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The Expression of Matrix Metalloproteinase-9 and Tumor Angiogenesis in Human Osteosarcoma

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Background : Matrix metalloproteinase-9 (MMP-9) is a matrix-degrading enzyme that's believed to play a crucial role not only for tumor invasion and metastasis, but also for a variety of stromal reactions, including neovascularization. The aim of this study was to investigate the expression of MMP-9 and to compare its expression with the angiogenesis activity in human osteosarcoma. **Methods :** Archival tumor tissue samples from 20 patients with osteosarcoma were analyzed by performing immunohistochemistry for the expression of MMP-9 and CD34. The vascularity was measured as the average microvascular density (MVD) of the CD34-positive vessels. The clinical information was obtained through searching the computerized retrospective database from the tumor registry. **Results :** MMP-9 was expressed in 90% (18/20) of the tumors we examined. The MVD ranged from 10.5 to 179.7 with a mean of 64.9. There was no significant correlation between the MMP-9 expression and the MVD (p=.613). The MMP-9 expression was not associated with any of the clinicopathologic variables, whereas the MVD showed an increasing tendency according to the metastasis status (p=.073). **Conclusions :** We demonstrated that MMP-9 activation is likely to occur in human osteosarcoma. However, there was no direct involvement of MMP-9 with tumor angiogenesis. It is noteworthy that MVD may aid physicians to predict the presence of distant metastasis in osteosarcoma patients.

Key Words : MMP-9; Angiogenesis; Osteosarcoma

Osteosarcoma is the second most common primary skeletal neoplasm, and it accounts for approximately 20% of all the primary malignant bone tumors.[1] Most osteosarcomas are aggressive, high- grade lesions, and approximately 80% of the osteosarcoma patients who are treated with only surgical excision will suffer metastasis.[2] There have been significant advances in the treatment of this disease in the past 20 years. Effective chemotherapy and limb-salvage surgery result in curing more than half of the patients. It is important to discover the prognostic indicators in order to estimate the outcome and to identify those patients for whom more vigorous therapy may be justified. Although elevated levels of alkaline phosphatase at the time of diagnosis and also prevalent necrosis (>90%) after neoadjuvant chemotherapy have been shown to be relevant factors, researchers are looking for more reliable prognostic factors.[3]

Invasion and metastasis of tumor cells are complex processes in which cell motility is accompanied by uncontrolled degradation of the basement membrane and components of the extracellular matrix.[4,5] Human MG-63 osteosarcoma cells have previously been characterized with respect to their invasiveness, as is reflected by their enhanced ability to efficiently degrade the extracellular matrix in vitro.[6] Matrix metalloproteinases (MMPs) are a family of zinc-containing proteinases that have the ability to degrade most parts of the extracellular matrix.[7,8] At present, the human MMP family is known to be composed of more than 20 members; these are classified into 5 major subfamilies: collagenases, gelatinases, stromelysins, membrane-type MMPs and those members not belonging to these subgroups.[9] MMP-9 (gelatinase B) contains fibronectin-like domains for collagen binding, and MMP-9 is capable of degrading type I, IV, V, VII and XI collagens and laminin. MMP-9's proteolytic ability suggests that it ultimately regulates cell migration, tumor growth and angiogenesis.[10] MMP-9 is overexpressed in many human malignancies including solid tumors and hematologic neoplasms.[8,11]

Angiogenesis is defined as the formation of new blood vessels from pre-existing ones.[12] For a tumor mass to grow, it is necessary for the tumor to develop its own vasculature; this vasculature serves not only to provide oxygen and nutrients, but it is also a means for metastasis. MMPs play multiple roles in the process of angiogenesis. Generation of null mice without MMP-2 or MMP-9 has emphasized the essential roles of these enzymes in angiogenesis. Tumor angiogenesis has been shown to be reduced

in the MMP-2-null mice,[13] whereas in the MMP-9-deficient mice, there is a defect in bone formation associated with a lack of angiogenesis.[14]

To date, there are only a few studies in the science literature on MMP-9 and angiogenesis in human osteosarcoma.[13-15] In this present study, we analyzed a group of osteosarcomas and we investigated the expression of MMP-9 and the microvascular density (MVD) in order to determine whether the MMP-9 expression is correlated with angiogenesis activity and the clinical outcomes.

