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Mizoribine-Mediated Apoptotic Signaling Pathway in Human T-Cell Line

K.W. Seo, H.K. Lee, S.J.N. Choi, B.J. So, S.K. Kim, and S.Y. Chung

ABSTRACT

Mizoribine (MZR), an inhibitor of inosine monophosphate dehydrogenase, which depletes cellular guanadine triphosphate, is an immunosuppressive drug. The aim of this study was to evaluate the mechanism by which MZR exerts cytotoxic effects on human Jurkat T cells. Our study showed that MZR-induced apoptotic death of human Jurkat T cells is dose-dependent and time-dependent, as revealed by chromatin condensation and H2AX phosphorylation. Furthermore, MZR increased the catalytic activity of caspase family cysteine proteases, including caspase-3, caspase-8, and caspase-9, in human Jurkat T cells. In conclusion, MZR induces the apoptotic death of human Jurkat T cells via activation of caspase family proteases as well as by mitochondrial dysfunction.

INOSINE MONOPHOSPHATE DEHYDROGENASE is an essential rate limiting enzyme in the purine metabolic pathway required for lymphocyte proliferation.[1] Mizoribine (MZR) inhibits lymphocyte proliferation via blockade of the IMPDH to guanosine triphosphate biosynthesis pathway.[2] This study was designed to elucidate the mechanism by which MZR exerts cytotoxic effects on human Jurkat T cells. After treatment with MZR, human Jurkat T cell line viability, H2AX phosphorylation, cytosolic release of cytochrome *c* from mitochondria, changes in mitochondrial transmembrane potential, enzymatic activity of caspase family proteases, and expression of Bcl 2 family proteins were measured using a variety of microscopic and biochemical techniques.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Human Jurkat T cells, a T-cell line, obtained from the Korean Type Culture Collection, were maintained in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum at 37°C with 5% carbon dioxide. Mizoribine (Sigma, St Louis, Mo) was dissolved at the concentration of 5 mg/mL in dimethylsufoxide, maintained at −20°C, and diluted in RPMI-1640 to final concentrations of 0.1 to 100 μmol/L. Guanosine (Sigma), melted to a concentration of 0.1 mmol/L in dimethylsulfoxide, was also diluted in the RPMI.

Measurement of Cell Viability

After cells were treated with ascending concentrations (0.5 to 100 μg/mL) of MZR, cell viability was measured using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay at 24 and 48 hours after MZR treatment. The absorbance at 540 nm in each well was determined with an automatic plate reader (Thermo Max).

Hoechst Staining of Nucleus

Cells were treated with 10 μg/mL of MZR for various periods. After staining with 10 μmol/L of Hoechst 33342, cells were visualized under ultraviolet light Leica MPS 60).

Enzymatic Activity of Caspase Family Proteases

Cells treated with 10 μg/mL of MZR for various periods were lysed to measure the activity of caspase proteases using fluorogenic peptide substrates (Ac-DEVD-AMC, caspase-3; Z-IETD-AFC, caspase-8; Ac-LEHD-AFC, caspase-9). Poly(adenosine diphosphate ribose) polymerase (PARP) cleavage was measured by Western blotting with anti-PARP antibody.

Western Blotting

Stimulated human Jurkat T cells were harvested and washed with phosphate-buffered saline solution, pH 7.4. Whole-cell lysate was quantified with a Bicinchroninic acid kit (Sigma), separated with sodium dodecylsulfate–polyscrylamide gel electrophoresis, and transferred onto nitrocellulose membranes. After blocking with 5% skim milk in TBS-T (25 mmol Tris, pH 7.6; 138 mmol sodium

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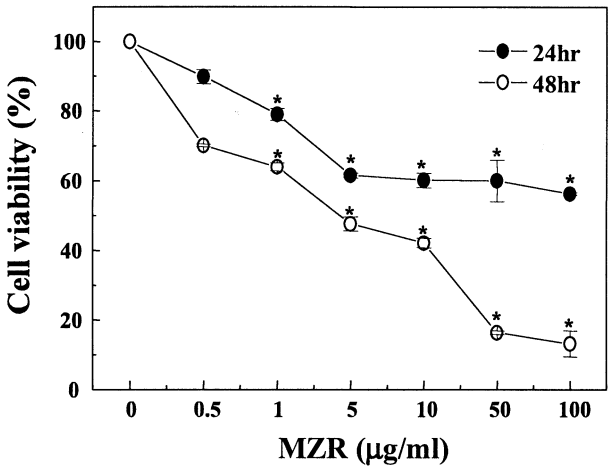

Fig 1. Mizoribine (MZR) decreased the viability of Jurkat T cells in dose-dependent and time-dependent manners. Cells were treated with various concentrations of MZR; then cell viability was measured by MTT assay at 24 and 48 hours after MZR treatment. Data represent the mean ± SD of quadruplicates. *$P < .05$ (Student *t* test), compared with control group.

chloride; 0.05% Tween 20), the membrane was incubated with anti-PARP, anti-phospho-H2AX, anti-Bcl-XL/S, anti-Bak, anti-Bim, anti-VDAC, and anti-cytochrome *c* antibodies at room temperature for 2 hours. Immunoblots were visualized by electrochemiluminescence.

