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ORIGINAL ARTICLE

**Binbin Zhong · Michiko Tajima · Hidenari Takahara
Hiromi Nochi · Koichi Tamoto · Naoto Tamura
Shigeto Kobayashi · Yoshifumi Tamura · Makoto Ikeda
Tomohiro Akimoto · Shinichi Yoshino
Hiroshi Hashimoto**

Inhibitory effect of mizoribine on matrix metalloproteinase-1 production in synovial fibroblasts and THP-1 macrophages

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Abstract To investigate the mechanism of antirheumatic action of mizoribine (MZR), we examined the expression of matrix metalloproteinase-1 (MMP-1) and MMP-3 utilizing THP-1 derived macrophage-like cells (THP-1 macrophages) and human synovial fibroblasts (SFs). The cells were respectively stimulated with lipopolysaccharide (LPS) and interleukin-1β in the presence or absence of MZR in vitro. The concentrations of MMP-1 and MMP-3 in the supernatant were measured by enzyme-linked immunosorbent assay. The secretion of MMP-1 from SFs, as well as THP-1 macrophages, was inhibited by MZR in a dose-dependent manner. Furthermore, a quantitative real-time polymerase chain reaction revealed that MZR decreased the expression of MMP-1 messenger RNA. These findings may be an explanation for the clinical effect of MZR in patients with rheumatoid arthritis.

Key words Matrix metalloproteinase (MMP) · Mizoribine · Rheumatoid arthritis (RA) · Synovial fibroblast · THP-1 derived macrophage-like cells

B. Zhong · M. Tajima · N. Tamura (✉) · S. Kobayashi · M. Ikeda · T. Akimoto · H. Hashimoto
Department of Rheumatology and Internal Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
Tel. +81-3-5802-1067; Fax +81-3-5800-4893
e-mail: tnaoto@med.juntendo.ac.jp

H. Takahara
Laboratory of Molecular Biology and Biochemistry, School of Agriculture, Ibaraki University, Inashiki, Ibaraki, Japan

H. Nochi · K. Tamoto
Department of Immunology and Microbiology, Faculty of Pharmaceutical Sciences, Health Sciences, University of Hokkaido, Ishikari-Toubetsu, Hokkaido, Japan

Y. Tamura
Department of Medicine, Metabolism and Endocrinology, Juntendo University School of Medicine, Tokyo, Japan

S. Yoshino
Department of Joint Disease and Rheumatism, Nippon Medical School, Tokyo, Japan

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial inflammation and the destruction of cartilage and bone. Fibroblast-like synoviocytes and macrophages are predominantly observed in RA synovial tissue containing a cartilage–pannus junction,[1] and matrix metalloproteinases (MMPs) secreted from these cells are generally believed to play a critical role in joint destruction.[2] Among the known MMPs, MMP-1 and MMP-3 were initially reported to be expressed in the synovial membranes of RA patients.[3] Recently, other MMPs, such as MMP-9, MMP-12, and MMP-13, have also been found in the synovial membranes of RA patients.[4–6] Furthermore, the serum levels of MMP-1 and MMP-3 are correlated with generalized clinical disease activity,[7] whereas synovial fluid MMP-1, MMP-3, and TIMP-1 activities are correlated with local joint inflammation.[8] Although the mechanisms of the antirheumatic actions of disease-modifying antirheumatic drugs (DMARDs) are not fully understood, inhibiting the production of MMPs has been postulated to contribute to the prevention of joint destruction.[9,10]

Mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate, MZR) is an immunosuppressive drug that has been used to treat patients with renal transplants and lupus nephritis since the 1980s.[11] Mizoribine suppresses the proliferation of synovial fibroblasts[12] and offers clinical advantages to patients with RA. However, the mechanism by which MZR benefits RA patients remains uncertain. To examine the antirheumatic action of MZR, we focused on MMP and tested whether MZR decreased the production of MMP-1 and MMP-3 in human synovial fibroblasts (SFs) and macrophage-like cells matured from the THP-1 cell line.

