Thrombin enhances the production of monocyte chemoattractant protein-1 and macrophage inflammatory protein-2 in cultured rat glomerular epithelial cells

Takeshi Fujita, Hideaki Yamabe, Michiko Shimada, Reiichi Murakami, Ryuichiro Kumasaka, Norio Nakamura, Hiroshi Osawa and Ken Okumura

Department of Nephrology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan

Abstract

Background. Glomerular crescents play an important role in progressive glomerular injury. The lesions consist of epithelial cells, macrophages and deposits of fibrin and extracellular matrix. Monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) are members of chemokine subfamilies. MCP-1 and MIP-2 are potent chemoattractant leukocyte cytokines, and they may be involved in crescent formation. Thrombin participates in fibrin formation. We hypothesized that thrombin stimulates the production of MCP-1 and MIP-2 by glomerular epithelial cells (GECs).

Methods. Cultured rat GECs from the 19th to the 24th passage were used. We incubated GECs with or without thrombin to examine the effect of thrombin on the production of MCP-1 and MIP-2. The levels of MCP-1 and MIP-2 were measured in the cell supernatants by enzyme-linked immunosorbent assay (ELISA). The mRNA expressions of MCP-1 and MIP-2 were analysed by real-time reverse transcriptase–polymerase chain reaction (RT-PCR). We also examined the inhibitory effect of argatroban, a synthetic thrombin inhibitor, and prednisolone in the production of MCP-1 and MIP-2 stimulated by thrombin.

Results. Thrombin stimulated the production of MCP-1 and MIP-2 in a dose- and time-dependent manner. Thrombin also enhanced the mRNA expressions of MCP-1 and MIP-2 in the GECs. The stimulating effect of thrombin on the production of MCP-1 and MIP-2 was inhibited by the addition of argatroban or prednisolone.

Conclusions. We demonstrated a novel role of thrombin: it stimulates the production of MCP-1 and MIP-2 by GECs. It is clinically important that the inhibition of these chemokines leads to the improvement of crescentic glomerulonephritis. Anti-thrombin drugs and prednisolone may be useful in treating crescentic glomerulonephritis.

Keywords: GEC; MCP-1; MIP-2; thrombin

Introduction

Crescentic glomerulonephritis clinically presents as a rapidly progressive form of glomerulonephritis. It is generally accepted that glomerular crescents play an important role in progressive glomerular injury. The lesions consist of epithelial cells, macrophages and deposits of fibrin and extracellular matrix. The process of leukocyte extravasation from the circulation to the inflammation site involves a cascade of interactions between soluble factors and surface molecules expressed by leukocytes and endothelial cells [1].

Monocyte chemoattractant protein-1 (MCP-1) is a member of the CC chemokine subfamily, and it is a potent chemoattractant of monocytes [2]. MCP-1 has been shown to be produced by various types of cells in culture. It is already reported that glomerular epithelial cells (GECs) produce MCP-1 [3].

Macrophage inflammatory protein-2 (MIP-2) is a member of the CXC chemokine subfamily, and it is a potent chemoattractant of neutrophils. In the rat, IL-8, a major neutrophil chemoattractant in humans, has not been identified with certainty, and MIP-2 may provide this function. MIP-2 is a major neutrophil chemoattractant contributing to the influx of neutrophils in antibody-induced glomerular inflammation in the rat [4]. In crescentic glomerulonephritis, it is suspected that acute necrotizing injury is primarily mediated by neutrophils.

Fibrin formation within the glomerular crescent has been observed in crescentic glomerulonephritis and is known to be involved in crescent formation. Intraglomerular generation of thrombin may result from the glomerular activation of tissue factor, which has been reported to occur in human proliferative glomerulonephritis [5] and in animal experimental glomerulonephritis [6–8]. Thrombin is a serine protease, and it has various biological effects besides its role in haemostasis. Thrombin stimulates the production of some
cytokines by lymphocytes [9] as well as the synthesis of tissue-type plasminogen activator and plasminogen activator inhibitor-1 by epithelial cells [10,11], vascular smooth muscle cells [12] and mesangial cells [13].

In the present study, we investigated the effect of thrombin on MCP-1 and MIP-2 production by cultured rat GECs.

