

THE ANTIPROLIFERATIVE EFFECT OF MIZORIBINE ON RHEUMATOID SYNOVIAL FIBROBLAST MEDIATED BY INDUCTION OF APOPTOSIS

Yasuhiko IMAIZUMI, Ryuichi SAURA, Kosaku MIZUNO,
and Keiji NAKAGAMI*

Department of Orthopaedic Surgery, Kobe University School of Medicine

*Institute for Life Science Research, Asahi Chemical Industry Co. Ltd.

KEY WORDS

rheumatoid arthritis; synovial fibroblast; mizoribine; apoptosis; Bcl-2

ABSTRACT

In order to investigate the mechanism of anti-rheumatic action of mizoribine (MZR), antiproliferative effect of MZR on synovial fibroblasts obtained from rheumatoid arthritis (RA) patients were examined. To examine the effect of MZR on DNA synthesis, total radioactivity of ³H-thymidine (³H-TdR) incorporated into the synovial fibroblasts was measured. Also quantification of DNA fragmentation of synovial fibroblasts in the cultured supernatant and cell associated Bcl-2 protein, which is suspected of interfering with apoptosis, were performed with enzyme-linked immunosorbent assay.

MZR suppressed ³H-TdR incorporation into synovial fibroblasts in a dose dependent fashion. Significant inhibition ($P < 0.01$) was attained at the concentration of more than 1 $\mu\text{g/ml}$ of MZR. However, induction of DNA fragmentation which is characteristic of apoptosis, were observed at only 10 $\mu\text{g/ml}$ of MZR over 72 h-incubation significantly. In terms of the Bcl-2 expression of synovial fibroblasts, up to 10 $\mu\text{g/ml}$ of MZR has no effect on the expression of this protooncogene bcl-2 expression. These results suggest that MZR might suppress the growth of rheumatoid pannus by inhibition of synovial fibroblast proliferation partially through the induction of apoptosis of synovial fibroblast without modulating Bcl-2 expression.

INTRODUCTION

In rheumatoid arthritis (RA), it is characterized by pronounced synovial hyperplasia composed of the extensive proliferation of synovial fibroblasts and inflammatory cell infiltration with neovascularization^{5, 28}. Pannus tissue that is the hyperplastic synovium invades the surface of articular cartilage and subchondral bone adjacent to the synovial cartilage junction lead to deteriorate the joint function. Also, it secretes various kinds of growth factors and inflammatory cytokines, which stimulate the growth of pannus tissue itself. Thus, synovial hyperplasia has a critical role in the propagation of rheumatoid synovitis¹⁹ and, therefore, interruption of synovial proliferation may reduce pannus growth and synovial fibrosis in RA .

Disease modifying anti-rheumatic drugs (DMARDs) are reported to not only ameliorate the symptoms of RA patients but also slow to down the progression of disease⁶. A variety of inhibitory effects of these drugs on immune competent cells such as macrophages or T/B lymphocytes are reported to be responsible for their therapeutic efficacy. Recently, immunosuppressive agents such as methotrexate (MTX) are also used in the treatment of RA with beneficial effect². As well as DMARDs, MTX is reported to suppress a variety of immune reactions due to cytotoxic effect on lymphocytes. Thus, it is suggested that immunosuppressive agents may present anti-rheumatic actions through the inhibition of immune competent cell function in vivo.

Since 1980, Mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate, MZR) has been administered to the patients with renal transplantation^{10, 25} or lupus nephritis as an immunosuppressive agent¹². It was reported to have beneficial effects in RA due to the suppression of lymphocyte function⁷. We have also found that MZR suppressed the proliferation of synovial fibroblasts obtained from RA patients through the inhibition of de novo synthesis of guanine ribonucleotides, which causes the depletion of intracellular guanosine triphosphate pool²². However, it is still unclear how MZR exerts its beneficial effects on patient's clinical courses in the treatment of RA .

