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PRODUCTION OF MATRIX METALLOPROTEINASES AND A METALLOPROTEINASE INHIBITOR BY SWARM RAT CHONDROSARCOMA

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SUMMARY: Chondrosarcoma was found to produce a heat-labile collagenase and a heat-stable collagenase inhibitor. Unlike its cartilage counterpart, the inhibitory activity in chondrosarcoma could only be detected after heat-treatment. Western blot analysis of chondrosarcoma-derived inhibitor showed that this inhibitor cross-reacted with a polyclonal antibody raised against purified cartilage-derived collagenase inhibitor (I) at a M.W. of about 33 kDa. In addition to the collagenase activity, which appears to be matrix metalloproteinase I (MMP-1), chondrosarcoma extracts were shown to contain four active gelatinase species which migrate at a molecular weight consistent with that reported for MMP-2 (72 kDa gelatinase, Type IV gelatinase) (2) and three active enzyme species which migrate at a molecular weight consistent with that reported for MMP-9 (92 kDa gelatinase, Type IV gelatinase) (3,4). In contrast, normal cartilage contained only two active and one latent form of MMP-2 in significantly lower amounts than in chondrosarcoma. In the case of MMP-9, the same three species were present in normal cartilage and in chondrosarcoma, but in lower amounts in the normal tissue. These results suggest that chondrosarcoma might develop *in vivo* because the inherent proteolytic balance between the protease(s) and its endogenous inhibitor(s) is shifted in favor of the enzyme.

Both normal cartilage and chondrosarcoma, a malignant tumor of cartilage, contain angiogenic factors (5,6). However, normal cartilage is an avascular tissue (7-12). Previous reports demonstrated that cartilage contained an inhibitor of angiogenesis (7-10, 12) suggesting that, under normal physiological conditions, the inherent balance between angiogenesis stimulators and inhibitors favors the inhibitors. We have reported the purification and characterization of a cartilage-derived angiogenesis inhibitor (CDI) which is also a collagenase inhibitor (1). More recently, we have reported that chondrocytes established and maintained in serum free culture produce an angiogenesis inhibitor which also inhibits MMP-1 activity (13). This inhibitory protein(s) may play an important role in maintaining the unique avascular nature of cartilage. In

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the current report, we have studied the activities of matrix metalloproteinases and their endogenous inhibitor(s) in chondrosarcoma, the vascularized counterpart of cartilage, and in normal cartilage.

MATERIALS AND METHODS

Extraction of Chondrosarcoma. Chondrosarcoma was grown subcutaneously on both hips of Sprague-Dawley CD male rats (100-125 g). When the tumors became about 5 cm in diameter, the rats were anesthetized and the tumors were excised as previously described (14). Approximately 30 g of tumor were homogenized in 100 ml of 2 M NaCl, 10 mM Tris, pH 7 and extracted by stirring at 4°C for 2 hours. The crude extract was centrifuged at 15,000 X g for 1 hour and the supernate was collected.

Heparin-Sepharose Chromatography. The supernatant solution of chondrosarcoma extract was diluted 20 times with 10 mM Tris, pH 7 and loaded onto a heparin-Sepharose column (1.5 X 12 cm) pre-equilibrated with 50 mM NaCl, 10 mM Tris, pH 7. After a wash with the equilibration buffer, the column was eluted with 340 ml of a gradient of 0.05 to 2 M NaCl in 10 mM Tris, pH 7, at a flow rate of 20 ml/hr. Fractions (8 ml) were collected and monitored for collagenase, collagenase inhibitor and endothelial cell growth factor activities.

Growth Factor Assay. Capillary endothelial cells (EC) were prepared from bovine adrenal glands and grown on gelatin-coated dishes as described by Folkman et al (15). Inhibition of growth factor activity was measured by [^{3}H]thymidine incorporation into the capillary EC as described previously (16).