Methods

GEC culture

A GEC culture was established through a modification of the method of Harper et al. [14]. Glomeruli were isolated from the kidneys of male Sprague-Dawley rats weighing 60–80 g (Charles River Japan, Inc., Tokyo, Japan) using stainless-steel sieves. Decapsulated glomeruli were placed on Vitrogen 100 gel (Collagen Corporation, Palo Alto, CA, USA), which is type-1 collagen derived from cattle skin. The glomeruli were nourished with a 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, USA). The K-1 medium is a preparation consisting of a 1:1 mixture of Dulbecco's modified Eagle's minimum essential medium (DMEM, Gibco Laboratories, Grand Island, NY, USA), Ham's nutrient mixture F-12 (Gibco) containing 2% Nu-Serum (Collaborative Research, Bedford, MA, USA) and ITS premix (I: insulin, T: transferrin, S: serum; Collaborative Research). Nu-Serum is an advanced, low-protein cell-growth medium supplement that includes epidermal growth factor, triiodothyronine, progesterone, estradiol, testosterone, hydrocortisone, etc. When the GECs grew from the glomeruli, they were harvested and subcultured. Cells between the 19th and the 24th passage were used for the study.

Characterization of cultured GECs

The cells used in this study satisfied the previously reported criteria for GECs [15]. They had a cobblestone appearance under a phase-contrast microscope. In the immunofluorescence study, they were positive for cytokeratin and FX1A. In addition, neither the marker for mesangial cells (Thy1.1) nor that for endothelial cells (factor VIII-related antigen) was detected in these cells. They were also susceptible to low doses (10–100 µg/ml) of aminonucleoside puromycin (Sigma, St Louis, MO, USA). Presently, it is not possible to determine specifically whether the GECs in the culture originated from the visceral or the parietal epithelium. We used confluent cells throughout the study.

Assay of MCP-1 and MIP-2

MCP-1 and MIP-2 were quantified by enzyme-linked immunosorbent assay (ELISA) using an immunoassay kit (Biosource International, Inc., Camarillo, CA, USA). This assay is a sandwich ELISA using an antibody specific for rat MCP-1 or MIP-2. No significant cross-reactivity with other cytokines was observed.

GECs were cultured in 12-well plates. Confluent cells were washed twice with Hanks’ balanced salt solution (Gibco) and incubated with DMEM containing 0.2% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) for 24, 48 or 72 h. Then, the levels of MCP-1 and MIP-2 were measured in the culture supernatants. After removing the culture supernatants, the cells in each well were dissolved in 1 N 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 GECs cultured on six-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) were incubated with or without 5 U/ml thrombin for 24, 48 or 72 h. The amount of MCP-1 and MIP-2 mRNA in the GECs was quantified by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The total RNA was extracted from the cells using an RNeasy Protect Mini Kit (QIAGEN, Valencia, CA, USA). 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, USA). 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 using GAPDH mRNA. We chose 5 U/ml of thrombin as the maximal dose because 5 U/ml of thrombin did not show the cell injury and we expected to get the certain results.

Stimulation of the production of MCP-1 and MIP-2 by thrombin

We measured the MCP-1 and MIP-2 levels in the supernatants of the GECs incubated with 0.5 to 5.0 U/ml α-thrombin (Sigma) for 72 h to determine its dose-dependent effect. The time effect of thrombin was also examined by incubating GECs with or without 5.0 U/ml thrombin for 24, 48 or 72 h. In addition, we also examined the inhibitory effect of argatroban, a synthetic thrombin inhibitor (Mitsubishi Kasei Corporation, Tokyo, Japan), and prednisolone sodium succinate (Shionogi & Co., Ltd, Osaka, Japan). GECs were incubated for 72 h with 5.0 U/ml thrombin alone, 5.0 U/ml thrombin plus argatroban (1 µM) or argatroban (1 µM) alone. GECs were also incubated for 72 h with 5.0 U/ml thrombin alone, 5.0 U/ml thrombin plus prednisolone (10−5 M) or prednisolone (10−5 M) alone. Then, the MCP-1 and MIP-2 levels were measured in the culture supernatants. We estimated the cytotoxicity of thrombin, argatroban and prednisolone to the cultured GECs based on the LDH release. We ascertained that these agents did not show cytotoxicity at the dosages used in this experiment.

