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Induction of fibroblast 92 kDa gelatinase/type IV collagenase expression by direct contact with metastatic tumor cells

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SUMMARY

Previous studies have correlated release of the 92 kDa type IV collagenase/gelatinase by tumor cells in culture with metastatic potential. We have now demonstrated that the ability of tumor cells that do not express the 92 kDa gelatinase to induce release of this metalloproteinase from normal fibroblasts may also be associated with the metastatic phenotype.

A transformed rat embryo cell line, 2.8, failed to release the 92 kDa gelatinase alone in culture, but gave rise to metastatic tumors whose explants contained the 92 kDa gelatinase. In contrast, a non-metastatic transformed cell line, RA3, did not express the 92 kDa gelatinase alone in culture or in tumor explants. To explore the mechanisms that might govern host-tumor cell interactions in this system, we have studied the effects of co-culture of these transformed cell lines with rat embryo fibroblasts (REF) in culture. 92 kDa gelatinase expression was induced by co-culture of 2.8 with REF, but co-culture of the non-metastatic line RA3 with REF did not result in induction of the 92 kDa gelatinase. The 92 kDa gelatinase in these co-cultures was released by the fibroblasts; methanol-fixed 2.8 cells induced 92 kDa gelatinase expression in REF, but fixed REF cells did not induce enzyme expression in 2.8 cells. This suggested that cell contact was required for induction, which was confirmed by showing that 92 kDa gelatinase induction in co-culture was abolished by separating REF from 2.8 by solute-permissive membranes. In addition, REF could not be stimulated to produce the 92 kDa gelatinase by 2.8-derived conditioned medium, by 2.8-derived extracellular matrix, or by isolated matrix components. These data indicate that metastatic tumor cells can induce 92 kDa gelatinase expression in fibroblasts through a mechanism dependent upon cell contact.

In situ hybridization of nude mouse tumors derived from these transformed cell lines revealed 92 kDa gelatinase expression in the stroma of tumors from 2.8, but not in tumors from RA3. Therefore, the experiments based on in vitro co-culture of tumor cells and fibroblasts, together with the in situ localization of mRNA to host cells, suggest that host production of the 92 kDa gelatinase may occur in response to direct contact with metastatic tumor cells.

Key words: MMP-9, 92 kDa type IV collagenase/gelatinase, fibroblast, metastasis

INTRODUCTION

Degradation of the extracellular matrix and basement membrane is an essential component of several steps in tumor invasion, angiogenesis and metastasis (Liotta and Stetler-Stevenson, 1991; Goldberg and Eisen, 1991; Hart et al., 1989). Different classes of enzymes have been implicated in this process, including members of the zinc-dependent proteinase family known collectively as matrix metalloproteinases (MMPs) (Matrisian, 1990). The ability of tumor cells to produce these metalloproteinases has been implicated in the process of tumor progression. Tumor production of a particular metalloproteinase, the 92 kDa type IV collagenase/gelatinase (MMP-9), has been associated with the metastatic phenotype (Bernhard et al., 1990, 1994; Ballin et al., 1988, 1991; Yamagata et al., 1988).

Production of MMPs by the host is also important in human tumor progression. Pyke and coworkers (1992) localized mRNA for MMP-9 to tumor-associated stromal cells, particularly tissue macrophages, as well as to tumor cells at the tumor-stromal interface in squamous cell and basal cell carcinomas. Karelina et al. (1993) also described prominent immunoreactivity for the 92 kDa gelatinase in stromal cells, but not in tumor cells, in human skin cancers. Similar findings of 92 kDa gelatinase expression in tissue macrophages surrounding invading malignant epithelium have been described in human colon adenocarcinoma (Pyke et al., 1993). Recent work in our laboratory and by others has also localized MMP-9 mRNA to tumor cells and to surrounding host elements in human squamous lung cancers (Urbanski, 1992; Canete-Soler et al., 1994). Other members of the matrix metalloproteinase family have been localized by immunocytochemistry or by in situ

hybridization to host elements in many different human tumors including oral squamous cell carcinoma, breast carcinoma, basal cell carcinoma, melanoma, epidermoid head and neck cancers, and colorectal neoplasms (Bauer et al., 1977; Woolley and Grafton, 1980; Huang et al., 1986; Basset et al., 1990; Hewitt et al., 1991; Polette et al., 1991; Gray et al., 1992; Poulsom et al., 1992). In fact, the metalloproteinase stromelysin 3 was identified because it was found only in the stroma of breast carcinomas but not in normal breast tissue (Basset et al., 1990).

Normal host cells in culture may produce MMP-9 constitutively, such as human synovial fibroblasts (Moll et al., 1990), while others may express MMP-9 in a developmentally regulated fashion or in response to trauma. For example, cytotrophoblasts express MMP-9 maximally at the time of blastocyst invasion into the uterine wall (Librach et al., 1991; Behrendtsen et al., 1992), while in vitro differentiation of human mononuclear phagocytes into monocyte-derived macrophages is accompanied by marked increases in the levels of MMP-9 production (Welgus et al., 1990; Campbell et al., 1991). Corneal burning in rats causes rapid upregulation of MMP-9 (Fini et al., 1992).

Host elements may also produce MMPs in response to a wide variety of exogenous stimuli. For example, interleukin-1β (IL-1β) induces MMP-9 expression in rabbit corneal stromal cells (Girard et al., 1991), while IL-1β, tumor necrosis factor-α (TNF-α), and lymphotoxin (TNF-β) all stimulate MMP-9 production by human dermal fibroblasts (Unemori et al., 1991). Transforming growth factor-β1 (TGF-β1) strongly induces expression of MMP-9 in human mucosal and epidermal keratinocytes (Salo et al., 1991). The tumor promoter phorbol ester (TPA) stimulates MMP-9 production in many cultured host cells, including human synovial and dermal fibroblasts (Moll et al., 1990; Unemori et al., 1991), monocyte-derived and alveolar macrophages (Welgus et al., 1990; Campbell et al., 1991), keratinocytes (Salo et al., 1991; Wilhelm et al., 1989), and rabbit corneal fibroblasts (Fini and Girand, 1990) and brain capillary endothelial cells (Herron et al., 1986). MMP-9 expression in host cells, however, in response to contact with tumor cells has not been previously demonstrated.