Radiometric Enzyme Assay for Collagenase and Collagenase Inhibitor. Enzyme and inhibitor activities were determined according to a modification (1) of the method previously described by Johnson-Wint (17). Briefly, samples were dialyzed against collagenase assay buffer (50 mM Tris-HCl, pH 7.6, 0.2 M NaCl and 1 mM $CaCl_2$). To test for the presence of active collagenase, aliquots of each fraction were added to wells containing ^{14}C-radiolabelled Type I collagen. To test for the presence of latent collagenase activity, samples were treated with trypsin (17) to activate the proenzyme before addition to the radiolabelled collagen. Aliquots of each fraction were incubated for 7 min at 37°C with one-tenth volume of trypsin (1 mg/ml). A one-tenth volume of soybean trypsin inhibitor (5 mg/ml) was added to quench the trypsin. After incubation with the radiolabelled collagen at 37°C for 2.5 hours, the supernates, which contained soluble radiolabelled collagen, were transferred to scintillation vials and counted in a Beckman model LS 3801 scintillation counter. To test for the presence of collagenase inhibitor(s), appropriately diluted samples (100 µl) were combined with 100 µl of bovine corneal collagenase, added to wells containing radiolabelled collagen and assayed as described above.

Substrate Gel Electrophoresis. Matrix metalloproteinase activity was visualized using SDS-polyacrylamide gels copolymerized with gelatin. As described by Herron and coworkers (2), Type I gelatin was added to the standard Laemmli acrylamide polymerization mixture at a final concentration of 1 mg/ml. Samples of normal cartilage and chondrosarcoma extracts were prepared as above, mixed with substrate gel buffer (10% SDS, 4% sucrose, 0.25 M Tris-HCl, pH 6.8 and 0.1% bromophenol blue) and loaded into wells of a 4% acrylamide Laemmli stacking gel on a Mini-Protean II apparatus and run as previously described (18). Following electrophoresis, gels were incubated in 2.5% Triton X-100 with gentle shaking for 30 min at room temperature with one change of detergent solution. Gels were rinsed and incubated overnight at 37° C in substrate

buffer (50 mM Tris-HCl buffer, pH 8, 5 mM $CaCl_2$ and 0.02% NaN_3). Following incubation, the gels were stained for 15-30 min with 0.5% Coomassie Blue R-250 in acetic acid:isopropyl alcohol:water (1:3:6), followed by destaining in water and photography. Proteolytic activity appears as clear zones (demonstrating lysis of the substrate in the gels) against a background of the dark-stained gel. For purposes of clarity, the photographs have been printed as negative images.

In order to verify that the gelatinase activities detected were, in fact, metalloproteinase activities, samples were electrophoresed as described above and the gels then incubated in substrate buffer in the presence or absence of 1, 10-phenanthroline (10 mM), an inhibitor of MMPs (2). To distinguish latent from active forms of MMPs, samples were exposed to 4-aminophenylmercuric acetate (APMA) (1mM) as previously described (2,19,20).

Western Blot Analysis. Samples (50 μg) enriched in collagenase inhibitor activity obtained from the heparin-Sepharose chromatography step were electrophoresed on SDS-15% polyacrylamide gels and transferred electrophoretically to a nitrocellulose sheet. The nitrocellulose sheet was incubated with CDI-specific IgG (21) and visualized by successive incubations with a conjugate of goat anti-rabbit IgG and alkaline phosphatase and enzyme substrate.

RESULTS

Chondrosarcoma Contains a Heat-labile Collagenase and a Heat-stable Collagenase Inhibitor. Normal avascular cartilage has been reported to contain collagenase inhibitor(s) (22-25). It has also been shown that chondrosarcoma chondrocytes produce an inhibitor of collagenase similar to that found in cartilage (26). However, we were unable to detect significant collagenase inhibitory activity in a vascularized chondrosarcoma. This suggested to us that chondrosarcoma in fact, may contain collagenase activity in excess of its *endogenous* inhibitor(s). Indeed, when fractionated chondrosarcoma samples from the heparin-Sepharose chromatography step were assayed for the presence of either active or latent MMP-1 activities, significant collagenase activity was observed localized in the fractions spanning 9 to 17 (Fig. 1). No significant inhibitory activity was detected. However, when the column fractions were heated at 60°C for 10 minutes to denature the enzyme activity and to dissociate bound inhibitor (23) and then assayed for the presence of collagenase inhibitor, a peak of collagenase inhibitory activity was found to comigrate with the enzyme (Fig. 1).

