Induction of 103-kDa Gelatinase/Type IV Collagenase by Acidic Culture Conditions in Mouse Metastatic Melanoma Cell Lines*

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Gelatinases/type IV collagensases have been shown to be involved in tumor invasion and metastasis. In this study, we examined the effect of culture medium pH on the secretion of the gelatinases from mouse B16 melanoma cell lines and human tumor cell lines using zymography analysis. The highly metastatic clone F10 of B16 melanoma did not secrete any gelatinase in neutral culture media (pH 7.1–7.3), whereas it secreted a high level of a 103-kDa gelatinase in an initial pH range of 5.4–6.1. The addition of an excess amount of glucose into a neutral culture medium also induced the gelatinase secretion from the cells by decreasing the medium pH during incubation. The extent of the acid-induced gelatinase secretion by the B16 melanoma cell lines was in the order of BL6 > F10 > F1 > the parent B16 line, in good agreement with the order of their metastatic potentials. Two human cell lines (A549 and HT1080) secreted a higher level of a 90-kDa gelatinase at pH 6.8 compared with pH 7.3. The acid-induced gelatinase secretion from B16-F10 cells was blocked by cycloheximide, indicating that the enzyme induction was due to de novo synthesis. When in vitro tumor cell invasion was assayed in Boyden chambers, B16-F10 cells incubated in an acidic medium exerted a more active migration through type IV collagen gel than those in a neutral medium. These results suggest that the acidic environment formed around tumor tissues may be an important factor in invasion and metastasis of some types of tumors.

Tumor metastasis is a multistep process in which complex tumor cell-host tissue interactions are involved. This process is often divided into several sequential steps, such as release from the primary tumor, invasion of the local connective tissue and a lymphatic or blood vessel, circulation, extravasation, and secondary growth at the target organ. Among these steps, the invasion of tumor cells through the basement membrane and their underlying interstitial stroma is thought to be one of the most critical steps in tumor metastasis. Recent studies have revealed a family of structurally related metalloproteinases that includes interstitial collagenase (EC 3.4.24.7) (1, 2), 92-kDa (or 90-kDa) gelatinase/type IV collagenase (gelatinase B, EC 3.4.24.35) (3, 4), 72-kDa (or 64-kDa) gelatinase/type IV collagenase (gelatinase A, EC 3.4.24.24) (5, 6), stromelysin 1 (EC 3.4.24.17) (7–9), and matrin/pump-1 (matrilysin, EC 3.4.24.23) (10, 11). They are secreted from various kinds of tumor cells and are capable of degrading extracellular matrix components with different specificities. Among them, type IV collagen-degrading enzymes are especially important because type IV collagen is the structural backbone of the basement membrane.

Liotta et al. (12) first reported that the secretion of a type IV collagen-degrading enzyme is well correlated with the metastatic potential of mouse B16 melanoma cell lines. The same correlation has also been observed in rat embryo cell lines transformed by various oncogenes (2). The same group purified a type IV collagenase with a M, of 68,000 from the conditioned medium of a mouse metastatic tumor cell line (6). Later, two kinds of type IV collagenases with M, values of 72,000 and 92,000 (~64,000 and ~90,000 on nonreducing SDS-polyacrylamide gel electrophoresis, respectively) were purified from H-ras-transformed human bronchial epithelial cells and SV40-transformed human lung fibroblasts, respectively; and their primary structures were determined by cDNA cloning (3, 5). These enzymes strongly digest gelatins as well as type IV and V collagens and are identical to gelatin-degrading metalloproteinases (gelatinases), which have been purified from several kinds of normal and malignant cells. Several groups have recently demonstrated that the secretion of the 92-kDa type IV collagenase is better correlated with the metastatic potential of tumor cells than that of the 72-kDa type IV collagenase (13–15).

In this study, we examined the secretion of these gelatinases/type IV collagenases from metastatic clones of mouse B16 melanoma cells and found that the highly metastatic B16 clones secreted no gelatinase in a neutral culture medium, whereas they secreted a high amount of a 103-kDa gelatinase in acidic culture media. Here, we report the acid induction of this matrix-degrading enzyme in the B16 melanoma cell lines and the possible importance of acidic environment in the invasion and metastasis of tumor cells.

