, with B-16 cells, or with conditioned medium from B-16 cultures. The conditioned medium was diluted to 40 or 80% prior to use. The culture media were collected at day 3 and assayed for collagenase activity as described in Materials and Methods. Data presented are the averages from two separate experiments. In experiment A, the conditioned medium was active, and in experiment B it was inactive. Of 20 experiments performed to date, 12 have given results similar to A and eight similar to B. CM, conditioned medium.

of B-16 cells that did not yield stimulatory conditioned medium, cultures were also performed where both cell types shared the same medium but were physically separated. This was done by attaching a perforated 35-mm culture dish to the inside of a 60-mm dish, thus creating two separated compartments sharing the same medium. When the B-16 cells and fibroblasts were plated together in one compartment, collagenase activity in the medium was high, but when each was plated into a separate compartment, collagenase activity was low, ie, similar to that of individual cultures plated in separate dishes (Table II). Thus, the stimulation obtained in cocultures appeared in some cases to depend on close cellular associations. This would most likely be mediated by direct cell contact or cell-associated matrix. In the above experiments, it was observed that B-16 cells plated with fibroblasts become more flattened and more tightly attached to the substratum than when they were plated alone. Thus, the influence of fibroblast matrix (SAM) on the ability of B-16 cells to condition their medium with the collagenase stimulatory factor was investigated.

The experimental protocol for testing the effect of fibroblast SAM on conditioning of B-16 culture medium is described in Materials and Methods. Three steps are involved, ie, preparation of fibroblast SAM, collection of conditioned medium from B-16 cells grown on this SAM (SAM/B-16), and measurement of collagenase stimulatory activity by culture of fibroblasts in the presence of SAM/B-16 conditioned medium. As shown in Figure 1, addition of SAM/B-16 conditioned medium stimulated fibroblast collagenase production to the level of 75% of that obtained in cocultures of fibroblasts and B-16. The maximum stimulation obtained varied between 30 and 75% from experiment to experiment. No significant amount of collagenase activity was detected in the SAM/B-16–conditioned medium itself (<20 m units/ml). Further experiments revealed that conditioning of B-16 media also depended on the initial plating density of the rabbit fibroblasts used for preparation of SAM. As shown in Table III, the maximum conditioning of B-16 culture medium occurred when SAM was prepared from fibroblast cultures plated at 0.5×10^5 cells/16-mm well. The requirement for FCS in conditioning the media of B-16 cells was also tested. It was observed that the conditioned media collected from B-16 cells plated on fibroblast SAM in the absence of serum did not stimulate fibroblast collagenase activity. To investigate which component of SAM might be responsible for enhancing the conditioning of B-16 medium, SAM was treated with enzymes before plating of the B-16 cells. Pretreatment of SAM with bacterial collagenase or chondroitinase ABC did not

TABLE II. Requirement for Close Cell Association for Stimulation of Collagenase Production*

Cell type	Compartment	Collagenase activity (m units/ml)
Rabbit synovial fibroblasts	Outer	< 20
B-16	Inner	< 20
Rabbit synovial fibroblasts + B-16	Inner	340
Rabbit synovial fibroblasts/B16	Outer/inner	< 20

*Cells were plated into a vessel comprised of a punctured 35-mm tissue culture dish attached inside a 60-mm tissue culture dish. B-16 cells and fibroblasts were plated into the inner 35-mm dish or the outer space between the walls of the 60- and 35-mm dishes at densities equivalent to 1×10^5 per 16-mm well. Collagenase activity was assayed in the medium harvested at day 3. Data presented are the averages from two separate experiments. The B-16 cells used in these experiments did not condition their medium with stimulatory factor when cultured on plastic.

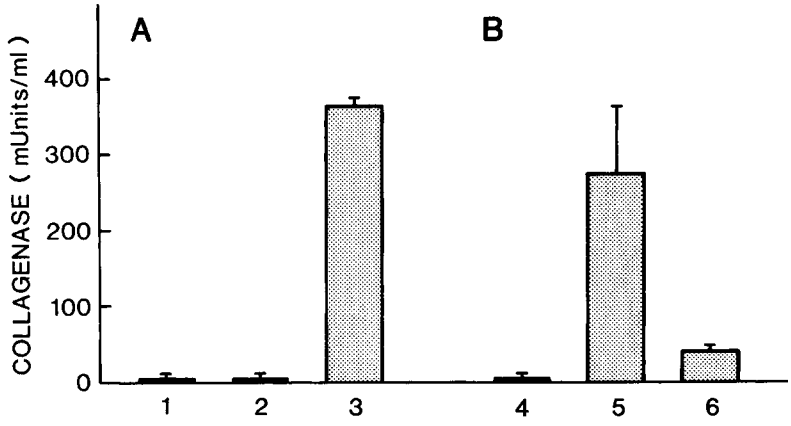

Fig. 1. Effect of fibroblast matrix (SAM) on the level of collagenase stimulatory factor in B-16-conditioned medium. A) Collagenase activity was assayed in 3-day culture medium from 1) fibroblasts alone, 2) B-16 alone, and 3) fibroblasts plus B-16. B) Collagenase activity was assayed in 3-day culture medium from fibroblasts incubated with conditioned medium from B-16 plated on 4) tissue culture plastic, 5) fibroblast SAM, and 6) collagen-coated petri dishes. None of these conditioned media contained collagenase activity when assayed directly. Experimental design is described in Materials and Methods. Results are expressed as m units of collagenase ± SEM per milliliter culture media.

