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Density-Dependent Induction of 92-kd Type IV Collagenase Activity in Cultures of A431 Human Epidermoid Carcinoma Cells

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We examined the* in vitro *regulation of the production of two type IV collagenases, MMP-2 and MMP-9, by A431 human epidermoid carcinoma cells. The A431 cells were cultured under sparse or confluent conditions. The addition of transforming growth factor-β (TGF-β) or phorbolester-TPA to sparse cultures induced low levels of MMP-9 secretion, whereas in confluent cultures only TGF-β produced this effect. Neither treatment altered the level of constitutive secretion of MMP-2. Treatment of sparse, actively growing cultures but not confluent stationary cultures with both TGF-β and TPA produced synergistic induction of MMP-9 but did not affect MMP-2. A431 cells were grown as discrete large monolayer colonies. Radiolabeling with [^{3}H]leucine or [^{3}H]thymidine followed by autoradiography revealed that all the A431 cells in the colonies were metabolically active and only those on the periphery were dividing. Only these dividing A431 cells stained positive by anti-MMP-9 antibodies. Our results demonstrate that the synergistic induction of MMP-9 secretion in A431 cells occurs subsequent to stimulation by external signals in only noncontact-inhibited dividing tumor cells. These regulatory mechanisms may account for the* in vivo *finding that many proteinases are localized at the invasion front of a neoplasm where tumor cells are dividing and accessible to various environmental signals. (Am J Pathol 1994, 144:1058–1067)

Metastasis consists of a series of interrelated steps whose outcome is dependent on both the intrinsic properties of tumor cells and the responses of the host.[1] To reach blood vessels or lymphatics, tumor cells must penetrate host stroma that includes basement membrane (BM) extracellular matrix (ECM). This interaction involves tumor attachment, matrix dissolution, motility induction, and penetration.[2,3] The degradation of ECM components surrounding malignant tumors is mostly mediated by metalloproteinases (MMPs), serine proteases, and cathepsins.[4]

The MMPs consist of a family of structurally similar enzymes with distinct substrate specificities.[5] MMP-1, or interstitial collagenase, degrades collagen types I to III.[6] MMP-3, or stromelysin, has broad proteolytic activity toward nonhelical domains of collagen types I to IV and noncollagenous ECM components such as proteoglycans, fibronectin, and laminin.[7] MMP-2 and MMP-9 (72- and 92-kd type IV collagenases/gelatinases, respectively) can attack BM collagen types IV and V at a single specific site within the triple helical region.[8,9] These two MMPs also possess potent proteolytic activity against gelatins.[10,11]

In most cell systems, the MMPs are secreted in latent forms and activated via a complex proteolytic cascade involving other MMPs,[12,13] urokinase-type plasminogen activator,[14] other proteinases,[15] and autoactivation.[16] The activities of the MMP family are specifically regulated by tissue inhibitors of metalloproteinase (TIMPs).[17,18] A direct correlation between the *in vitro* expression of MMP-2 and MMP-9 and metastatic potential *in vivo* has been reported for tumor cells[19–23] and oncogene-transfected cells.[24–29] Moreover, under *in vitro* and *in vivo* conditions tumor invasion (and metastasis) can be inhibited by TIMPS.[30–32]

Our understanding of the biological functions of MMPs and TIMPs has been significantly advanced by recent *in vivo* studies. Specifically, *in situ* hybridization and immunolocalization techniques produced

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evidence that the expression of MMP-2 and MMP-9 *in vivo* is enhanced in cancers of the skin,[33] colon,[34,35] breast,[35] and prostate[36] during progression from the benign to the malignant state. Histological studies of tumor sections revealed that these collagenases appeared either in the tumor cells[34,36–40] or in normal stromal cells such as fibroblasts[40–43] or infiltrating macrophages.[40,41] Regardless of the tumor type, both MMP-2 and MMP-9 have been found to localize at the invasion front where tumor cells are actively proliferating and are adjacent to host tissues. Similar localization to the leading edge of the tumor has been reported for other MMPs,[44,45] urokinase-type plasminogen activator,[46] and cathepsin B.[47] This restricted localization of MMPs contrasts sharply with the distribution of TIMPs throughout the same tumor.[42,48,49] Why collagenase expression is associated with the invasion edge of a tumor is unclear.

Because the regulatory mechanisms for MMPs and TIMPs in tumor cells are still unclear, we studied the regulation of MMP-2 and MMP-9 in human epidermoid carcinoma A431 cells treated *in vitro* with transforming growth factor-β (TGF-β) and the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The data demonstrate that the production of MMP-9 but not MMP-2 stimulation by exogenous signals of cells capable of dividing suggest that distinct regulatory mechanisms control the expression of these two MMPs.

Materials and Methods

Cell Culture

The A431 human squamous carcinoma cell line[50] was maintained as a monolayer culture in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, L-glutamine, 2x vitamin solution, and penicillin-streptomycin (Flow Laboratories, Rockville, MD). The cultures were incubated at 37 C in a humidified atmosphere of 5% CO_2-95% air. The cell line was tested and found free of mycoplasma and pathogenic mouse viruses (assayed by M.A. Bioproducts, Walkersville, MD).

Cell Treatment and Medium Collection

A431 cells were maintained as monolayer cultures in 6- or 48-well cell culture clusters (Costar Co., Cambridge, MA). After the monolayer cultures reached 30% or full confluence (dense culture), the cells were washed with Hanks' balanced salt solution and treated with porcine TGF-β1 (1 ng/ml) (R&D Systems, Minneapolis, MN) or TPA (25 ng/ml) (Sigma Chemical Co., St. Louis, MO) or TGF-β1 and TPA for 24 hours in serum-free Dulbecco's modified Eagle medium (DMEM-F12) (GIBCO-BRL, Grand Island, NY). The control group consisted of cells incubated in serum-free medium alone. After the incubation period, the conditioned medium was harvested and spun at 3000 *g* at 4 C for 30 minutes to remove cell debris. Protein content (in medium from various treatments) was estimated according to a standard protein assay procedure recommended by the manufacturer (Bio-Rad Laboratories, Hercules, CA), where bovine serum albumin (BSA) was used to plot the standard concentration curve. The medium was normalized before analysis by gelatin zymography or Western blotting.