Previous work in our laboratory characterized a group of transformed rat embryo cell tumor lines whose metastatic phenotype correlated with their ability to secrete the 92 kDa gelatinase either in culture or in nude mouse tumor explants. 92 kDa gelatinase activity was detectable in tumor explants of a metastatic v-*myc*- and H-*ras*-transformed cell line, 2.8, but was not found when these cells were grown alone in tissue culture. RA3, an H-*ras* and E1A transfectant, was non-metastatic and did not produce the 92 kDa gelatinase in culture or in tumor explants (Bernhard et al., 1990).

Speculating that the metastatic capacity of 2.8 tumor cells was related to their ability to induce expression of the 92 kDa gelatinase by host cells, we devised an in vitro primary rat embryo fibroblast and tumor cell co-culture model with which to examine potential tumor-host interactions. We have now demonstrated in vitro that normal rat embryo fibroblasts in vitro express the 92 kDa gelatinase in response to cell-cell contact with metastatic tumor cell line 2.8, but not when in contact with the non-metastatic cell line RA3. Examination of nude mouse tumors derived from these cell lines by in situ hybridization revealed MMP-9 mRNA within both the tumor cells and the adjacent stromal cells in tumors from 2.8, as seen in human tumors (Pyke et al., 1992, 1993; Canete-Soler et al., 1994; Karelina et al., 1993). The tumors from the non-metastatic cell line RA3 did not show MMP-9 mRNA nor did the stromal elements adjacent to these tumors. Therefore, the in vitro production of the 92 kDa gelatinase by fibroblasts in response to cell-cell interaction with metastatic tumor cells may serve as a model for one important component of the metastatic process demonstrated in these tumor sections.

MATERIALS AND METHODS

Cell lines and cell culture

2.8, 2.3, 2.10.1 and 2.10.10 are H-*ras*- and v-*myc*-transformed rat embryo cell lines and RA1, RA3 and RA4 are H-*ras*- and E1A-transformed rat embryo cell lines, as previously described (Bernhard et al., 1990). REF are primary rat embryo fibroblasts. Cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin and 5% fetal calf serum (FCS) (Upstate Biotechnology; Lake Placid, NY) for tumor cells or 10% FCS for fibroblasts.

Substrate gel electrophoresis

Substrate gel electrophoresis was performed essentially as described (Bernhard et al., 1990). Briefly, harvested serum-free supernatants were thawed, centrifuged, and resolved under non-reducing conditions in 7% polyacrylamide gels containing 0.1% porcine gelatin (Sigma Chemical Co., St Louis, MO). Gels were washed in 0.05 M Tris-HCl, pH 7.4, 2% Triton X-100 for 30 minutes, rinsed in 0.05 M Tris-HCl, pH 7.4, for 15 minutes, and incubated at 37°C overnight in 0.05 M Tris-HCl, pH 7.4, 5 mM $CaCl_2$, 1% Triton X-100, 0.2 M NaCl, 0.02% sodium azide. Gels were stained with Coomassie Blue for one hour and destained in methanol, acetic acid, water 2:1:7 (v/v). Prestained high molecular mass protein markers (Gibco-BRL; Gaithersburg, MD) were resolved on the same gel, separated after electrophoresis, and fixed in destain buffer. Clear bands indicate gelatinase activity.

Generation of conditioned media

2.8 (5×10^4) or REF were plated in 16 mm tissue culture wells (Corning; Corning, NY) in 1 ml DMEM, 10% FCS for 24 hours. Medium was replaced with 0.5 ml fresh DMEM, 10% FCS, for 48 hours and then harvested, centrifuged to remove cellular debris, filter-sterilized, and used immediately as conditioned medium. A 0.5 ml portion of serum-free DMEM was subsequently added to the same plated cells and collected 24 hours later, centrifuged, filter-sterilized, and used immediately as conditioned serum-free medium.

Cocultivation experiments

Tumor cells (10^5) and REF (10^5) were grown together in 1 ml DMEM, 10% FCS in 16 mm tissue culture wells for 48 hours. Alternatively, 10^5 tumor cells or REF were grown in 16 mm wells to confluence, and then cultured with 10^5 of the second cell line in 1 ml DMEM, 10% FCS for 48 hours. After 48 hours of co-culture, growth medium was removed, the wells were washed with serum-free DMEM, and 0.2 ml serum-free DMEM was added for 24 hours. These supernatants were harvested, centrifuged, and frozen at −20°C until use for substrate gel electrophoresis.

For experiments testing the effects of culture in conditioned growth medium on MMP-9 induction, 10^5 cells were plated in 1 ml DMEM, 10% FCS for 24 hours, after which medium was replaced with 0.5 ml cell-conditioned medium 3:1, 1:1, 1:3 (v/v, with DMEM, 10% FCS) or in 0.5 ml cell-conditioned medium for 24 hours, and changed to 0.5 ml serum-free medium for an additional 24 hours. For experiments

testing the effects of conditioned serum-free medium, 10^5 cells were grown in 1 ml DMEM, 10% FCS for 48 hours, and growth medium was replaced with either 0.5 ml cell conditioned serum-free medium or 0.5 ml cell conditioned serum-free medium, 1:1 (v/v, with serum-free DMEM) for 24 hours. These supernatants were collected and processed as above.

Co-culture experiments separating cells with tissue culture-treated polycarbonate membrane inserts with pore sizes of 0.4, 3, 5 and 8 μm (Costar Transwell; Cambridge, MA) were carried out after pre-soaking the inserts in DMEM, 10% FCS, for 2 hours at 37°C. 10^5 cells were plated in 16 mm tissue culture wells in 0.5 ml DMEM, 10% FCS, membrane inserts were added, and 10^5 cells of a second cell line were added to the upper portion of the insert in 0.5 ml DMEM, 10% FCS. After 48 hours, wells and inserts were washed with serum-free DMEM, then incubated for 24 hours in 0.5 ml serum-free DMEM, which was subsequently processed as above. The control 2.8 and REF co-culture was also incubated in 0.5 ml serum-free DMEM.

Methanol fixation

Confluent monolayers of cells in 16 mm tissue culture wells were treated with 1 ml 80% (v/v) methanol for 5 minutes at room temperature, and were washed extensively with serum-free DMEM prior to addition of 10^5 cells of the second cell line in 1 ml DMEM, 10% FCS for 24 hours. Medium was replaced with 0.2 ml serum-free DMEM for 24 hours and subsequently collected and processed as above. Non-viability of fixed cells was confirmed by trypan blue dye exclusion.

Preparation of extracellular matrix.