It is generally accepted that there is a close relationship between cell proliferation and cell death, and that there might be an imbalance between the growth and death of synovial cell in RA, which lead to synovial hyperplasia¹⁸. Therefore, induction of apoptosis in RA synovial fibroblasts is proposed to be one of the promising ways for the treatment of RA by way of the reduction of synovial hyperplasia in situ. MZR is reported to induce DNA fragmentation in CEM leukemia cell³, which suggest the MZR might induce DNA fragmentation of synovial fibroblasts in RA patients. Therefore, in the present study, we have examined the effect of MZR on DNA fragmentation in synovial fibroblasts obtained from RA patients in vitro.

MATERIALS AND METHODS

1. Preparation of synovial fibroblast monolayer cultures

Synovial tissue samples were surgically obtained, after consent, from the patients with RA at the time of total knee arthroplasty. Diagnosis of RA was based on

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the revised criteria for the classification of rheumatoid arthritis by American Collage of Rheumatology in 1987. Synovial fibroblasts were isolated as described previously with some modifications²¹. Briefly minced synovial tissue were enzymatically dissociated using 0.2 % of collagenase (Sigma Chemical Co., St. Louis, MO) and 0.25 % of trypsin-EDTA (DIFCO LABORATORIES, Detroit, MI) at 37 °C for 2 hours with gentle stirring. The dissociated cells were suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL., Grand Island, NY, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Biowhittaker., Walkersville, ML USA), 100units/ml penicillin and 100 ug/ml streptomycin and cultured in tissue culture flask (Corning Incorporated, Corning, NY) at cell density of 10^5 cell/cm². The next day, culture flasks were vigorously rinsed to remove non-adherent lymphoid cells with fresh DMEM, and resultant adherent synovial fibroblasts were then cultured with synovial fibroblast culture medium. When the primary cultures reached confluence, cells were trypsinized, resuspended in synovial fibroblast culture medium, and seeded into 75-cm² tissue culture flask at cell density of 10^5 cell/cm² for further passage. All the experiments described below were conducted using cells at the second or third passage.

2. Assay for tritiated thymidine (³H]-TdR) incorporation into synovial fibroblasts

In order to examine the effect of MZR on DNA synthesis of synovial fibroblasts, ³H-TdR incorporation into the synovial fibroblasts was quantified. Ten thousand of synovial fibroblasts were allowed to attach in every well of 96-well flat-bottomed culture plates (Corning Incorporated, Corning, NY, USA) in DMEM with 10%FBS and cultured for 72 hours. Fifteen hours before terminating proliferation assay of incubation time, 37 kBq of ³H-TdR (DuPont/NEN Research Products, Boston, MA) were added to each well. At the end of proliferation assay, synovial fibroblasts were washed three times with distilled water. One hundred µl of scintillation liquid were added and cell associated ³H-TdR was determined using scintillation counter (PACKARD INSTRUMENT COMPANY, Mariden, CT, USA).

3. Quantification of DNA fragmentation of synovial fibroblasts in the cultured supernatant by enzyme linked immunosorbent assay (ELISA)

One hundred thousand of synovial fibroblasts were seeded in each well of 24-well flat bottomed culture plate and cultured for 1 days in DMEM supplemented with 10% FBS. On the following day, after gently washed with 0.05 M phosphate buffered saline (PBS; pH 7.4), medium was replaced to DMEM with 10%FBS, containing varying concentrations of MZR and

incubated for additional 72 h. After the incubation periods, the cultured supernatant were collected and centrifuged at 15000 rpm for 10 minutes to remove cell debris and high molecular weight DNA.

Fragmented DNA in the culture supernatant was measured by quantitation of cytosolic oligonucleosome-bound DNA with a Cell Death Detection ELISA kit (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions. This assay was based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histone. This allowed the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysate.