MATERIALS AND METHODS

Cells and Culture Conditions—Three metastatic clones (F1, F10, and BL6) have been established from the mouse B16 melanoma by Fidler and co-workers (16–18). F1 and F10 were obtained from the American Type Culture Collection through Dainippon Seiyaku (Osaka, Japan). The parent B16 cell line was kindly provided by the Collection of Transplantable Tumor Cell Lines (Tohoku University), and BL6 by Bioscience Research Laboratories (Nippon Mining, Saitama, Japan). Human tumor cell lines tested for the secretion of

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acids, the cultures were then rinsed three times with Dulbecco's Ca2+/Mg2+-free phosphate-buffered saline and incubated in serum-free DMEM/F-12 media at different pH values. The serum-free conditioned media were harvested after incubation for 3 days and clarified for 30 min. The protein present in each clarified conditioned medium was precipitated by 80% saturated ammonium sulfate and collected by centrifugation at 15,000 × g for 30 min. The protein precipitate was dissolved in a small volume of 10 mM Tris-HCl (pH 7.5), dialyzed against the same buffer, and used as "concentrated conditioned medium" (0.2 ml).

Gelatin Zymography of Proteinases—Gelatinolytic activities of secreted proteinases were analyzed by zymography on gelatin-containing gels as described previously (9, 10). Concentrated conditioned media to be analyzed (6.7 ml/lane unless otherwise noted) were mixed with equal volumes of concentrated sample buffer (4% (w/v) SDS, 12% (w/v) Tris-HCl (pH 6.8), 10% (v/v) glycerol, 50 mM CaCl2, and 0.02% NaN3 at room temperature for 1 h, followed by incubation in 50 mM Tris-HCl (pH 7.5) containing 10 mM CaCl2 and 0.02% NaN3 at 37 °C for 20 h. The resultant gels were stained with Coomassie Brilliant Blue R-250. The molecular weight markers used were myosin (M, 200,000), Escherichia coli β-galactosidase (M, 116,250), rabbit muscle phosphorylase b (M, 97,400), bovine serum albumin (M, 66,200), and hen egg white ovalbumin (M, 42,690).

In Vitro Invasion Assay of B16-F10 Cells—The in vitro invasion assay was carried out in Boyden chambers with polyvinylpyrrolidone-free polycarbonate porous filters (diameter, 8 mm; pore size, 8 μm) (Nuclepore, Pleasanton, CA) by the method of Albin et al. (19) with modifications. Each filter was previously coated with 100 μg of type IV collagen (Koken Co. Ltd., Tokyo). Serum-free NIH/3T3 conditioned medium, which had been prepared as described previously (20), was placed into the lower compartment of the chamber as a chemotactrant. When B16-F10 cells were grown to ~90% saturation, they were washed three times with Dulbecco's Ca2+/Mg2+-free phosphate-buffered saline and then incubated in serum-free DMEM/F-12 with an initial pH of 7.3 or 6.1. After incubation for 1 day, the cells in each culture were harvested by a rubber policeman while the serum-free conditioned medium was collected. The harvested cells were suspended in the respective conditioned media at a density of 1 × 105 cells/ml. The cell suspension (0.5 ml) was placed onto the type IV collagen-coated filters in the upper compartment of the Boyden chambers. After the indicated periods of incubation in a CO2 incubator, filters were fixed in methanol and stained with Giemsa stain. Migrated cells under the filters were counted in eight randomly selected microscopic fields/filter (magnification × 200). The results are expressed as the mean ± S.D. of three separate experiments.

RESULTS

Secretion of Gelatinase from B16-F10 Cells—It has been reported that the highly metastatic clone F10 of mouse B16 melanoma cells secrete type IV collagenase activity into culture medium (12). In our preliminary experiments, however, no activity was detected. We tested gelatinase secretion with various culture media and found that the melanoma cells secreted a gelatinase activity with a M, of 105,000 in a culture medium with a high glucose concentration (5.9 mg/ml) (Fig. 1). In this medium, the pH decreased from 7.3 to 6.4 during incubation for 8 days, whereas such a pH drop was not noticed in the lower glucose medium (1.4 mg/ml). Further investigation revealed that induction of gelatinase secretion was caused by the pH drop rather than by the high glucose concentration.

