Spontaneous activation of the NF-κB signaling pathway in isolated normal glomeruli

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FOR MANY YEARS, ISOLATED GLOMERULI have been used to elucidate a variety of glomerular function. Explantation of isolated glomeruli enables the determination of environmental factors that modulate proliferation, matrix metabolism, and generation of bioactive substances by “intact” glomerular cells (1, 3, 17, 30). It is also useful to identify factors that regulate glomerular vascular permeability and contraction (7, 28, 31). Combination with microperfusion techniques allows for elucidation of roles of vasoactive substances in the regulation of glomerular circulation and filtration (6). Despite the extensive use of isolated glomeruli for investigation, little has been understood about whether and how isolation and explantation affect the structure and function of the normal glomerulus.

We previously reported that ex vivo incubation of isolated normal glomeruli resulted in apoptosis of podocytes, activation of MAP kinases, and expression of genes regulated by activator protein 1 (AP-1) (10, 11). For example, ex vivo incubation of isolated glomeruli showed DNA fragmentation and positive staining for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling within 15 min (10). Histological analysis of isolated glomeruli revealed that a number of podocytes (>80%) showed condensed nuclear chromatin that is typical of apoptosis (10). Exploration of isolated glomeruli also affects the transcriptional profile of glomeruli. It is known that expression of stromelysin and monocyte chemoattractant protein 1 (MCP-1) is regulated by AP-1 (14, 21, 32, 34). Ex vivo incubation of isolated glomeruli showed spontaneous induction of stromelysin and MCP-1 (11), which was preceded by rapid upregulation of c-fos and c-jun as well as activation of MAP kinases including ERK, p38 MAP kinase, and JNK, without any external stimuli (11). These results indicated that, immediately after explantation, MAP kinase pathways are spontaneously activated, leading to consequent induction of the AP-1-dependent gene expression.

Ex vivo incubation of isolated glomeruli may also mobilize signal transduction other than the MAP kinase-AP-1 pathways. Recently, we found some evidence indicating that NF-κB may be spontaneously activated in explanted normal glomeruli. We previously reported that ex vivo incubation of isolated normal glomeruli resulted in apoptosis of podocytes, activation of MAP kinases, and expression of genes regulated by activator protein 1 (AP-1) (10, 11). For example, ex vivo incubation of isolated glomeruli showed DNA fragmentation and positive staining for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling within 15 min (10). Histological analysis of isolated glomeruli revealed that a number of podocytes (>80%) showed condensed nuclear chromatin that is typical of apoptosis (10). Exploration of isolated glomeruli also affects the transcriptional profile of glomeruli. It is known that expression of stromelysin and monocyte chemoattractant protein 1 (MCP-1) is regulated by AP-1 (14, 21, 32, 34). Ex vivo incubation of isolated glomeruli showed spontaneous induction of stromelysin and MCP-1 (11), which was preceded by rapid upregulation of c-fos and c-jun as well as activation of MAP kinases including ERK, p38 MAP kinase, and JNK, without any external stimuli (11). These results indicated that, immediately after explantation, MAP kinase pathways are spontaneously activated, leading to consequent induction of the AP-1-dependent gene expression.

MATERIALS AND METHODS

Cells and reagents. Mesangial cells (SM43) were established from isolated renal glomeruli of a male Sprague-Dawley rat and identified as being of the mesangial cell phenotype, as described before (18). Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 in air.

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SEAP28 cells seeded in 96-well plates (2 cross-feeding studies, as follows. SM/NF inhibitors (TMI-1 or TMI-2). The media were collected and used for

Twenty-four hours later, media were collected for SEAP assay.

Isolation and explanation of glomeruli. Normal glomeruli were isolated from adult male Sprague-Dawley rats (250–300 g body wt) using the conventional sieving method (11, 29). The animal experiments included in our paper had been approved by The Animal Experiment Committee at the University of Yamanashi. In brief, kidneys were minced well and forced through sequential steel sieves, and glomeruli were collected with the use of cold PBS. Ex vivo incubation of isolated glomeruli (7, 500 glomeruli/1.5 ml medium) was performed at 37°C for up to 24 h in the presence of 1% FBS. After the incubation, culture media and glomeruli were used for SEAP assay and Northern/Western blot analyses, respectively.

Creation of glomeruli transferred with NF-κB reporter cells. SM/NFxB-SEAP5 and SM/SV-SEAP28 control cells were established, as described before (8, 23). SM/NFxB-SEAP5 cells express SEAP under the control of κB enhancer elements (23). SM/SV-SEAP28 cells express SEAP under the control of the simian virus 40 early promoter and enhancer (8).

