Mizoribine Suppresses Proliferation of Rat Glomerular Epithelial Cells in Culture and Inhibits Increase of Monocyte Chemoattractant Protein-1 and Macrophage Inflammatory Protein-2 Stimulated by Thrombin

Hideaki Yamabe,* Michiko Shimada, Reiichi Murakami, Takeshi Fujita, Yuko Shimaya, and Norio Nakamura

Department of Nephrology, Graduate School of Medicine, Hirosaki University; 5 Zaifu-cho, Hirosaki 036–8562, Japan. Received December 16, 2011; accepted February 10, 2012; published online February 28, 2012

Glomerular crescents play an important role in progressive glomerular injury. The lesions consist of epithelial cells, macrophages and fibrin deposition. Macrophage chemoattractant protein-1 (MCP-1) is a chemoattractant of monocytes, which has a potential of procoagulant activity. Macrophage inflammatory protein-2 (MIP-2) is a chemoattractant of neutrophils and acute necrotizing injury is primarily mediated by neutrophils in crescentic glomerulonephritis. Mizoribine (MZR) is an immunosuppressive drug and it has been used for organ transplantation and treatment of various autoimmune diseases. The aim of this study is to investigate the effects of MZR on glomerular epithelial cells (GEC). Rat GEC were cultured with K1 medium and used from 12th to 14th passage. GEC proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MCP-1 and MIP-2 were quantified by enzyme-linked immunosorbent assay (ELISA) in culture supernatants and mRNA expressions of MCP-1 and MIP-2 were analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The proliferation of GEC was suppressed by MZR in a dose-dependent manner in the range of 1.0–100.0 μg/mL. These concentrations of MZR had no toxic effect to GEC. Thrombin (1.0–5.0 U/mL) enhanced the production of MCP-1, MIP-2 and the mRNA expressions of MCP-1 and MIP-2. The stimulatory effect of thrombin was inhibited by addition of MZR (10 μg/mL). It is concluded that MZR may be useful for the treatment of crescentic glomerulonephritis.

Key words mizoribine; glomerular epithelial cell; monocyte chemoattractant protein-1; macrophage inflammatory protein-2

Crescentic glomerulonephritis clinically presents a rapidly progressive glomerulonephritis. It is generally accepted that glomerular crescents play an important role in progressive glomerular injury. The lesions consist of proliferated epithelial cells, infiltrated macrophages and leucocytes, and deposits of fibrin and extracellular matrix. The process of leucocyte extravasation from the circulation to the site of inflammation involves a cascade of interactions between soluble factors and surface molecules expressed by leucocytes and endothelial cells.

Monocyte chemoattractant protein-1 (MCP-1) is a member of CC chemokines subfamily and it is a potent chemoattractant of monocytes. MCP-1 production has been shown by various types of cells in culture including glomerular epithelial cells (GEC).

Macrophage inflammatory protein-2 (MIP-2) is a member of CXC chemokines subfamily and it is a potent chemoattractant of neutrophils. In the rat, interleukin-8 (IL-8), a major neutrophils chemoattractant in humans, has not been identified with certainty, and MIP-2 may provide this function. MIP-2 is a major neutrophils chemoattractant contributing to influx neutrophils in antibody-induced glomerular inflammation in the rat. In crescentic glomerulonephritis, presumably, acute necrotizing injury is primarily mediated by neutrophils.

Fibrin formation within glomerular crescent has been observed in crescentic glomerulonephritis and is known to be involved in crescentic formation. Intraglomerular generation of thrombin may result from the glomerular activation of tissue factor, which has been reported to occur in human proliferative glomerulonephritis and in animal experimental glomerulonephritis. Thrombin is a serine protease and it has various biological effects besides its role in hemostasis. Thrombin stimulates the production of some cytokines by lymphocytes as well as the synthesis of tissue-type plasminogen activator and plasminogen activator inhibitor 1 by mesangial cells and epithelial cells, vascular smooth muscle cells and mesangial cells.

Mizoribine (MZR) is an immunosuppressive drug, which inhibits selectively inosine monophosphate dehydrogenase. It has been used for organ transplantation and treatment of various diseases such as autoimmune diseases. The aim of this study is to investigate the effects of MZR on cultured GEC.

MATERIALS AND METHODS

GEC Culture GEC culture was established by a modification of the method of Harper et al. Glomeruli were isolated from the kidneys of male Sprague-Dawley rats weighing 60–80 g (Charles River Japan Inc., Tokyo, Japan) with stainless steel sieves. Decapsulated glomeruli were placed on Vitrogen 100 gel (Collagen Corporation, Palo Alto, CA, U.S.A.), which is type 1 collagen derived from cattle skin. The glomeruli were nourished with K1-3T3 medium, which is a 1:1 mixture of K1 medium and conditioned medium of Swiss mouse 3T3 fibroblasts (American Type Culture Collection, Rockville, MD, U.S.A.). K-1 medium is a 1:1 preparation of Dulbecco’s modified Eagle’s minimum essential medium (DMEM, Gibco Laboratories, Grand Island, NY, U.S.A.) and Ham’s nutrient mixture F-12 (Gibco) containing 2% Nu-Serum (Collaborative Research, Bedford, MA, U.S.A.), and ITS premix (I: insulin, T: transferrin, S: serenium, Collaborate Research). Nu-Serum is an advanced, low-protein, cell growth medium supplement including epidermal growth factor, triiodothyronine, progesterone, estradiol, testosterone, hydrocortisone etc. When GEC

* To whom correspondence should be addressed. e-mail: yamabe@cc.hirosaki-u.ac.jp
grew up from the glomeruli, GEC were picked up and subcultured. The cells between 19th and 24th passage were used for the study.