The procedure of Biswas (1985) was modified as follows: 0.5×10^5 cells were plated for 24 hours in 16 mm tissue culture wells, medium was replaced for an additional 24 hours, and cells were then washed twice with phosphate-buffered saline (PBS) before incubation at room temperature with 0.25 M NH_4OH or 0.5% Triton X-100 in sterile water for 5 minutes. Cells were removed by gentle pipetting, and the matrix was washed extensively with PBS prior to addition of 10^5 cells of the second cell line in 1 ml DMEM, 10% FCS. Medium was changed to serum-free DMEM after 24 hours, and these supernatants were harvested and processed as above 24 hours later. Methanol fixation of extracellular matrix was carried out as described above for cell monolayers.

Preparation of cell conditioned medium on extracellular matrix

Extracellular matrix of REF was prepared as above and washed extensively with PBS prior to the addition of 10^5 2.8 in 1 ml DMEM, 10% FCS. After 24 hours, medium was replaced with 0.5 ml DMEM, 10% FCS and incubated for 72 hours. This conditioned medium was harvested, centrifuged and filter sterilized prior to addition to monolayers of REF in 16 mm tissue culture dishes at dilutions of 1:2.5, 1:1 and 2.5:1 (v/v, with DMEM, 10% FCS). Medium was replaced with 0.5 ml serum-free DMEM 24 or 48 hours later and processed as above. Similar experiments were performed with conditioned medium from REF grown on 2.8-derived extracellular matrix.

Preparation of tissue culture substrates

Tissue culture plates (24-well) were coated with 1 ml 0.1, 0.2, 0.5 or 1.0% porcine gelatin (Sigma) in sterile water overnight at 37°C. After extensive washing with serum-free DMEM, 10^5 cells were plated in these wells in 1 ml DMEM, 10% FCS for 48 hours, and medium was replaced with 0.2 ml serum-free DMEM, which was harvested and processed as above 24 hours later. Alternatively, type IV collagen-coated 24-well plates (Collaborative Research BioCoat; Bedford, MA) or plates coated with 0.1 ml Matrigel (Collaborative Research; Bedford, MA) at 8.9 mg/ml overnight at 37°C were washed, and 2×10^4 cells were added in 0.5 ml DMEM, 1% FCS for 24 to 48 hours. These supernatants were assayed directly for gelatinase activity.

Metalloproteinase characterization

A substrate gel was run with duplicate lanes of pooled serum-free supernatants derived from co-cultures of REF with methanol-fixed 2.8 cells, as described above. Sections of this gel were incubated overnight in the developing buffer described above, in the presence or absence of 10 mM EDTA or of recombinant TIMP-1 (a generous gift from Edward Hughes) at a final concentration of 10 μg/ml. Serum-free supernatants were also treated with TIMP-1 at a final concentration of 10 μg/ml for 15 minutes at room temperature or with 1 mM *p*-aminophenyl-mercuric acetate (APMA; Sigma) for 12 hours at 37°C prior to loading on the gel. Purified human MMP-9 and monoclonal antibodies to human MMP-9 were generous gifts from Deborah L. French.

Preparation and characterization of rat MMP-9 cDNA.

A primer based on the published human MMP-9 sequence (Wilhelm et al., 1989) was prepared in the antisense direction from bases 1778-1803. This primer was annealed to total RNA isolated from the transformed rat cell line 2.10 at 52°C, followed by reverse transcription. The generated cDNA was amplified in a PCR reaction with the above primer and a sense primer for bases 1146-1171 of the human MMP-9 sequence. The resultant product of 560 bases was used to screen a rat macrophage cDNA library. Ten clones were identified and sequenced, yielding three overlapping nucleotide sequence regions. The nucleotide sequence has 79% similarity to the human MMP-9 sequence, and the predicted amino acid sequence has 86% similarity (Bernhard et al., 1993, and unpublished data). The cDNA probe for northern blot analysis was derived from the 1.3 kb *Eco*RI insert fragment released from cDNA clone p8P2a, which extends from nucleotide 125 to 1408 of the homologous human MMP-9 gene.

Tumor preparation and in situ hybridization

Cells were grown to subconfluence, trypsinized, washed and resuspended at 5×10^5/0.1 ml PBS for subcutaneous injection into nude mice as previously described (Bernhard et al., 1990). Mice were killed by cervical dislocation and resected tumors were fixed either in Bouin's fixative or in 1% glutaraldehyde, 50 mM phosphate buffer containing 0.475% NaCl for 90 minutes at 4°C followed by embedding in paraffin. Sections (5 μm) were cut from each embedded sample and placed on Superfrost Plus Slides (Fisher Scientific; Springfield, NJ). One section was stained with hematoxylin and eosin. Sections were deparaffinized by two 10-minute immersions in 100% xylene followed by two 5-minute immersions in 100% ethanol and then air-dried. The tissue was permeabilized by pepsin treatment (2.5 mg/ml in 0.12 M HCl, 0.3% Brij 35, pH 2) at 45°C for 20 minutes. The pepsin reaction was stopped by washing the slides twice with PBS at room temperature.

In situ hybridization was performed using riboprobes labelled with digoxigenin and then detected with antibodies to digoxigenin coupled to alkaline phosphatase (Dooley et al., 1988). Sections were prehybridized for 2 hours at 50°C with moderate shaking in a Coplin jar in hybridization buffer (50%, v/v, formamide (Fisher Scientific), 5× SSC, 2% (w/v) blocking reagent (BMB; Indianapolis, IN), 0.1% (w/v) *N*-lauroylsarcosine, 0.02% (w/v) SDS). RNA (4 μg/ml) of the indicated probe was preheated to 85°C for 5 minutes, followed by hybridization on each section in 25 μl of hybridization buffer in a moist chamber for at least 6 hours at 50°C. The slides were then washed twice for 5 minutes with 2× SSC, 0.1% SDS at room temperature followed by two 15-minute washes with 0.1× SSC, 0.1% SDS at 57°C.

For color detection, slides were pretreated with 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, and then incubated for 30 minutes at room temperature with 20 ml of 1% blocking buffer (BMB) containing 50 mg/ml tRNA (Gibco-BRL), followed by a 30 minute incubation at room temperature with 1:3500 alkaline phosphatase-conjugated anti-digoxigenin antibody (BMB). Excess antibody was removed by washing twice with

0.1 M maleic acid, 0.15 M NaCl, pH 7.5. Slides were then incubated for 2 minutes in 100 mM Tris-HCl, 50 mM $MgCl_2$, pH 9.5, followed by incubation in 15 ml of 100 mM Tris-HCl, 50 mM $MgCl_2$, pH 9.5, with 0.1 mM levamisole (Sigma), 0.338 mg/ml 4-nitroblue tetrazolium chloride (BMB), 0.173 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BMB) in the dark at 37°C for 10-15 hours. The reaction was stopped by washing for 5 minutes at room temperature with 10 mM Tris-HCl, 1 mM EDTA, pH 8. Slides were counterstained with Hematoxylin Stain Gill's Formulation no. 2 (Fisher) for 30 seconds and mounted with Cristal Mount (Biomeda Corp; Foster City, LA).