Materials and methods

Cell culture and measurement of MMP-1 and MMP-3 in the supernatant

THP-1, a human acute myelomonocytic leukemia cell line, was cultured in RPMI supplemented with 10% fetal calf serum (FCS) containing 100 ng/ml of phorbol myristate acetate (PMA; Sigma, Tokyo, Japan) at a concentration of 1×10^5 cells/well in a 24-well plate for 48 h, allowing the cells to mature into macrophage-like cells (THP-1 macrophages). The cells were washed once with the medium and stimulated with 1 μg/ml of lipopolysaccharide (LPS; Sigma) for 48 h. The cells were untreated or treated with 1, 3, 10 and 30 μg/ml of MZR (kindly donated by Asahikasei Pharma, Tokyo, Japan) during the stimulation. In another experiment, the same amount of MZR was added 24 h prior to LPS for pretreatment and coincubated during the stimulation. Human SFs were derived from the synovial tissues of healthy volunteers (CS-ABI-479 cells, purchased from Dainippon Pharmaceutical, Osaka, Japan), cultured using a CS-C complete medium kit (Dainippon), and stimulated with 0.1 ng/ml of interleukin (IL)-1β (R&D Systems, Minneapolis, MN, USA) for 48 h. Mizoribine, at concentrations of 1 and 10 μg/ml, was added 24 h prior to IL-1β or simultaneously, and coincubated during the stimulation. The viability of cells after the stimulation was examined with 0.4% trypan blue staining. The supernatants were stored at –80°C, and the concentrations of MMP-1 and MMP-3 were measured using enzyme-linked immunosorbent assay (ELISA) kits (Fuji Chemical Industries, Toyama, Japan).

Quantitative real-time polymerase chain reaction

mRNA was isolated from the SFs using an RNA extraction kit (Qiagen, Hilden, Germany) and quantified using a spectrophotometer. One microgram of total RNA was reverse transcribed into cDNA for use as a polymerase chain reaction (PCR) template. The RNA samples were then denatured at 65°C for 5 min and reverse transcribed at 42°C for 60 min. The PCR products were analyzed using 2% agarose gel electrophoresis. Quantitative real-time PCR was performed using an ABI Prism 7700 sequence detection system (Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The β-actin gene was used to control the amount of template in each sample. The PCR amplifications were conducted in 25-μl reactions using 40 cycles with 1 μM of the appropriate primers (forward 5′-CTGAAGGTGATGAAGCAGCC-3′ and reverse 5′-AGTCCAAGAGAATGGCCGAG-3′ for MMP-1,[13] forward 5′-ATGAAGAGTCTTCCAATCCTACTGT-3′ and reverse 5′-CATTATATCAGCCTCTCCTTCATAC-3′ for MMP-3,[14] and forward 5′-GGTCTCAAACATGATCTGGG-3′ and reverse 5′-GGGTGAGAAGGATTCCTATG-3′ for β-actin) and 12.5 μl of SYBR Green Master Mix (ABI). Each cDNA sample was tested in triplicate. The PCR conditions for MMP-1 consisted of a 5-min hot start at 95°C, followed by 40 cycles for 1 min at 95°C and 1 min at 55°C,[13] while those for MMP-3 consisted of a 5-min hot start at 95°C, followed by 40 cycles at 95°C for 45 s and 45 s at 55°C.[14] The results of the real-time PCR were analyzed using the ABI Prism 7700HT sequence detection system.

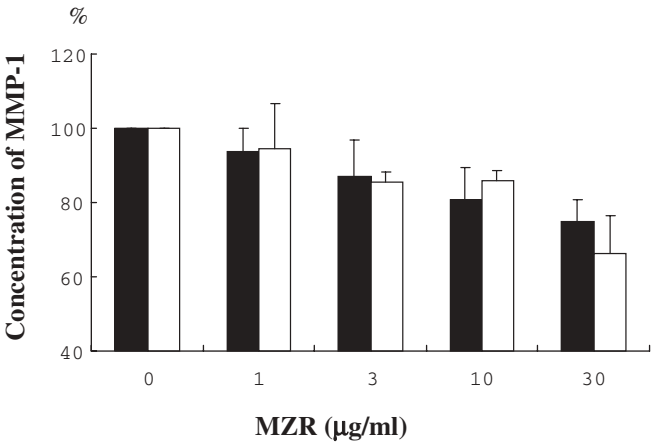

Fig. 1. Effect of mizoribine (*MZR*) on the secretion of matrix metalloproteinase-1 (*MMP-1*) from THP-1 macrophages. Macrophages derived from 1×10^5 THP-1 cells were stimulated with lipopolysaccharide (LPS) for 48 h. The indicated concentrations of MZR were added 24 h prior to LPS (*black columns*) or simultaneously (*white columns*), and coincubated during the stimulation. The concentrations of MMP-1 in the supernatant were measured using an enzyme-linked immunosorbent assay (ELISA). Results were calculated into percentages based on the amount of MMP-1 in the sample without MZR. *Bars* show the mean ± standard deviation (SD) of three independent experiments

Results

Effect of MZR on the secretion of MMP-1 and MMP-3 from stimulated THP-1 macrophages

To examine whether MZR decreases the release of MMP-1 and/or MMP-3 from THP-1 macrophages, we stimulated the cells with LPS in the absence or presence of various concentrations of MZR and measured the concentrations of MMP-1 and MMP-3 in the supernatant using an ELISA. When the MZR and LPS were added simultaneously, the release of MMP-1 was inhibited by approximately 20%–30% in a dose-dependent manner (Fig. 1). The inhibitory effect was also equally observed when the cells were pretreated with MZR prior to LPS stimulation. On the other hand, the release of MMP-3 from the THP-1 macrophages was inhibited much weakly and not dose dependently (Fig. 2). The addition of MZR did not affect the cell viability and form of the cells even at 30 μg/ml, the maximum concentration used in this study.