Characterization of Cultured GEC The cells used in this study satisfied previously reported criteria as GEC. They showed cobblestone appearance under phase-contrast microscopic examination. In immunofluorescence study, they were positive for cytokeratin and FXIa. In addition, neither markers for mesangial cells (Thy1.1) nor endothelial cells (Factor VIII-related antigen) were detected in these cells. They had also susceptibility to low doses (10–100 µg/mL) of aminonucleoside puromycin (Sigma, St. Louis, MO, U.S.A.). Presently, it is not possible to determine specifically whether GEC in culture originate from visceral or parietal epithelium.

Evaluation of GEC Proliferation Proliferation of GEC was evaluated by 3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide (MTT) assay as described before. GEC were plated in wells of 96 well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, U.S.A.) at a density of 5000 cells/well. After overnight incubation with K1 medium, the cells were washed twice with Hank's balanced salt solution (HBSS, Gibco). The cells were incubated for 48h under various experimental condition. During the last 1.5h, 20 µL MTT solution (Promega, Madison, WI, U.S.A.) was added. Then optical density (OD) at 490nm with a reference of 650nm was measured by a microplate reader.

Effects of MZR on GEC Proliferation To examine the inhibitory effect of MZR on GEC proliferation, GEC were incubated in DMEM with 0.2% bovine serum albumin (BSA, Sigma) containing various concentrations of MZR (1.0–100.0 µg/mL) for 48h. Then GEC proliferation was evaluated by using MTT method. The cytotoxicity of MZR was evaluated by assessing the release of lactate dehydrogenase (LDH) from GEC. GEC were incubated in 24 well plates (Falcon, Becton Dickinson) with MZR (1.0–100.0 µg/mL) for 72h and the amount of LDH in the cell supernatants was measured. After removing the cell supernatants, GEC were lysed by melitin (50 µg/mL, Sigma) and the amount of LDH in the cells was also measured. LDH was quantified by a colorimetric method using LDH assay kit (Roche Diagnostics, Mannheim, Germany).

Assay of MCP-1 and MIP-2 MCP-1 and MIP-2 were quantified by an enzyme-linked immunosorbent assay (ELISA) using Immunoassay Kit (Biosource International, Inc., Camarillo, CA, U.S.A.). This assay is a sandwich ELISA using an antibody specific for rat MCP-1 and MIP-2. No significant cross-reactivity with other cytokines was observed. GEC were cultured in 12-well plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Confluent cells were washed twice with Hank’s balanced salt solution (Gibco) and incubated with DMEM containing 0.2% BSA for 24, 48, or 72h. Then the levels of MCP-1 and MIP-2 were measured in the culture supernatants. After removing culture supernatants, the cells in each well were dissolved in 1N NaOH and the protein content was measured by the method of Lowry et al. using BSA as the standard. Then the levels of MCP-1 and MIP-2 were expressed as pg per µg of GEC protein.

Quantitative PCR The confluent GEC cultured on the six-well plates (Becton Dickinson) were incubated with or without 5.0 U/mL thrombin for 24, 48 and 72h. The amount of MCP-1 and MIP-2 mRNA in GEC was quantified by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from the cells using an RNeasy Protect Mini Kit (QIAGEN, Valencia, CA, U.S.A.). The RNA was transcribed into the first-strand cDNA with an Omniscript RT kit (QIAGEN). Quantitative RT-PCR was performed using an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA, U.S.A.). Rat-specific primers for MCP-1 (Rn00580555_m1), MIP-2 (Rn00586403_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) were obtained from Applied Biosystems. The relative amounts of MCP-1 and MIP-2 mRNA in the samples were normalized by GAPDH mRNA.

Effect of MZR for the Production of MCP-1 and MIP-2 Stimulated by Thrombin We stimulated the production of MCP-1 and MIP-2 by GEC using 1.0–5.0 U/mL of α thrombin (Sigma) and examined the inhibiting effect of MZR (10 µg/mL) alone. The amount of MCP-1 and MIP-2 were measured in the culture supernatants.

Effect of MZR for the mRNA Production of MCP-1 and MIP-2 Stimulated by Thrombin We enhanced the mRNA of MCP-1 and MIP-2 in GEC using 5.0 U/mL of α thrombin (Sigma) and examined the inhibiting effect of MZR (10 µg/mL) alone. The mRNA of MCP-1 and MIP-2 were quantified in the GEC. We chose 5.0 U/mL of thrombin as the maximal dose because 5.0 U/mL of thrombin did not show the cell injury and we expected to get the certain results.