For riboprobe preparation, a fragment extending from nucleotides 898 to 1234 of a rat 92 kDa gelatinase cDNA (p8P2a) was subcloned into the *Xho* and *Eco*RI sites of the pBluescript SK polylinker. Plasmid DNA was purified using the Magic Megapreps DNA purification system (Promega; Madison, WI). A 20 μg sample of plasmid DNA was cleaved with *Pst*I or *Kpn*I and purified by treatment with 150 mg/ml Proteinase K (BMB), 0.5% SDS for 1 hour at 37°C followed by phenol/chloroform extraction and ethanol precipitation. Run-off sense and antisense transcripts incorporating digoxigenin-UTP (BMB) were synthesized from 1 μg of purifed template with T7 and T3 RNA polymerases (Gibco-BRL). The transcription reaction was stopped by precipitation with 4 M LiCl and ethanol. Typical riboprobe yield was 6-8 μg of RNA. Probe size was confirmed by denaturing polyacrylamide gel electrophoresis. Sense and antisense probes were adjusted to the same concentration and applied to slides as above. Sense and antisense riboprobes derived from human gamma actin cDNA (provided by Dr Mats Gafuel) were used as controls.

RESULTS

In order to characterize the interaction of transformed rat embryo cell tumor line 2.8 with host elements that may be involved in induction of 92 kDa gelatinase expression, an in vitro co-cultivation system was devised. 10^5 REF and 10^5 2.8 cells were co-cultivated for 48 hours, followed by incubation in serum-free medium for 24 hours. Serum-free incubation was necessary because FCS also contains gelatinase activity. As shown in Fig. 1, a gelatinase of approximately 92 kDa was detectable by substrate gel electrophoresis in serum-free medium harvested from these co-cultures, but serum-free media derived from cultures of 2.8 or REF alone did not contain this gelatinase. 2.8 and REF were also grown alone in culture at densities of 10^5 and 10^6 cells/ml for up to 96 hours without enzyme induction (data not shown). These experiments demonstrated that 92 kDa gelatinase production in these co-cultures did not result from increased plating density or time in culture.

Similar experiments were performed with the non-metastatic rat embryo cell tumor line RA3 to determine whether expression of the 92 kDa gelatinase in co-culture was a specific response to metastatic cells. RA3 did not produce this enzyme alone or in co-culture with REF (Fig. 1). Cocultures were done with additional similarly transformed rat embryo cell lines. As shown in Fig. 2, induction of the 92 kDa gelatinase was seen in response to several metastatic v-*myc*- and H-*ras*-transformed cell lines, but not in response to non-metastatic H-*ras*- and E1A-transformed lines. None of these cell lines alone in culture produced this 92 kDa gelatinase. These data strongly suggest that the ability of tumor cells to induce host 92 kDa gelatinase expression may also be associated with the metastatic phenotype.

Using this in vitro co-culture system, we sought to characterize the intercellular interaction responsible for induction of the 92 kDa gelatinase. We first tested whether a soluble factor

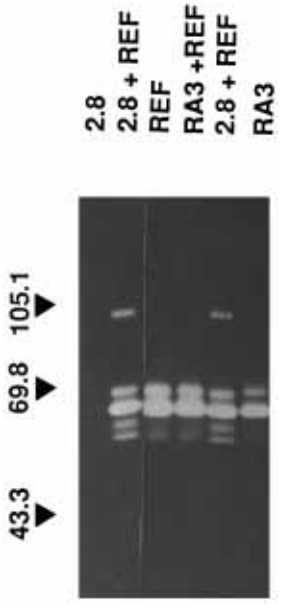

Fig. 1. A 92 kDa gelatinase was produced by co-culture of REF with metastatic tumor cell line 2.8 but not with nonmetastatic cell line RA3. The designated cell lines were cultured alone or together as described in Materials and Methods. Serum-free supernatants were harvested after 24 hours and subjected to substrate gel electrophoresis; cleared bands indicate gelatinolytic activity. Duplicate lanes are shown for 2.8 + REF. Location of molecular mass standards in kDa is shown at left.

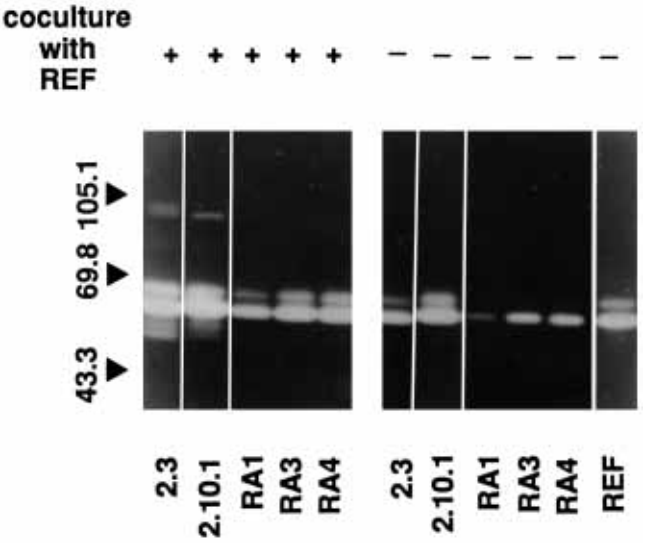

Fig. 2. 92 kDa gelatinase induction occurred in response to other metastatic tumor cell lines. Co-cultures were performed as described in Materials and Methods. The left panels demonstrate cell lines 2.3 and 2.10.1 (v-*myc*- and H-*ras*-transformed metastatic cell lines) and cell lines RA1, RA3 and RA4 (H-*ras*- and E1A-transformed non-metastatic cell lines) co-cultured with REF. The right panel demonstrates the same cell lines cultured alone. A control lane for REF alone is shown at the far right. Location of molecular mass standards in kDa is shown at far left.

produced by one of the cell lines was involved in enzyme induction. REF and 2.8 were grown together in culture separated by solute-permissive polycarbonate membranes with pore sizes 0.4, 3, 5 and 8 μm. As shown in Fig. 3, this culture arrangement abrogated 92 kDa gelatinase secretion into serum-free medium. The diminished 92 kDa gelatinase signal in the control co-culture when compared with that shown in Fig. 1 is due to a 2.5-fold dilution with serum-free DMEM (see Materials and Methods). An insignificant number of cells (<0.02%) passed through the largest-pore membrane in the time of co-culture (data not shown). Conditioned medium and conditioned serum-free medium from tumor cells did not induce 92 kDa gelatinase expression by REF, and similar media from REF did not induce 92 kDa gelatinase expression in 2.8 (data not shown). These experiments provide no evidence to support the involvement of a soluble factor in the induction of the 92 kDa gelatinase in the co-cultures, and suggest that cell-cell contact may trigger 92 kDa gelatinase induction in this system.

