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Matrix Metalloproteinases Participate in Osteosarcoma Invasion[1]

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Background. **Osteosarcoma (OS) is a highly malignant bone tumor and is the most frequent malignant bone tumor in children and adolescents. Metastases are the major cause of death, and patients with relapse have poor prognosis. Several solid tumors display enhanced expression of matrix metalloproteinases (MMPs), and recently MMP-inhibitors have entered clinical trials. A disturbance of the MMP system in favor of enhanced proteolytic activity may be suspected in OS because OS growth is accompanied by both enhanced local bone destruction and bone formation, two processes that are dependant on proteolytic enzymes. Thus, the aim of the present study was to evaluate the involvement of MMPs in a panel of human OS cell lines, xenografts and biopsies.**

Material and methods. **Expression of MMPs and their endogenous inhibitors were studied by zymography and Northern blot analyses. *In vitro* invasion of OS cell lines and effects of MMP-inhibitors (Marimastat and doxycycline) were assessed in the transwell chamber assay.**

Results. In vitro **invasiveness was compared with gelatinase activity, and the most invasive cell line secreted the highest amounts of MMP-2 and MMP-9. Two different MMP-inhibitors significantly reduced OS cell invasion. The majority of the OS xenografts expressed both the inactive and active form of MMP-2 and in some cases also MMP-9. The biopsies from primary and metastatic OS also expressed MMP-2 mRNA. However, MMP-9 levels were higher in the biopsies than in the xenografts.**

Conclusion. **The obtained results support the hypothesis that MMPs and their endogenous inhibitors participate in the invasive process of human OS.**

Key Words: **Marimastat; tissue inhibitor of metalloproteinases; TIMP-1; TIMP-2; gelatinase; MMP-2; MMP-9; p53; docycycline; osteosarcoma.**

INTRODUCTION

In normal bone, matrix is constantly degraded and replaced by new matrix, a process requiring a proper balance between bone degradation and synthesis [1]. Proteolytic enzymes like matrix metalloproteinases (MMP), plasminogen activators and cathepsins are required in this process [2–4]. Recent reports on MMP knockout mice and on human genetic disorders have drawn the attention to the importance of MMPs for normal skeletal development [5, 6]. The human MMP family consists of more than 20 proteinases and can degrade various components of the bone matrix, like collagen, proteoglycans, fibronectin, and laminin. Furthermore, MMPs can induce alterations in the extracellular environment and thereby affect cell activities, for instance by release of TGF-β from the bone matrix and degradation of IL-β and calcitonin. The control systems regulating the biological activity of these proteinases are complex and occur at three different levels; transcription, activation of the enzymes from their latent to the active form and inhibition by endogenous inhibitors, like tissue inhibitors of MMPs (TIMPs) and plasminogen activator inhibitors.

Tumor growth, invasion, and metastasis are processes that include tumor cell proliferation, proteolytic digestion of the extracellular matrix (ECM), cell migration through basement membranes into the circulatory system, extravasation, and growth at the metastatic site [7]. MMPs are involved in the metastatic process as a consequence of their capacity to degrade basement

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membranes, thus facilitating invasion and metastasis. In addition, the MMPs can, by their proteolytic activity, promote tumor growth by increasing the bioavailability of growth factors residing in the ECM [8]. Furthermore, angiogenesis is a prerequisite for tumor growth, as all tumors larger than a few mm require their own blood supply, and tissue-remodeling enzymes are necessary for penetration of the newly formed blood vessels into the tumor [9]. Overexpression of MMPs in malignant tissue compared to corresponding normal tissue has been demonstrated in a large variety of malignant tumors like lung, colon, breast, prostate, and pancreatic carcinoma, and clinical evidence indicates that overproduction of MMPs confers a poor prognosis in several of these tumor types. In general, the gelatinases (MMP-2 and -9) are the two MMPs most consistently overexpressed in malignant tissues and, thus, associated with tumor aggressiveness, metastatic potential, and poor prognosis.

Osteosarcoma (OS) is a highly malignant bone tumor characterized by formation of neoplastic bone tissue and is the most frequent malignant bone tumor in children and adolescents [10, 11]. Radiological evidence of both bone destruction and bone formation is characteristic, the latter representing neoplastic bone. Patients with localized disease are reported to have an event-free 5-year survival between 44% and 78%. Metastases, most commonly to the lungs, are the major cause of death, and patients with relapse are reported to have 5-year-survival rates between 14% and 50%. Additionally, a disturbing number of long-term survivors of OS have severe late effects, including second cancers and anthracycline-induced cardiomyopathy. Therefore, novel treatment modalities to ensure better outcome for OS patients are warranted.