MATERIALS AND METHODS

From January 2000 to November 2004, twenty osteosarcoma patients had their bone lesions biopsied. None of the patients had received chemotherapy or radiation therapy before the biopsies were taken. The specimens were fixed in 10% buffered formalin and embedded in paraffin. Four-μm thick sections were then cut and stained with hematoxilyn-eosin for the diagnosis. The patients' clinical information was obtained through a computerized retrospective search of the database of the tumor registry.

Immunohistochemical studies were performed by using the streptavidin-biotin method. The serial 4 μm sections were dewaxed in xylene and subsequently hydrated in a graded series of ethanol solutions. The anti-MMP-9 and anti-CD34 monoclonal antibodies were purchased from Zymed (San Francisco, USA). The MMP-9 monoclonal antibody was reactive for intact human MMP-9, and it recognized both the latent and active forms of the enzyme. The deparaffinized and hydrated tissue sections were immersed for 30 min in methanol that contained 0.03% H_2O_2 to block the endogenous peroxidase activity. All of the sections were treated for 5 min with 0.1 N citrate buffer to retrieve the unmasked antigens. The sections were then incubated in normal horse serum (diluted 1:20) for 30 min to block the nonspecific antibody binding sites. The monoclonal antibodies for MMP-9 and CD34 were diluted in phosphate-buffered saline that was supplemented with 5% normal horse serum and 1% bovine serum albumin at a dilution ratio of 1:200. The sections were incubated overnight with each monoclonal antibody at 4℃. The sections were next treated with biotinylated anti-mouse IgG horse serum for 30 min and then with avidin DH-biotinylated horseradish peroxidase complexes for 30 min. Afterwards, the sections were colored with 3,3'-diaminobenzidine tetrahydrochloride for 10 min, and they were next counterstained with 1% Mayer's hematoxylin.

All the slides that were stained by immunohistochemistry were scored separately by two authors to exclude any interobserver variability. Only the cytoplasmic staining for both molecules was regarded as positive. For MMP-9, the sections were scanned at low magnification (×100) with using a light microscope. Areas with the predominant staining pattern were chosen for further evaluation at a higher magnification (×200). The cases showing immunoreactivity in more than 10% of tumor cells were interpreted as being positive. The methods for determining the microvessel staining and counting were the same as those described previously.[16] Any single brown-stained cell or cluster of endothelial cells that was clearly separate from the adjacent microvessels, tumor cells and other connective tissue elements was considered to be a vessel. The branching structures were counted as a single vessel unless there was discontinuity in the structure. The vessels were counted in the 5 areas of highest vascular density at ×200 magnification (a ×20 objective and a ×10 ocular, 0.785 mm^2 per field). The individual MVD was expressed as the mean of the vessels in these areas.

Statistical analysis was performed using Pearson's χ^2 test and Fisher's exact test for the evaluation of the background factors. A p-value less than 0.05 was considered to be statistically significant.

RESULTS

We studied the biopsy specimens from 20 patients suffering with osteosarcoma. Table 1 shows the patients' clinicopathologic data along with the results of the immunohistochemical staining. The patients ranged in age from 9 to 58 years (average age: 22.9 years); there were 6 men and 14 women. Fourteen patients displayed disease that involved the femur, 3 patients' disease involved the tibia, 1 involved the humerus, 1 the iliac wing and 1 the sacrum with the tumor sizes ranging from 2-15.5 cm and the mean tumor size was 6.9 cm. Two patients had lymph node involvement. Four patients showed local recurrence and three of them experienced metastasis. A total of six patients had metastasis at a mean interval of 26 months (range: 9-51 months) (data not shown).

