Role of MMP-9 and its tissue inhibitor TIMP-1 in human osteosarcoma

Findings in 42 patients followed for 1–16 years

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Background **The activity of matrix metalloproteinases (MMPs) in degrading extracellular matrix is controlled by activation of proenzymes and inhibition of MMP tissue inhibitors (TIMPs).**

Patients and methods **To assess the proteolytic cascade imbalance in malignancy progression, tissue expression and serum levels of MMP-2, MMP-9 and of their inhibitors TIMP-2 and TIMP-1 respectively were evaluated in 42 selected patients with high-grade osteosarcoma (OS). MMP-2, MMP-9, TIMP-2 and TIMP-1 were studied in biopsies by immunohistochemistry and in serum by ELISA test. Patients were subdivided into 3 groups according to their follow up: continuously disease-free, diagnosis of metastasis during follow-up, and metastasis at diagnosis.**

Results **Immunohistochemistry demonstrated an imbalance between MMPs and TIMPs, with a more evident role for MMP-9 than for MMP-2 in tumor progression. TIMP-1 inhibitor in plasma was higher in patients with osteosarcoma than in a control group. This high value of TIMP-1 was particularly evident in the group of patients who later developed metastases and/or local recurrences, and in those with metastases at diagnosis.**

Interpretation **Our findings confirm the protective action of TIMP-1, as MMP inhibitor, but also show its activity as a growth factor underlining its multifunctional role in OS.**

During the past three decades, the survival of high-grade OS patients has significantly improved, thanks to chemotherapy (Link et al. 1986). However, there is still a need for prognostic factors which might be useful in planning new therapeutic strategies. Apart from P-glycoprotein (Serra et al. 2003), few reliable biological prognostic factors for OS have been identified.

Matrix metalloproteinases (MMPs) are associated with degradation of the extracellular membrane (ECM), including the basement membrane. The human MMP gene family contains more than 20 zinc-dependent enzymes (Nelson et al. 2000) and of these, MMP-2 and MMP-9 have been associated with aggressiveness in human cancers (Davies et al. 1993). In melanomas the expression of MMP-9 is associated with metastatic spread of tumor cells (MacDougall et al. 1995), whereas MMP-2 expression increases with increasing tumor grade (Vaisanen et al. 1996). Moreover, a clear relationship between production of MMP-2 and MMP-9 and malignant breast cancer has been demonstrated (Davies et al. 1993). Besides transcriptional regulation, MMP activity is regulated by proenzyme activation and by four different MMP tissue inhibitors (TIMPs). TIMPs form non-covalent complexes with MMPs.

Metastatic cells secrete a large amount of MMPs which can degrade ECM components (Ray and Stetler-Stevenson 1994). An increased expression of MMPs in tumor cells was found to correlate with metastatic potential (Liotta et al. 1991, Tsuchiya et al. 1994). In both in vitro (Albini et al. 1991) and experimental models (Reich et al. 1988),

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the presence of TIMPs can suppress metastasis, thus preserving ECM integrity.

Some reports suggest a multifunctional role for TIMP-1 (Hayakawa et al. 1992, Soloway et al. 1996) which, by promoting growth of a variety of cells (Gomez et al. 1997, Guedez et al. 1998), may also act as a growth factor.

To assess the significance of proteolytic cascade imbalance in progression of OS malignancy, we evaluated the expression of MMP-2, MMP-9 and of their inhibitors TIMP-2 and TIMP-1, in patients with high-grade OS in relation to their clinical outcome.

Patients and methods

Patient selection

The study was carried out on biopsies and plasma of primary high-grade OS from 42 patients (30 males), who underwent surgery at the Rizzoli Institute. 32 were osteoblastic, 5 chondroblastic, 4 were mixed, and 1 was fibroblastic. The median patient age was 16 (7–57) years and the most frequent site was the femur (23/42). Mean follow-up time, dated from the histological diagnosis, was 61 (10–194) months. We divided the patients into 3 groups: 16 who never had metastasis or local recurrence, 14 who had metastases during follow-up, and 12 who had had metastases at diagnosis. The mean follow-up time for the disease-free group was 92 (59–194) months, and for the groups with metastases it was 45 (10–178) months.

The choice of cases was dictated by the need to have (for each patient) a biopsy of an untreated specimen and a blood sample taken on the first day of hospitalization, before any treatment.

45 healthy individuals who had a routine blood test were chosen as the healthy control group (median age 30 years, 27 females). No one in the control group had a tumor when the blood test was taken. The plasma values in the different groups were compared by analysis of variance.

