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Gelatinases of Metastatic Cell Lines of Murine Colonic Carcinoma as Detected by Substrate-Gel Electrophoresis

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SUMMARY: Gelatinases were detected in the conditioned medium of murine colonic carcinoma cells by SDS-polyacrylamide gel electrophoresis using gels copolymerized with gelatin. Several gelatinase activities differing in molecular weight were detected but the major activities migrated with molecular weights of 60,000 and 95,000. The enzymes did not hydrolyze bovine serum albumin or casein, and required calcium for activity. All of the gelatinase activities were inhibited by EDTA, 1,10-phenanthroline and dithiothreitol but not by N-ethylmaleimide and phenylmethylsulfonyl fluoride. The 95,000 dalton gelatinase was separated from the 60,000 dalton gelatinase by affinity chromatography on Ricinus communis agglutinin-agarose, and the former activity was markedly increased in highly metastatic cell lines as compared with its activity in poorly metastatic cell lines.

Destruction or penetration of the basement membrane is thought to be an essential step in successful metastasis by tumor cells and thus, the enzymes which degrade extracellular matrices are believed to play important roles in the process of tumor invasion and metastasis (1,2). Many proteases have been reported to be involved in the degradation of extracellular matrices including: interstitial collagenase, type IV collagenase, gelatinase, stromelysin and elastase (3). Of these, attention has been focused on the enzymes which can degrade type IV collagen, a major component of basement membrane. The presence and activity of type IV collagenase has been correlated with the metastatic potential of tumor cells in a variety of animal model systems (4-6).

Other proteases such as gelatinase (7), stromelysin (8) or elastase (9) can also degrade type IV collagen. Thus, it is important to determine if these enzymes are expressed in tumor cells and if so, how their activity correlates with the metastatic capability of the tumor cells. Recently, a metalloprotease capable of degrading gelatin, type I and type IV collagens was

Abbreviations: RCA, Ricinus communis agglutinin; SDS, sodium laurylsulfate; PAGE, polyacrylamide gel electrophoresis

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purified from mouse melanoma cells and the authors suggested that this enzyme may be important in the process of tumor invasion (10). We now report the detection and characterization of a secreted gelatinase isolated from conditioned culture medium of murine colonic carcinoma cells.

MATERIALS AND METHODS

Reagents: Fetal calf serum was obtained from Boehringer Mannheim Biochemicals (Indianapolis,IN). RPMI 1640 and HB101 medium were the products of Nissui Pharmaceutical Co., Ltd (Tokyo, Japan) and Hana Media, Inc. (Berkeley, CA), respectively. RCA-agarose was purchased from Seikagaku Kogyo (Tokyo, Japan). Other reagents were the purest grade available from commercial sources.

Cell lines: Colon 26 cells derived from a chemically induced undifferentiated colonic carcinoma in Balb/c mouse (11) were used in this study. Clonal cell lines were isolated using the procedures described by Tsuruo et al (12) and tested for their ability to metastasize to the lung after transplantation to the anterior wall of the abdomen of Balb/c mice (6-8 week old, female). LuM1 and LuM4 cell lines were highly metastatic in this assay system and NM11 and NM16 cell lines were poorly metastatic. Details of the isolation and characterization of the cell lines will be published elsewhere. Cells (2.5×10^5) were inoculated in Falcon culture dishes (6 cm) and cultured in 5 ml of RPMI 1640 medium with 10% fetal calf serum at 37°C under 5% CO_2 in air. After overnight culture, the cells were washed twice with 3 ml of serum-free medium (HB101 or RPMI 1640) and maintained in 3 ml of medium. At appropriate time intervals, the medium was harvested and tested for gelatinase activity.

Detection of gelatinase activity: Gelatinase activity was detected in SDS-PAGE (13) using 10% gel of 1 mm thickness to which 300 µg/ml of gelatin had been added and copolymerized (14). The serum-free, conditioned medium was mixed with SDS sample buffer and after electrophoresis, the gel was gently shaken in 2.5% Triton X-100 for 1 h at room temperature before being incubated in 0.05M Tris-HCl buffer, pH 7.4 containing 0.01M $CaCl_2$ and 0.02% NaN_3 for 20 h at 37°C (8). The gel was then stained with 0.2% Coomassie brilliant blue R in 50% methanol-10% acetic acid for 1 h, and washed in 20% methanol-10% acetic acid. The gelatinase activities were detected as unstained bands.