Changes of Mitochondrial Membrane Potential Transition

Cells were treated with 10 μg/mL of MZR for 12 hours and stained with JC-1 (Molecular Probes, Eugene, Ore), and mitochondrial membrane potential transition was measured by flow cytometry.

Flow Cytomery Measurement

After propidium iodide staining, cells were examined by flow cytometry (FACS Vantage Flow; Becton Dickinson Immunocytometry System, San Jose, Calif).

Two-Dimensional Gel Electrophoresis

After cells were treated with 10 μg/mL of MZR for 24 hours, protein extracts were subjected to 2-dimensional (2-D) electrophoresis. Protein spots visualized by silver staining were analyzed using software (PDQue at, Bio-Rad).

Statistical Analysis

All results are expressed as mean values ± SD of quadruplicates.

RESULTS

Treatment of MZR decreased human Jurkat T cell viability in dose-dependent and time-dependent manners (Fig 1). Mizoribine-induced cell death was confirmed as apoptosis, characterized by chromatin condensation and H2AX phosphorylation. Mizoribine increased the catalytic activity of caspase-3, caspase-8 and caspase-9 proteases (Fig 2). Activation of caspase-3 protease was further confirmed by

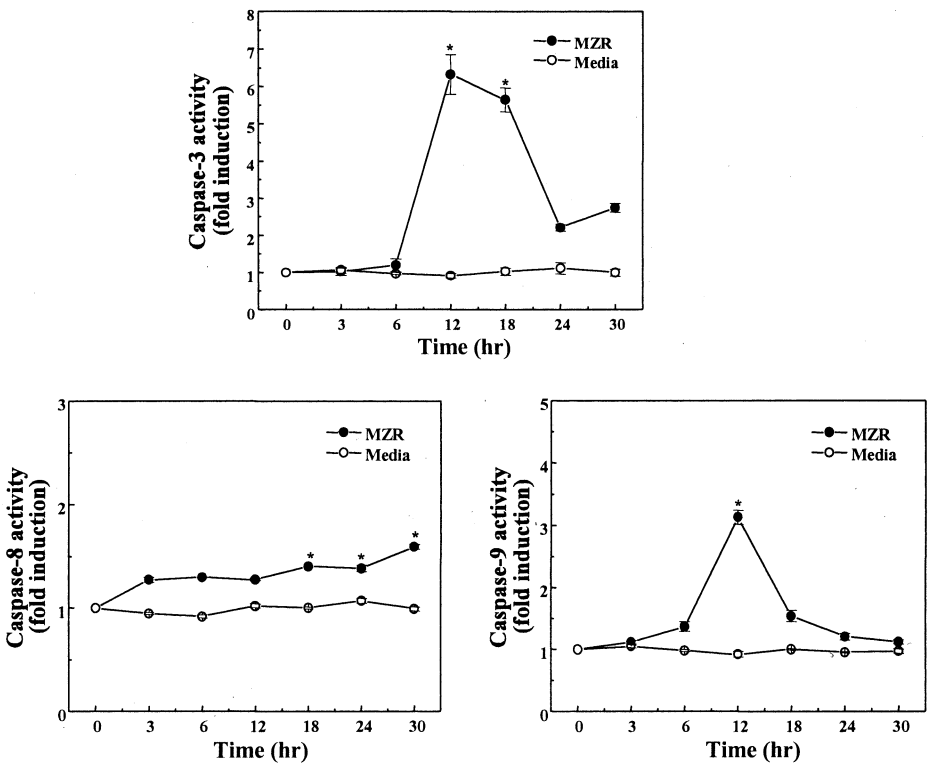

Fig 2. Mizoribine (MZR) increased the catalytic activity of caspase proteases of human Jurkat T cells. Cells were treated with 10 μg/mL of MZR for various periods, and lysed to measure the activity of these proteases by using fluorogenic biosubstrates. Lysate was reacted with Ac-DEVE-AMC as a substrate of caspase-3 protease **(A)**, Ac-VEID-AFC for caspase-8 protease **(B)**, and Ac-LEHD-AFC for caspase-9 protease **(C)**. Data represent the mean ± SD of quadruplicates. *$P < .05$ (Student *t* test), compared with control group.