Experiments utilizing methanol-fixed monolayers of one cell line as a culture substrate for the second line were next carried out to determine which cell line was producing the 92 kDa gelatinase. As shown in Fig. 4, a methanol-fixed monolayer of 2.8 induced REF to express the 92 kDa gelatinase, while a fixed monolayer of REF did not induce 2.8. Methanol fixation did not in itself induce 92 kDa gelatinase secretion in either cell line. Induction was also abrogated by separation of REF from fixed 2.8 monolayers by polycarbonate membranes (data not shown), as shown above for co-cultures of REF with live 2.8 cells. These results demonstrate that induction of 92 kDa gelatinase expression was not due to secretion of a stimulatory factor from fixed cells, and that REF produced this enzyme in co-culture with 2.8 cells. Taken together, these results support the hypothesis that a cell surface-associated factor unique to metastatic tumor cells possesses the capacity to induce fibroblast expression of the 92 kDa gelatinase in co-culture, and that cell contact is required for gelatinase induction in this model.

The next question asked was whether this stimulatory factor could be a component of the extracellular matrix or a substrate for the 92 kDa gelatinase. Experiments were therefore performed to evaluate the ability of deposited cell matrix or purified enzyme substrates to induce 92 kDa gelatinase expression. REF were grown on a substrate of extracellular matrix derived from 2.8 cell monolayers by ammonium hydroxide or detergent lysis and 92 kDa gelatinase production was not induced. Similar experiments failed to demonstrate a tumor cell-inducing capacity in REF-derived matrix on either REF or 2.8. Methanol treatment of similarly derived matrix was also carried out to test for the possibility that methanol exposed an occult gelatinase activating stimulus in the matrix.

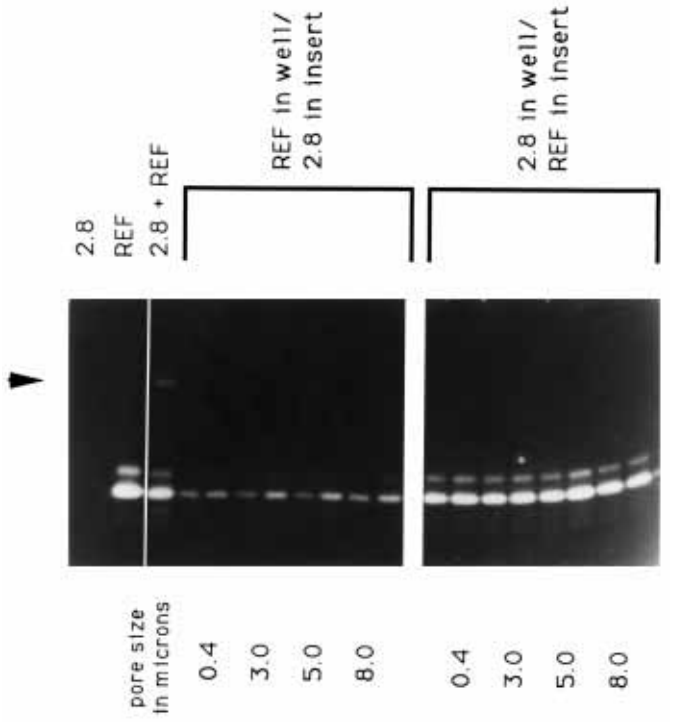

Fig. 3. The factor responsible for MMP-9 induction was not soluble. REF were placed in tissue culture wells, membrane inserts were added (membrane pore size indicated in μm), and 2.8 cells were added to the upper portion of the insert. Wells and inserts were incubated with serum-free DMEM after 48 hours in serum-containing medium. Similar experiments were carried out with cells in the reverse configuration. Supernatants were subjected to substrate gel electrophoresis. Controls for 2.8, REF and the co-culture of 2.8+REF are shown at left. Results from duplicate experiments are shown for each membrane pore size. The decreased signal at 92 kDa for 2.8+REF compared to that shown in Fig. 1 is due to a 2.5-fold dilution in serum-free medium (see Materials and Methods). Arrowhead indicates 92 kDa band.

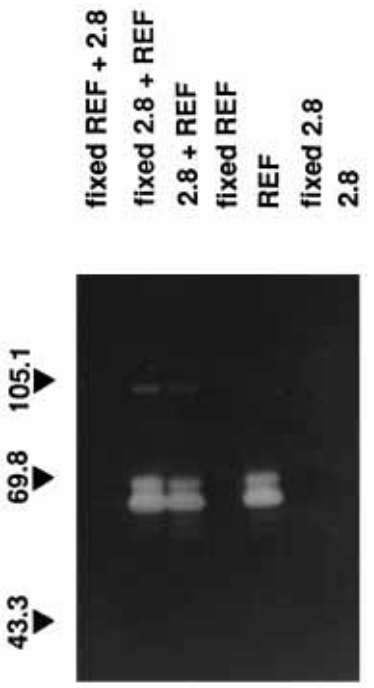

Fig. 4. REF produced the 92 kDa gelatinase in co-culture. REF were cultured on methanol-fixed monolayers of 2.8 tumor cells, or 2.8 were cultured on methanol-fixed monolayers of REF, followed by incubation in serum-free DMEM. Harvested supernatants were subjected to substrate gel electrophoresis. Serum-free incubations of live or fixed cell monolayers alone are also shown. Location of molecular mass standards in kDa is shown at left.

These methanol-treated matrix substrates were also incapable of 92 kDa gelatinase induction in REF or 2.8. 92 kDa gelatinase expression could not be induced by culture of either REF or 2.8 on the matrix analog Matrigel, type IV collagen, or gelatin (data not shown). These results suggest that the extracellular matrix does not contain the 92 kDa gelatinase inducing agent.

