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Matrix Metalloproteinase and Tissue Inhibitor of Metalloproteinase in Human Bone Marrow Tissues

An Immunohistochemical Study

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Abstract

Unlike other tissues, bone marrow (BM) seldom displays fibrosis after injury, suggesting a possible suppressive mechanism against secondary myelofibrosis in BM tissues. We investigated if fibrosis-related factors, such as matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP), were expressed in BM tissues in vivo. We attempted immunohistochemical studies on specimens of 16 BM aspiration materials with normal hematological findings and 21 BM tissues from autopsy cases who had succumbed to acute heart failure or cerebrovascular diseases without any BM injuries. Identification of immunohistochemically reactive MMP-2, MMP-9 and TIMP-2 in BM tissue samples revealed for the first time that MMP-2 was localized in the myeloid cells, erythroblasts and megakaryocytes, MMP-9 in the myeloid cells and megakaryocytes. In addition, expression of TIMP-2 in the megakaryocytes as well as in the histiocytes within the stroma was verified. In the non-pathological condition, MMP/TIMP expressions were not encountered in BM stromal cells, such as fibroblasts, vascular endothelial cells, reticulum cells on adipocytes, except for TIMP-2 identification in stromal histiocytes. It is highly possible that these MMP and TIMP expressions in the BM hematopoietic cells and stromal histiocytes are significantly associated with suppression or induction of myelofibrosis. (J Nippon Med Sch 2000; 67: 235—241)

Key words: human bone marrow, myelofibrosis, MMP-2, MMP-9, TIMP-2

Introduction

Injuries frequently induce fibrosis or cicatrisation in many organs and tissues[1]. However, while secondary myelofibrosis is frequently induced in patients with malignant myelodisorders, such as myeloproliferative diseases, malignant lymphoma and metastatic tumors in bone marrow (BM), it is rarely encountered in non-malignant myelodisorders such as drug-induced BM injuries. Moreover, few reports have documented myelofibrosis after intensive chemotherapy for acute leukemia[2]. These findings indicate that the BM tissue is different from other tissues, suggesting certain suppressive mechanisms of myelofibrosis are at work.

Myelofibrosis is usually associated with the abnormal release of growth factors including transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and calmodulin, which enhances syntheses and inhibitory degradation of extracellular matrices[3-5]. Rameshwar et al. have demonstrated an increase in TGF-b in

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blood and BM tissues of patients with primary and secondary myelofibrosis[6]. TGF−β displays inhibitory effects on synthesis of matrix metalloproteinase-1 (MMP-1) and matrix metalloproteinase-2 (MMP-2), and also facilitates synthesis of tissue inhibitor of metalloproteinase (TIMP)[5,7].

It has been well established that matrix metalloproteinases degrade protein components of the extracellular matrix and that their activity is regulated by inhibitory interaction of TIMPs[8]. The balance between MMP and TIMP activity regulates the turnover of extracellular matrices including collagen fibers. Therefore, it is highly probable that myelofibrosis is also regulated by MMP/TIMP interactions.

Janowska-Wieczorek et al.[9,10] and Ries et al.[11] have shown that BM progenitor cells (CD 34+cells) and BM mononuclear cells of healthy humans produce MMPs and/or express mRNA of TIMP-1 and TIMP-2 in in vitro studies. By using aspirated BM cells, Borregaard et al.[12] have further established that MMP-9 appears in myeloid cells on maturation in vitro. However, expression of MMP/TIMP in hematopoietic and stromal cells in BM tissues in vivo have not yet been reported.

The aim of our study is to investigate the possible existence of a MMP / TIMP-related suppressive mechanism of myelofibrosis. As a first step in the investigation, we studied the immunohistochemical aspects of MMP and TIMP in hematologically normal BM tissues.

Materials and Methods

(1) *Tissue samples*

Tissue samples were obtained from 16 BM aspiration and 21 autopsy cases in the Tokyo Metropolitan Police Hospital with informed consent.