Statistical analysis

All data were expressed as mean ± SD. The results were compared using one-way factorial ANOVA and multiple comparison tests, and two-way repeated-measures ANOVA. P < 0.05 was considered to be significant.
Results

Stimulation of the production of MCP-1 and MIP-2 by thrombin

The levels of MCP-1 and MIP-2 in the GEC-culture supernatants after 72 h of incubation with thrombin are shown in Figure 1. The mean level of MCP-1 from three wells was 2.60 ± 0.35 pg/µg cell protein (thrombin: 0 U/ml), 4.72 ± 0.92 pg/µg cell protein (thrombin: 0.5 U/ml), 6.55 ± 1.68 pg/µg cell protein (thrombin: 2.0 U/ml) and 7.67 ± 1.45 pg/µg cell protein (thrombin: 5.0 U/ml) (Figure 1A). The mean level of MIP-2 from three wells was 15.97 ± 6.11 pg/µg cell protein (thrombin: 0 U/ml), 44.43 ± 1.96 pg/µg cell protein (thrombin: 0.5 U/ml), 42.20 ± 3.81 pg/µg cell protein (thrombin: 2.0 U/ml) and 52.20 ± 5.14 pg/µg cell protein (thrombin: 5.0 U/ml) (Figure 1B). Thrombin at 2.0 U/ml and 5.0 U/ml significantly increased MCP-1 production in a dose-dependent manner. Thrombin at 0.5 U/ml, 2.0 U/ml and 5.0 U/ml also significantly increased the level of MIP-2. The time effect of thrombin was also examined by incubating GECs with or without 5.0 U/ml thrombin for 24, 48 or 72 h. Thrombin significantly stimulated the production of MCP-1 (Figure 2A) and MIP-2 (Figure 2B) in a time-dependent manner.

Enhancement of the mRNA expression of MCP-1 and MIP-2 by thrombin

The relative MCP-1 mRNA expressions after 24 and 48 h of incubation were not different between the control and the GECs stimulated with thrombin (24 h: 1 ± 0.37 in the control versus 3.53 ± 1.42 with thrombin; 48 h: 1.68 ± 1.43 in the control versus 8.32 ± 5.91 with thrombin). However, after 72 h of incubation, thrombin significantly stimulated MCP-1 mRNA expression as compared with the control (72 h: 3.20 ± 0.81 in the control versus 8.32 ± 1.52 with thrombin, P < 0.05, Figure 3A). The relative MIP-2 mRNA expressions after 24 and 48 h were not different between the control and the GECs stimulated with thrombin (24 h: 1 ± 0.07 in the control versus 3.00 ± 1.93 with thrombin; 48 h: 0.91 ± 0.51 in the control versus 8.16 ± 2.57 with thrombin). After 72 h of incubation, thrombin significantly stimulated MIP-2 mRNA expression as compared with the control (72 h: 2.23 ± 2.49 in the control versus 68.98 ± 17.39 with thrombin, P < 0.05, Figure 3B).

Fig. 1. Thrombin stimulated the production of monocyte chemoattractant protein-1 (MCP-1) (A) and macrophage inflammatory protein-2 (MIP-2) (B) by GEC. Values are mean ± SD for three wells and representative data from one of two experiments are shown, respectively.

Fig. 2. Time course of the production of monocyte chemoattractant protein-1 (MCP-1) (A) and macrophage inflammatory protein-2 (MIP-2) (B) in the absence of thrombin (○), 5.0 U/ml thrombin (●). Thrombin stimulated the production of MCP-1 and MIP-2 in a time-dependent manner (P < 0.05). Values are the mean ± SD for three wells and representative data from one of two experiments are shown, respectively.
Thrombin enhances MCP-1 and MIP-2 of GECs

Fig. 3. Thrombin (5.0 U/ml) significantly stimulated mRNA expressions of monocyte chemoattractant protein-1 (MCP-1) (A) and macrophage inflammatory protein-2 (MIP-2) (B) compared with control in 72 h of incubation. Values are the mean ± SD for three wells and representative data from one of two experiments are shown, respectively.

Fig. 4. Argatroban (1 µM) inhibited the increasing effect of thrombin (5.0 U/ml) in the production of monocyte chemoattractant protein-1 (MCP-1) (A) and macrophage inflammatory protein-2 (MIP-2) (B) by GEC. Values are the mean ± SD for three wells and representative data from one of two experiments are shown, respectively.

Inhibitory effect of argatroban and prednisolone on the increased production of MCP-1 and MIP-2 with thrombin

Argatroban inhibited thrombin’s effect of stimulating the production of MCP-1 and MIP-2 by GECs (Figure 4). Prednisolone also inhibited thrombin’s effect of stimulating the production of MCP-1 and MIP-2 by GECs (Figure 5).