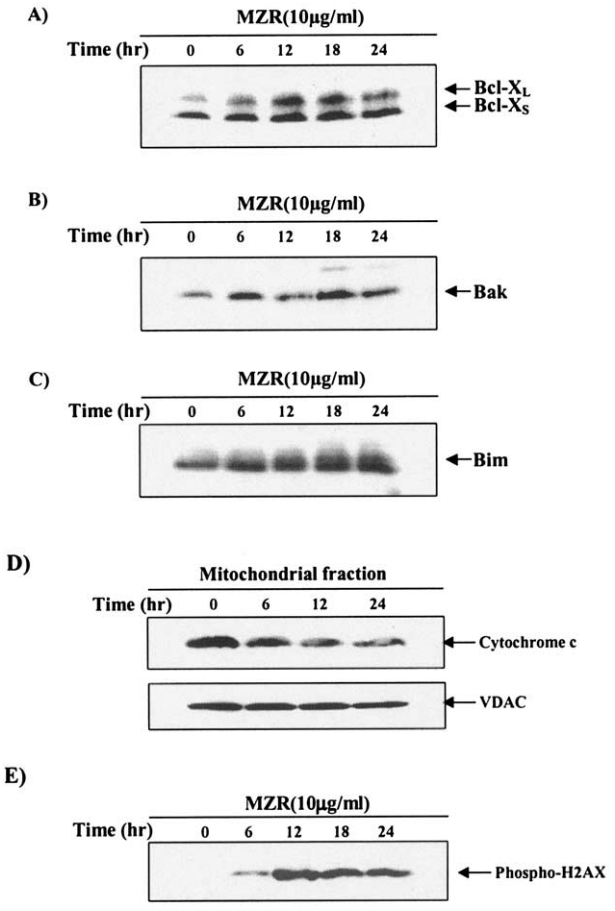

Fig 3. Expressions of Bcl-XL/S **(A)**, Bak **(B)**, and Bim **(C)** proteins in mizoribine (MZR) induced the cytosolic release of cytochrome *c* from mitochondria in Jurkat T cells **(D)** and the phosphorylation of H2AX in human Jurkat T cells **(E)**.

degradation of PARP, a substrate of capase-3 protease by MZR in Jurkat cells. In addition, MZR induced decreased Bcl-XL expression, whereas Bcl-XS, Bak, and Bim expression were increased, (Fig 3, A-C)). Guanosine markedly inhibited cell viability and apoptosis, with consistent suppression of the activity of caspase-8 protease, which is an upstream caspase among the caspase family, and PARP cleavage in MZR-treated cells. Furthermore, MZR induced changes in mitochondrial membrane potential transition and in the cytosolic release of cytochrome *c* from mitochondrial H2AX phosphorylation. Mizoribine also induced mitochondrial membrane potential transition of Jurkat T cells (Fig 4).

We screened the expression profile of proteins in Jurkat T cells using 2-D gel electrophoresis. Among 300 spots resolved in 2-D gels, comparison of control versus apoptotic cells revealed that the signal intensity of 10 spots was decreased and of 5 spots was increased (Fig 5).

DISCUSSION

Mizoribine, a potent inhibitor of IMPDH, is an effective immunosuppressive drug in human kidey transplantation.[3] Mizoribine inhibits both humoral and cellular immunities by blocking guanosine synthesis, and induces monocyte differentiation.[4] In addition, MZR has been proposed to have an immunosuppressive effect by induction of apoptosis.[5] The clinical application of MZR is to prevent rejection after organ transplantations;[6,7] however, the detailed mechanism is not yet clarified.

This study was designed to elucidate the mechanisms by which MZR exerts cytotoxic effects on human T lymphocytes. Our study showed that MZR-induced apoptotic death of human Jurkat T cells is dose-dependent and time-dependent, which was revealed by chromatin condensation and H2AX phosphorylation. Furthermore, MZR increased the catalytic activity of caspase family cysteine proteases, including caspase-3, caspase-8, and caspase-9 proteases in Jurkat T cells.

In conclusion, our findings suggest that MZR induces the apoptotic death of Jurkat T cells via activation of caspase family proteases, as well as by mitochondrial dysfunction. Further studies of target proteins are needed to identify targets to develop better immunosuppressive agents for clinical application.