The effect of medium pH on the secretion of the 105-kDa gelatinase is shown in Fig. 2. The gelatinase activity became detectable as the initial pH of the culture medium was decreased to 7.0, and it reached the maximal level between pH 6.1 and 6.4. In this pH range, three additional proteolytic bands of M, 120,000, 90,000, and 70,000 were observed on the zymogram (Fig. 2A). The growth of F10 cells was maximal at an initial pH of ~6.8 and gradually declined as the pH was further decreased (Fig. 2B). The cells were still viable at an initial pH of 4.7, whereas a further pH decrease completely killed them.

To show that the apparent induction of the gelatinase activities in the acidic media was due to increased enzyme secretion, two experiments were carried out (data not shown). First, when the conditioned medium obtained from the culture at pH 7.3 was adjusted to pH 6.0 and incubated at 37 °C for 2 days, no gelatinolytic activity appeared in the conditioned medium. Second, when the conditioned medium from the culture at pH 5.9 was divided into two portions; adjusted to pH 6.0 and pH 7.4, respectively; and incubated at 37 °C for 2 days, the gelatinolytic activities did not significantly change at both pH values. These results indicated that the high gelatinolytic activities in the acidic media resulted from the increase in the secretion of the enzyme proteins, but not from the activation of latent enzymes at low pH or from the inactivation of active enzymes at neutral pH.

![Fig. 1. Effect of D-glucose concentration on secretion of gelatinase from mouse B16-F10 melanoma cells. F10 cells were cultured in 10% FCS-supplemented DMEM/F-12 medium with 1.4 mg/ml (left) or 5.9 mg/ml (right) glucose for 3 days, and the gelatinase activities present in the respective conditioned media were analyzed by gelatin zymography as described under "Materials and Methods." Arrow, 105-kDa gelatinolytic activity.](image-url)
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**Fig. 2.** Effect of pH of culture medium on gelatinase secretion and growth of mouse B16-F10 melanoma cells. B16-F10 cells were grown to confluence in the standard serum-containing medium, and the medium was replaced with serum-free media at the indicated pH values. After incubation for 3 days, gelatinase activities in the resultant conditioned media and the numbers of cells in the cultures were analyzed. A, zymogram of gelatinolytic activities. **Arrow,** 103-kDa gelatinolytic activity. **B,** relative activity of 103-kDa gelatinase (A), number of viable cells (O), and protein concentration of conditioned media before concentration (C). The 103-kDa gelatinolytic activity was estimated by densitometric scanning at 530 nm of each zymogram shown in A and is expressed as relative activity (percent of maximum activity). The number of viable cells was counted with a hemocytometer by the trypan blue exclusion method. The number of cells at the time of the pH shift was 2 × 10^5 cells/dish. Other experimental conditions are described under "Materials and Methods."

The pH change in the culture medium during cultivation was examined by incubating serum-free culture media at various pH values with confluent B16-F10 cells for four different time periods. The pH values of the acidic media increased considerably during the 2-day incubation, e.g. from pH 4.7 to 5.8 (Fig. 3). This seemed to be the reason why the cells were viable in the culture medium at pH 4.7.

The growth and morphology of B16-F10 cells were examined by incubating nearly confluent cells in serum-free media at pH 7.3, 6.8, 5.9, and 4.7. At pH 7.3, the cells rapidly divided during the initial 24-h incubation, but the number of the cells markedly decreased after the initial 2 days because of cell death (Fig. 4A). In contrast, when the cells were incubated in the medium at pH 5.9, the number of the cells decreased to 40% of the initial cell number during the initial 24-h incubation, but gradually recovered to 114% after a further 2-day incubation. Lower but significant cell growth was also observed at an initial pH of 4.7. At pH 6.8, the cells reached the highest cell density after 3 days of incubation and maintained the level for at least 3 days.

The morphology of B16-F10 cells at four different pH values is shown in Fig. 4B. At pH 6.8, the confluent cells piled up on the dish, exhibiting compact and spindle-shaped morphology. When the pH was lowered to 5.9 or 4.7, they became much longer and larger in cell shape, with decreased cell density. The morphological differences between the neutral and acidic media were essentially reproduced in sparse cultures (data not shown).

It is well known that the extracellular matrix-degrading activities of metalloproteinases are regulated by two kinds of metalloproteinase inhibitors, TIMP and TIMP-2 (21, 22). We also analyzed the secretion of these inhibitors from B16-F10 cells...
cells cultured at pH 7.3 and 5.9 using the reverse zymography reported by Apodaca et al. (25). The analysis showed that this cell line secreted only TIMP-2 and that there was no significant difference in its amount in the neutral and acidic culture media (data not shown).