Chondrosarcoma-derived Collagenase Inhibitor Cross-reacts With CDI-specific IgG. Fractions enriched in collagenase inhibitory activity were analyzed by Western blot using CDI-specific IgG. A major band corresponding to an approximate m.w. of 33,000 (Fig. 2) was observed. This is consistent with the molecular weight reported for the anti-angiogenic collagenase inhibitor isolated from the conditioned medium of normal chondrocytes (13). A minor band at approximately 19,000 was also observed. This band might correspond to another collagenase inhibitor found in normal cartilage extracts (24).

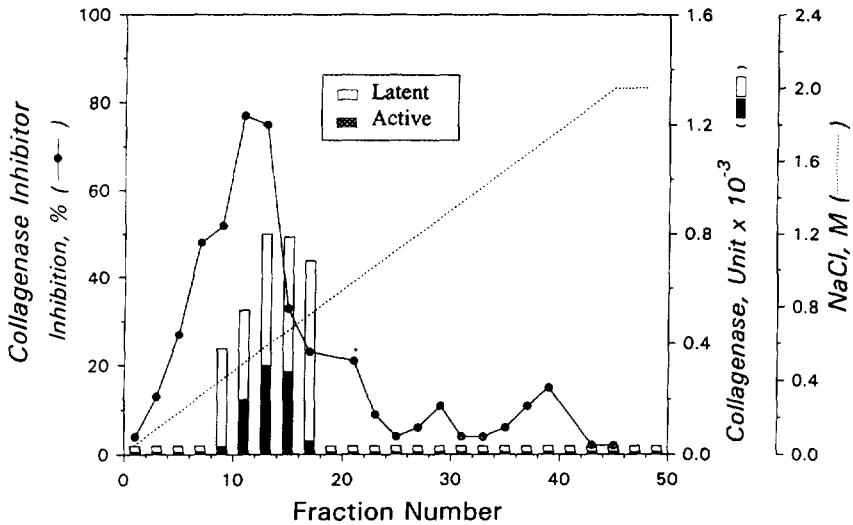

Figure 1. Collagenase and collagenase inhibitory activities in chondrosarcoma. Fractions from the heparin-Sepharose column were tested for their ability to inhibit collagenase. No inhibition was detected. However, when aliquots of these fractions were heat treated at 80°C for 10 min, a potent collagenase inhibitory activity was found (•—•). Fractions were also tested for the presence of both active (open bar) and latent (solid bar) collagenase activities before and after heat treatment. Before heat treatment, a peak of collagenase activity was found to coincide with the peak of collagenase inhibitory activity that was detected only after heat treatment. Collagenase activity was not observed after heat treatment. One unit of collagenase is defined as that amount of enzyme activity which produces 10% cleavage of total collagen in 2.5 hours at 37°C.

Chondrosarcoma Production of MMPs is Qualitatively and Quantitatively Different from That of Normal Cartilage. In light of the apparent difference in production of MMP-1 between chondrosarcoma and normal cartilage, we then compared the gelatinase activities in these two different tissues. In contrast to normal avascular cartilage which contains three forms of gelatinase activity which migrate at a molecular weight consistent with their being members of the MMP-2 family of proteolytic enzymes, chondrosarcoma produces an additional proteolytic activity (Fig. 3a). Importantly, APMA treatment of these enzymes revealed that one of the MMP-2 species in cartilage (~66 kDa) represented the latent form of the enzyme, whereas all of the species detected in chondrosarcoma extracts appeared to be active species (data not shown). Additionally, chondrosarcoma contained higher quantities of all of these enzyme species as well (Fig. 3a). With respect to the MMP activity migrating at the molecular weight reported for MMP-9, zymography revealed that although both chondrosarcoma and normal cartilage contained the same three species of active MMP-9, all three species were present in significantly greater amounts in chondrosarcoma (Fig. 3b). Treatment with 1, 10-phenanthroline demonstrated that all of the gelatinase activities detected by zymography, were in fact, metalloproteinase activities.