Gelatin Zymography

Aliquots of conditioned medium harvested from A431 cells with or without treatment were subjected to substrate gel electrophoresis.[52] The samples were normalized by protein content and applied without reduction to a 7.5% polyacrylamide slab gel impregnated with 1 mg/ml gelatin (Sigma). After electrophoresis, the gel was washed at room temperature for 30 minutes in washing buffer (50 mM Tris-Cl, pH 7.5, 15 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5% Triton X-100) and incubated overnight at 37 C with shaking in the same buffer except using 1% Triton X-100. The gel was stained with a solution of 0.1% Coomassie brilliant blue R-250. Clear zones against the blue background indicated the presence of gelatinolytic activity.

Western Blotting

Proteins in conditioned medium collected from A431 cells in either sparse or dense cultures were precipitated by 3.3% trichloracetic acid (TCA) at 4 C overnight and pelleted by centrifuging at 19,500 × *g* at 4 C in MTX-150 high speed refrigerated microcentrifuge (Tomy Tech USA, Inc., Palo Alto, CA). TCA-containing supernatants were removed as completely as possible and protein pellets were resuspended in TNC solution (50 mM Tris-Cl, pH 7.5, 0.15 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3). The protein concentration in samples was measured by a standard protein assay (Bio-Rad) as described above. The same amount of protein from each sample was applied to 7.5% polyacrylamide slab gel. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) under reducing conditions and electrotransferred onto a nitrocellulose filter according to procedures described by Burnette.[53] The blot was then

blocked with 22% (weight/volume) fat-free dry milk solution and treated with anti (human 92-kd type IV collagenase)-rabbit serum, kindly provided by Dr. W. Stetler-Stevenson, NIH, Bethesda, MD. After extensive washing with TTBS buffer (50 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 0.05% Tween 20), the bound anti-collagenase antibodies were complexed with goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad). The collagenase band was visualized by using 165 μg/ml of 5-bromo-4-chloro-3-indolylphosphate and 333 μg/ml of nitro blue tetrazolium (Bio-Rad) in 0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$.[53]

Cell Labeling and Autoradiography

To allow cells to grow into discrete monolayer colonies, A431 cells were seeded into 8-well glass chamber slides (Nunc, Inc., Naperville, IL) at a density of 50 cells/well. After 7 to 10 days of culture (the medium was replaced every 3 days), the colonies were treated with TGF-β (1 ng/ml) plus TPA (25 ng/ml) for 24 hours. At that time, we added 0.5 μCi/ml [^{3}H]-leucine (specific activity, 131 Ci/mmol) in 80% leucine-free DMEM, 10% DMEM-F12, 10% FBS (ICN Biomedicals, Inc., Irvine, CA) for 8 hours or 0.5 μCi/ml [^{3}H]thymidine (specific activity, 2 Ci/mmol) (ICN Biomedicals, Inc.) to the culture medium for 24 hours. Radiolabeling of the cells was determined by liquid scintillation counting.

Autoradiography of the labeled A431 colonies was conducted after several extensive washings with cold PBS. The cells were then fixed with 95% ethanol plus 5% acetic acid for 30 minutes at room temperature. After fixation, the cells were washed again and air-dried and coated with NTB-2 autoradiography emulsion (IBI Kodak, New Haven, CT) that had been pre-warmed to 45 C, diluted 1:1 (volume/volume) with warm distilled water, and maintained at 45 C throughout the entire coating process. The slides were allowed to dry at room temperature, placed in black boxes, and kept in the dark at 4 C for 2 to 5 days. The slides were then developed in Kodak D-19 developer for 2 minutes and fixed in Kodak rapid fixer for 4 minutes. The slides were air-dried, mounted with Permount, and examined with a bright field microscope. Duplicate slides were counterstained with aqueous hematoxylin before drying.

Immunogold Staining

A431 cells were maintained as either sparse cultures or monolayer colonies in 8-well glass chamber slides as described above. The cells were incubated in medium (control) or medium containing 1 ng/ml TGF-β and 25 ng/ml TPA for 24 hours. Monensin is known to block the release of MMPs from cells.[54] We therefore added 1 μM monensin (Sigma Chemical Co.) to the culture medium during the last 4-hour incubation period. The cells were then washed with PBS and fixed with 2% paraformaldehyde for 10 minutes and rinsed with PBS. Cell membranes were permeabilized with 0.1% Triton X-100 and cells were blocked with 5% BSA containing 1% normal horse serum and 1% normal goat serum (Vector Laboratories, Inc., Burlingame, CA) and then given an application of anti-human 92-kd type IV collagenase rabbit serum. After an overnight incubation at 4 C with the primary antibody, the cells were washed with 1× PBS, briefly blocked with 5% BSA, and treated with gold-labeled goat anti-rabbit IgG (Amersham Co., Arlington Heights, IL). The cells were then washed and post-fixed with 2% glutaraldehyde, followed by color development using silver intense kit (Amersham Co.). In this assay, a positive reaction is visualized as brown/black staining.

Results

Induction of MMP-9 by TGF-β and TPA

Because TGF-β and TPA have been shown to modulate the expression of MMP-2 and MMP-9 in normal fibroblasts[55] or keratinocytes,[56] we used an epidermoid carcinoma line in our experiments. We plated different numbers of A431 human epidermoid carcinoma cells into 6-well plates to produce sparse (30% confluence) and dense (full confluence) cultures. These cultures were then incubated for 24 hours in serum-depleted medium containing TGF-β, TPA, or both. The conditioned medium was normalized and subjected to gelatin zymographic analysis (Figure 1).