Characterization of 103-kDa Gelatinase Secreted from F10 Cells—To characterize the 103-kDa gelatinase secreted by B16-F10 cells, this enzyme was partially purified from 3-day serum-free conditioned medium, with an initial pH of 5.9. When the conditioned medium was applied to a gelatinagarose column, the gelatinase was adsorbed onto the column and eluted with 7.5% dimethyl sulfoxide, as reported for the human 92-kDa gelatinase (3).

Effects of various proteinase inhibitors on the activity of the partially purified 103-kDa gelatinase were examined by the zymography assay. 1,10-Phenanthroline (1 mM) and di-thiothreitol (1 mM) perfectly inhibited the gelatinase activity in the reaction mixture containing 10 mM CaCl2, whereas diisopropyl fluorophosphate, p-chloromercuribenzoic acid, and pepstatin showed no effect (data not shown). This indicated that the purified enzyme was a metalloproteinase.

It is well known that the matrix metalloproteinases are secreted in proenzyme forms, which are inactive in free solutions but capable of degrading gelatin in the zymography assay. The latent proenzymes can be converted to mature active enzymes with lower molecular weights when treated with organomercurials, proteinases, or some denaturing reagents (24). When the partially purified 103-kDa gelatinase was treated with 1 mM p-aminophenylmercuriacetic acid at 37°C for 1 h and then analyzed by gelatin zymography, the molecular weight of the gelatinase shifted from 103,000 to 90,000 (data not shown). The purified gelatinase could degrade 3H-labeled type IV collagen in free solution in the presence of the organomercurial, but hardly in its absence (data not shown). These results indicated that the M, 103,000 form was a proenzyme (zymogen) and that the M, 90,000 form, which was also observed in the acidic conditioned medium of B16-F10 cells (see Fig. 2A), was its active form.

The above-mentioned properties of the 103-kDa gelatinase suggested that it was most likely the same enzyme as the human 92-kDa gelatinase/type IV collagenase, which has been purified and characterized from SV40-transformed human lung fibroblasts and other sources (3, 4). The difference in their molecular weights is probably due to the difference in species because NIH-3T3 and some other mouse cell lines secreted the 103-kDa gelatinase instead of the 92-kDa gelatinase, the latter of which was found in the conditioned media of human and rat cell lines (see Fig. 9).

The optimum pH for the gelatinolytic activity of the 103-kDa enzyme was determined by the zymography assay (Fig. 5). It exerted the maximum activity in a pH range of 7.0–8.2, but it still maintained ~60% of the maximum activity even at pH 6.0, the optimum pH for enzyme secretion.

Mechanism of Gelatinase Induction by Acidic Culture—The two kinds of gelatinases with M, values of 92,000 and 72,000 are known to be localized on the plasma membrane in some human tumor cell lines (25). At least two mechanisms can be considered for the acid induction of the 103-kDa gelatinase in F10 cells: the de novo synthesis and the simple release of intracellular or plasma membrane-bound enzyme. To test these possibilities, the following experiments were carried out. The time course of gelatinase accumulation in the conditioned medium was examined after replacement with the culture medium at pH 5.9 (Fig. 6A). The enzyme activity became detectable 24 h after the pH shift and increased gradually during further incubation, indicating that its release or secretion from the cells was a relatively slow process. When the acidic medium was again replaced with the neutral medium, the secretion of the 103-kDa gelatinase was gradually diminished and became undetectable after 3 days (Fig. 6B). This indicated that the acid-induced secretion of the gelatinase was reversible.

To analyze cell-associated gelatinases, F10 cells cultured at pH 7.3 and 5.9 were harvested with a cell scraper, dissolved in SDS, and subjected to the zymography. The analysis showed a high molecular weight gelatinolytic band (M, >200,000), but hardly showed the 103-kDa gelatinase (data not shown). There was no significant difference in these cellular gelatinolytic activities in the neutral and acidic cul-

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Fig. 5. Effect of pH on gelatinolytic activity of acid-induced 103-kDa enzyme. The relative activity of the partially purified 103-kDa gelatinase was determined by gelatin zymography analysis with the following reaction mixture at the indicated pH values: 50 mM 2.2'-dimethylglutaric acid, 50 mM Tris, and 50 mM 2-amino-2-methyl-1,3-propanediol containing 10 mM CaCl2 and 0.02% NaN3. Relative gelatinolytic activity was determined by densitometric scanning at 530 nm of each zymogram. Other experimental conditions are described under "Materials and Methods."