TABLE III. Effect of Fibroblast Density on SAM-Induced Conditioning of B-16 Medium*

Cell density- (cell No./16-mm well)	Collagenase activity (m units/ml ± SEM)
0.1×10^5	<20
0.2×10^5	145 ± 34
0.5×10^5	195 ± 19
1.0×10^5	20 ± 10

*Fibroblasts plated at the different densities were used for preparation of SAM as described in Materials and Methods. B-16 cells were plated on these preparations of SAM, incubated for 3 days under tissue culture conditions, and their conditioned media were assayed for collagenase stimulatory activity as described in Materials and Methods. Collagenase activity was also measured directly in conditioned media of the fibroblasts plated at a density of 0.2×10^5 or 1×10^5 per 16-mm well and found to be <20 m units per milliliter of media.

reduce the effect of SAM (Fig. 2), suggesting that neither collagen nor one of the glycosaminoglycans, hyaluronic acid, chondroitin, or the chondroitin sulfates, is the active component in SAM. The increased effect of collagenase-treated SAM compared to untreated SAM was not due to contamination by bacterial collagenase but presumably was the result of increased exposure of the active factor in SAM.

Attempts have also been made to reproduce the effect of SAM with purified extracellular matrix components. As shown in Figure 1, collagen is ineffective, a result that has been reproduced in four experiments. Other materials, however, have

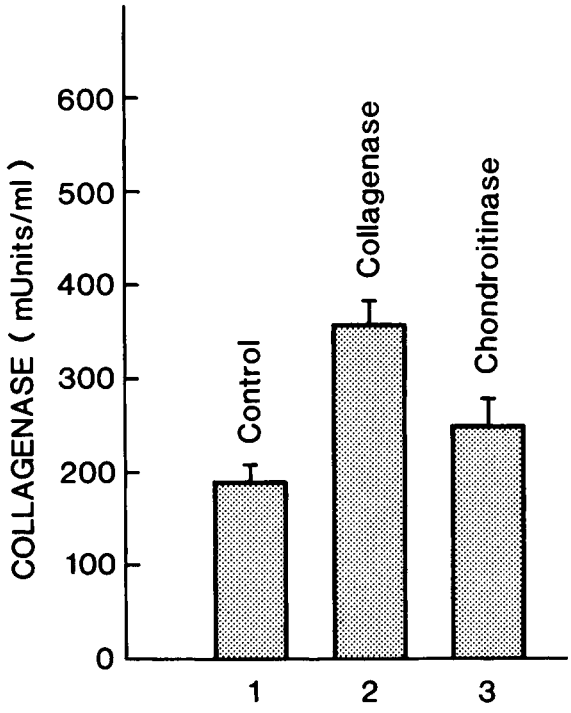

Fig. 2. Effect of enzyme-treated SAM on the level of collagenase stimulatory factor in B-16–conditioned medium. The matrix was treated either with bacterial collagenase (50 μ/16-mm well) or chondroitinase ABC (0.5 μ/16-mm well) at 37°C for 1 hr prior to the preparation of B-16–conditioned medium. Collagenase activity was assayed in 3-day culture medium from fibroblasts after incubation with conditioned medium from B-16 grown on fibroblast SAM which was 1) untreated, 2) pretreated with collagenase, or 3) pretreated with chondroitinase ABC. Experimental design is the same as in Figure 1. Controls in this experiment included assay of medium from cultures of fibroblasts or B-16 cells alone, cocultures of fibroblasts and B-16, and cultures of fibroblasts in the presence of conditioned medium from B-16 cells plated on plastic. These controls gave similar results to those shown in panels 1 to 4 in Figure 1.

had variable effects, notably fibronectin which alone or in combination with collagen was stimulatory in some preliminary experiments. Further experimentation with various combinations of these and other SAM components will be necessary to clarify the nature of the active factor in SAM.

DISCUSSION

The present study indicates that the production or release of collagenase stimulatory factor from tumor cells is influenced by fibroblast matrix. Since cocultures of fibroblasts and B-16 always showed high levels of collagenase activity, it seems likely that the release of the tumor cell stimulatory factor into the media of these cocultures is affected by fibroblast matrix present in the cocultures. Supporting this is the observation that conditioned medium from cocultures of fibroblasts and B-16 stimulates collagenase production in cultures of fibroblasts alone even when conditioned

medium from the same B-16 cells cultured alone on plastic is not stimulatory (data not shown).

Although exposure of B-16 cells to SAM clearly causes secretion of the collagenase-stimulatory factor into the culture medium, secretion was also shown to occur in the absence of SAM in many cultures of B-16 cells [1] (Table I). Thus, it appears that the mechanism of release of the factor(s) into the medium depends not only on fibroblast matrix but also on other environmental factors. Recent observations in my laboratory suggest that the stimulatory factor is bound at the cell surface of tumor cells and is hydrophobic in nature [8,9]. Thus, it is reasonable to assume that the factor is released from the tumor cell membrane into the culture medium under the influence of SAM or other variables in the culture conditions. Attempts to release the factor from the surface of tumor cells by cytochalasin B or cold shock, agents known to induce vesiculation of cells [10,11] have been unsuccessful. It is not yet known which component in SAM induces the release of factor into the medium. Since SAM is composed of fibronectin, proteoglycans, hyaluronate, and many other substances [12], any of these components alone or in complex associations might influence factor release.

Extracellular matrix components have been shown by others to influence various aspects of tumor cell behavior that might promote their invasiveness, eg, tumor cell adhesion [13,14] and migration [15,16]. The results herein demonstrate that extracellular matrix also stimulates production of the collagenase stimulatory factor, which may in turn cause increased matrix degradation and thus facilitate invasion of tumor cells through connective tissue barriers.

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