Both sparse and confluent cultures of A431 cells constitutively secreted the latent form of MMP-2 and trace amounts of MMP-9 gelatinolytic activity. Treatment with either 1 ng/ml TGF-β or 25 ng/ml TPA induced low levels of MMP-9 production in sparse cultures (Figure 1) but further increases in concentration of TGF-β or TPA had no additional effect (data not shown). In confluent cultures, TGF-β but not the mitogen, TPA, induced low levels of MMP-9 production. When sparse cultures of A431 cells were incubated with both TGF-β (1 ng/ml) and TPA (25 ng/ml), we observed a synergistic induction of MMP-9 production. This synergism was not found in A431 cells after they reached full confluence (dense cultures). The secretion of MMP-2 was not significantly affected by either the treatment or the state of confluence (Figure

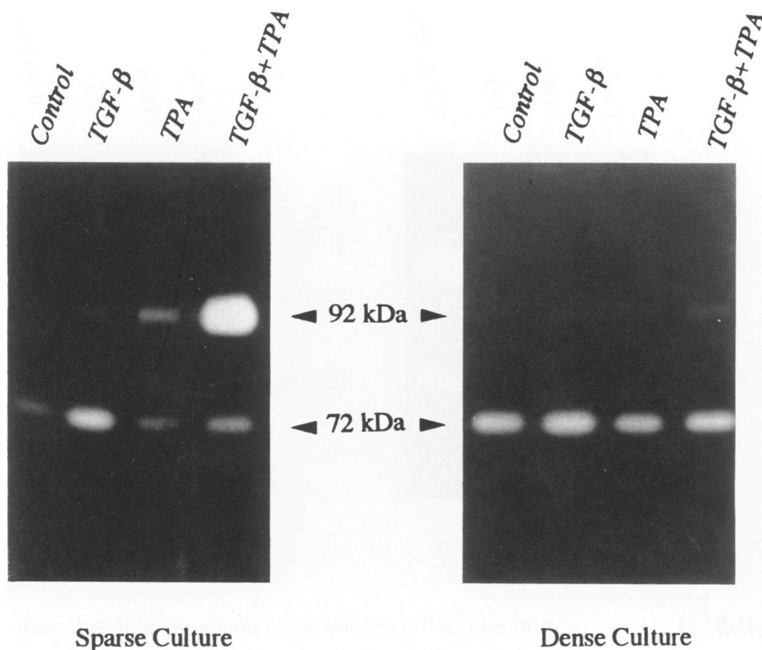

Figure 1. *Effect of cell density on induction of MMP-9 by TGF-β and TPA. A431 cells were plated in 6-well cluster plates and maintained in either sparse culture (30% confluence) or dense culture (full confluence). Cells were treated without (control) or with TGF-β (1 ng/ml) or TPA (25 ng/ml) or both for 24 hours in serum-free DMEM-F12. After incubation, the conditioned medium was collected, normalized, and analyzed by gelatin zymography (see Materials and Methods).* **Arrows** *indicate the positions of latent forms of MMP-2 (72 kd) and MMP-9 (92 kd).*

1). Similar results were obtained when the same number of cells were plated into either small (30 mm) or large (100 mm) dishes to yield sparse or confluent cultures. Collectively, these data suggest that in A431 cells MMP-9 and MMP-2 are differentially regulated.

Culture Density and Induction of MMP-9

To the best of our knowledge, the synergistic induction of MMP-9 by TGF-β and TPA has not been previously reported. Furthermore, its abolishment in confluent cultures was of great interest. A decrease in gelatinolytic activity as shown by zymography could have resulted from either a genuine suppression of protein biosynthesis or an inhibition by TIMP-1, because latent MMP-9 can form a complex with TIMP-1,[57] which is also secreted by A431 cells (data not shown). To examine this question, a Western blotting analysis was conducted using a polyclonal anti-MMP-9 antibody (Figure 2). A431 cells grown to sparse or full confluence were treated with TGF-β plus TPA for 24 hours and the conditioned medium was precipitated by 3.3% TCA. MMP-9 was expressed in treated cells under sparse culture conditions (lane 1) but not in treated dense cultures (lane 2), suggesting that in dense cultures induction of MMP-9 biosynthesis did not occur.

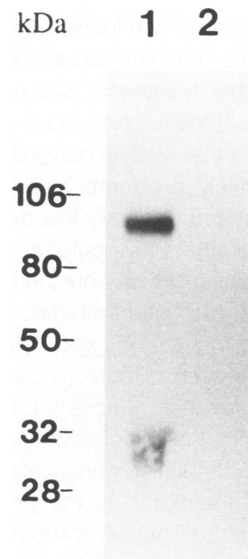

Figure 2. *Western blot analysis of density-dependent induction of MMP-9 by TGF-β and TPA. A431 cells were plated in 6-well cluster plates and maintained in sparse (lane 1) or dense (lane 2) culture. Cells under both culture conditions were treated with TGF-β (1 ng/ml) plus TPA (25 ng/ml) in serum-free DMEM-F12 for 24 hours. The proteins in conditioned medium were precipitated by 3.3% TCA and applied to polyacrylamide gels (SDS-PAGE) under reducing conditions. After electrophoresis, the proteins were electrotransferred to nitrocellulose filter and probed with anti-MMP-9 antibody. Molecular weight markers (Bio-Rad) used (data not shown) were phosphorylase B, 106 kd; BSA, 80 kd; ovalbumin, 50 kd; carbonic anhydrase, 32 kd; and soybean trypsin inhibitor, 28 kd. This is a representative experiment of three.*

To rule out the possibility that A431 cells at confluence excrete soluble factors that antagonize the stimulatory function of TGF-β and/or TPA, A431 cells in sparse cultures were exposed to TGF-β, TPA, or both suspended in fresh medium (panel A) or medium conditioned by dense A431 cell cultures for 24 hours (panel B). As shown in Figure 3, the dense culture-conditioned medium had little inhibitory effect on MMP-9 expression induced by TGF-β and TPA.