Effect of MZR on release and mRNA expression of MMP-1 and MMP-3 in stimulated SF

Next, we tested the inhibitory effect of MZR on MMP-1 and MMP-3 secretion from SFs, thought to be a major

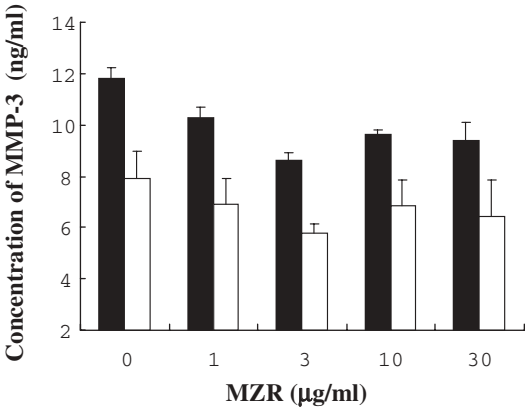

Fig. 2. Effect of mizoribine (*MZR*) on the secretion of matrix metalloproteinase-3 (*MMP-3*) from THP-1 macrophages. THP-1 macrophages were stimulated with LPS for 48 h. The indicated concentrations of MZR were added 24 h prior to LPS (*black columns*) or simultaneously (*white columns*), and coincubated during the stimulation. The concentrations of MMP-3 in the supernatant were determined using an ELISA. *Bars* express the mean ± SD of three independent experiments

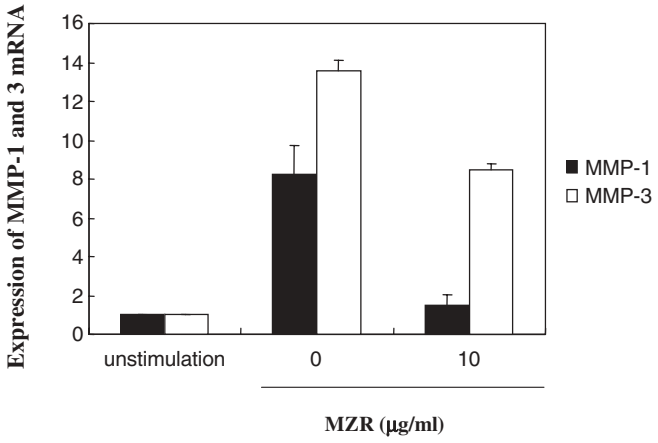

Fig. 4. Effect of mizoribine (*MZR*) on the expression of matrix metalloproteinase (*MMP*)-1 mRNA in SFs. RNA was extracted from SFs treated as described in Fig. 3. The mRNA of MMP-1 and MMP-3 were quantified using real-time polymerase chain reaction. The results were calculated as -fold based on the amount of unstimulated samples. *Bars* indicate mean ± SD in triplicate

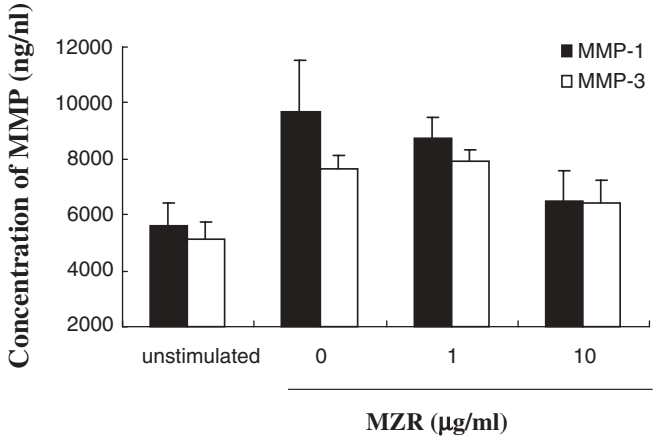

Fig. 3. Effect of mizoribine (*MZR*) on the secretion of matrix metalloproteinase (*MMP*)-1 and MMP-3 from synovial fibroblasts (SFs). The SFs were stimulated with 0.1 ng/ml of interleukin-1β (IL-1β) and cultured for 48 h. The indicated concentrations of MZR were added 24 h prior to IL-1β and coincubated during the stimulation. The concentrations of MMP-1 and MMP-3 were determined using an ELISA. *Bars* express the mean ± SD of triplicate wells

source of the serum MMPs found in patients with RA. The cells were pretreated with MZR for 24 h and stimulated with IL-1β for another 48 h. The concentrations of MMP-1 and MMP-3 in the supernatant were then measured using the ELISA. As shown in Fig. 3, MMP-1 secretion from the SFs was inhibited by approximately 60% by the addition of 10 μg/ml of MZR. In contrast, the secretion of MMP-3 was not significantly decreased by an equivalent dose of MZR. The viability and form of the cells did not change by addition of MZR.