Fractionation of gelatinase: Gelatinases in the serum-free, conditioned medium were fractionated by column chromatography on RCA-agarose. The RCA-agarose column (10 x 40 mm) was equilibrated with 0.01M Tris-HCl, pH 7.4 buffer containing 0.05% Tween 20 and 0.9% NaCl. The serum-free, conditioned medium (0.1-1.0 ml) of LuM1 cells was added to the column and the column was washed first with 6 ml of the buffer and then with 6 ml of the same buffer containing 1M NaCl. Gelatinases bound to the column were eluted with a buffer containing 0.05% Tween 20, 1M NaCl and 0.3M galactose. Fractions (0.3 ml) were collected and proteins and gelatinase activities were monitored by SDS-PAGE. Fractions having gelatinase activities were pooled, concentrated and dialyzed against 0.05M Tris-HCl, pH 7.4 containing 0.9% NaCl.

RESULTS AND DISCUSSION

Clonal cell lines differing in their metastatic potential were derived from an undifferentiated mouse colonic carcinoma cell line (see materials and methods). One highly metastatic cell line, LuM1, secreted two gelatinase activities which were detected in the serum-free medium after 6 hours of culture. Using SDS-PAGE gels copolymerized with gelatin, these gelatinase activities were observed to have molecular weights of 60,000 and 95,000 (Figure 1A, lane 1). The gelatinase activities in the medium increased with time

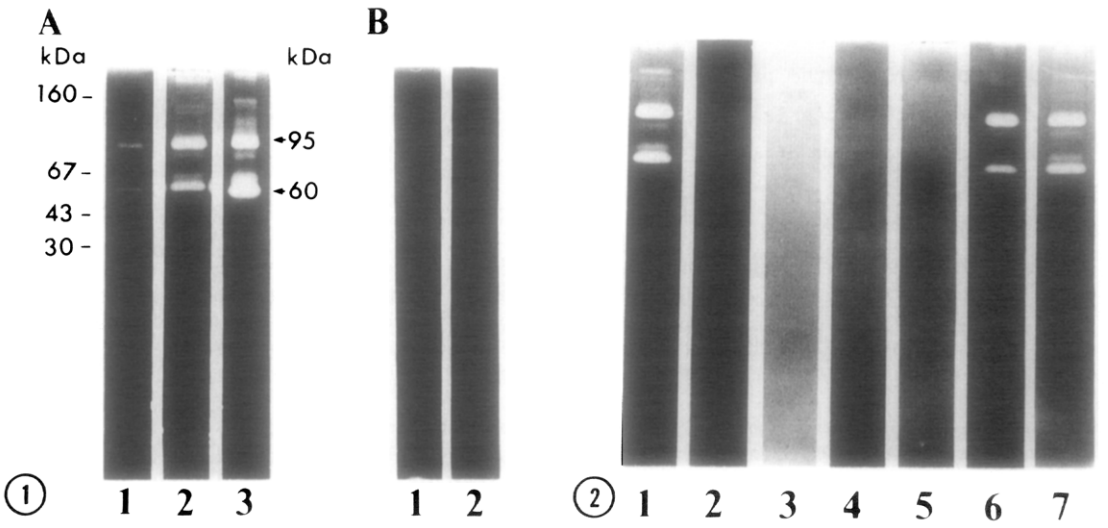


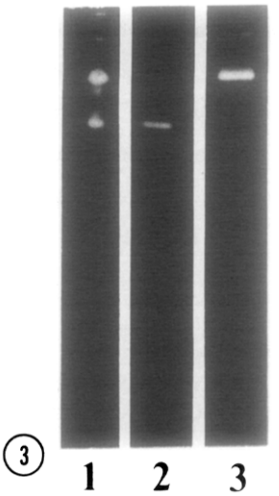
Figure 1. Gelatin degrading activities of the serum-free, conditioned medium (HB101) of LuM1 cells as detected by substrate-gel electrophoresis. A; 7.5 µl of the medium from 6 (lane 1), 24 (lane 2) and 48 (lane 3) h of culture was submitted to SDS-PAGE in gelatin-gel and gelatinase activity was tested. B; the medium from 24 h of culture was tested in a gel containing bovine serum albumin (lane 1) or casein (lane 2).