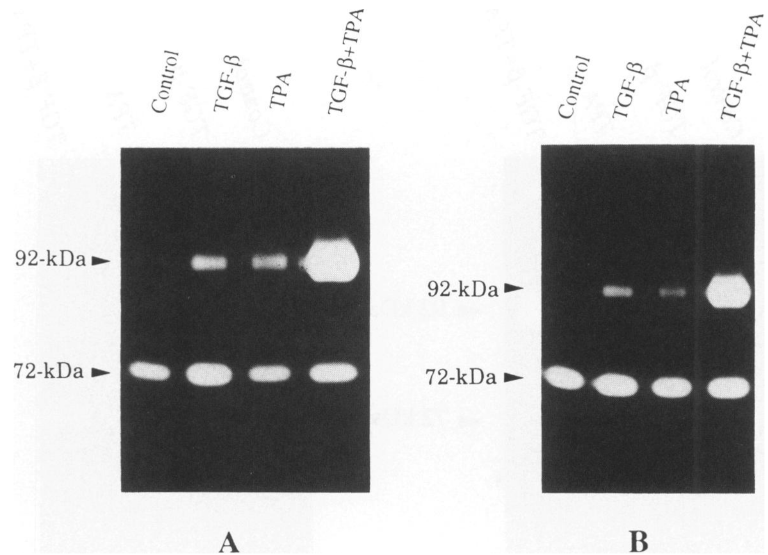

Figure 3. *Effect of dense culture-conditioned medium on induction of MMP-9 by TGF-β and TPA. A431 cells were plated in 6-well cluster plate and maintained in sparse culture when treated without (control) or with TGF-β (1 ng/ml) or TPA (25 ng/ml) or both, either in fresh DMEM-F12* (**A**) *or medium conditioned by dense culture for 24 hours* (**B**). *After a 24-hour incubation, the supernatants were harvested and analyzed by gelatin zymography.*

Growth Arrest and Induction of MMP-9

The observation that the synergistic activity of TGF-β/TPA was abolished in confluent cultures suggested that the synergistic induction was associated with cell division. To test this hypothesis, we had to prove that the A431 cells cultured to confluence were not metabolically inert and that MMP-9 biosynthesis was only inducible in actively proliferating cells. A431 cells were therefore plated at a very low density in 8-well chamber slides so that they could grow into discrete monolayer colonies in which only the cells at the periphery were free of contact inhibition and thus expected to divide. In the study shown in Figure 4, the colonies were exposed to medium containing TGF-β and TPA in the presence or absence of monensin (1 μM), an ionophore that blocks MMP secretion and thus allows its accumulation within cells.[54] One group of colonies was incubated in the presence of 0.5 μCi/ml of [^{3}H]leucine (Figure 4, A and D) to assess the status of protein synthesis or [^{3}H]thymidine (Figure 4, B and E) to assess cell division. Distribution of ^{3}H was visualized by autoradiography. Figure 4 shows that only the cells at the periphery of the colony actively incorporated [^{3}H]thymidine into their nuclei (Figure 4, B and E), whereas all the cells in the colony were uniformly labeled with [^{3}H]leucine (Figure 4, A and D). These results demonstrate unequivocally that although the entire colony remained metabolically active, only the cells at the periphery were incorporating [^{3}H]thymidine. To determine whether only these dividing cells could be induced to produce MMP-9, a parallel group of treated colonies was examined for MMP-9 expression using immunolocalization techniques. As shown in Figure 4, C and F, only cells at the perimeter of the colony were positively stained, demonstrating that cell division is necessary for the induction of MMP-9 by TGF-β and TPA.

In the last set of experiments, we determined the presence of collagenase in sparse cultures by immunohistochemistry using antibodies against MMP-9. A431 cells were cultured under sparse conditions for 20 hours in medium alone (Figure 5A) or in medium containing TGF-β and TPA (Figure 5B). Monensin (1 μ) was added to both cultures for an additional 4 hours and the sparse cultures were processed for immunostaining. A431 cells in medium alone did not show positive staining with the anti-MMP-9 antibody (Figure 5A). In A431 cells treated with TGF-β and TPA, the cytoplasm was densely stained positive for MMP-9 (Figure 5B). The dense perinuclear staining pattern (Figures 4F and 5B) is characteristic of monensin treatment and indicates the efficacy of the reagent.

Discussion

These results demonstrate that the induction of high levels of MMP-9 in human epidermoid carcinoma A431 cells is associated with cell proliferation and is dependent on exogenous stimulation. Treatment of A431 cells with TGF-β and TPA synergistically induced MMP-9 but not MMP-2 secretion. This suggested that the regulation of MMP-2 and MMP-9 production is independent. The synergistic induction of MMP-9 production by TGF-β and TPA was dependent on cell proliferation. In contact-inhibited growth-arrested cultures of A431, the synergistic induction of MMP-9 did not occur.