A disturbance of the MMP/TIMP balance in favor of enhanced proteolytic activity may be suspected in OS since OS growth is accompanied by both enhanced local bone destruction and bone formation, two processes that are dependant on proteolytic enzymes. Thus, the aim of the present study was to evaluate the importance of MMPs and TIMPs in a panel of human OS cell lines, xenografts and biopsies. Given enhanced MMP activity in OS, inhibition of MMPs could be a target for treatment in OS patients.

MATERIALS AND METHODS

In Vitro Cell Culture

The human OHS and KPDX cell lines were established at Department of Tumor Biology, The Norwegian Radium Hospital [12, 13], whereas the U2OS and SaOS cell lines were purchased from the American Type Culture Collection. The cells were cultivated in RPMI 1640 (Life technologies Inc., Middlesex, United Kingdom), supplemented with 10% heat-inactivated fetal calf serum (Life technologies Inc.) and L-glutamine (2.0 mM; Life technologies Inc.).

Animals and Establishment of Tumor Xenografts

Tumor tissue from altogether 21 patients treated for OS at the Norwegian Radium Hospital were collected. From these, 11 biopsies were implanted in nude mice for establishment of xenografts, while two of the same biopsies, together with ten others were snap frozen in liquid nitrogen and subsequently stored at −70°C for molecular analysis. For growing human tumors as xenografts, male and female BALB/c or NIH3 nu/nu mice, bred at the Norwegian Radium Hospital's nude rodent facility and kept under specific pathogen free conditions, were used. Food and water were supplied *ad libitum*. Housing and all procedures involving animals were performed according to protocols approved by The Animal Care and Use Committee at the Norwegian Radium Hospital in compliance with guidelines of the National Ethical Committee for Animal Experimentation on animal welfare. The animals were at age 4 to 8 weeks when fragments of tumor tissues from biopsies or surgically removed tumors were implanted s.c. into the flanks to establish the xenografts. Tumor tissue was harvested, snap frozen in liquid N_2 and kept at −70°C until being processed for further analysis.

Gelatin Zymography

For zymography analyses cells were cultivated in serum free media for 48 h. Conditioned media were harvested, centrifuged and stored at −70°C until being assayed. Protein lysates form xenografts were prepared in 50 mM Tris-HCl (pH 7.5), containing 150 mM NaCl and 0.1% NP-40 with 2 μg/ml pepstatin, aprotinin (Sigma Chemical Co., St. Louis, MO) and leupeptin (Roche Diagnostics, Mannheim, Germany). Protein quantification was done by Bradford analysis, and 5 μg of the protein lysate or 15 μl of the conditioned media sampled from 250,000 cells were assayed for gelatinase activity using 10% sodium dodecyl sulfate-polyacrylamide gels (PAGE) containing gelatin (0.1% Bloom 300; Sigma). The gelatin zymograms were calibrated with human gelatinase standard from capillary whole blood (Chemicon, Hampshire, United Kingdom) and activated with 0.2% Brie 35 (Sigma). Gels were stained with 0.2% Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Hercules, CA) and destained in a solution containing 30% methanol and 10% acetic acid. Gelatinase activity was visualized as cleared regions in the blue gels.

Northern Blot Analysis

Total cellular RNA from tumor tissue was prepared by the guanidinum-thiocyanate-caesium chloride method [14]. Subsequently 5 μg of total RNA was separated by an agarose gel containing 6.6% formaldehyde and transferred onto Hybond-N+ membranes (Amersham Pharmacia Biotec, Uppsala, Sweden). After baking and UV crosslinking, the membranes were hybridized with probes labeled with 32P according to the random primer technique [15]. The hybridizations were carried out in a buffer containing 0.5M disodium phosphate pH 7.2 and 1% SDS. For repeated hybridizations bound probes were stripped off by incubating the filters twice in 0.1% SDS, 0.1 × SSC for 5 min at 95°C. To correct for uneven amounts total RNA loaded in each lane, the filters were rehybridized with a kinase-labeled oligonucleotide probe specific for human 18S rRNA. The mRNA levels were measured using a Storm Phospho-Imager and quantified using the Image Quant software package.

The cDNA probes specific for MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 were kindly provided by Dr. Elizabeth Bona at British Biotech Inc., while the probe against MT1-MMP was a generous gift from Dr. H. Sato [16].

Evaluation of *in Vitro* Invasive Properties of OS Cells

The *in vitro* invasive properties of OS cells were evaluated in the transwell chamber invasion assay (Costar, Cambridge, MA). Each transwell filter (8 μm pore size) was coated with 50 μg/filter of the