The BM aspirates (11 males, 5 females; age range: 20−72 y.o., mean: 51 y.o.) were either with acute leukemia in complete remission (n=7) or malignant lymphoma without BM infiltration (n=9). In all cases, peripheral blood counts and BM cell counts were within the normal hematological range. All BM aspirates, obtained from the sternum of the patients, were fixed with U-fix (Sakura Seiki Co. Ltd., Tokyo) and embedded in paraffin prior to sectioning at a thickness of 3-μm. Tissue sections were stained with hematoxylin-eosin or followed up with immunohistochemical studies. Cases with 30% cellularity or less were omitted from our study.

The autopsy cases (8 males, 13 females; age range: 45−84 y.o., mean: 69 y.o.) died of severe cerebrovascular attack (n=2), acute heart failure (n=19), acute myocardial infarction (n=12), dissecting aortic aneurysm (n=2), acute pulmonary infarction (n=3), rupture of iliac aneurysm (n=1) and asthmatic multiple attacks (n=1) with a post-mortem interval ranging from 1 hr 8 min to 16 hr 3 min (mean: 6 hr 8 min). Of these, 14 cases were nonresponsive to resuscitation, while 7 died within 12 hr post-resuscitation. The 4 x 4 mm wide BM tissue specimens were obtained from the vertebra, fixed with U-fix and decalcified before embedding in paraffin. Sections with 3-μm thickness were stained with hematoxylin-eosin. Other 3-μm sections for follow-up immunohistochemical studies were repeated according to treatments for BM aspirates. Cases with 30% cellularity or less in BM tissues and those complicated with malignant neoplasm, infection or hematological abnormalities were omitted from our study.

(2) *Immunohistochemistry*

In studies with immunohistochemistry, the following primary antibodies (Fuji Pharmaceutical Co Ltd, Toyama, Japan) were diluted accordingly before use: mouse anti-human MMP-2 antibody (JR 0222, 1: 200), mouse anti-human MMP-9 antibody (LS 02, 1: 100), and mouse anti-human TIMP-2 antibody (LS 0205, 1: 100). Deparaffinized and dehydrated tissue sections were pretreated with 8.0 M guanidine (Wako Pure Chemicals, Tokyo, Japan) in 0.1 M Tris buffer (pH 7.6) overnight to unmask the antigenic sites[13].

For MMP-9 detection, sections were treated with 170-W microwave heating in distilled water prior to guanidine treatments. As for MMP-2 detection, sections were further incubated in 0.1% pepsin (Sigma Chem. Co., St. Louis, Mo, USA) at 37℃ for 10 min after microwave heating and guanidine treatments. After treatment with normal goat serum, pretreated sections were incubated overnight with the respective primary antibodies at 4℃ in a humidified room. The sections were then treated with biotinized goat anti-mouse IgG antibody (E 0433, 1: 300-dilution; Dako Ja-

pan, Kyoto Japan) and avidin-biotin-peroxidase complex, followed by reaction with 3, 3'-diaminobenzidine before nuclear staining with hematoxylin.

(3) *Evaluation of immunohistochemical findings*

Cell lineages of positively stained cells in the sections were morphologically identified. Sections that indicated either false-positive or false-negative identification as a whole were categorized as invalid and therefore obsolete.

Positive identification incidences of the respective cell types were accordingly calculated, except for the invalid cases. The intensity of staining reactivity observed in tissue components was categorized as follows: negative (−), mild (+), moderate (++) and strong (+++).

Results

Immunohistochemical reactivities of MMP-2, MMP-9 and TIMP-2 in BM aspirates and BM tissues from autopsy cases revealed distribution of the respective enzymes in different cell types of subjects studied (**Table 1**).

MMP-2: Erythroblasts in 10 BM aspirates (72%) and 17 BM tissues from autopsies (94%) were strongly reactive for MMP-2 (**Fig. 1-A**). Partial reactivities of mature myeloid cells were encountered in 5 cases each of BM aspirates (36%) and BM tissues (28%). Moreover, positive MMP-2 expression in megakaryocytes was distinctly localized in 1 BM aspirates (7%) and 5 BM tissues (28%), accordingly (**Fig. 1-B**). Although some cases showed reactivities in two cell lineages, reactivities limited to erythroblasts were observed in 8 of 14 BM aspirates (57%) and 13 of 18 BM tissues (72%). Assessments of 2 BM aspirates and 3 BM tissues were invalid.