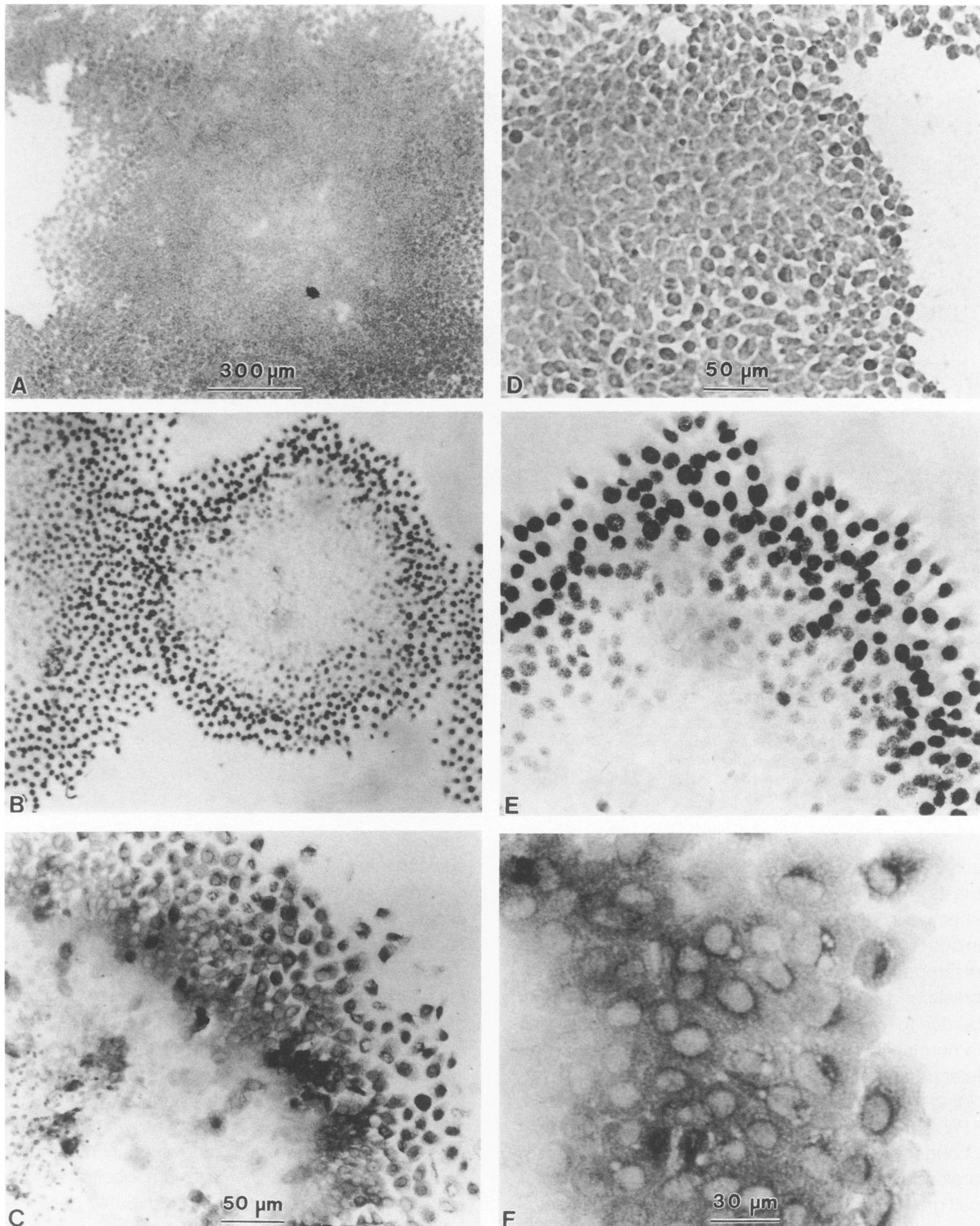

Figure 4. *Peripheral localization of MMP-9 in dividing cells. A431 cells were plated in 8-well chamber slides and allowed to grow into discrete monolayer colonies as described in Materials and Methods. The colonies were then treated with TGF-β (1 ng/ml) plus TPA (25 ng/ml) for 24 hours and in the presence of 1 μ monensin during the last 4 hours. The cells in colonies were labeled with 0.5 μCi/ml [³H]leucine for 8 hours* (**A**,**D**) *or with 0.5 μCi/ml [³H]thymidine for 24 hours* (**B**,**E**) *and autoradiographed (see Materials and Methods). Alternatively, the colonies were immunostained with anti-MMP-9 antibody* (**C**,**F**). **B** *and* **E** *show labeled nuclei.* **C** *and* **F** *show cytoplasmic staining of cells at the periphery of the colony.* **A**,**B**, ×*59;* **C** *to* **E**, ×*233;* **F**, ×*455.*

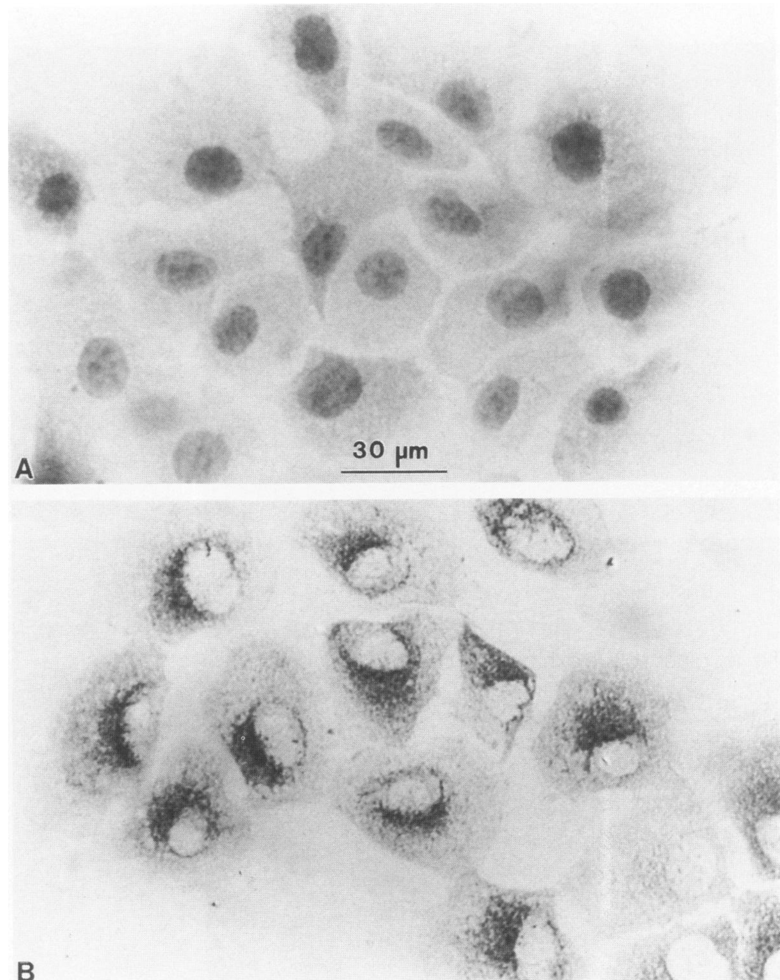

Figure 5. *Immunostaining for MMP-9 in sparse cultures of A431 cells. A431 cells were plated in 8-well chamber slides and maintained in sparse culture. To induce MMP-9, cells were exposed to a combined treatment of TGF-β (1 ng/ml) and TPA (25 ng/ml) for 24 hours with the presence of 1 μ monensin for the last 4 hours* (**B**). *As a negative control, cells were treated with monensin alone without addition of TGF-β and TPA* (**A**). *Cells in* **A** *and* **B** *were both stained for 92-kd type IV collagenase using anti-MMP-9 antibody. Cells in* **A** *were counterstained with hematoxylin to show nuclei. Cells in* **B** *were not counterstained. Cells in* **A** *show no positive cytoplasmic staining, whereas cells in* **B** *show dense perinuclear cytoplasmic staining. Magnification ×544.*

