Autocrine Factor Enhancing the Secretion of Mr 95,000 Gelatinase (Matrix Metalloproteinase 9) in Serum-free Medium Conditioned with Murine Metastatic Colon Carcinoma Cells

Sumiko Hyuga, Yohko Nishikawa, Keita Sakata, Hidekazu Tanaka, Sadako Yamagata, Kenji Sugita, Shinsuke Saga, Mutsushi Matsuyama, and Satoru Shimizu

Pathophysiology Unit, Aichi Cancer Center Research Institute, 1–1 Kanokoden, Chikusa-ku, Nagoya 464 [S. H., Y. N., S. Y., S. S.]; Second Department of Pathology, Nagoya University School of Medicine, Showa-ku, Nagoya 466 [K. S., S. S., M. M.]; and Shionogi Research Laboratory, Shionogi and Company, Ltd., Segisus, Fukushima-ku, Osaka 533 (H. T., K. S., M. MI; and Shionogi Research Laboratory, Shionogi and Company, Ltd., Segisus, Fukushima-ku, Osaka 533 (H. T., K. S., M. MI; and Shionogi Research Laboratory, Shionogi and Company, Ltd. , Segisus, Fukushima-ku, Osaka 533)

ABSTRACT

We previously reported that murine tumor cells with a high spontaneous metastatic potential to the lung secrete higher amounts of Mr 95,000 gelatinase (matrix metalloproteinase 9, MMP9) than do poorly metastatic cells. The present study, conducted to clarify the mechanisms underlying the increase in MMP9, revealed an autocrine factor that enhances the secretion of Mr 95,000 gelatinase (MMP9). The secretion of MMP9 by highly metastatic colon carcinoma LuM1 cells, detected by zymography, was augmented 10-fold when cultured in medium supplemented with serum-free medium conditioned with LuM1 cells. Because the secretion of Mr 60,000 gelatinase (MMP2), as well as total protein, by the same cells was not affected under these conditions, the augmentation appears specific for MMP9. The steady-state level of MMP9 mRNA was elevated in LuM1 cells cultured in the presence of the supernatant. The amount of the factor in the culture medium increased with time in culture, indicating that it was produced by the LuM1 cells. It was found to be heat stable but sensitive to trypsin digestion. Conditioned medium from poorly metastatic NM11 cells did not stimulate the secretion of gelatinases by NM11 cells, suggesting that autocrine stimulation of MMP9 secretion is a characteristic of metastatic cells. This factor could account for the augmented secretion of MMP9 by murine tumor cells with spontaneous metastatic potential to the lung.

INTRODUCTION

The process of tumor metastasis consists of many complex steps, each involving specific interaction of tumor cells with host cells or the extracellular matrix. Migration of tumor cells across the basement membrane is considered to be essential for the tumor cells to achieve successful metastasis (1). Thus, the secretion of enzymes that can degrade basement membrane components would clearly favor metastasis. The basement membrane extracellular matrix separating epithelium, endothelium, and epithelium from underlying stroma is predominantly made up of type IV collagen, laminin, and heparan sulfate proteoglycans. These components of basement membranes can all be degraded by MMPs, a family of proteinases acting specifically on extracellular matrix proteins and therefore playing important roles in normal and pathological processes involving degradation of basement membranes. They hydrolyze type IV collagen, laminin, heparan sulfate proteoglycans, and gelatinases (MMP2 and MMP9) on type IV collagen (3).

can; and gelatinases (MMP2 and MMP9) on type IV collagen (3). Increased expression of stromelysins, matrixins, or gelatinases has been reported in tumor cells with an invasive or metastatic phenotype (4–12). The observed association between secretion of MMPs and invasive or metastatic properties of tumor cells implies that their expression could be crucial for progression of a tumor to malignancy. Elucidation of the mechanisms of how the secretion of these MMPs is regulated is therefore of prime importance to understanding of malignant progression.

We previously reported that cultured murine tumor cells secrete Mr 95,000 and 60,000 gelatinases/type IV collagenases (MMP2 and MMP9, respectively) and that those with high spontaneous metastatic potential to the lung produce remarkably higher amounts of MMP9 than their counterparts with poorly metastatic potential (6). The present study was undertaken to elucidate mechanisms underlying the augmented secretion of MMP9 by murine metastatic colon carcinoma colon 26 cells. As a result, an autocrine factor enhancing secretion of MMP9 was detected in medium conditioned with metastatic cells.