The next set of experiments was designed to determine whether the cell surface-associated factor behaved similarly to a previously described membrane-associated tumor cell collagenase stimulatory factor (TCSF). Release of this factor, which stimulates fibroblast interstitial collagenase (MMP-1) production from tumor cells into conditioned medium, was found to be enhanced by 'conditioning' of the tumor cells on fibroblast-derived extracellular matrix (Biswas, 1985; Nabeshima et al., 1991). We performed similar experiments by conditioning 2.8 cells on REF-derived matrix, collecting the resultant tumor cell conditioned medium, and assaying its ability to induce REF production of gelatinase. Such 2.8-derived conditioned medium also failed to induce 92 kDa gelatinase expression by REF. Likewise, REF-derived conditioned medium failed to induce 92 kDa gelatinase expression by cell line 2.8 (data not shown). These experiments provided evidence that the factor associated with cell line 2.8 could not be released from the cell surface in the same manner as TCSF.

Experiments were next performed to characterize this inducible gelatinase activity. As shown in Fig. 5, the 92 kDa gelatinase activity induced in response to contact with either live or fixed tumor cells was found to be comparable to the level of activity induced by treatment with phorbol ester (TPA), which is known to be an effective inducer of 92 kDa gelatinase expression in REF. Also, as demonstrated in Fig. 6, this activity was inhibitable by incubation of the zymogram in the presence of EDTA, a chelating agent that inhibits metalloproteinases, or with recombinant TIMP-1, a specific inhibitor of metalloproteinases. As a control, TIMP-1 added to the serum-free supernatant just prior to gel loading did not affect gelatinase detection; similarly, TIMP expressed in any of these experiments would be dissociated from MMPs prior to zymography and would not affect the results. A shift in gel mobility to a slightly lower molecular mass was observed following treatment of serum-free supernatants with the organomercurial APMA, and the inducible band co-migrated with purified human MMP-9 on zymograms (data not shown). Northern analysis and ribonuclease protection assay using probes derived from a rat MMP-9 cDNA of RNA derived from cell line 2.10.10, which makes the 92 kDa gelatinase constitutively, from REF stimulated with TPA, and from REF with 2.8 co-

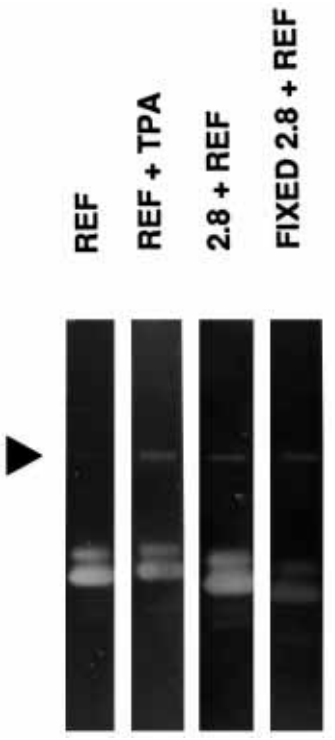

Fig. 5. REF produced the 92 kDa gelatinase equally in response to tumor cell contact and to treatment with TPA. Cocultures were performed as described in Materials and Methods. For REF+TPA, following growth to subconfluence, REF were treated with 10 ng/ml TPA in serum-free medium for 24 hours. Results shown are derived from the same experiment run on separate gels. Arrowhead indicates 92 kDa band.

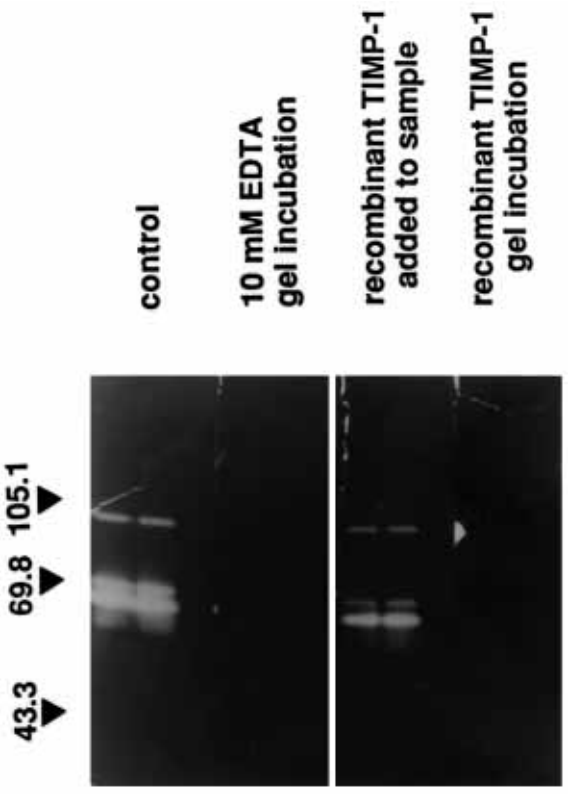

Fig. 6. The inducible 92 kDa gelatinase activity is a metalloproteinase. Duplicate lanes are shown for each experiment. In the left panel, a substrate gel of serum-free supernatants derived from co-cultures of fixed 2.8 cells with REF is shown. In the second panel, 10 mM EDTA was added to the developing buffer during overnight incubation of the gel at 37°C. In the third panel, 10 μg/ml recombinant TIMP-1 was added to the serum-free supernatant just prior to gel loading. In the right panel, 10 μg/ml recombinant TIMP-1 was added to the overnight incubation in developing buffer. Location of molecular mass standards in kDa is shown at left.