Mitogenic agents such as TPA have been shown to stimulate the expression of MMPs in normal fibroblasts.[58] Unlike our findings, however, TPA was quite effective in stimulating type IV collagenase secretion in both sparse and confluent fibroblast cultures.[58] The discrepancy between the results with normal fibroblasts and A431 carcinoma cells at confluence may be due to the fact that TPA caused transient changes in normal fibroblasts that resembled those from cellular transformation by oncogenic viruses, thus overriding the effects of contact inhibition.[58] In A431 cells, TPA did not induce any apparent morphological changes nor did it stimulate cell proliferation. Actually, prolonged treatment with TPA inhibited A431 tumor cell growth (data not shown). Because TPA mediates most of its regulatory function via protein kinase C (PKC),[59] it is possible that TPA did not activate PKC in confluent cultures; that was the case for 10T½ mouse fibroblasts. TPA failed to induce translocation of cytosolic PKC to plasma membrane in confluent cultures.[60] Direct assays of PKC activity in sparse versus confluent cultures could test this possibility.

Endothelial BM and its underlying interstitial stroma serves as a physical scaffolding for tissues and is important in the maintenance of normal tissue architecture.[61] Therefore, disturbance of the ECMs integrity may contribute to the transition of a benign tumor or *in situ* carcinoma to a malignant neoplasm.[61] At the invasion front of a malignant tumor, tumor cells are in immediate contact with host tissue ECM and thus with high concentrations of cytokines and growth factors. These biologically active substances, which are usually released and activated on degradation of matrix components,[62,63] can stimulate tumor cells to proliferate.[61] As suggested by our study, only cells capable of division (noncontact-inhibited cells) can produce collagenases in response to exogenous stimuli. This mechanism may be applied to the regulation of autocrine growth factors[64] and their cell surface receptors.[65]

Tumor cells also produce several paracrine factors that influence the proliferation and functioning of stromal cells such as fibroblasts and macrophages.[66] The synthesis of these paracrine modulators could well be affected by the proliferative state of the cells. For example, epithelial cells from the adult rabbit cornea can secrete both stimulators and inhibitors that modulate proliferation and collagenase production of fibroblasts from the same source. Stimulators were found in low density epithelial cell cultures, whereas high density epithelial cultures secreted inhibitory molecules.[67] In general when cells enter quiescence, they exhibit major alterations in cell surface receptors, expression of intracellular regulating molecules such as tran-scription factors, cytochemical enzymes, and cellular ultrastructures.[64,68] Our finding that proliferating A431 cells respond differently from contact-inhibited growth-arrested A431 cells to external stimulation agrees with these observations. These data may explain why tumor cells at the invading front of a neoplasm often exhibit high levels of collagenase activity.[43,44] First, cells at the leading edge of a neoplasm are more likely to divide than those cells in the center of the lesion. Second, the gradient of host cytokines is likely to be higher at the tumor/host edge than at the center of the tumor. The required combination of external stimuli may vary with different tumor cells and different organ environments. Obviously, our proposed mode for the production of MMP-9 is in sharp contrast with the production of MMP-2. MMP-2 in our system, as in many others, is constitutively expressed and its production is by and large refractory to external stimuli.[55] Nevertheless, it is certain that expression of MMP-9 and MMP-2 is subject to distinct regulatory modes, which suggests that these two MMPs have different roles in both normal and pathological invasion.

In summary, we show that the induction of MMP-9 in A431 cells requires stimulation with exogenous signals and sparse culture conditions. These *in vitro* data may explain the *in vivo* findings that collagenase production by colon carcinoma cells depends on the organ environment[69] and the widespread observation that collagenase expression is localized to the invasion front of most tumors.[33–45]

Acknowledgments

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References

1. Fidler IJ: Critical factors in the biology of human cancer metastasis: twenty-eight G H A Clowes Memorial Award Lecture. Cancer Res 1990, 50:6130–6138
2. Liotta LA, Rao CN, Barsky SH: Tumor invasion and the extracellular matrix. Lab Invest 1983, 49:639–649
3. Barsky SH, Siegal GP, Jannotta F, Liotta LA: Loss of basement membrane components by invasive tumors but not by their benign counterparts. Lab Invest 1983, 49:140–147
4. Werb Z: Proteinases and matrix degradation. Textbook of Rheumatology, vol 34. Edited by Kelly WN. Philadelphia, W B Saunders Co, 1989, pp 300–321
5. Matrisian LM: The matrix-degrading metalloproteinases. Bioessays 1992, 14:455–463
6. Goldberg GI, Wilhelm SM, Kronberger A, Bauer EA, Grant GA, Eisen AZ: Human fibroblast collagenase: complete primary structure and homology to an oncogene transformation-induced rat protein. J Biol Chem 1986, 261:6600–6605
7. Okada Y, Nagase H, Harris ED Jr: A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components: purification and characterization. J Biol Chem 1986, 261: 14245–14255
8. Fessler L, Duncan K, Tryssvason K: Identification of the procollagen IV cleavage products produced by a specific tumor collagenase. J Biol Chem 1987, 259: 9783–9789
9. Hibbs MS, Hasty KA, Seyer JM, Kang AH, Mainardi C: Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. J Biol Chem 1985, 260:2493–2500
10. Murphy G, Ward R, Hembry RM, Reynolds JJ, Kuhn K, Tryggvason K: Characterization of gelatinase from pig polymorphonuclear leukocytes. Biochem J 1989, 258: 463–472
11. Okada Y, Morodomi T, Enghild JJ, Suzuki K, Yasui A, Salvesen G: Matrix metalloproteinase-2 from human rheumatoid synovial fibroblasts: purification and activation of the precursor and enzymatic properties. Eur J Biochem 1990, 194:721–730
12. Murphy G, Cockett MI, Stephens PE, Smith BJ, Docherty AJP: Stromelysin is an activator of procollagenase: a study with natural and recombinant enzymes. Biochem J 1987, 248:265–268
13. Ogata Y, Enghild JJ, Nagase H: Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. J Biol Chem 1992, 267:3581–3584
14. He C, Wilhelm SM, Pentland AP, Marmer BL, Grant GA, Eisen AZ, Goldberg G: Z. Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. Proc Natl Acad Sci USA 1989, 86:2632–2636
15. Okada Y, Nakanishi I: Activation of matrix metalloproteinase 3 (Stromelysin) and matrix metalloproteinase 2 ('gelatinase') by human neutrophil elastase and ca-

