NOTE

Matrix Metalloproteinases (MMPs) Activity in Cultured Canine Bone Marrow Stromal Cells (BMSCs)

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ABSTRACT: Autologous bone marrow stromal cells (BMSCs) infusion therapy improves the hepatic fibrosis. To investigate the mechanism of remission, we evaluated the matrix metalloproteinase (MMP)-2 and -9 activity in canine BMSCs and the effect of pro-inflammatory cytokines on their expression. The activity and the gene expression of MMPs were analyzed by gelatin zymography and quantitative RT-PCR, respectively. The specific gelatinase bands were indicative effect of MMP-2 and -9 in canine BMSCs. MMP-2 expression seemed to be increased by TNF-α and IL-1β while MMP-9 was enhanced by TNF-α and IL-6. These results suggested that remissive effect on liver fibrosis might be partly attributable to the MMP-2 and -9 activity in BMSCs under the inflammatory condition.

KEY WORDS: bone marrow stromal cell (BMSC), canine, culture, inflammatory cytokines, matrix metalloproteinase (MMPs).

Medical therapies are not eradicate for hepatic fibrosis, and so the liver transplantation is an only radical surgery available both for the human and animal patients. However, the transplantation is also limited, because of the many problems, including the lack of donors, the stressful surgery, and tissue rejection. Recent clinical papers in human medicine present the effective therapeutic regime using autologous bone marrow stromal cells (BMSCs) for the patients with severe hepatic fibrosis [21]. Effect of the cell therapy has been explained by differentiation of the bone marrow cells into a variety of non-hematopoietic cell lineages [15] including hepatocytes [1, 14, 17, 23]. Bone marrow cells are an attractive cell source for regenerative medicine compared to tissue-specific stem cells [7]. Transfusion of BMSCs would be able to restore the biochemical function in recipients with progressive liver failure; furthermore, based on the some previous data, hepatic cells differentiated from BMSCs protect against or ameliorate CCl4-induced fibrosis in recipient rats [24] and mice [18]. Matrix metalloproteinase (MMP) -9 production of BMSCs, and the promotion in the liver resident cells contribute to the spontaneous regression of liver fibrosis in mice [5]. MMPs are a family of enzymes implicated in the degeneration and remodeling of extracellular matrix, as well as vascularization [9, 10]. Inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, initiate or enhance the gelatinase gene expression, however the expression of MMPs in canine BMSCs is poorly understood and less information is available in the veterinary literature. Aim of this study was to evaluate the activities of MMPs in canine BMSCs and the effects of cytokine on the expression.

Bone marrow cells were collected from humeri and femora of clinically healthy dog (2-years-old male Beagle) under anesthesia. The animal received humane care complying with the guidelines for treatment of experimental animals at Yamaguchi University. The bone marrow cells were seeded onto Nunclon® Falcon surface (NUNC, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) and cultured in Dulbecco’s modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, U.S.A.), supplemented with fetal bovine serum (FBS, 5%), streptomycin (50 µg/ml) and amphotericin B (2.5 µg/ml), in a 5% CO2 incubator at 37°C. After 2 days, non-adherent cells were washed away with medium and adherent cells were used as canine BMSCs. The culture medium was changed twice a week and cells in the second or third passage were used.

To evaluate gelatinase activity, we used gelatin zymography. BMSCs (1.5 × 105 cells/cm²) were seeded onto an 8 well dish, adhered overnight, washed and incubated with DMEM for 24 hr. TNF-α, IL-1β and IL-6 (Steinheim, Germany) were diluted with DMEM to yield the final concentrations (100 ng/ml). After 24 hr treatment with or without TNF-α, IL-1β and IL-6, the supernatants were collected and stored at −80°C until use. The remaining adherent cells were immediately dissolved with lysis buffer (from a Fastpure RNA kit, Takara Biomedicals, Shiga, Japan) and

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were then kept at −80°C until use for RNA isolation. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Tokyo, Japan). Supernatants containing an equal amount of protein (25 µg) were mixed with a sample buffer consisting of 0.25 M Tris–HCl (pH 6.8), 8.6% sodium dodecylsulfate (SDS), 30% glycerol and 5% (w/v) bromophenol blue. Gelatin zymography was performed as previously described with modifications. Briefly, the samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) co-polymerized with gelatin (1%) as the substrate. Following electrophoresis, the gel was incubated for 1 hr at room temperature in a 2.5% Triton X-100 solution, and subsequently incubated at 37°C for 18 hr in Tris–HCl buffer (pH 7.4), with or without 10 mmol/l CaCl₂. The gels were stained with 0.05% Coomassie Brilliant Blue R-250 in 45% methanol plus 10% glacial acetic acid, and destained with 25% methanol plus 8% glacial acetic acid and 5% methanol plus 7.5% glacial acetic acid. Gelatinase activities were detected as unstained bands against a background of Coomassie blue-stained gelatin. Enzyme activity was assessed by densitometry using a Kodak 1D Image Software™ (Eastman Kodak, Rochester, NY, U.S.A.). A purified human MMP-2/MMP-9 mixture (COSMO BIO Co., Ltd., Tokyo, Japan) was used as a positive control. Human MMP-2, the pro form of MMP-2, and pro-MMP-9 were identified as bands at 62, 72 and 92 kDa, respectively.