4. Bcl-2 ELISA

Synovial fibroblasts were cultured in a 24-well plate at cell density of 1.0×10^5 /well for 1 days in DMEM supplemented with 10% FBS. On the following day, after gently washed with 0.05 M PBS, medium was replaced to DMEM with 10% FBS, containing varying concentrations of MZR and incubated for 72 h. After the incubation periods, synovial fibroblasts were lysed in PBS containing 1% sodium dodecyl sulfate and 10% Tween 20 (Wako Pure Chemical Industries LTD., Osaka, Japan) at 4 °C for 30 min. The cell lysate were centrifuged at 15000 rpm for 10 minutes and the supernatant was collected. Bcl-2 protein concentration in the synovial fibroblasts were then measured by using commercially available ELISA kit (T Cell Diagnostics, Inc., Woburn, MA, USA) according to the manufacture's instruction.

5. Data analysis

All data were analyzed by a one-way analysis of variance followed by a Student's-Newman-Keuls post hoc test using the InStat statistical program (Graph Pad, San Diego, CA, USA). To confirm the reproducibility of the experiments performed in this study, assay for [³H]-TdR incorporation into synovial fibroblasts were repeated more than three times and ELISA was employed twice for measuring either DNA fragmentation or Bcl-2 protein concentration in culture medium. Data represent means \pm standard deviation of at least 6 samples and were considered significantly different when P value < 0.05.

RESULTS

1. Effects of MZR on ³H-Tdp incorporation into synovial fibroblasts

In order to investigate whether MZR suppresses DNA synthesis, synovial fibroblasts were treated with MZR at concentrations ranging from 0.1 to 10 μ g/ml. Addition of MZR to synovial fibroblast cultures suppressed ³H-TdR incorporation as reported previously²². Incubation with a varying concentration of MZR demonstrated that ³H-TdR uptake into synovial fibroblasts was decreased by MZR in a dose dependent manner up to 10 μ g/ml. At 1 μ g/ml of MZR, ³H-TdR

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incorporation of synovial fibroblast was reduced in comparing to the control. Up to 10 $\mu\text{g/ml}$ of MZR, incorporation of $^3\text{H-TdR}$ was further reduced and statistical significance of the results was observed at more than 1 $\mu\text{g/ml}$ of MZR (Fig. 1). This concentration is attainable in the serum of rheumatoid patients treated by MZR⁷.

2. Effect of MZR on DNA fragmentation of SFB

Mizoribine is reported to induce guanine nucleotide depletion, which results in inhibition of the entry of T cell into S from G1 phase of the cell cycle and leads to inhibition of DNA replication²⁴. It is also reported that inosine monophosphate (IMP) dehydrogenase inhibition by MZR induced a DNA fragmentation of CEM leukemia cell³ for over 4 hour incubation. Therefore, the effect of MZR on DNA fragmentation of synovial fibroblast was investigated in order to examine whether or not MZR induced synovial fibroblast-apoptosis in vitro.