MATERIALS AND METHODS

Cells. Highly and poorly metastatic LuM1 and NM11, respectively, cells were isolated by in vivo selection from murine colon carcinoma colon 26 cells according to the method described by Tsuruo et al. (13). Briefly, cultured colon 26 colon cells (10⁶ cells) were implanted intradermally into the abdominal skin of a 5-week-old BALB/c female mouse. After 4 weeks, the lung of the mouse bearing colon 26 tumors was dissected out and transplanted to another mouse. This was then repeated, and cloned metastatic cells in culture (LuM1) were obtained from metastatic lung nodules after 7 cycles of lung transplantation. Poorly metastatic cells (NM11) were cloned from cultured cells obtained from the primary tumor. Average numbers of metastatic nodules in the lungs of mice 5 weeks after intradermal implantation of 5 × 10⁵ LuM1 or NM11 cells to 6-week-old BALB/c female mice were 98 and 4, respectively. Cell lines were maintained in RPMI 1640 medium with 10% FCS.

Detection and Quantitation of Gelatinase. Zymography with gelatin as a substrate was used to detect gelatinases in serum-free culture medium (6). Quantitation was performed by densitometric tracing of zymograms using a Shimadzu CS-930 densitometer. Gelatin activity, detected as a band of reduced Coomassie blue staining, showed a peak in the negative direction from background staining, and the peak area of the detected band was proportional to the amount of gelatinase. Calculated values calibrated with the numbers of cells in culture were used for relative amounts of gelatinase. In all experiments, two independent culture supernatants were obtained and used for measurement of gelatinase activity. All data are means ± range of points and represent the results of at least three independent experiments. For immunochemical detection of Mr 95,000 gelatinase in the culture, 100 µl of supernatant were obtained and used for measurement of gelatinase activity. All data are means ± range of points and represent the results of at least three independent experiments. For immunochemical detection of Mr 95,000 gelatinase in the culture, 100 µl of supernatant were obtained and used for measurement of gelatinase activity. All data are means ± range of points and represent the results of at least three independent experiments.
remove cell debris. To eliminate gelatinases, the centrifuged supernatant (1.5 liters) was subjected to column chromatography using gelatin-cellulose (bed volume of 90 ml; Seikagaku Kogyo, Tokyo), and the fraction passing through the column was obtained. This procedure was repeated three times to remove any trace amounts of remaining gelatinase, and the remaining passing-through fraction was concentrated to 50 ml. The concentrated fraction (fraction G), after testing for the absence of gelatinase activity, was used for detection of factor(s) enhancing gelatinase secretion. Amounts of protein were determined with a protein assay reagent from Bio-Rad Laboratories using BSA as a standard.

Detection of a Factor Enhancing the Secretion of $M_{95,000}$ Gelatinase (MMP9). Trypsinized cells were washed once with 10% FCS-RPMI 1640 medium and twice with serum-free RPMI 1640 medium. They were then suspended in serum-free RPMI 1640 medium at the concentration of $10^6$ cells/ml, and 1 ml of cell suspension was seeded in a culture dish of 30 mm diameter. After culture overnight, medium was changed to RPMI 1640 medium containing 100 μg/ml of BSA with or without fraction G, and culture supernatant was collected after 24 h. The gelatinase activities in the supernatant with fraction G were measured and compared with those in the culture supernatant without fraction G. To examine the effect of fraction G on the secretion of total protein, $10^6$ cells/ml, and 1 ml of cell suspension was seeded in a culture dish of 30 mm diameter. After culture overnight, medium was changed to RPMI 1640 medium containing 100 μg/ml of BSA with or without fraction G, and culture supernatant was collected after 24 h. The amount of secreted protein was expressed as total radioactivity in the precipitate, and the steady-state level of MMP2 mRNA was not affected by the presence of fraction G.

RESULTS

Time courses of secretion of $M_{60,000}$, 60,000 and 95,000 gelatinases (MMP2 and MMP9, respectively) by LuM1 and NM11 cells into serum-free RPMI 1640 medium are shown in Fig. 1. The amount of $M_{95,000}$ gelatinase in the serum-free culture medium of LuM1 cells increased exponentially, not linearly, with time in culture, contrasting with that of $M_{60,000}$ gelatinase, which increased linearly with time. Secretion of both $M_{60,000}$ and 95,000 gelatinases by NM11 cells also occurred linearly. These results suggested the presence of some factor(s) that enhanced the secretion of $M_{95,000}$ gelatinase in the culture medium of LuM1 cells.