thepsin G. FEBS Lett 1989, 249:353–356
16. Stetler-Stevenson WG, Krutzsch HC, Wacher MP, Margulies IM, Liotta LA: The activation of human type IV collagenase proenzyme. J Biol Chem 1989, 264: 1353–1356
17. Docherty AJP, Lyons A, Smith BJ, Wright EM, Stephens PE, Harris TMF: Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. Nature 1981, 318:66–69
18. Stetler-Stevenson WG, Krutzch HC, Liotta LA: Tissue inhibitor of metalloproteinase (TIMP-2). J Biol Chem 1989, 264:17374–17378
19. Liotta LA, Tryggvasson K, Garbisa S, Hart IR, Folts CM, Shafie S: Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 1980, 284:67–68
20. Turpeenniemi-Hujanen T, Thorgeirsson VP, Hart IR, Grant SS, Liotta LA: Expression of collagenase IV (basement membrane collagenase) activity in murine tumor cell hybrids that differ in metastatic potential. J Natl Cancer Inst 1985, 75:99–103
21. Nakajima M, Welch DR, Belloni PN, Nicolson GL: Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma cell clones of differing metastatic potentials. Cancer Res 1987, 47:4869–4876
22. Yamagata S, Tanaka R, Ho Y, Shimizu S: Gelatinases of murine metastatic tumor cells. Biochem Biophys Res Commun 1989, 158:228–234
23. Sato H, Kida Y, Mai M, Endo Y, Sasaki T, Tanaka J, Seiki M: Expression of genes encoding type IV collagen-degrading metalloproteinase and tissue inhibitors of metalloproteinases in various human tumor cells. Oncogene 1992, 7:77–83
24. Garbisa S, Pozzatti R, Muschel RJ, Saffiotti U, Ballin M, Goldfarb RH, Khoury G, Liotta LA: Secretion of type IV collagenolytic protease and metastatic phenotype: induction by transfection with c-Ha-ras but not c-Ha-ras plus Ad2-Ela. Cancer Res 1987, 47:1523–1528
25. Ura H, Bonfil D, Reich R, Reoldel R, Pfeifer A, Harris CC, Klein-Szanto AJP: Expression of type IV collagenase and procollagen genes and its correlation with the tumorigenic, invasive, and metastatic abilities of oncogene-transformed human bronchial epithelial cells. Cancer Res 1989, 49:4615–1621
26. Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant G, Seltzer JL, Kronberger A, He C, Bauer EA, Goldberg GI: H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement membrane collagen. J Biol Chem 1988, 263:6579–6587
27. Ballin M, Gomez DE, Sinha CC, Thorgeirsson UP: Ras oncogene mediated induction of a 92-kDa metalloproteinase: strong correlation with the malignant phenotype. Biochem Biophys Res Commun 1988, 154:832–838
28. Wilhelm SM, Collier IE, Marmer BL, Eisen AZ, Grant GA, Goldberg GI: SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. J Biol Chem 1989, 264:17213–17221
29. Bernhard EJ, Muschel RJ, Hughes EN: M_r92,000 gelatinase release correlates with the metastatic phenotype in transformed rat embryo cells. Cancer Res 1990, 50:3872–3877
30. Schultz RM, Silberman S, Persky B, Bajkowski AS, Carmichael DF: Inhibition by human recombinant tissue inhibitor of metalloproteinases of human amnion invasion and lung colonization by murine B16-F10 melanoma cells. Cancer Res 1988, 48:5539–5545
31. Khokha R, Waterhouse P, Yagel S, Lala PK, Overall CM, Norton G, Denhardt DT: Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science 1989, 243:947–950
32. Albini A, Melchiori A, Santis L, Liotta LA, Brown PD, Stetler-Stevenson WG: Tumor cell invasion inhibited by TIMP-2. J Natl Cancer Inst 1991, 83:775–779
33. Karelina TV, Hruza GJ, Goldberg GI, Eisen AZ: Localization of 92-kDa type IV collagenase in human skin tumors: comparison with normal human fetal and adult skin. J Invest Dermatol 1993, 100:159–165
34. Levy AT, Cioce V, Sobel ME, Garbisa S, Grigioni WF, Liotta LA, Stetler-Stevenson WG: Increased expression of the M_r72,000 type IV collagenase in human colonic adenocarcinoma. Cancer Res 1991, 51:439–444
35. D'Errico A, Garbisa S, Liotta LA, Castronovo V, Stetler-Stevenson WG, Grigioni WF: Augmentation of type IV collagenase, laminin receptor and Ki67 proliferation antigen associated with human colon gastric and breast carcinoma progression. Modern Pathol 1991, 4:239–246
36. Stearns ME, Wang M: Type IV collagenase (M_r72,000) expression in human prostate: benign and malignant tissue. Cancer Res 1993, 53:878–883
37. Barsky SH, Togo S, Garbisa S, Liotta LA: Type IV collagenase immunoreactivity in invasive breast carcinoma. Lancet 1983, 1:296–297
38. Barsky SH, Grossman DA, Bhuta S: Desmoplastic basel cell carcinomas possess unique basement membrane-degrading properties. J Invest Dermatol 1987, 88:324–329
39. Monteagudo C, Merino MJ, San-Juan J, Liotta LA, Stetler-Stevenson WG: Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. Am J Pathol 1990, 136:585–592
40. Pyke C, Ralfkiaer E, Huhtala P, Hurskainen T, Dano K, Tryggvason K: Localization of messenger RNA for Mr 72,000 and 92,000 type IV collagenases in human skin cancers by in situ hybridization. Cancer Res 1992, 52:1336–1341
41. Pyke C, Ralfkiaer E, Tryggvason K, Dano K: Messenger RNA for two type IV collagenases is located in stromal cells in human colon cancer. Am J Pathol 1993, 142:359–365