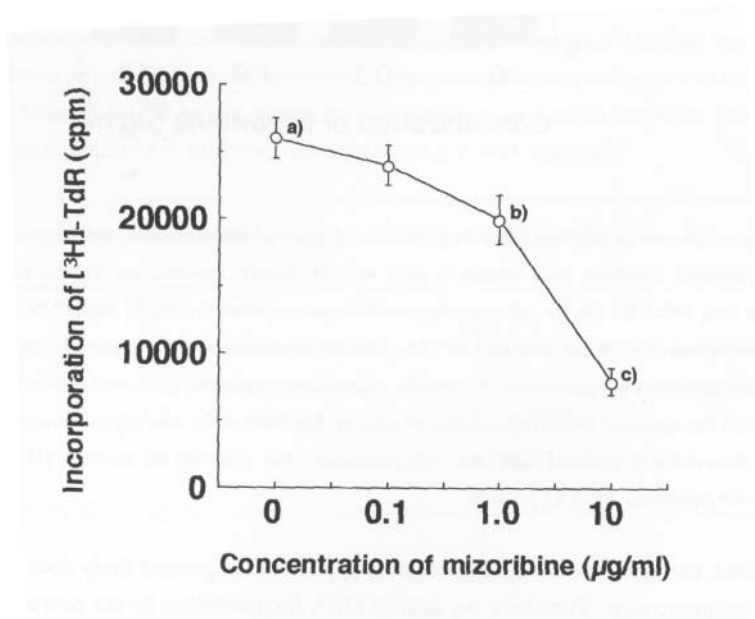


Figure 1. Effect of MZR on ^3H -thymidine incorporation into synovial fibroblast from RA patients. Ten thousand of synovial fibroblasts were allowed to attach in every well of 96-well flat-bottomed culture plates in DMEM with 10% FBS and cultured for 72 hours. Mizoribine suppressed $^3\text{H-TdR}$ incorporation into the synovial fibroblast in a dose dependent fashion. Significant inhibition ($P < 0.01$) was observed at the concentration of over 1 $\mu\text{g/ml}$ MZR. Data represent the means \pm SD from ten samples. a) vs. b): $p < 0.05$. a) vs. c): $p < 0.01$.

MZR has increased DNA fragmentation in the dose dependent manner shown in Figure 2 . At 1 μ g/ml of MZR, DNA fragmentation of synovial fibroblast was promoted in comparing to the control. A maximal 1.3-fold increase in DNA fragmentation was detected at a concentration of 10 μ g/ml of MZR.

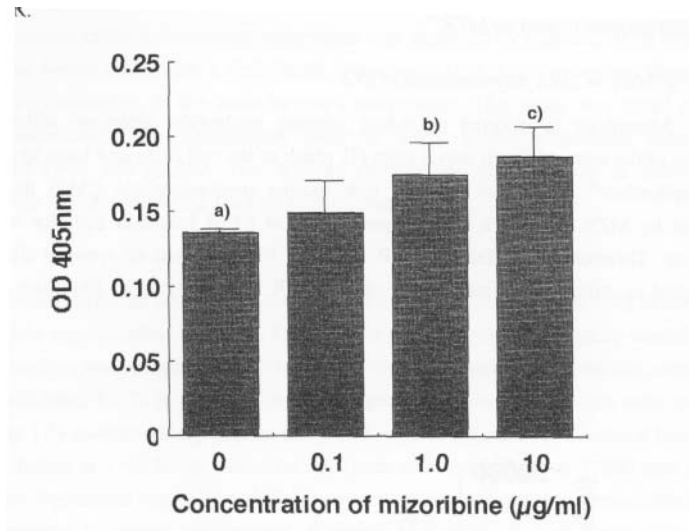


Figure 2. Dose-responses of MZR on DNA fragmentation of synovial fibroblast from RA patients. One hundred thousand of synovial fibroblast were seeded in each well of 24-well flat-bottomed culture plate in DMEM supplemented with 10%FBS. On the following day, medium was replaced to DMEM with 10%FBS, containing varying concentrations of MZR and incubated for 72 h. After the incubation periods, fragmented DNA in the culture supernatant was measured by quantitation of cytosolic oligonucleosome-bound DNA with a Cell Death Detection ELISA kit. MZR has increased DNA fragmentation of synovial fibroblast in the dose dependent manner. Significant increase was observed at 10 μ g/ml of MZR over 72-h incubation. Data represent the means \pm SD from 6 samples. a) vs. b): p = not significant, a) vs. c): p < 0.05.

Indeed, the assay for DNA fragmentation applied in the present study does not define the number of apoptotic cells. Therefore, the data on DNA fragmentation do not provide information on the percentage of cells that underwent apoptosis in a given culture. The percentage of cells that synthesize DNA and incorporate 3 H-TdR is also not indicated. It is possible that MZR may affect distinct sub-populations of synovial fibroblasts such as in G1 phase and suppress DNA synthesis in one subset and induce apoptosis in another. In order to detect which cells in culture as apoptotic, further experiments should be performed with an assay that allows localization and quantification of the number of cells that undergo apoptosis in the presence of MZR.