In order to detect the factor enhancing $M_{95,000}$ gelatinase secretion, the effect of serum-free culture medium conditioned with LuM1 cells, after included gelatinases were eliminated (fraction G), was examined on gelatinase secretion by LuM1 cells. When cultured in medium supplemented with fraction G, LuM1 cells showed enhanced secretion of $M_{95,000}$ gelatinase (MMP9) as shown in Fig. 2, A and B, the amount being about 10-fold higher than in control culture without fraction G. Enhancement of gelatinase secretion by fraction G was specific for $M_{95,000}$ gelatinase, and the secretion of $M_{60,000}$ gelatinase was not affected by its addition to the culture medium. Immunochemical detection of $M_{95,000}$ gelatinase using a monoclonal antibody raised against mouse $M_{95,000}$ gelatinase also showed a remarkable increase of the amount of secreted $M_{95,000}$ gelatinase in the culture supernatant with fraction G (Fig. 2C); total protein secreted by LuM1 cells, however, was not seriously affected by the addition of fraction G (Table 1). The effect of fraction G on the secretion of $M_{95,000}$ gelatinase was dose dependent up to a maximum of 20–50 μg protein/ml (Fig. 3). The amount of the enhancing activity of $M_{95,000}$ gelatinase secretion in the culture supernatant increased with time in culture (Fig. 4), specific activity (enhancing activity/mg protein) being almost the same during the same time, suggesting that LuM1 cells continuously secrete the autocrine factor involved. Results of Northern analysis are illustrated in Fig. 5. LuM1 cells cultured in serum-free medium expressed mRNAs of both MMP9 and MMP2. Elevated steady-state levels of both 3.5- and 2.7-kilobase pair mRNAs of MMP9 were observed in LuM1 cells cultured in the presence of fraction G, which is consistent with the enhanced secretion of $M_{95,000}$ gelatinase (MMP9). In contrast, the steady-state level of MMP2 mRNA was not effected by the presence of fraction G in the culture medium.

Effects of heating or trypsin digestion of fraction G on the enhancement of the gelatinase secretion are shown in Fig. 6. The activity in fraction G was relatively heat stable, 80% being retained after heating in boiling water for 10 min. After digestion with trypsin, however, fraction G lost a considerable proportion of its enhancing activity on gelatinase secretion. In the process of preparing fraction G, serum-free culture supernatant was concentrated using a membrane filter with a molecular sieve size of 30,000. These results suggest that the factor having an enhancing activity on gelatinase secretion is a heat-stable protein with a molecular weight of >30,000.

To compare autocrine enhancement of $M_{95,000}$ gelatinase secretion between LuM1 and NM11 cells, G fractions were prepared from both, and enhancing activity was assessed in the two cell types (Fig. 7). The $M_{95,000}$ gelatinase secretion of LuM1 cells was enhanced by fraction G from either LuM1 or NM11 cells, the extent being lower in the latter case. With NM11 cells, only the LuM1 cell fraction G enhanced gelatinase secretion, and no stimulation by fraction G from NM11 cells was evident, indicating that the autocrine-enhancing mechanism of MMP9 secretion observed in LuM1 cells does not operate in the NM11 case.

DISCUSSION

In the present study, aimed at clarifying the mechanism underlying higher amounts of secretion of MMP9 ($M_{95,000}$ gelatinase) by metastatic LuM1 cells than poorly metastatic NM11 cells, both derived from murine colon carcinoma colon 26 cells (6), there was clear evidence that an autocrine factor might be responsible.
Matrix metalloproteinases are secreted aszymogens that have no activity without activation in vivo by proteolytic cleavage of the NH2-terminal peptide or in vitro by treatment with various reagents such as amino phenyl mercuric acetate and SDS. Activated metalloproteinases degrade the components of extracellular matrix according to their substrate specificities: interstitial collagens by interstitial collagenase (MMP1); type IV collagen by gelatinases (MMP2 and MMP9), stromelysins (MMP3 and MMP10), and matrilysin (MMP7); and proteoglycans by MMP3, MMP10, and MMP7 (2). However, the activity of these metalloproteinases is inhibited by inhibitors such as α2-macroglobulin in serum or TIMP in the body fluid (2). The TIMPs are secreted by the same cells that secrete the metalloproteinases. The activity of metalloproteinases is thus strictly controlled throughout the processes of secretion, activation, and inhibition. This means that there are various difficulties in estimating the activity of metalloproteinases in crude samples, because they must be activated at the same time as inhibitors are eliminated. Zymography overcomes these difficulties and, therefore, is considered to be the best method for detection of gelatinases, with proteins in the samples being first separated by SDS-PAGE so that the TIMPs and the molecular species of gelatinases are located according to their molecular size. The inactive form of gelatinases,zymogens, are activated by SDS in the process. After samples are renatured with Triton X-100, all of the molecular species of gelatinases can be easily detected without any special treatment to activate gelatinases or to eliminate the inhibitors in the crude samples.