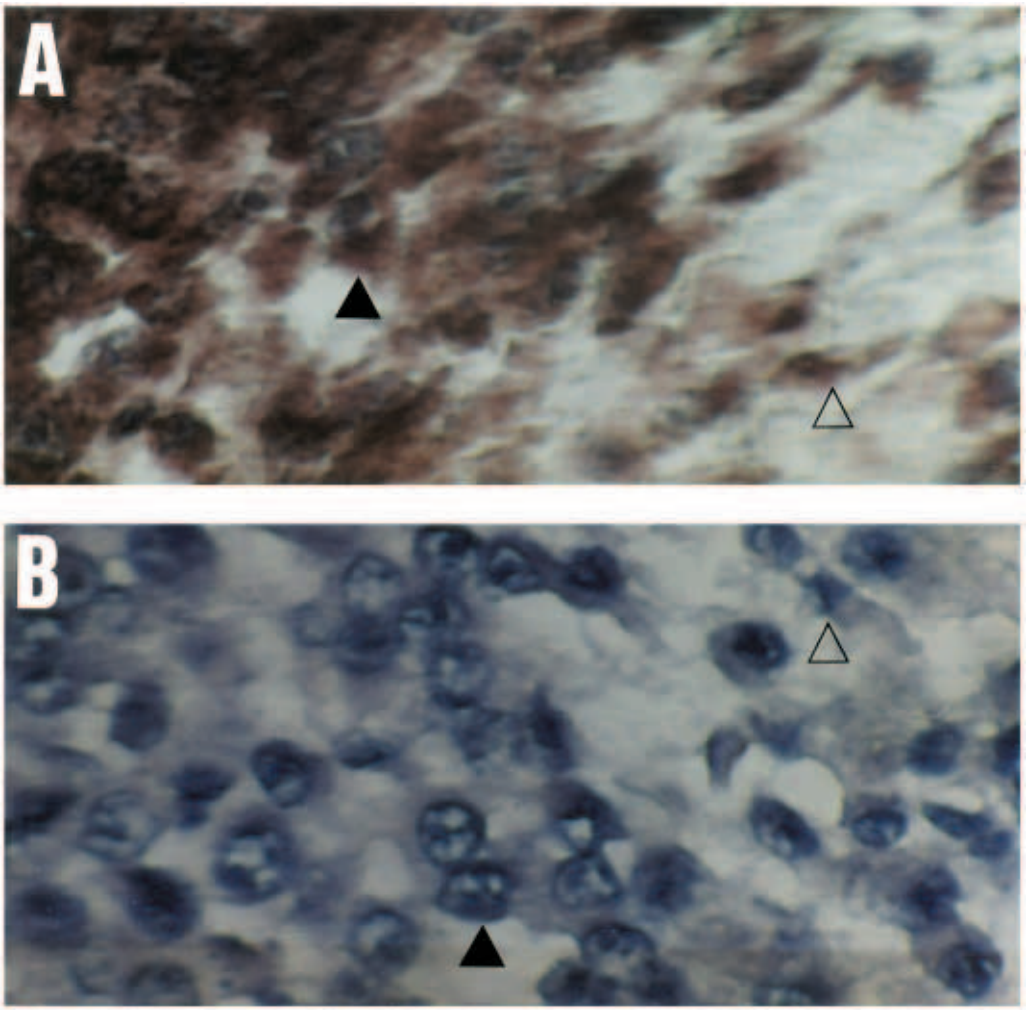

Fig. 7. Localization of MMP-9 mRNA to stromal and tumor cells. In situ hybridization was performed with sense and antisense riboprobes. Brown stain is positive; the counterstain is blue. Sections are shown at ×400. Hybridization with antisense probe (A): tumor cells at left (filled arrowhead) with large nuclei and multiple nucleoli were strongly positive, as were the tumor stromal cells seen at right (open arrowhead). Hybridization with sense probe (B): No positive staining is seen in either tumor cells (filled arrowhead) or in tumor stromal cells (open arrowhead).

cultures, demonstrated similar band patterns (data not shown). By western blotting, two monoclonal antibodies to human MMP-9, as well as a polyclonal human MMP-9 antibody developed in our own laboratory, did not cross-react with the inducible 92 kDa gelatinase activity in rat cell cultures (data not shown). Together, these results confirm that this 92 kDa gelatinase activity is a metalloproteinase, and strongly suggest that it is, in fact, MMP-9.

In all of the experiments above, another prominent gelatinase activity was seen at approximately 65 kDa. As shown in Fig. 6, this activity also appears to be a metalloproteinase. This lower molecular mass gelatinase was present in all co-cultures containing REF, as well as in cell line RA3 alone, but did not appear to be altered by co-cultivation of tumor cells with fibroblasts or by treatment with TPA. This metalloproteinase could be an activated form of MMP-9 (Okada et al., 1992; Watanabe et al., 1993) or it may be the 72 kDa gelatinase. In the absence of available antibodies to either enzyme in the rat, this activity could not be identified further.

Having demonstrated that 2.8 tumor cells were capable of inducing MMP-9 expression from normal fibroblasts in cell culture, we performed in situ hybridization on tumors from the transformed rat embryo cell lines to determine if the in vivo pattern of 92 kDa gelatinase expression might be consistent with these observations in cultured cells. Sense and antisense riboprobes derived from a rat MMP-9 cDNA were applied to tumor sections to look for stromal production of the 92 kDa gelatinase. As shown in Fig. 7, antisense riboprobe clearly demonstrated both stromal and tumor cell signal for the 92 kDa gelatinase in tumors from the 2.8 cell line. Although the stromal cells that stain for MMP-9 mRNA have a fibroblastic appearance, we cannot determine from these studies whether they are histiocytes or fibroblasts or perhaps both. Control hybridization with the sense probe was negative. RA3 tumors, which were non-metastatic and did not produce the 92 kDa gelatinase in vitro, were negative in both the sense and antisense hybridizations (data not shown). Examination of in vitro cell-cultures by in situ hybridization was technically unsuccessful due to background problems.

DISCUSSION

We have demonstrated a novel, cell contact-dependent mode of induction of the 92 kDa gelatinase in normal primary rat embryo fibroblasts by metastatic tumor cells. The induction of expression of this 92 kDa gelatinase in fibroblasts was shown to occur as a response to co-culture with the metastatic cell line 2.8, but not with the non-metastatic cell line RA3. Cocultures of REF on methanol-fixed 2.8 monolayers contained the 92

kDa gelatinase, while co-cultures of 2.8 on methanol-fixed REF did not, indicating that REF produced the 92 kDa gelatinase in the co-cultures. Further experiments demonstrated that fibroblast expression of the 92 kDa gelatinase in co-cultures was also associated with the metastatic phenotype in other similarly transformed rat embryo cell lines that did not themselves express this enzyme. These results together support the hypothesis that metastatic 2.8 cells, but not non-metastatic RA3 cells, can induce normal fibroblasts to express the 92 kDa gelatinase, and that cell-cell contact may be necessary for this induction. They do not answer the question of whether 2.8 cells may also be induced in vitro, bearing in mind that RNA for MMP-9 was detected by in situ hybridization in both tumor and stromal cells in tumors derived from this cell line.

This inducible 92 kDa enzyme was shown to be a metalloproteinase by its characteristic responses to treatment with a chelating agent, EDTA, with an organomercurial agent, APMA, and with TIMP-1, which stoichimetrically binds to and inhibits metalloproteinases. The level of induction was also shown to be similar to that seen in response to TPA, a known inducer of MMP-9 expression. In the absence of available antibodies to rat MMP-9, we could not conclusively prove that the gelatinolytic enzyme detected by zymography was, in fact, MMP-9, although data from Northern analysis and ribonuclease protection assay suggest that MMP-9 RNA is also inducible in the co-cultures. It is unlikely that a 92 kDa metalloproteinase different from MMP-9 is being produced in these co-cultures.