To further investigate the inhibitory effect of MZR on MMP-1 secretion in stimulated SFs, the expression of MMP-1 mRNA was quantified using real-time PCR. Both protein secretion and the expression of MMP-1 mRNA were decreased in the presence of 10 μg/ml of MZR (Fig. 4), whereas the inhibition of MMP-3 mRNA was weaker.

Discussion

In the inflamed synovial tissue of patients with RA, fibroblast-like synoviocytes and macrophages are the dominant cell populations in areas adjacent to the cartilage–pannus junction,[1] and the secretion of proteolytic enzymes from these cells plays a critical role for cellular invasion and the degradation of cartilage and subchondral bone.[15,16] Among the proteases, the abundant production of MMP-1, which can digest collagen types I, II, III, VI, and X, and gelatins, was first demonstrated at the sites of joint erosion;[17] later, MMP-3 was also reported to be involved in the degradation of articular cartilage and synovium.[18–20] The expression of these protease genes has been observed in tissues obtained only a few weeks after the onset of symptoms,[21] emphasizing the very early potential for joint destruction, and indicating the importance of MMP inhibition in the treatment of RA.

Mizoribine is an imidazole nucleoside isolated from the culture medium of the mold *Eupenicillum brefeldianum* M-2166, found in the soil of Hachijo island, Tokyo, Japan in 1974.[11] Mizoribine was approved in Japan for the clinical treatment of RA in 1992 after the marked amelioration of adjuvant arthritis via the suppression of T-cell function was demonstrated in rats[22] and a low incidence of adverse clinical effects was reported.[23] Moreover, MZR treatment was reported to improve bone lesions in the hind legs of animals with adjuvant arthritis.[24] Here, we examined whether MZR could inhibit MMP-1 and MMP-3 production in stimulated SFs and macrophage-like cells. The concentration of MZR in body fluids has been reported to reach 5–10 μM (approximately equivalent to 2.0–4.0 μg/ml) at its peak,[25] and MZR has been shown to partially inhibit MMP-1 production in

vitro at approximately this concentration in THP-1 macrophages and at 3–5-fold higher concentration in SFs. We expected MZR pretreatment to increase the reduction of MMP-1 production on macrophages due to cell growth inhibition; however, such an effect was not recognized. Others have reported that mepacrine, an antimalarial drug, inhibited the release of MMP-1 dose dependently in stimulated human fibroblast-like synoviocytes.[13] Methotrexate also inhibited the synthesis of MMP-1, but not of MMP-3, in SFs.[26] More recently, leflunomide was reported to inhibit the production of MMP-1, MMP-3, and MMP-13 secretion from stimulated SFs, and this effect was suggested to be induced by the suppression of the mitogen-activated protein kinase signaling pathway.[10,27,28] In fact, methotrexate and leflunomide have been reported to be capable of preventing joint destruction in patients with RA.[29–31]

Although the mechanisms responsible for the inhibitory effects of MZR are unclear, nuclear factor-κB (NF-κB)-mediated transcription in synovial macrophages and activator protein 1 (AP-1), prominent in SFs, were reported to induce MMPs synthesis in the synovia of patients with RA,[32–36] suggesting that MZR may inhibit part of the signal transduction pathway conducted to NF-κB and/or AP-1. Several existing drugs, include glucocorticosteroids, gold thiolates, and D-penicillamine, have actions that directly or indirectly inhibit NF-κB and/or AP-1.[37] Moreover, in RA synovial fibroblasts, IL-6 and MMP-1 are regulated by the cyclin-dependent kinase inhibitor p21, and alterations in p21 expression may activate AP-1 leading to enhanced proinflammatory cytokine and MMP production.[38] It was reported that MZR could inhibit IL-6 production in RA synovial fibroblasts,[39] suggesting that MZR may inhibit IL-6 and MMP-1 production in the same signal transduction pathway.

Safety of MZR was assessed between two dosage groups, 150 mg/day and 300 mg/day, of a report from post-marketing surveillance, and no statistical difference was observed (1.3% and 7.1%, respectively).[40] Furthermore, administration of 25 mg/kg per day of MZR induced only mild reduction of splenic lymphocytes, and even 100 mg/kg per day of MZR did not decrease the number of bone marrow cells in mice.[41] In our study, the dose-dependent inhibitory effect of MZR to MMP-1 production was recognized without affecting the cell forms and number of dead cells. These findings indicate that a higher dose of MZR may be tolerated and required to obtain a sufficient clinical effect.

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