Quantitative measurements reflecting secretion of Mr 60,000 and 95,000 gelatinases (MMP2 and MMP9, respectively) by metastatic LuM1 cells showed that the accumulation of the latter in serum-free medium was not linearly proportional to time in culture but rather exponential, in contrast to the secretion of MMP2 by LuM1 cells and both MMP2 and MMP9 by poorly metastatic NM11 cells. Because the amount of MMP9 secreted in 24 h by LuM1 cells, tested after daily exchange of the culture medium, did not change for several days (data
AUTOCRINE FACTOR ENHANCEMENT OF GELATINASE SECRETION

Because it is well known that tumor cells can also, themselves, secrete growth factors or cytokines, the possibility of autocrine stimulation must be taken into account as demonstrated by the present study. Paracrine stimulation by factors derived from tumor cells has been reported for production of MMPs by fibroblasts. The paracrine factor, tumor cell-derived collagenase stimulatory factor, was originally found in conditioned medium of human tumor cells and, when purified from the cell surface, strongly stimulated the secretion of MMP1, MMP3, and MMP2 (40). While tumor cell-derived collagenase stimulatory factor does not act on the tumor cells themselves, autocrine factors stimulating MMP secretion have been reported. Rabbit synovial fibroblasts, when stimulated by 12-O-tetradecanoylphorbol-13-acetate or IL-1β, synthesize and secrete autocrine factors, which in turn induce secretion of MMP1. Purification of the factors revealed them to be serum amyloid A and β2-microglobulin (41, 42). In addition, autocrine intermediate stimulating factors have been reported in SPARC (osteonectin)-treated fibroblasts (43).

Microenvironmental conditions have been shown to regulate the expression of MMPs: cell-cell or cell-extracellular matrix interactions and transport of soluble factors such as growth factors and cytokines are involved. Signal transduction through the fibronectin receptor has been reported to induce collagenase and stromelysin expression (19, 20). Augmented secretion of type IV collagenase due to interaction of tumor cells with extracellular matrix proteins such as laminin, collagen, and Matrigel has been reported (21–23), and perturbation of cell-extracellular matrix contacts with antibodies to cellular receptors is known to influence the secretion of type IV collagenase (24, 25).

Soluble factors such as growth factors and cytokines have been shown to stimulate the secretion of matrix metalloproteinases: collagenase (MMP1) and stromelysin (MMP3) by IL-1β, tumor necrosis factor-α, EGF, basic fibroblasts growth factor, and platelet-derived growth factor (26–30), and Mr 92,000 gelatinase (MMP9) by EGF, IL-1β, and tumor necrosis factor-α (31–33). TGF-β suppresses the secretion of MMP1 and MMP3 (30, 34) but augments that of MMP2 and MMP9 (35–37). Thus, the expression of MMPs by tumor cells appears to depend on what kinds of receptors for growth factors or cytokines they possess or whether the soluble factors enhancing or inhibiting secretion of MMPs are supplied from outside. For example, compounds from fibroblasts in the stroma surrounding tumor tissues augment their secretion of MMP2 and MMP9, influencing the metastatic potential of tumor cells (38, 39).

Fig. 5. Northern analysis of mRNA levels of MMP9 and MMP2. Total cellular RNA was prepared by acid guanidine thiocyanate-phenol-chloroform extraction from LuM1 cells cultured in the presence (+) or absence (−) of fraction G. RNA (20 μg) was fractionated on a 1% agarose gel containing 2.2% formaldehyde, transferred to Hybond N (Amersham), and hybridized with DNA probes randomly labeled with [α-32P]dCTP. cDNAs used as probes were for murine MMP9 and human MMP2.

Fig. 6. Stability of enhancing activity of gelatinase secretion after heating or trypsin digestion. Fraction G (20 μg protein in 200 μl) was incubated with 1 μg trypsin for 24 h at 37°C and then tested for activity after 5 μg soybean trypsin inhibitor was added. For the heat stability test, fraction G was kept in boiling water for 10 min. Columns (bars), means (± ranges of points) from duplicate determinations.