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3 . Effect of MZR on Bcl-2 expression of synovial fibroblasts

Members of the Bcl-2 family are involved in the regulation of apoptosis in a variety of cell types ^{11, 20}. It is also reported that Bcl-2 expressing synovial cells could be detected only in the upper lining layers and might contribute to lack of apoptosis in the lining layer of proliferating synovium¹⁵. Therefore, in order to study whether MZR affect the expression of protooncogene bcl-2 in synovial fibroblasts, quantification of cell associated anti-apoptosis molecule, Bcl-2 protein was performed by ELISA.

When synovial fibroblasts were incubated with up to 10 µg/ml of MZR, it has failed to affect the Bcl-2 expression in synovial fibroblasts (Table), by which concentration MZR markedly induced DNA fragmentation of synovial fibroblast from RA patients. These observations suggest that MZR might induce DNA fragmentation without modulating the Bcl-2 expression in synovial fibroblast.

Table. Effect of MZR on Bcl-2 expression of synovial fibroblast. Synovial fibroblasts were cultured in a 24-well plate at cell density of 1.0×10^5 /well for 1 days in DMEM supplemented with 10% FBS. On the following day, medium was replaced to DMEM with 10% FBS, containing varying concentrations of MZR and incubated for 72 h. After the incubation periods, Bcl-2 protein concentration in the synovial fibroblast cell lysate were then measured by ELISA. Up to 10 µg/ml of MZR has not affected the Bcl-2 expression on synovial fibroblasts. Data represent the means \pm SD from 6 samples. a) vs. b): p = not significant. a) vs. c): p = not significant.

Mizoribine (µg/ml)	Bcl-2(U/ml)
0	14.6 \pm 0.797 ^{a)}
0.1	15.6 \pm 1.67
1.0	15.1 \pm 0.636 ^{b)}
10	15.5 \pm 0.912 ^{c)}

DISCUSSION

Rheumatoid arthritis is consider to be a chronic inflammatory disorders accompanied by neovascularization, massive infiltration of inflammatory cell. Tumor-like growth of synovial fibroblasts yields to pannus tissue. Invasion of articular cartilage and subchondral bone by these pannus tissue results in the deterioration of joint functions ^{5, 28}

Recently, immunosuppressive agents have been used in the treatment of RA with

beneficial effect on patient's clinical course¹³. However, the mechanisms of anti-rheumatic action of these drugs have not been fully understood. MZR is a new imidazole nucleoside isolated from the culture medium of the mold, *Eupenicillium brefeldianum* M-2166 found in the soil of Hachijo Island, Tokyo Japan in 1974⁷. It inhibits the de novo biosynthesis of purines. Phosphorylated to MZR-5'P (MZR-5'-monophosphate) by adenosine kinase in the cells, MZR inhibits GMP synthesis by the antagonistic blocking of IMP dehydrogenase, which reduced intracellular XMP concentration. GMP synthetase is also inhibited competitively in the pathway from IMP to GMP of purine synthesis system. MZR was reported to suppress both humoral and cellular immunity by selectively inhibiting the proliferation of lymphocyte through depletion of intracellular GMP. MZR has been approved in Japan to have a clinical efficacy for the treatment of RA in 1992, because it has been shown to markedly inhibit the adjuvant arthritis in rat by suppression of T cell function⁹ and be associated with a low incidence of adverse effect clinically. Thus, MZR is used for the treatment of RA patients with beneficial effects. However, only the suppression of lymphocyte proliferation was reported as a possible mechanism underlying its effect. Therefore, in an attempt to elucidate the mechanisms of action of MZR in RA, we have investigated its effects on DNA synthesis and apoptosis of synovial fibroblast from RA patients. Our data demonstrated that MZR inhibited DNA synthesis by synovial fibroblasts in vitro in a concentration-dependent manner as previously reported²². In terms of SFB proliferation, significant inhibitory effect of MZR was observed at a concentration of 1 µg/ml. This concentration of MZR is attainable clinically in the sera of RA patients administered with MZR. These observations strongly suggest that MZR may play an inhibitory role in the growth of rheumatoid pannus by inhibiting synovial fibroblast proliferation in vivo.