Figure 2. Characterization of gelatin degrading activities. The serum-free, conditioned medium (HB101) from 24 h culture of LuM1 cells was tested for gelatinase activities in the presence (lane 1) and absence (lane 2) of 0.01 M calcium chloride, and in the presence of 10 mM EDTA (lane 3), 1 mM 1,10-phenanthroline (lane 4), 1 mM dithiothreitol (lane 5), 10 mM N-ethylmaleimide (lane 6), and 1 mM phenylmethylsulfonyl fluoride (lane 7).

and in addition to the two major activities, at least 6 bands were detected in the medium by 48 hours (Figure 1A, lanes 2 and 3). The activity was specific for gelatin and hydrolysis of bovine serum albumin or casein was not observed by this procedure (Figure 1B). Characterization of these gelatinase activities suggested that they were metalloproteases; they required calcium ion for activity (Figure 2, lanes 1 and 2), and were inhibited by 10mM EDTA, 1mM 1,10-phenanthroline and 1mM dithiothreitol but not by 10mM N-ethylmaleimide or 1mM phenylmethylsulfonyl fluoride (Figure 2, lanes 3-7). Almost all of the gelatinase activity was in the supernatant fraction of centrifugation at 100,000 x g for 1 h, and the optimum pH for enzyme activity was at pH 7-8.

Presence of gelatinase and its synergism with collagenase in the degradation of collagens have been reported in normal and pathological tissues and cells (15-18). Recently, enzymes that degrade both gelatin and native collagens or other extracellular matrix proteins have been purified (7,8,10,19). Their molecular weights range from 59,000 to 150,000 (7,10,15,18,19) which suggests that there are a variety of gelatin degrading enzymes differing in substrate specificity and molecular size.

The present study demonstrates that LuM1 cells secreted gelatinases with molecular weights of 60,000 to over 200,000. Chan et al reported similar



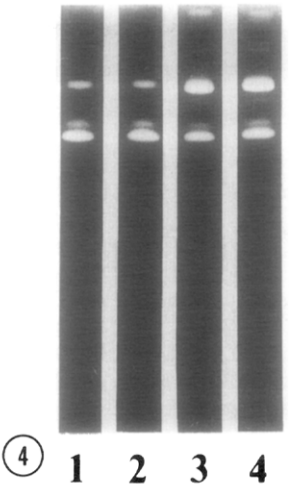


Figure 3. Fractionation of gelatin degrading activities on RCA-agarose. 100 µl of the serum-free, conditioned medium (RPMI 1640) from 24 h culture of LuM1 cells was fractionated as described in materials and methods. Lane 1, the conditioned medium; lane 2, the fraction not adsorbed to the column; lane 3, the fraction eluted from the column by 0.3 M galactose.

Figure 4. Comparison of gelatin degrading activities among the cell lines with different metastatic potential. 7.5 µl of the serum-free, conditioned medium (HB101) from 24 h culture of NM11 (lane 1), NM16 (lane 2), LuM1 (lane 3) and LuM4 (lane 4) was submitted to SDS-PAGE and gelatinase activity was tested.

patterns of multiple gelatinase activities in the normal and Raus sarcoma virus-transformed primary avian tendon cells (20). The multiple activities detected may have resulted from the substrate-gel procedure which has been reported to activate latent forms of collagenase without changing their molecular weight (21). Alternatively, differences in post-translational modifications (such as glycosylation) of the enzymes could account for the multiplicity of gelatinase forms. To examine this latter possibility, we attempted to fractionate the gelatinase bands by lectin-affinity chromatography. The 95,000 dalton, but not the 60,000 dalton, gelatinase bound to and was eluted with galactose from a RCA-agarose column (Figure 3). Thus, LuM1 cells must secrete at least two types of gelatinases which differ in their oligosaccharide side chains. Purification and characterization of the enzymes are under way to elucidate the structural and functional relationship of these two forms of gelatinase.

To determine if the presence of specific gelatinases correlated with metastatic potential, we compared the gelatinases secreted by cloned cell lines derived from the colon 26 carcinoma cells (Figure 4). It is apparent that the cell lines with low metastatic capability (NM11 and NM16) secreted fewer gelatinases (Figure 4, lanes 1 and 2), and that the amount of gelatinases of 95,000 molecular weight and greater, increased markedly in the highly metastatic cell lines (LuM1 and LuM4, Figure 4, lanes 3 and 4). Recent

studies have shown that metastatic cells contain metalloproteases which can degrade both gelatin and native collagen (22,23). We are not yet sure whether or not the gelatinases detected here can degrade native collagens. However, increased secretion of the 95,000 dalton gelatinase by metastatic colonic carcinoma cells could facilitate the penetration of the extracellular matrix by these cells and, thus, promote spontaneous metastasis.

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