The expression of MMP-9 was observed in 90% (18/20) of the tumors (Fig. 1). There was no significant correlation between the MMP-9 expression and the clinicopathological factors. The MVD ranged from 10.5 to 179.7 with a mean of 64.9 (Fig. 2). No apparent differences were seen in the MMP-9 expression and

Table 1. Clinicopathologic data of 20 patients with osteosarcoma

Case	Age/ Gender	Location	Size (cm)	Subtype	Stage	MMP-9	MVD	LN involvement	Recurrence	Metastasis	Status (mo)
1	22/M	Distal femur	8.5×5.6×5	osteoblastic	IIB	+	26.1	–	–	–	NED/114
2	26/M	Proximal femur	15.5×5.5×4	osteoblastic	III	+	18.6	–	+	Lung	DOD/6
3	31/F	Distal femur	10×9×5	fibroblastic	IIB	+	16.9	–	–	–	NED/96
4	16/F	Proximal tibia	5×4×4	low-grade	IA	+	49.2	–	–	–	NED/78
5	9/M	Distal femur	9×7.5×5.5	osteoblastic	IIB	–	10.5	–	–	–	NED/65
6	16/M	Proximal humerus	6.5×2×2	osteoblastic	IIA	–	92.2	–	–	–	NED/69
7	20/F	Proximal tibia	6×4×3	osteoblastic	IIA	+	12.3	–	–	–	NED/72
8	10/F	Femur shaft	8×5×3	osteoblastic	III	+	97.3	–	+	Lung	AWD/66
9	11/F	Distal femur	12×8×5	osteoblastic	III	+	113.8	–	+	Lung	AWD/60
10	42/F	Distal femur	2×1×0.3	osteoblastic	IIA	+	28.5	–	–	–	NED/59
11	28/F	Iliac wing	5×4×3	osteoblastic	IIA	+	80.1	–	–	–	NED/57
12	28/F	Distal femur	7×5×4	osteoblastic	IIA	+	81.6	–	–	–	NED/55
13	31/F	Distal femur	2.5×2×2	osteoblastic	IIA	+	16.7	–	–	–	NED/57
14	58/F	Distal femur	9×6×5	telangiectatic	III	+	179.7	–	–	Lung	DOD/24
15	16/M	Distal femur	10×6×4	osteoblastic	III	+	70.3	–	–	Scapula	AWD/34
16	19/M	Proximal tibia	1.5×1.5×1	osteoblastic	IIA	+	143.5	–	–	–	NED/42
17	18/F	Distal femur	6×5×2	osteoblastic	III	+	92.2	+	–	Lung	AWD/32
18	27/F	Distal femur	2.5×2×2	osteoblastic	IIA	+	15.1	–	–	–	NED/29
19	17/F	Distal femur	6.5×4.5×3	osteoblastic	IIA	+	90.5	–	–	–	NED/26
20	12/F	Sacrum	6×4×3.5	chondroblastic	IIA	+	62.6	+	+	–	AWD/60

NED, no evidence of disease; DOD, died of disease; AWD, alive with disease.

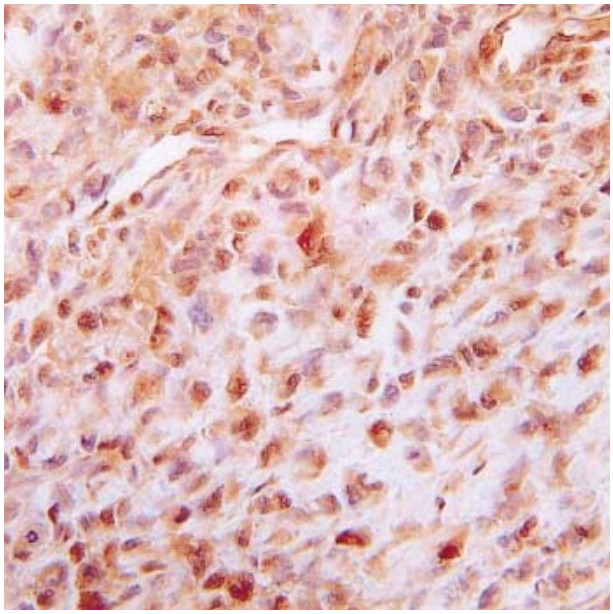

Fig. 1. Immunohistochemistry with matrix metalloproteinase-9 shows cytoplasmic staining in most of tumor cells.