MMP-9: Positive MMP-9 identification of all mature myeloid cells (**Fig. 1-C**) was established in all BM aspirates and 18 BM tissues from autopsies (95%). Most BM tissues indicated markedly strong reactivity for MMP-9 in isolated mature myeloid cells. Although all BM aspirates revealed reactivity only in mature myeloid cells, megakaryocytes in 6 BM tissues (32%) also expressed MMP-9 (**Fig. 1-D**). Two BM tissues from autopsies were assessed as invalid.

TIMP-2: TIMP-2 was expressed in histiocytes in all BM aspirates and 4 BM tissues from autopsies (50%) (**Fig. 1-E**). Reactivity for TIMP-2 in morphologically identified immature myeloid cells and monocytes were confirmed in 10 BM aspirates (63%) and 4 BM tissues (50%). Additional positive expressions were observed in megakaryocytes in 3 BM aspirates (19%) and 2 BM tissues (25%) (**Fig. 1-F**). In most BM aspirates, reactivities for TIMP-2 peaked in histiocytes, immature myeloid cells and monocytes. Evaluations of 13 autopsy materials were invalid.

Invalidated autopsies: The mean postmortem interval of 13 autopsies for invalid TIMP-2 identification was 6 hr 50 min, and assessment was valid in 8 autopsies within 4 hr 55 min. In cases where expressions of MMP-2 or MMP-9 were invalid, identification of 2 of 3 items (MMP-2, MMP-9 and TIMP-2) was impractical.

Differences between BM aspirates and BM tissues from autopsies: Reactivities for MMP-2 in megakaryocytes were higher in BM aspirates than in BM tissues, while MMP-9 localization in megakaryocytes was restricted to the autopsy cases.

Discussion

In this study, immunohistochemical reactivities of MMP-2, MMP-9 and TIMP-2 were, for the first time, revealed in hematopoietic cells of bone marrow tissues in vivo. It has been reported that cultured BM mononuclear cells, which correspond to myeloid granular cells and monocytes, release MMP-9 but no MMP-2 or TIMP-2[9,10]. In our present in vivo study, we demonstrated that myeloid granular cells and monocytes express MMP-9, and some of them express MMP-2 and TIMP-2 as well, in the bone marrow tissue. Besides, we were able to prove reactivities in erythroblasts for MMP-2 as well as in megakaryocytes for MMP-2, MMP-9 and TIMP-2. To our knowledge, the findings of erythroblasts and megakaryocytes in cultured cells have not been documented. Moreover, TIMP-2 expression in histiocytes was dis-

Table 1 Immunohistochemical reactivity for matrix metalloproteinase-2(MMP-2), MMP-9 and tissue inhibitor of metalloproteinase(TIMP-2) in human bone marrow tissues

MMP-2	Erythroblasts	Mature myeloid cells	Megakaryocytes	Number of BMA cases	Number of autopsy cases
	(+++)	(−)	(−)	8	13
	(−)	(+++)	(−)	4	0
	(+++)	(−)	(++)	1	4
	(+++)	(+++)	(−)	1	0
	(−)	(+++)	(++)	0	1
Invalid				2	3
Positive rate					
BMA	10/14(71%)	5/14(36%)	1/14(7%)		
Autopsy	17/18(94%)	5/18(28%)	5/18(28%)		
MMP-9	Erythroblasts	Mature myeloid cells	Megakaryocytes	Number of BMA cases	Number of autopsy cases
	(−)	(+++)	(−)	16	13
	(−)	(+++)	(+++)	0	5
	(−)	(−)	(+++)	0	1
Invalid				0	2
Positive rate					
BMA		16/16(100%)			
Autopsy		18/19(95%)	6/19(32%)		
TIMP-2	Histiocytes	Immature myeloid cells or monocytes	Megakaryocytes	Number of BMA cases	Number of autopsy cases
	(+++)	(+++)	(−)	8	2
	(+++)	(−)	(−)	5	2
	(+++)	(+++)	(+)	2	0
	(−)	(+++)	(+)	0	2
	(−)	(+++)	(−)	0	2
	(+++)	(−)	(+)	1	0
Invalid				0	13
Positive rate					
BMA	16/16(100%)	10/16(63%)	3/16(19%)		
Autopsy	4/8(50%)	4/8(50%)	2/8(25%)		

BMA: bone marrow aspiration, MMP: matrix metalloproteinase, TIMP: tissue inhibitor of metalloproteinase. Positive rate of the respective cell types are calculated except for the invalid cases. The intensity of staining is categorized as follows: negative(−), mild(+), moderate(++) and strong(+++).

covered in the BM stroma. The results have led to the suggestion that these cell components may be concerned with the mechanism relating to promotion or inhibition of myelofibrosis in the BM tissue.