Fig. 6. Time course of gelatinase secretion after pH shift. A, F10 cells were grown to near confluence in serum-containing medium at pH 7.3, and then the medium was replaced with serum-free medium at pH 5.9. The conditioned medium was collected after the indicated periods of incubation, and the gelatinolytic activities of the conditioned media were analyzed by gelatin zymography. B, F10 cells were incubated in serum-free medium at pH 5.9 for 3 days. Gelatinolytic activity of this conditioned medium is shown in lane 1 (left). The medium was then replaced with serum-free medium at pH 7.3 (day 0) and thereafter was changed every 3rd day (lane 2 (center), days 0–3 at pH 7.3; lane 3 (right), days 3–6 at pH 7.3).
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In this study, the three tumor lines and the parent B16 melanoma line were compared for the secretion of gelatinases into serum-free culture media at three different initial pH values, 7.3, 6.8, and 5.9 (Fig. 8). Like F10, BL6 and F1 did not secrete gelatinolytic activity at pH 7.3, whereas at pH 5.9, they secreted high levels of the 103-kDa gelatinase. In contrast, the parent B16 line in the medium at pH 7.3 secreted a high level of a 64-kDa gelatinolytic activity but not 103-kDa activity. When the same cell line was incubated at pH 5.9, the secretion of the 64-kDa gelatinase slightly decreased, whereas the 103-kDa gelatinase was only slightly secreted. At pH 5.9, the relative activity of the 103-kDa gelatinase was in the order of 100 (BL6), 78 (F10), 59 (F1), and 9 (the parent B16 line) as estimated by densitometric scanning of the zymogram at 530 nm. The order of the acid inducibility of the 103-kDa gelatinase in the four cell lines correlated well with the order of their metastatic potentials.

The effect of culture pH on the secretion of gelatinases was also tested with 11 human tumor cell lines. The HT1080 fibrosarcoma cell line secreted a higher level of a 90-kDa gelatinolytic activity (probably the 92-kDa gelatinase) at pH 6.8 than at pH 7.3, although gelatinase secretion was reduced at pH 5.9 (Fig. 9). In HT1080 cells, the secretion of gelatinolytic bands with a Mr, values of 64,000 and 57,000 (probably the proenzyme and mature form of the 72-kDa gelatinase, respectively) and an unidentified gelatinolytic band with a Mr of >200,000 was also markedly induced at pH 6.8. The lung adenocarcinoma cell line A549 showed a slight induction of the 90-kDa gelatinolytic activity in the medium at pH 6.8. No induction was observed in the other cell lines (A-431, EJ-1, HLE, HSC-4, NY, T24, T98G, YST-2, and YST-3) (data not shown).

Effect of Medium pH on Tumor Cell Invasion through Type IV Collagen Gel—It has been reported that B16-F10 cells are capable of invading the reconstituted basement membrane matrigel in Boyden chambers (19). We tested the effect of the acidic culture medium on the in vitro invasive capacity of the cell line using type IV collagen-coated porous filters. The cells were previously incubated in serum-free medium at pH 7.3 or 6.1 for 1 day, harvested, and suspended in the respective conditioned media. When the cell suspensions were applied to the type IV collagen-coated filters in Boyden chambers and incubated, the cells migrated through the filters toward the lower chambers containing NIH-3T3 conditioned medium as a chemoattractant. Cell migration was significantly faster at pH 6.1 than at pH 7.3 (Fig. 10). This result indicated that the
that acidic culture media hardly affected the secretion of the tinase is activated by acid treatment. It seems possible that the low pH in tumor tissues also favors the autolytic activation of time, the migrated cells on the lower surface of the filters were own conditioned media, and placed onto type coated filters.

of secreted latent gelatinases. In addition, it should be noted a marked pH decrease in the culture medium, stimulating the that in normal tissues. The elevated sugar uptake and subse-
quent glycolysis with the insufficient supply of oxygen in human cells, whereas it was hardly observed with the parent B16 cell line, which has a very low metastatic potential. Although the parent cell line secreted a high level of the 64-kDa gelatinase, its secretion was suppressed by the pH shift of the culture medium. Two human tumor cell lines (HT1080 and A549) also secreted higher levels of the 92-kDa gelatinase at pH 6.8 than at pH 7.5. Thus, the acid induction of the 103- or 92-kDa gelatinase is not specific to B16 melanoma cells. Furthermore, this study demonstrated that the growth rate of the B16 melanoma cells was better at pH 6.8 than at pH 7.3 and that they could grow even below pH 6.0. This implies that some types of cells are unexpectedly resistant to or even stimulated for cell growth by acidic environment.