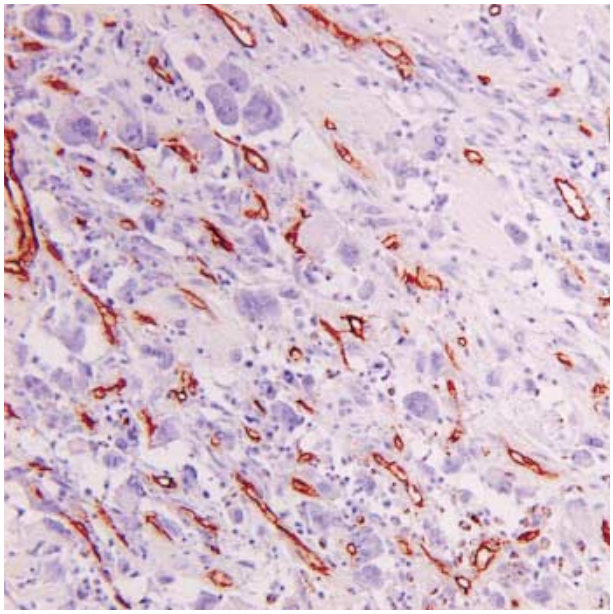

Fig. 2. Immunohistochemistry with CD34 shows the endothelial cells in intratumoral stroma. Vascularity was measured by the average microvascular density (MVD) of CD34-positive vessels.

the MVD according to the histologic subtype. The relationship between the MVD and the other prognostic parameters or MMP-9 is listed in Table 2. There was no association between the MVD and patients' age, gender, stage, tumor size, lymph node involvement or tumor recurrence. The mean MVD was greater in tumors that expressed MMP-9 than in the tumors that were negative for MMP-9, but the difference was not statistically significant. The mean MVD showed an increasing tendency in the tumors showing metastasis compared to those tumors without metastasis (p=.073) (95.3 vs. 51.8, respectively). The MVD also correlated with a trend for an advanced stage (p=0.084). In addition,

Table 2. Relationship of MVD with clinicopathologic variables

	No.	MVD (mean±SD)	p-value
Age			NS
<22.9	12	71.7±20.5	
≥22.9	8	54.7±13.2	
Gender			NS
Male	6	60.2±16.0	
Female	14	66.9±9.3	
Tumor size			NS
<6.5	9	55.6±16.4	
≥6.5	11	67.0±26.1	
Stage			NS*
I	1	49.2	
II	13	52.0±11.6	
III	6	96.5±22.8	
Recurrence			NS
−	16	73.1±18.4	
+	4	62.8±24.3	
LN involvement			NS
−	18	63.5±20.1	
+	2	77.4±14.5	
Distant metastasis			NS†
−	14	51.8±8.9	
+	6	95.3±31.8	
MMP-9			NS
−	2	51.4±22.0	
+	18	66.4±13.1	

*p=.084, †p=.073.

the mean MVD was higher in tumors ≧6.5 cm in size, and also in the tumors with lymph node involvement, although this was statistically insignificant.

DISCUSSION

Over 80% of the osteosarcoma patients who are treated with excision alone will develop pulmonary metastasis, and this suggests that the majority of patients with this disease harbor micrometastasis at the time of diagnosis.[2] There are not yet any histologic or molecular variables that can help predict the presence or absence of micrometastasis. MMPs are a class of matrix- and basement membrane-degrading enzymes, and they are known to be associated with tumor invasion and metastasis. MMP-2 and MMP-9 are closely related in their structures and they show similar substrate specificities, i.e., for type IV collagen, which is the major component of the basement membrane.[17] They have been reported to be important for degrading the basement membrane and thus, they are critical for the invasion and metastasis seen in colorectal adenocarcinoma.[18] In particular, MMP-9 is expressed in the developing and remodeling bone and also in the osteosarcoma cell lines.[19-21] These findings prompted us to investigate the status of MMP-9 in human osteosarcoma.