Immunohistochemistry (IHC)

IHC was performed on deparaffinized sections using the streptoavidin-biotin complex method (AutoProbe III, Biomeda, Foster City, CA). Sections were deparaffinized and rehydrated through graded alcohols. After treatment with Endo Blocker reagent (Lab-Vision, Fremont, CA) to eliminate endogeneous peroxidase, blocking serum (Biomeda) and citric acid solution (10 mM), the sections were incubated at room temperature for 30 min with primary antibodies MMP-2, MMP-9, TIMP-1 and TIMP-2 (NeoMarkers, Union City, CA; 1:5 dilution). Then secondary biotinylated antibody and sABC complex were applied, and the slides were developed in 3-amino 9-ethylcarbazole and counterstained with hematoxylin.

The percentage intensity of immunoreactivity was evaluated according to the following scale: 0–20% (negative or weakly positive), 21–50% (moderately positive) and > 50% (strongly positive).

Characteristics of 42 patients with osteosarcoma

Variables	Number of patients
Gender	
Male	30
Female	12
Age	
≤ 16	22
>16	20
Anatomical site	
Femur	23
Tibia	8
Humerus	5
Fibula	3
Pelvis	3
Histological types	
Osteoblastic OS	32
Chondroblastic OS	5
Osteoblastic and chondroblastic OS	4
Fibroblastic OS	1
Follow-up	
No metastasis	16
Metastasis during follow-up	14
Metastasis at diagnosis	12

Enzyme-linked immunosorbent assay (ELISA)

Serum concentrations of MMP-2, MMP-9, TIMP-1 and TIMP-2 were measured using a two-site ELISA "sandwich" format (Fuji Chemical Industries, Toyama, Japan) with specific anti-human antibodies which recognized both inactive and active forms of MMPs. 100 µL of a diluted unknown sample was pipetted into appropriate microplate wells that had already been coated with primary

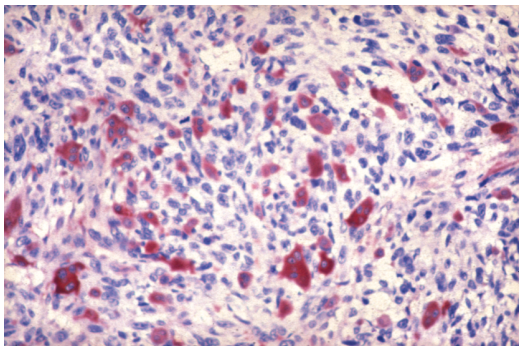

Figure 1. MMP-2 immunoreactivity in osteosarcoma (IHC, ×20). Some plurinucleated cells may have been reactive benign cells, but these were not considered to be part of the positive percentage by the pathologist.

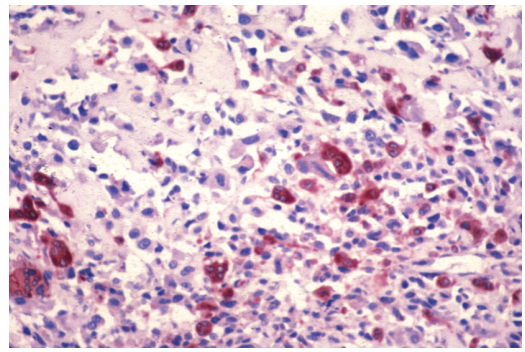

Figure 2. Intense expression of MMP-9 in osteosarcoma (IHC, ×20). Some plurinucleated cells may have been reactive benign cells, but these were not considered to be part of the positive percentage by the pathologist.

antibodies. The plates were then incubated with 100 µL horseradish peroxidase conjugated secondary antibodies at room temperature (RT) for 1–2 h. The plates were washed four times and 100 µL of RT equilibrated 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide, in 20% dimethylformamide, was dispensed and incubated for 30 min at RT. The reaction was stopped by the addition of 100 µL 1M sulfuric acid, and absorbance was measured at 450 nm. The concentrations of MMPs and TIMPs were calculated using standard curves. For MMP-2 and TIMP-1 analysis, before adding the secondary antibodies, an incubation period of 2 hours at RT was necessary, followed by washing with the appropriate buffer.

Results

IHC

MMP and TIMP expression was classified as positive when more than 20% of tumor cells were positively stained and positivity was further subdivided into moderate (21–50%) and strong (> 50%).

Of the 42 tumors studied, 12 showed a moderate to strong positive immunostaining for MMP-2 (Figure 1), and 24 for MMP-9 (Figure 2). There was no immunoreactivity against the tissue inhibitor of metalloproteinases TIMP-2, and immunoreactivity against TIMP-1 was seen in only 3 cases.