Soluble factors did not appear to be sufficient for 92 kDa gelatinase induction in fibroblasts by tumor cells. Tumor cell-derived conditioned media did not induce expression of the 92 kDa gelatinase by REF, and co-culture of tumor cells and fibroblasts separated by solute-permissive membranes abolished the 92 kDa gelatinase induction in REF. These experiments support the hypothesis that cell-cell contact may be sufficient for this enzyme induction in REF.

In other cell lines, components of the extracellular matrix have been shown to induce other members of the MMP family (Turpeenniemi-Hujanen et al., 1986; Teale et al., 1988; Azzam and Thompson, 1992). Further experiments were therefore performed in which REF were cultured on tumor cell-derived extracellular matrix, Matrigel, type IV collagen, or gelatin. None of these culture substrates stimulated fibroblast MMP-9 expression, suggesting that the inducing factor associated with cell line 2.8 is not a component of the extracellular matrix. In addition, culture conditions previously shown to result in release of TCSF, the factor that stimulates fibroblast interstitial collagenase production, from tumor cell membranes failed to induce fibroblast MMP-9.

The production of the 92 kDa gelatinase in vitro in response to tumor cells may provide a model for studying production of metalloproteinases in the cells of the host. Host production of MMP-9 appears to occur in many naturally occurring tumors (Karelina et al., 1993; Pyke et al., 1992; Urbanski et al., 1992; Canete-Soler et al., unpublished data; Pyke et al., 1993). The in vitro co-culture of tumor cells and fibroblasts provides a model system for studying one mechanism for host-tumor cell interaction that leads to induction of the 92 kDa gelatinase in stromal cells.

Our results demonstrate that cell to cell contact between tumor and normal cells was sufficient for the induction of the 92 kDa gelatinase seen when normal fibroblasts were plated on methanol-fixed tumor cell monolayers. The only previously described tumor cell surface-associated factor that is similarly capable of inducing fibroblast metalloproteinase expression is TCSF, a transmembrane protein that bears structural homology to members of the immunoglobulin superfamily. This factor has been shown to induce only MMP-1 expression in both its membrane-bound and soluble forms (Biswas, 1985; Nabeshima et al., 1991). Cell contact-dependent induction of a matrix metalloproteinase by a tumor cell has not been previously described.

In situ hybridization of tumors derived from cell lines 2.8 or RA3 using sense and antisense riboprobes demonstrated both stromal and tumor cell signal for MMP-9 in 2.8 tumors, but not in RA3 tumors, suggesting that the in vitro specificity of host MMP-9 expression in response only to metastatic tumor cells is maintained in vivo. However, these findings warrant further explanation.

A strong signal for MMP-9 RNA was seen in 2.8 tumor cells. Although the in vitro experiments clearly showed that the 92 kDa gelatinase is inducible in the REF, we cannot rigorously determine whether any of the 92 kDa gelatinase was made by 2.8 during the live co-cultures. However, in vitro, we have been unable to demonstrate 92 kDa gelatinase production by cell line 2.8 alone. It is interesting to note that agents previously shown to induce MMP-9 expression in tumor cells, such as IL-1 and TPA, were not capable of inducing 2.8 92 kDa gelatinase expression, while REF treated with TPA produced this enzyme (unpublished observations).

The strength of the MMP-9 mRNA signal in in situ hybridization might appear to be out of proportion to the strength of the gelatinase activity detected on our zymograms. The in situ procedure is not quantitative and in our hands has been sensitive at levels where mRNA gave only weak signals in northern blot analysis. Unfortunately, due to the lack of available antibodies to rat gelatinases, we could not examine the expression of the 92 kDa gelatinase protein in situ. Therefore, any conclusions regarding the correlation of 92 kDa gelatinase protein levels in vitro with levels of RNA expression detected by in situ hybridization must be made cautiously. We did demonstrate that the level of gelatinase expression in co-cultures was comparable to the level seen in REF in response to stimulation with phorbol ester, and was comparable to the level described for 2.8-derived tumor explants previously (Bernhard et al., 1990).

It is reasonable to speculate that other cells or cytokines present only in the intact host participate in stimulating MMP-9 activity in both 2.8 tumor cells or in fibroblasts. An alternative, albeit very unlikely, explanation for these findings would be that the enzyme detected by zymography is not the same as the enzyme detected by in situ hybridization with a rat MMP-9 cDNA-derived probe. Until further mechanistic studies are completed, it is only safe to conclude that fibroblasts produced the 92 kDa gelatinase in response to tumor cell contact in vitro, and that stromal cells adjacent to tumor cells produced RNA for MMP-9 in vivo.

These results also suggest that divergent signalling pathways may mediate expression of the 92 kDa gelatinase in response to different stimuli in REF and in 2.8. Prescott et al. (1989), in fact, demonstrated that different fibroblast lines express collagenase in response to either TCSF or TPA, to both factors, or

to neither, suggesting the coexistence of divergent regulatory pathways for these two signals. At the level of transcription, promoter motifs homologous to the binding sites for AP-1, common to several metalloproteinases, as well as SP-1, c-ets-1, and NF-κB, may mediate these signals that induce MMP-9 expression (Huhtala et al., 1991; Sato and Seiki, 1993).

Protein kinase C (PKC), an enzyme essential to the cellular response to TPA (Nishizuka, 1984), may be an important participant in MMP signal transduction. The PKC inhibitor H7 blocks stimulation by both TPA and TNF-α of plasma membrane PKC activity and collagenase expression in fibroblasts (Brenner et al., 1989). Conversely, TPA-mediated, but not IL-1-mediated, collagenase induction in synovial fibroblasts could be blocked by the PKC inhibitors staurosporine, sphingosine and H7, suggesting that another PKC-independent signalling pathway may be active in the response to IL-1, but not to TPA or to TNF-α (Hulkower et al., 1991).

Our in vitro system appears to recapitulate an aspect of host 92 kDa gelatinase induction involving cell-cell contact. Further studies are under way to examine the signal transduction pathway for induction of the 92 kDa gelatinase in rat embryo fibroblasts in response to metastatic tumor cells and to characterize the cell surface-associated component of metastatic tumor cell line 2.8, which activates this pathway.

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