Total RNA was isolated from adherent bone marrow cells by using a Fastpure RNA kit (Takara Biomedicals) following the manufacturer’s instructions. For the reverse transcriptase reaction, SuperscriptTM first strand synthesis for RT-PCR (Invitrogen Corp., Carlsbad, CA, U.S.A.) was used. Specific oligonucleotide primers for canine MMP-2 and MMP-9 were purchased from Takara (Takara Biomedicals), primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was designed from Genbank sequences (Table 1). Quantitative RT-PCR was performed using the SYBR® PremixTM Ex Taq II (Tli RNaseH Plus) (Takara Biomedicals) with a Thermal Cycler Dice Real Time II (Takara Biomedicals) according to the manufacturer’s instructions. Quantitative RT-PCR date are presented as mean±SD. Significant differences were determined using the Student’s t-test, with a P<0.05 considered statistically significant.

In the gelatin zymography, the specific gelatinase bands were observed from the analysis of canine BMSCs. Compared with standard bands, based on human pro MMP-9, pro-MMP-2 and MMP-2 molecules (92, 72 and 62 kDa, respectively), the gelatinase bands associated with the highest activity was estimated to be a canine pro-MMP-2 and pro-MMP9. When the gelatinolytic reactions were performed in the absence of CaCl₂, gelatinase activity was not detected, indicating that the observed reactions were the result of zinc-dependent metal MMPs. Furthermore, to identify the contribution of matrix serine proteinases (MSPs) to the gelatinase activity of canine BMSCs, we assessed whether MSP inhibitors, such as phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphates (DFP), suppress gelatinase activity. These MSP inhibitors were ineffective against gelatinase activity in canine BMSCs, which further confirms the function of MMPs produced by canine BMSCs. TNF-α and IL-6 enhanced gelatinase activity of pro-MMP9, whereas IL-1β did not 24 hr after treatment. On the other hands, IL-1β enhanced the gelatinase activity of pro-MMP2 24 hr after treatment (Fig. 1).

Quantitative RT-PCR revealed that canine MMP-2 and MMP-9 mRNA were expressed in BMSCs. TNF-α and IL-6 enhanced MMP-9 mRNA expression 24 hr after treatment. TNF-α and IL-1β seemed to enhance MMP-2 mRNA expression 24 hr after treatment, although it was not significantly different (Fig. 2).

In the present study, we detected functional MMPs in canine BMSCs that were enhanced by inflammatory cytokines. Using gelatin zymography, we confirmed the production of pro MMP-2 and pro MMP-9 in canine BMSCs, that seemed to be enhanced by IL-1β and by TNF-α or IL-6, respectively. And also the quantitative RT-PCR supported the results. Although MMP-2 and MMP-9 have similar substrate specificities, there is difference in the regulation of their expression. MMP-2 is constitutively expressed by several cell types and not inducible [4]. In contrast, MMP-9 production is inducible by pro-inflammatory cytokines including TNF-α and IL-1β released from leukocyte, airway resident cells, endothelial cells, smooth muscle cells and fibroblasts [2, 13, 19]. In a study using a mouse model of chronic liver failure, transplanted BMSCs migrates to the fibrotic area and expresses MMP-9 there, that indicative of the resolution of fibrosis [22]. The degradation of the fibrous extracellular matrix could improve the liver function, and survive the diseased mice, following BMSC transplantation [22]. In this study, MMP-9 increased in the cells treated by inflammatory cytokines such as TNF-α and IL-6. IL-6 plays a crucial role during liver regeneration [11, 16]. Interestingly, down-regulation of MMP-2 induced by IL-6 contributes hepatocyte protection from CCl₄ treatment [3]. In the liver, IL-6 is synthesized by tissue macrophages in response to stimulation by TNF-α [12]. Furthermore, two components of the complement system, C3α and C5α, bind to receptors on the surface of Kupffer cells, triggering the release of IL-6 and TNF-α [8]. IL-6 also plays a key role in inhibiting apoptosis by activating several genes, including FLIP, Bcl-2 and Bcl-xL, which code for proteins that block the action of caspase [6]. Stat-3 is a key mediator in the antiapoptotic response initiated by IL-6 [20].

### Table 1. Primer used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Dog gene</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>MMP-2</td>
<td>(F) 5’AATCTCGGAGCCTGCACCTCCTG &lt;br&gt;(R) 5’TGCACCTGTCCGCACGATGAA</td>
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<tr>
<td>MMP-9</td>
<td>(F) 5’GTGAAGACCGACGACCGTGGA &lt;br&gt;(R) 5’CGAGAATTCACCCGGCAGTGAAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>(S) 5’CCACCCCAATGATCAGTT &lt;br&gt;(AS) 5’TGGAGAGTGTTGGTTCAGT</td>
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**References:**

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2. In a study using a mouse model of chronic liver failure, transplanted BMSCs migrates to the fibrotic area and expresses MMP-9 there, that indicative of the resolution of fibrosis [22].
3. The degradation of the fibrous extracellular matrix could improve the liver function, and survive the diseased mice, following BMSC transplantation [22].
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6. Stat-3 is a key mediator in the antiapoptotic response initiated by IL-6 [20].
in liver injury may play a role not only antiapoptosis of hepatocytes but also MMP-9 production which resulting in improvement of liver fibrosis following BMSC transplantation therapy. In conclusion, canine BMSCs produce MMPs, and inflammatory cytokines enhance the MMP’s activities. The results give some speculation that invasion and lives of transplanted BMSCs at fibrous liver tissue could be partly due to the MMP’s activities of them, and that the cell therapy would be useful to improve the liver function.

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