Over recent years, evidence has accumulated indicating that apoptosis is not limited to immature thymocytes but can be similarly triggered in mature cells in physiological and pathological conditions^{8, 27}. Cell proliferation and apoptosis regulate the homeostasis of cells and tissues. Dysregulation of this cycle is critical to development of disease resulting in cancer and autoimmunity. In RA, synovial cells are reported to be capable of synthesis of various kinds of cytokines and growth factors and proliferate by themselves^{5, 28}. Due to the proliferative nature of RA synovium, it can be speculated that the continuing growth of synovial fibroblasts is due to an impaired balance of growth (cell proliferation) and death (apoptosis) by various stimuli including cytokines and protooncogene, such as c-fos and c-myc induction¹. It is, in particular, revealed that IL-1 down-regulates expression of Fas (AP0-1/CD95) antigen, which is a widely expressed membrane-anchored protein inducing apoptosis, and inhibit apoptosis of synovial cells resulting in synovial hyperplasia²³. Thus, very few Fas molecule appears to be capable of triggering apoptosis in RA synovial fibroblasts and a certain percentage of Fas molecules expressed on

synovial fibroblasts in functional because Anti-Fas antibodies can induced apoptosis of RA synovial cells in vitro¹⁷. Also, various kinds of stimuli such as growth factor deprivation and Fas/FasL system are reported to induce apoptosis of synovial fibroblast²⁶. Although it is difficult

to induce apoptosis in terminally differentiated cells, the use of a potent induced of apoptosis of rheumatoid synovium may serve as a novel and effective strategy for the

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treatment of RA.

In the present study, MZR is shown to suppress DNA synthesis and promote a small but significant amount of DNA fragmentation of synovial fibroblast originated from rheumatoid patients in the dose dependent fashion. These observations suggest that MZR might induce

apoptosis of synovial fibroblast in RA in vivo. In terms of the induction of cell death by immunosuppressive agents, it is demonstrated that inhibition of IMP dehydrogenase by MZR induced an apoptosis of CEM leukemia cell. It is also reported that MZR impeded ³H-TdR incorporation after as little as 1 h of treatment and greater degree of depletion of cellular GTP by the incubation with MZR for longer duration caused cell death with characteristics of apoptosis in insulin-secreting β -cell lines¹⁴.

The detail intracellular mechanism of apoptosis of synovial fibroblast is, however, still unknown. It is suspected that Bcl-2 interferes induction of apoptosis mediated by various stimuli such as Fas/FasL mediated pathway in lymphoid cells^{11, 20}. The expression of bcl-2 of synovial fibroblast from RA patient is reported not to be affected by IL-1 β . Apoptosis of synovial fibroblast seems to be mediated by modulation of Fas antigen expression but not Bcl-2 expression²³. In this study, MZR has no effect on the expression of protooncogene bcl-2 of synovial fibroblast up to 10 μ g/ml. These findings indicate that MZR might induce an apoptosis of synovial fibroblast as insulin-secreting β -cell lines due to inhibition of IMP dehydrogenase, which lead to GTP depletion without modulation of bcl-2 protein expression. These observations suggest that MZR may suppress the process involved in synovial hyperplasia due to not only the inhibition of DNA replication by GTP depletion but also induction DNA fragmentation of synovial fibroblasts. Further investigation is required and efforts are underway to determine whether MZR may modulate Fas/FasL system, which is another important pathway for triggering apoptosis in synovial fibroblasts.

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