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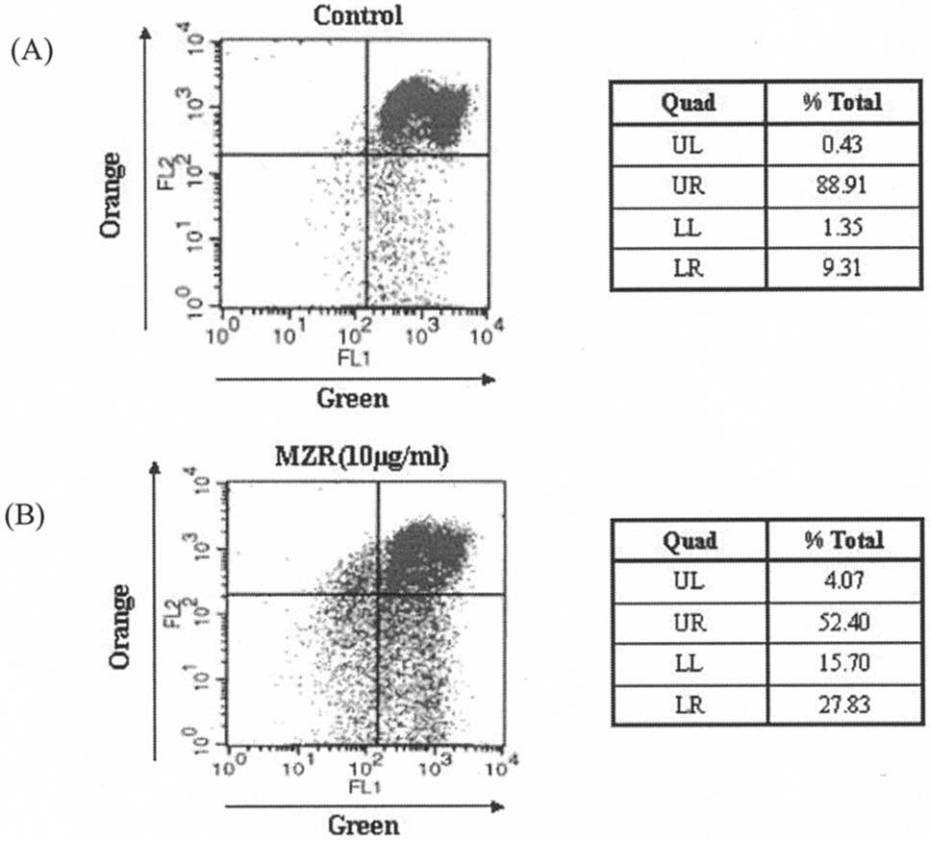

Quad	% Total
UL	0.43
UR	88.91
LL	1.35
LR	9.31

Quad	% Total
UL	4.07
UR	52.40
LL	15.70
LR	27.83

Fig 4. Mizoribine (MZR) induced the mitochondrial membrane potential transition of human Jurkat T cells. Cells were treated with 10 μg/mL of MZR for 12 hours and stained with JC-1, and then membrane potential transition was measured by flow cytometry. The data were 1 of 3 independent experiments. **A,** Control cells. **B,** Cells treated with MZR for 12 hours.

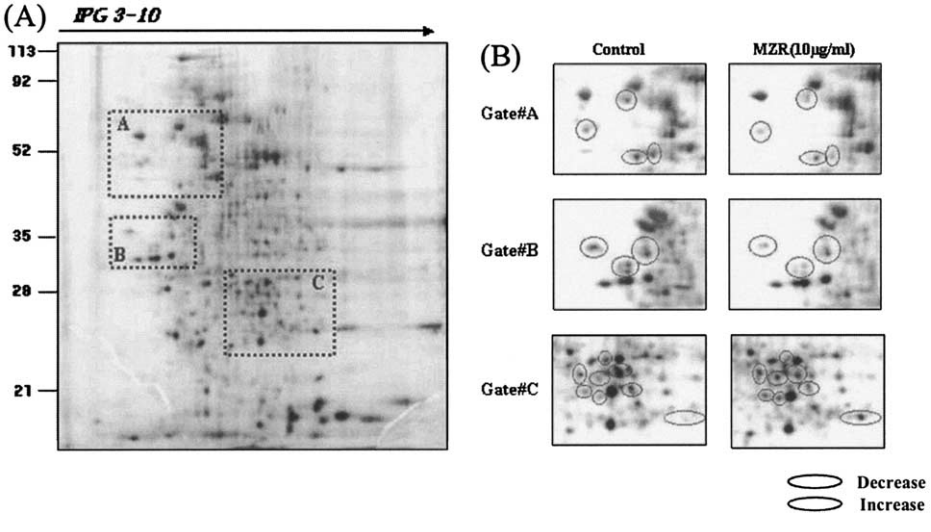

Fig 5. **A,** Silver staining of 2-dimensional polyacrylamide gel electrophoresis in Jurkat T cells treated with 10 μg/mL of mizoribine (MZR) for 24 hours. **B,** Comparison of respective gel sections (Gate#A, Gate#B, Gate#C) in human Jurkat T cells treated with 10 μg/mL of MZR for 24 hours.