Discussion

Glomerular crescents are usually observed in rapidly progressive glomerulonephritis. It is well known that the blood coagulation process participates in the formation of crescents, because crescents consist of GECs, macrophages and fibrin. GECs 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 blood coagulation occurs continuously. Moreover, inflammation occurs when blood coagulates.

In this study, we showed that thrombin stimulated the production of MCP-1 and MIP-2 by GECs and enhanced the mRNA levels of these cytokines. It is reported that thrombin stimulates cell proliferation. In our study we used fully confluent cells and we did not observe a significant cell proliferation after 72 h of incubation with thrombin. Furthermore, we evaluated the levels of cytokines per the amount of cell protein and the levels of cytokine mRNA per the amount of GAPDH mRNA. Therefore we excluded that the increase in cytokine mRNA expressions is due to the increase in cell number. It is well known that neutrophils are involved in the acute phase of inflammation and the subsequent macrophage infiltration. MIP-2 (the rat counterpart of IL-8 in humans) is a potent chemoattractant of neutrophils. MCP-1 is a potent chemoattractant of macrophages. The measurement of these chemokines is useful for predicting the seriousness of inflammation. We measured these chemokines as markers of inflammation in the supernatants of cultured GECs. MCP-1 has been shown to be produced in various types of cells, such as vascular endothelial cells [16], smooth muscle cells [17], cardiomyocytes, fibroblasts [18], mesangial cells [19] and GECs [3]. Zhi-Hong et al. have reported that the expression of MCP-1
was detected in glomeruli with crescents by immunohistochemistry [20]. The biopsy specimens were taken from 32 patients and eight normal kidneys. MCP-1 was undetectable in the glomeruli of normal kidneys. Among the crescents, MCP-1 was detected in fibrocellular crescents and was more prominent in cellular crescents, but was undetectable in fibrous crescents. MCP-1 was 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. have reported that the predominant glomerulus-infiltrating cells are macrophages and lymphocytes, and that granulocytes appeared in the interstitium in ANCA-associated vasculitis [21]. MCP-1 and MIP-2 produced by GECs may participate not only in glomerular but also in interstitial cell infiltration.

Thrombin has multiple biological functions in addition to its role in haemostasis [22]. We have also reported that thrombin mediated various factors [23–28]. This study demonstrates that thrombin stimulates the production of both MCP-1 and MIP-2 by GECs. Thrombin is generated at sites of glomerular injury upon the activation of the intrinsic or extrinsic coagulation pathway, and it may then accelerate the production of MCP-1 and MIP-2. GECs may subsequently produce excessive extracellular matrix through a TGF-β-dependent mechanism and play a role in crescent formation.

We also demonstrated that argatroban and prednisolone inhibit the stimulating effect of thrombin on the production of MCP-1 and MIP-2. The protease activated receptor (PAR)-1 is a cellular receptor for thrombin. The contribution of PAR-1 to inflammatory cell-mediated renal injury has been assessed in murine crescentic glomerulonephritis [29]. The treatment of wild-type mice with a selective PAR-1-activated peptide (TRAP) augmented the histological and functional indices of glomerulonephritis [29]. Thrombin receptor activating peptide (TRAP) treatment did not alter the severity of GN in PAR-1-deficient (PAR-1−/−) mice [29]. These results indicate that the activation of PAR-1 by thrombin or TRAP amplifies crescentic glomerulonephritis. Argatroban competitively inhibits the binding of PAR-1 to thrombin. Therefore, our study suggests that anti-thrombin therapy may be effective in the treatment of crescentic glomerulonephritis. Some anti-thrombin drugs may reduce the magnitude of crescent formation via the inhibition of chemokine production. Natori et al. have reported that MCP-1 protein was detected in the conditioned medium from cultures of interleukin-1β-treated GECs, and that level was reduced by the prior addition of dexamethasone [30]. Recently, it has been shown that glucocorticoids are potent inhibitors of transcription factor NF-κB [31], which has been shown to control the expression of the human MCP-1 gene [32]. All of these findings, including our data, suggest that it is reasonable to use anti-thrombin drugs and corticosteroids in the treatment of crescentic glomerulonephritis.

It is concluded that thrombin stimulates the production of MCP-1 and MIP-2 and enhances the mRNA levels of these cytokines in GECs. It is clinically important that the inhibition of these chemokines leads to the improvement of crescentic glomerulonephritis: anti-thrombin drugs or prednisolone may mitigate the seriousness of crescentic glomerulonephritis.

Conflict of interest statement. None declared.

References


Received for publication: 26.3.08
Accepted in revised form: 29.5.08