The selective localization of MMP-9 in mature myeloid cells in the bone marrow tissue corresponds with the findings of MMP-9 expression in cultured BM mononuclear cells[9,10] and immunocytochemically reactive MMP-9 in mature myeloid cells of BM aspirates[11]. This fact confirms the reliable identification of MMP-releasing cells in BM tissues by employing an immunohistochemical approach. Positive reactivities for MMP-2, MMP-9 and TIMP-2 in erythroblasts and megakaryocytes were proven for the first time by this methodology and the facts suggest the functional regulation of these cells in myelofibrosis via MMP/TIMP interaction.

While secretions of MMP-2 and TIMP-2 were not confirmed in cultured BM mononuclear cells, our results indicate positive identification of MMP/TIMP in

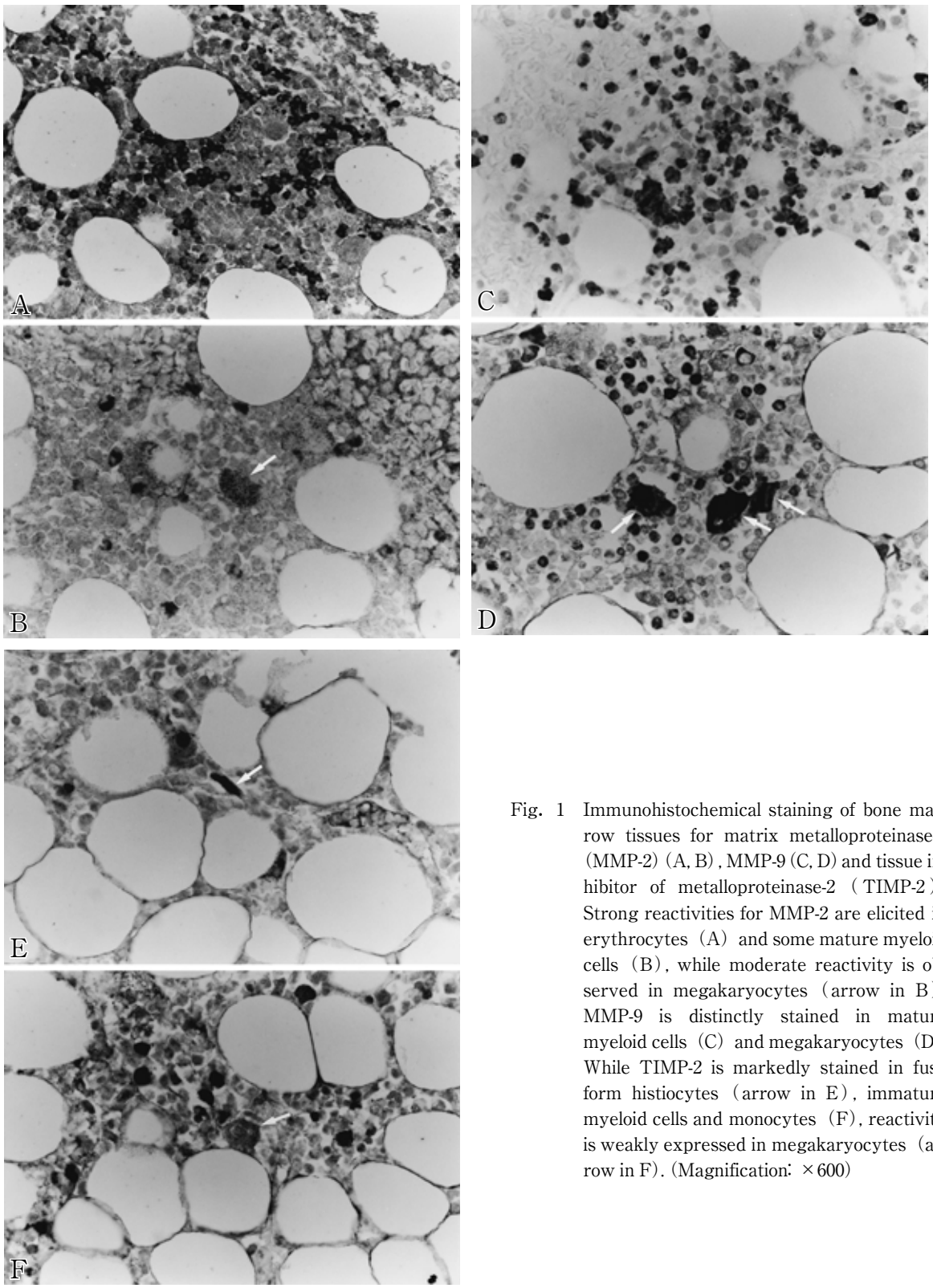

Fig. 1 Immunohistochemical staining of bone marrow tissues for matrix metalloproteinase-2 (MMP-2) (A, B), MMP-9 (C, D) and tissue inhibitor of metalloproteinase-2 (TIMP-2). Strong reactivities for MMP-2 are elicited in erythrocytes (A) and some mature myeloid cells (B), while moderate reactivity is observed in megakaryocytes (arrow in B). MMP-9 is distinctly stained in mature myeloid cells (C) and megakaryocytes (D). While TIMP-2 is markedly stained in fusiform histiocytes (arrow in E), immature myeloid cells and monocytes (F), reactivity is weakly expressed in megakaryocytes (arrow in F). (Magnification: ×600)

the myeloid granular cell/monocyte system in BM tissues. This discrepancy might be attributable to the difference between in situ and in vitro treatments. Since mRNA of TIMP-2 has been expressed in cultured BM mononuclear cells[9], it is highly possible that plausible secretion occurs, depending naturally on the given conditions. Furthermore, mutual interactions between BM mononuclear cells and erythroblasts/

megakaryocytes in vivo can not be discounted.

The significance of analyzing MMP/TIMP expression within BM tissues is as follows: (i) individual cell identification is rendered possible when staining of various cell systems, and hematopoietic cells at different maturing stages is concurrently performed under a similarly specified condition; and (ii) comparative study based on our present data is rendered possible on pathological changes in BM tissues, such as acute drug-induced myelodisorders or myelofibrosis due to varied causes.