Among the patients who developed metastases during follow-up, 11/14 had a moderate to strong positivity for MMP-9, whereas the rate was 6/14 for MMP-2. In the group of 16 patients who never developed metastases, only 3/16 showed a moderate to strong positivity for MMP-2, while 8/16 were positive for MMP-9. However, the percentage positivity in the tissue for the two proteases was lower in the 12 cases with metastasis at diagnosis (3/12 for MMP-2 and 5/12 for MMP-9).

ELISA

Plasma concentration of MMP-2, MMP-9 and TIMP-2 in the 42 OS patients showed no statistically different values compared to the healthy control population. On the other hand, plasma concentration of TIMP-1 showed values that were higher than the control (1425 (SD 588) ng/mL vs. 1155 (SD 261) ng/mL; $p = 0.004$). Considering the three groups of patients, TIMP-1 showed higher mean values in the two groups with metastases (1747 (SD 839) ng/mL and 1484 (SD 443) ng/mL, respectively), compared to the group that did not develop metastasis (1144 (SD 417) ng/mL). However, the analysis of variance showed a statistically significant difference only between the mean values of the group with metastases at onset compared to the one that never developed metastases ($p = 0.01$).

We found no correlation between TIMP-1 expression and sex or age in both the OS and control group.

Discussion

MMPs and their inhibitors, which are involved in the degradation and remodelling of extracellular matrix, have important roles in tumor invasion and metastasis (Kahari and Saarialho-Kere 1999). Thus, correlations between protease expression and tumor progression have often been observed (Sier et al. 1996, Talvensaa-Mattila et al. 1998). Furthermore, MMP expression has been found to be significantly stronger in tumors than in normal tissues (Stearns and Wang 1993).

Studies on expression of MMPs and TIMPs in musculoskeletal tumors are rare. MMP-2 expression seems to play an important role in epithelial differentiation of tumor cells in synovial sarcoma but, despite its strong expression, MMP-2 appears to have no effect on prognosis in this tumor (Saito et al. 2002). Our previous studies on a series of soft tissue sarcomas have shown that increased MMP-2 reactivity and lack of TIMP-2 expression are associated with poor prognosis (Benassi et al. 2001). Another report (Soderstrom et al. 2001) found that MMP-2, MMP-13, MMP-14 and TIMP-2 mRNAs are more strongly expressed in human chondrosarcomas than in benign cartilage tumors and in normal adult cartilage. Also, protease expression may be useful for identification of patients at risk of local recurrence and to assess survival in patients with chondrosarcomas. Other authors have demonstrated that in human chondrosarcomas the ratio of MMP-1 to its tissue inhibitor appears to be related to the degree of malignancy, local recurrence, metastasis and survival (Berend et al. 1998). Studies on childhood OS showed a strong MMP-9 immunoreactivity in most untreated biopsies and pulmonary metastases, whereas positivity was lower in resected samples after chemotherapy (Himelstein et al. 1998). In our series of high-grade OS, IHC analysis detected a high percentage of tumors with moderate to strong staining for MMP-9, and minimal or no detection of TIMP-1 and TIMP-2 expression. Furthermore, the higher grade of MMP-9 immunoreactivity seemed to be associated with patients who would develop metastases during follow-up. These data emphasize the possible prognostic role of MMP-9 in an early phase of osteosarcoma progression, while, when metastatic foci are already clinically present at diagnosis and the disease is thus at a more advanced stage, other mechanisms appear to be involved in regulating tumor dissemination. On the other hand, the lack of TIMP expression at the protein level in almost all high-grade OS samples indicates deregulation of the MMP/TIMP pathway, with possible pathogenetic significance.

On the contrary, determination of plasma levels of MMPs and TIMPs has indicated that average levels of TIMP-1 are higher in patients with OS than in controls. In particular, TIMP-1 reached its highest values in the group of patients with metastases at onset, which agrees with a previous study that associated enhanced TIMP-1 with advanced tumor stage and worse prognosis (Airola et al. 1999). This result may seem to contrast with our recent paper (Benassi et al. 2003) in which the value of TIMP-1 was found to be low in the blood of patients with soft tissue sarcomas, but this is probably due to the different histological diagnosis and different patient ages. Whether or not circulating TIMP-1 is produced by tumor cells to protect themselves against ongoing proteolysis is still under investigation, and these results are important in that they will give a better understanding of the role of TIMP-1 in OS cell dissemination.

In conclusion, although they have different roles and are of significance at different stages of the disease, both MMP-9 and TIMP-1 can be considered to be biological markers of poor prognosis in OS, and they may have the potential to better define subtypes of high-risk patients.

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