42. Poulsom R, Pignatelli M, Stetler-Stevenson WG, Liotta LA, Wright PA, Jeffery RE, Langcroft JM, Rogers K, Stamp GWH: Stromal expression of 72 kDa type IV collagenase (MMP-2) and TIMP-2 mRNAs in colorectal neoplasia. Am J Pathol 1992, 141:389–396
43. Tryggvason K, Hoyhtya M, Pyke C: Type IV collagenase in invasive tumors. Breast Cancer Res Treat 1993, 24:209–218
44. Woolley DE: Collagenolytic mechanisms in tumor cell invasion. Cancer Metastasis Rev 1984, 3:361–372
45. Basset P, Bellocq JP, Stoll WI, Hutin P, Limacher JM, Podhajcer OL, Chenard MP, Rio MC, Chambon P: A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature 1990, 348:699–704
46. Skriver L, Larsson LI, Kielberg V, Nielsen LS, Anderson PB, Kristensen P, Dano K: Immunocytochemical localization of urokinase-type plasminogen activator in Lewis lung carcinoma. J Cell Biol 1984, 99:752–757
47. Graf M, Baici A, Strauli P: Histochemical localization of cathepsin B at the invasion front of the rabbit V_2 carcinoma. Lab Invest 1981, 45:587–596
48. Childers JW, Hernandez AD, Kim JH, Stricklin GP: Immunolocalization of collagenase inhibitor in normal skin and basel cell carcinoma. J Am Acad Dermatol 1987, 17:1025–1032
49. Hewitt RE, Leach IH, Powe DG, Clark IM, Cawston TE, Turner DR: Distribution of collagenase and tissue inhibitor of metalloproteinases (TIMP) in colorectal tumours. Int J Cancer 1991, 49:666–672
50. Giard PJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, Parkes WP: In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 1973, 51:1417–1421
51. Birkedal-Hansen H, Taylor RE: Detergent-activation of latent collagenase and resolution of its component molecules. Biochem Biophys Res Commun 1982, 107: 1173–1178
52. Burnette WN: Western blotting: electrophoretic transfer of proteins from sodium dodecylsulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection and radioiodinated protein A. Anal Biochem 1981, 112:195–203
53. Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC: A rapid, sensitive method for detection of alkaline phosphatase-conjugated antibody on western blots. Anal Biochem 1984, 136:175–179
54. Nagase H, Brickerhoff CE, Vater CA, Harris ED Jr: Biosynthesis and secretion of procollagenase by rabbit synovial fibroblasts. Biochem J 1983, 214:281–288
55. Overall CM, Wrana JL, Sodek J: Transcriptional and post transcriptional regulation of 72 kDa gelatinase type IV collagenase by transforming growth factor β in human fibroblasts. J Biol Chem 1991, 266:14064–14071
56. Salo T, Lyons JG, Rahemtulla F, Birkedal-Hansen H, Larjava H: Transforming growth factor-1β upregulates type IV collagenase expression in cultured human keratinocytes. J Biol Chem 1991, 266:11436–11441
57. Goldberg GI, Marmer BL, Grant GA, Eisen AZ, Wilhelm SM, He C: Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of MMPs designated TIMP-2. Proc Natl Acad Sci USA 1989, 86:8202–8211
58. Salo T, Turppeenniemi-Hyjanen T, Tryggvason K: Tumor-promoting phorbol esters and cell proliferation stimulate secretion of basement membrane (type IV) collagen-degrading metalloproteinase by human fibroblasts. J Biol Chem 1985, 260:8526–8531
59. O'Brian CA, Ward NE: Biology of the protein kinase C family. Cancer Metastasis Rev 1989, 8:199–214
60. Miloszewska J, Trawicki W, Janik P, Moraczewski J, Przybyszewska M, Szaniawska B: Protein kinase C translocation in relation to proliferative state of C3H 10T½ cells. FEBS Lett 1986, 206:283–286
61. Liotta LA, Rao CN, Wewer UM: Biochemical interactions of tumor cells with the basement membrane. Annu Rev Biochem 1986, 55:1037–1057
62. Baird A, Ling N: Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implications for a role of heparanase-like enzymes in the neovascular response. Biochem Biophys Res Commun 1987, 142:428–435
63. Andres JL, Stanley K, Cheifetz S, Massague J: Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-β. J Cell Biol 1989, 109:3137–3145
64. Mulder KM: Differential regulation of c-myc and transforming growth factor-α messenger RNA expression in poorly differentiated and well-differentiated colon carcinoma cells during the establishment of a quiescent state. Cancer Res 1991, 51:2256–2262
65. Rizzino A, Kazakoff P, Nebelsick J: Density-induced down regulation of epidermal growth factor receptors. In Vitro Cell Dev Biol 1990, 26:537–542
66. Hernandez AD, Hibbs MS, Postlethwaite AE: Establishment of basal cell carcinoma in culture: evidence for a basal cell carcinoma-derived factor(s) which stimulates fibroblasts to proliferate and release collagenase. J Invest Dermatol 1985, 85:470–475
67. Johnson-Wint B: Regulation of stromal cell collagenase production in adult rabbit cornea: in vitro stimulation and inhibition by epithelial cell products. Proc Natl Acad Sci USA 1980, 77:5331–5335
68. Williams GM, Stromberg K, Kroes R: Cytochemical and ultrastructural alterations associated with confluent growth in cell cultures of epithelial-like cells from rat liver. Lab Invest 1973, 29:293–303
69. Nakajima M, Morikawa K, Fabra A, Bucana CD, Fidler IJ: Influence of organ environment on extracellular matrix degradative activity and metastasis of human colon carcinoma cells. J Natl Cancer Inst 1990, 82: 1890–1898