It is well known that the pH in tumor tissues is lower than that in normal tissues. The elevated sugar uptake and subsequent glycolysis with the insufficient supply of oxygen in tumor cells, which convert glucose to lactic acid, are considered to be the main cause of the low pH in tumor tissues (26, 27). This study indicates that such an acidic environment may stimulate the secretion of the high molecular weight gelatinase/type IV collagenase from some kinds of tumor cells in vivo. Indeed, the culture of a highly metastatic clone of mouse B16 melanoma cells in a high glucose medium caused a marked pH decrease in the culture medium, stimulating the secretion of the 103-kDa gelatinase in a latent precursor form. Davis et al. (28) have reported that a latent 94-kDa progelatinase is activated by acid treatment. It seems possible that the low pH in tumor tissues also favors the autolytic activation of secreted latent gelatinases. In addition, it should be noted that acidic culture media hardly affected the secretion of the important metalloproteinase inhibitors TIMP and TIMP-2.

Recent reports have shown a close correlation between the secretion of the 92-kDa gelatinase and the metastatic poten-
tial of tumor cells, suggesting the involvement of its type IV collagen-degrading activity in tumor invasion and metastasis (13–15). We have recently found that the 92-kDa gelatinase has an ~25 times higher activity than the 72-kDa gelatinase and that the former enzyme is capable of degrading type I collagen as well as type IV collagen (29). Therefore, the secretion of the 92-kDa enzyme seems more influential in the proteolytic degradation of the extracellular matrix than that of the 72-kDa enzyme.

In this study, B16-F10 cells incubated in acidic medium exhibited a more active chemotactic migration through a type IV collagen-coated filter than those in neutral medium. This fact suggests a possibility that the acidic environment formed by tumor cells or by other mechanisms increases the invasive potential of tumor cells by stimulating the secretion of the 103- or 92-kDa gelatinase. The matrix metalloproteinases have been considered to be involved in various physiological and pathological conditions besides tumor invasion, such as embryonic development, leukocyte migration, arthritis, angiogenesis, and tissue repair. Under these conditions, microenvironmental pH may also affect gelatinase secretion from normal cells, although this possibility was not tested in this study. In addition, there is a possibility that the 103- or 92-kDa gelatinase may be a kind of stress protein that acts to protect cells from the pH shock.

It has been reported that growth factors such as epidermal growth factor, platelet-derived growth factor, interleukin-1, and tumor necrosis factor-α stimulate gene expression of several metalloproteinases (30). The 92-kDa gelatinase has been reported to be induced by epidermal growth factor, interleukin-1, and tumor necrosis factor-α (31, 32). Similar metalloproteinase induction is also seen with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (3, 30). A 12-O-tetradecanoylphorbol-13-acetate-responsive element sequence has been identified in the regulatory region of metalloproteinase genes (30, 33, 34). Therefore, the expression of these metalloproteinase genes is thought to be regulated by external signals mediated by the protein kinase C pathway. In addition to growth factors and the tumor promoter, laminin and a synthetic peptide of 19 amino acids from the laminin A chain have been reported to stimulate the secretion of type IV collagenase activity from B16-F10 cells and from two human tumor cell lines (25).

To our knowledge, induction of metalloproteinase production by acidic culture conditions has not been previously reported with any kinds of cells. Acid induction of 103-kDa gelatinase secretion in mouse B16 melanoma cell lines seemed to be due to its de novo synthesis rather than to the simple stimulation of its release from the cells because cycloheximide effectively inhibited gelatinase secretion. We also tested the effect of 12-O-tetradecanoylphorbol-13-acetate on gelatinase secretion from B16-F10 cells, but it showed no stimulatory effect (data not shown). Therefore, acid induction of gelatinase secretion does not seem to be mediated by the protein kinase C pathway. Further studies are required to understand the molecular mechanism of acid-induced gelatinase secretion.

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