The expression of MMP-9 was analyzed in 20 patients suffering with different stages of osteosarcoma, and their MMP-9 expressions were evaluated for possible correlations with a variety of clinicopathological parameters (age, gender, tumor size, lymph node involvement, stage, metastasis and recurrence) and angiogenesis. We found the expression of MMP-9 in most of the osteosarcomas (90%), which is in keeping with the previous data.[22] For the childhood osseous osteosarcomas, all the samples of the pretreated tumors demonstrated intense immunostaining for MMP-9.[22] Seventy-three percent positivity for MMP-9 was also reported for the osteosarcomas that developed around the knee.[3]

Low-levels of MMP-9 activity in osteosarcomas were reported to be associated with fewer metastatic pulmonary lesions, whereas increased activity was associated with a high metastatic potential, and this suggests that MMP-9 is associated with the development of metastatic disease.[23,24] Another study on MMP-9 in osteosarcomas revealed that the patients who were positive for MMP-9 had a shorter overall survival and their tumors had a high metastatic potential.[3] In contrast, the expression of MMP-9 in our samples did not correlate with the patients' disease metastasis and survival. These conflicting results may be due to definable factors such as the differences in the analytic methodologies that were used, and the histologic subtypes and progression (early vs. late) of the tumors that were included in our study. In the study by Foukas *et al*,[3] low-temperature antigen retrieval was applied instead of the heat treatment that was used in this study. Other factors that should also be taken into consideration are the type and nature of the analyzed specimens. The tissues used for the previous studies were all surgically resected specimens that were obtained after chemotherapy, whereas all the tissues in our study were biopsy samples taken before treatment. Since chemotherapy may change the status of the tumors' molecular biology, further studies that are based on a larger series with using both pre- and post-chemotherapy osteosarcoma specimens are warranted, and such studies are currently in progress at our laboratories.

The metastatic process depends on several important factors, including angiogenesis.[25] The intratumoral MVD correlated with metastasis and patient survival for the colorectal adenocarcinomas.[26] In this study, we found that the MVD was higher in the primary tumors from the patients with metastasis than in those tumor from patients without metastasis. This may suggest that an enhanced vascular supply reflects an increased risk of metas-

tasis for that type of tumor. Tumor cells are rarely observed to shed into the circulation before the primary tumor has been vascularized. It has been documented that a greater number of tumor vessels increases the opportunity for tumor cells to enter the circulation. Furthermore, the newly formed capillaries have fragmented basement membranes, which allow them to be more easily penetrated by tumor cells than the mature vessels.[27] Thus, in the hypervascular osteosarcomas, metastasis may be enhanced by the leaky nature of the newly formed blood vessels, and this facilitates vascular invasion and subsequent metastasis.

There was no statistically significant relationship between the expression of MMP-9 and angiogenesis in this study. MMP-9 expression has been reported to be associated with angiogenic factors such as vascular endothelial growth factor in a variety of human tumors.[3] This might have occurred because both angiogenesis and the function of MMP-9 are directly blocked by tissue inhibitors of matrix metalloproteinases (TIMPs).[28]

MMPs can serve as potential targets for therapeutic intervention. Several investigations have demonstrated the effectiveness of anti-MMP therapy; a synthetic small molecule has been shown to inhibit the endotoxin-induced and the cytokine-induced synthesis of MMP-9, and the inhibition of MMP-9 has shown activity against metastasis in a rat sarcoma model.[29] MMPs as the targets for therapy were also described in osteosarcoma.[9] In the present study, we detected MMP-9 expression in 90% of the samples. The fact that MMP-9 activation is likely to occur in osteosarcoma may make this type of tumor particularly suitable for treatment with an emerging class of drugs that block MMP-9 secretion and matrix invasion. Using MMP-9 inhibition as one arm of a multitargeted approach will provide benefits for the treatment of osteosarcomas, and a larger scale study is needed to confirm the MMP-9 expression in these tumors.

In summary, we observed that MMP-9 was expressed in a majority of osteosarcomas. The present study also demonstrated that MMP-9 alone may have no effect on tumor angiogenesis, and there may be other factors or mechanisms involved in the process. However, MVD may serve as a useful predictor for identifying the patients who are at high risk for tumor metastasis.

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