Statistical Analysis All data were expressed as mean ± standard deviation. Results were compared using one-way factorial analysis of variance (ANOVA) and multiple comparison tests p<0.05 was considered to be significant.

RESULTS

Effects of MZR on GEC Proliferation The proliferation of GEC was suppressed by MZR in a dose-dependent manner in the range of 1.0–100.0 µg/mL (Fig. 1). These concentrations of MZR had no toxic effect to GEC evaluated by LDH release (not shown).

Effect of MZR for the Increased Production of MCP-1 and MIP-2 Stimulated by Thrombin The stimulatory effect of thrombin (1.0 U/mL) on the production of MCP-1 and MIP-2 was inhibited by addition of MZR (MCP-1, control: 0.20±0.07 pg/µg cell protein, thrombin: 0.52±0.03 pg/µg cell protein, thrombin+MZR: 0.40±0.02 pg/µg cell protein, p<0.01 vs. thrombin, MIP-2, control: 4.06±0.08 pg/µg cell protein, thrombin: 11.67±0.54 pg/µg cell protein, thrombin+MZR: 8.77±1.40 pg/µg cell protein, p<0.001 vs. thrombin) (Fig. 2). The relative mRNA expressions of MCP-1 and MIP-2 stimulated by thrombin (5.0 U/mL) were also inhibited by addition of MZR (MCP-1, control: 1.00±0.1, thrombin: 9.75±0.4, thrombin+MZR: 7.52±0.8, p<0.01 vs. thrombin, MIP-2, control: 0.99±0.2, thrombin: 17.9±1.0, thrombin+MZR: 9.53±1.0, p<0.001 vs. thrombin) (Fig. 3).

DISCUSSION

Glomerular crescents are usually observed in rapidly
progressive glomerulonephritis. It is well known that blood coagulation process participates in the formation of crescents, because crescents consist of glomerular epithelial cells, macrophages and fibrin. Glomerular epithelial cells and macrophages are potent providers of tissue factor, which is activated by several cytokines. Therefore the association between coagulation and inflammation is generally accepted. In inflammation, vascular endothelial cells are activated and are injured, and consequently, blood coagulation occurs. Besides, inflammation occurs when blood coagulates. Thrombin has multiple biological functions in addition to its role in haemostasis. We have also reported that thrombin mediated various factors. In the previous study, we showed that thrombin increased the production of MCP-1 and MIP-2 by GEC and enhanced mRNA of these cytokines.

It is well known that neutrophils are involved in acute phase of inflammation and following macrophages infiltrate. MIP-2 (counterpart of IL-8 in human) is a potent chemoattractant of neutrophils. MCP-1 is a potent chemoattractant of macrophages. Liu et al. reported that the expression of MCP-1 was detected in glomeruli with crescents by immunohistochemistry. The biopsy specimens were taken from 32 patients and 8 normal kidneys. MCP-1 was undetectable in glomeruli of normal kidneys. In crescents, MCP-1 was detected in fibrocellular crescents and was more prominent in cellular crescents, but was undetectable in fibrous crescents. MCP-1 expressed mainly by CD68-positive macrophages and parietal epithelial cells in crescents. These observations suggest that MCP-1 may mediate the inflammatory process of crescent formation and progression to fibrosis. There is no report describing the role of MIP-2 in crescent formation. Weidner et al. reported that predominant glomerular infiltrating cells are macrophages and lymphocytes and granulocytes appeared in interstitium of ANCA-associated vasculitis. MCP-1 and MIP-2 produced by GEC may participate in not only glomerular but also interstitial cell infiltration. It seems important to inhibit GEC proliferation and the production of these cytokines for the treatment of glomerulonephritis.

MZR is an immunosuppressive drug, which inhibits selectively inosine monophosphate dehydrogenase. It has been used for organ transplantation and treatment of various diseases such as autoimmune diseases. Our study demonstrated that MZR clearly inhibits GEC proliferation and blocked the increase of MCP-1 and MIP-2 production enhanced by thrombin. The mechanism by which MZR inhibits GEC proliferation is unknown. Recently Liu et al. reported MZR inhibits mesangial cell proliferation through the S phase kinase-associated protein 2 dependent mechanism. Therefore the similar mechanism may exist in GEC.

MZR has been reported to attenuate tubulointerstitial fibrosis in rat models of unilateral obstruction, noninsulin-dependent diabetes, and peritoneal fibrosis via suppression of macrophages infiltration in the interstitium. Recently, Tanaka et al. reported MZR attenuates renal injury and intraglomerular infiltration of macrophages in patients with severe lupus nephritis. It is suggested that MZR inhibits MCP-1 production by GEC and may block the infiltration...
of intraglomerular macrophages. Nozu et al. reported that the blood concentration became 1.33 µg/mL or higher, when 300 mg/d MZR orally in two divided dose was administered. Thus 10 µg/mL MZR in our study is not so irrelevant dose. In conclusion, MZR suppressed the GEC proliferation and inhibited the enhancement of MCP-1 and MIP-2 production stimulated by thrombin. MZR may be useful for the treatment of crescentic glomerulonephritis.

REFERENCES