The results of this study in the non-pathological condition indicate that MMP/TIMP expressions were not encountered in fibroblasts, vascular endothelial cells, reticulum cells and adipocytes, which were regarded as hematopoiesis-related stromal cells in BM, apart from TIMP-2 identification in stromal histiocytes. As quantitative and functional changes occurring in the stromal cells of BM tissues during tumor proliferation of hematopoietic cells have been reported[14], informative identification of MMP/TIMP in stromal cells in pathological BM tissues remains to be challenged.

The fact that they were deat coupled with postmortem systemic degeneration meant that autopsy cases were sometimes futile for the identification of MMP-2, MMP-9 and TIMP-2 when compared with biopsy cases. Cases with futile TIMP-2 evaluation displayed a mean postmortem interval longer than that of validated cases. Compared with MMP-2 and MMP-9 expressions, TIMP-2 reactivity was more susceptible to postmortem effects. However, our results indicate that even autopsy cases can be sufficiently effective for examination of MMP/TIMP expression in BM tissues, if the postmortem interval is within five hours. Effects of primary diseases in BM aspiration cases can not be ignored, as differences were noted between BM aspirates and autopsies on the incidences of MMP-2 and MMP-9 in megakaryocytes. Comparative studies on parallel findings in healthy donors are now in progress at our laboratory.

In conclusion, our present results of positive expressions of MMP-2, MMP-9 and TIMP-2 in hematopoietic cells and stromal histiocytes in the BM tissue strongly suggest their associated influences in the production and degradation of fibrogenic extracellular-matrix proteins which relate to myelofibrosis in the pathological condition.

Acknowledgment: The authors wish to thank Ms. Mitsue Kataoka and Ms. Arimi Ishikawa of the Department of Pathology (I), Nippon Medical School for their technical assistance in our experiments.

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(Received, March 3, 2000)
(Accepted, April 18, 2000)