Fig. 7. Comparison of autocrine enhancement of Mr 95,000 gelatinease secretion between LuM1 and NM11 cells. G fractions (Fr.G) were prepared from LuM1 and NM11 cells, and enhancing activity of Mr 95,000 gelatinease secretion of each was examined. Secretion of Mr 95,000 gelatinease of LuM1 cells was stimulated by fraction G preparations from both LuM1 and NM11 cells, but that of NM11 cells was enhanced only by the fraction G from LuM1 cells. Fraction G from NM11 cells did not stimulate Mr 95,000 gelatinease secretion by NM11 cells, in contrast to the clear stimulation in the LuM1 case. Points (bars), means (± ranges of points) from duplicate determinations.
The autocrine factor, with an $M_0$ of 70,000, released by tumor cells has been characterized (44). A unique autocrine factor with an $M_0$ of 70,000, releasing factor that stimulates secretion of MMP2 and invasion of tumor cells, has been purified from cell culture medium conditioned with metastatic human colon cancer cells (45). Invasion-stimulating factor did not directly stimulate an increase in mRNA levels of MMP2 but was suggested to regulate the secretion of proMMP2. The steady-state level of MMP9 mRNA was elevated, indicating that transcription or post-transcriptional processes controlling protease release (46). The description of an autocrine stimulation of MMP9 secretion by TGF-β released from fibrosarcoma cells transfected with a plasmid containing a porcine TGF-β1 promoter has been reported (47), and the present results further suggest that autocrine control of MMPs is a widespread phenomenon, occurring in a variety of cells.

The factor detected in the culture medium of metastatic murine colon carcinoma LuM1 cells in the present study was characterized for specificity for MMP9 with no stimulation of MMP2. The steady-state level of MMP9 mRNA was elevated, indicating that transcription or stabilization of mRNA is switched on in metastatic cancer cell case. The comparison of autocrine enhancement of MMP9 secretion between LuM1 and NM11 cells revealed a complex nature for the factor stimulation. The fact that the LuM9-secretion of LuM1 cells was stimulated by fraction G from both sources, but in the NM11 case, only that from LuM1 cells demonstrated activity, precludes a widespread phenomenon, occurring in a variety of cells.

We gratefully acknowledge the kind gift of human MMP2 cDNA from Dr. G. I. Goldberg, Washington University School of Medicine.

REFERENCES

5. Garbisa, S., Pozzatti, R., Muschel, R. J., Saffiotti, U., Ballin, M., Goldfarb, R. H., Khoury, O., and Uotta, L. A. Secretion of type IV collagenase/gelatinase and metalloendoproteinase inhibitor expression in human prostatic cancer cells (45). Invasion-stimulating factor did not directly stimulate an increase in mRNA levels of MMP2 but was suggested to regulate the secretion of proMMP2. The steady-state level of MMP9 mRNA was elevated, indicating that transcription or post-transcriptional processes controlling protease release (46). The description of an autocrine stimulation of MMP9 secretion by TGF-β released from fibrosarcoma cells transfected with a plasmid containing a porcine TGF-β1 promoter has been reported (47), and the present results further suggest that autocrine control of MMPs is a widespread phenomenon, occurring in a variety of cells.

The factor detected in the culture medium of metastatic murine colon carcinoma LuM1 cells in the present study was characterized for specificity for MMP9 with no stimulation of MMP2. The steady-state level of MMP9 mRNA was elevated, indicating that transcription or stabilization of mRNA is switched on in metastatic cancer cell case. The comparison of autocrine enhancement of MMP9 secretion between LuM1 and NM11 cells revealed a complex nature for the factor stimulation. The fact that the LuM9-secretion of LuM1 cells was stimulated by fraction G from both sources, but in the NM11 case, only that from LuM1 cells demonstrated activity, precludes a widespread phenomenon, occurring in a variety of cells.

We gratefully acknowledge the kind gift of human MMP2 cDNA from Dr. G. I. Goldberg, Washington University School of Medicine.

ACKNOWLEDGMENTS

We gratefully acknowledge the kind gift of human MMP2 cDNA from Dr. G. I. Goldberg, Washington University School of Medicine.


Autocrine Factor Enhancing the Secretion of $M_r$ 95,000 Gelatinase (Matrix Metalloproteinase 9) in Serum-free Medium Conditioned with Murine Metastatic Colon Carcinoma Cells

Sumiko Hyuga, Yohko Nishikawa, Keita Sakata, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/54/13/3611

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.