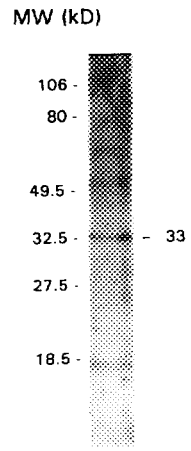

Figure 2. Western blot analysis of chondrosarcoma-derived collagenase inhibitor. Heparin-Sepharose column fractions which were enriched for collagenase inhibitory and capillary EC DNA synthesis inhibitory activities were analyzed by Western blot using CDI-specific IgG. The chondrosarcoma-derived inhibitor cross-reacted with anti-CDI antibody at a position of 33 kDa. Prestained SDS-PAGE standards (low molecular weight range) were used as molecular weight markers.

DISCUSSION

Proteolysis is a key component of many important biochemical and physiological events such as tissue remodelling, angiogenesis, tumor invasion, embryonic morphogenesis and trophoblast implantation, among others (27-31). One of the most important enzyme families involved in these processes is the matrix metalloproteinase family (32-35). Under normal conditions, proteolysis is strictly regulated by an inherent proteolytic balance between enzyme and endogenous inhibitor (35,36). Under pathological conditions, however, these regulatory controls fail. For example, deregulated metalloproteinase activity has been causally related to malignant conversion, the stage

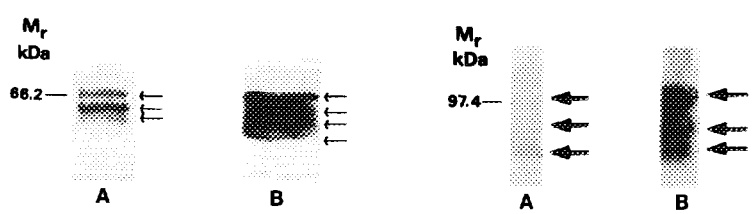

Figure 3. Metalloproteinase activities in cartilage and chondrosarcoma. Tissue extracts of cartilage and chondrosarcoma were subjected to substrate gel electrophoresis. MMP activities are presented as negative images of zones of clearance in a polyacrylamide gel impregnated with gelatin and stained with Coomassie R-250. The left lanes show the molecular mass of the protein standards. The arrows in the right lanes indicate the location of the proteolytic zones representing MMP-2 species (4a) and MMP-9 species (4b). Lanes (A) represent cartilage extracts and lanes (B) represent chondrosarcoma extracts.

in cancer progression when tumorigenic cells acquire the ability to invade and metastasize (35).

With respect to the matrix metalloproteinase family of enzymes, we have found that in Swarm rat chondrosarcoma, in contrast to normal cartilage, the inherent proteolytic balance appears to be shifted in favor of the enzyme activity as demonstrated both by radiometric enzyme assays and zymography. The MMP profile observed in chondrosarcoma versus normal cartilage is qualitatively and quantitatively different in that chondrosarcoma contains significantly greater amounts of MMP-1, MMP-2 and MMP-9, and unlike normal cartilage, these enzyme species appear to be present in their active state.

With respect to MMP inhibitors, we have found that although both normal cartilage and chondrosarcoma contain a collagenase inhibitor (1,13,21,24-26), heat-treatment was not necessary for the inhibitor to be detected in normal cartilage (1,22-24). This study also demonstrates that, in addition to our previously reported chondrosarcoma-derived capillary endothelial cell growth factor (6), chondrosarcoma also contains a potent inhibitor of mammalian collagenase. Immunoblot analysis using CDI-specific IgG suggests that this inhibitor may be CDI, the cartilage-derived inhibitor.

In light of the importance of MMP activity in both angiogenesis and tumor growth and development, future studies will focus on the identification and characterization of the metalloproteinases and the inhibitor(s) involved in the development of, and the transition to, the angiogenic state during tumorigenesis.

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