Coculture. Normal glomeruli (5,000 glomeruli/well) were seeded onto confluent cultures of SM43 cells or SM/NFxB-SEAP5 cells (2 × 10^5 cells/well) in 12-well plates. After 24 h, glomeruli were removed, and mesangial cells were harvested and subjected to Northern blot analysis. For SEAP assay, 1,500 glomeruli/well were seeded onto confluent cultures of mesangial cells (2 × 10^5 cells/well) in 96-well plates in the presence or absence of PD098059 (50 μM; MAP kinase kinase 1 inhibitor) or SB203580 (25 μM; p38 MAP kinase inhibitor). Twenty-four hours later, media were collected for SEAP assay.

Cross-feeding. To prepare media conditioned by glomeruli (GCM), 10,000 normal glomeruli were incubated for 24 h in 1 ml of culture medium containing 1% FBS, as described before (23). In some experiments, GCM were prepared in the presence of 1 μM TACE inhibitors (TMI-1 or TMI-2). The media were collected and used for cross-feeding studies, as follows. SM/NFxB-SEAP5 cells or SM/SV-SEAP28 cells seeded in 96-well plates (2 × 10^4 cells/well) were exposed to undiluted or two- to three-times diluted GCM for 24 h in the presence or absence of MG132 (25 μM; NF-κB inhibitor), PD098059 (50 μM), or SB203580 (25 μM). Twenty-four hours later, media were harvested and subjected to SEAP assay. For Northern blot analysis, confluent SM43 cells or SM/NFxB-SEAP5 cells in 12-well plates (2 × 10^5 cells/well) were exposed to GCM in the presence or absence of MG132 for 6 h and subjected to the assay. Fractionation of GCM was performed using Microcon Centrifuge Filters YM-50 (molecular wt <50 kDa, Nihon Millipore, Tokyo, Japan), as described before (8). After centrifugation at 10,000 g for 10 min, the filtered GCM was collected and used for studies.

Northern blot analysis. Total RNA was extracted from mesangial cells and isolated glomeruli by a single-step method, and Northern blot analysis was performed, as described before (16). cDNAs for SEAP (BD Biosciences), MCP-1 (27), and TNF-α (25) were used to prepare radiolabeled probes for hybridization. Expression of GAPDH was used as a loading control.

Western blot analysis. Western blot analysis was performed by the enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK), as described before (35). Primary antibodies used were anti-iκBα antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-iκBβ antibody (1:200 dilution; Santa Cruz Biotechnology). As a loading control, levels of β-actin were evaluated using anti-β-actin antibody (1:30,000 dilution; Sigma-Aldrich Japan, Tokyo, Japan).

SEAP assay. SEAP activity in culture media was evaluated by a chemiluminescent method using a Great EscAPe SEAP detection kit (Clontech, San Jose, CA) (12, 13, 35).

Statistical analysis. Experiments (reporter assays, formazan assay) were performed in quadruplicate. All experiments including Western/ Northern blots and DNA binding assay were repeated at least two to three times with consistent results. Data are expressed as means ± SE. Statistical analysis was performed using the nonparametric Mann-Whitney U-test to compare data in different groups. P < 0.05 was considered to be a statistically significant difference.

RESULTS

Spontaneous expression of MCP-1 in explanted glomeruli via autocrine/paracrine factors. As we previously reported (11), ex vivo incubation of isolated normal glomeruli exhibits spontaneous expression of MCP-1 (Fig. 1A). This induction is observed in blood-free glomeruli and even in the absence of FBS (11). The expression of MCP-1 in explanted glomeruli is dependent on the MAP kinase-AP-1 pathway, because 1) MAP kinases are activated in explanted glomeruli, and 2) treatment with MAP kinase inhibitors suppresses MCP-1 expression (11). Using coculture and cross-feeding, we first examined whether the spontaneous induction of MCP-1 is mediated by autocrine/paracrine factors produced by glomerular cells. Isolated normal rat glomeruli were seeded onto confluent culture of SM43 mesangial cells. After 24 h, glomeruli were removed, and mesangial cells were subjected to Northern blot analysis. As shown in Fig. 1B, significant induction of MCP-1 mRNA was observed in SM43 cells exposed to isolated normal glomeruli. This was further confirmed by cross-feeding studies. SM43 cells were exposed to media conditioned by normal glomeruli (GCM) for 6 h and subjected to assay. Northern blot analysis showed that GCM markedly induced expression of MCP-1 in mesangial cells (Fig. 1C). These results support the idea that isolated normal glomeruli produce autocrine/paracrine factors that trigger MCP-1 expression.
Spontaneous activation of NF-κB in explanted glomeruli.

Expression of MCP-1 in mesangial cells is known to be dependent on both AP-1 and NF-κB (22). Interestingly, like inhibitors of MAP kinases (11), MG132, an inhibitor of NF-κB, dramatically suppressed the induction of MCP-1 in explanted glomeruli (Fig. 2A). This result raised a possibility that the spontaneous induction of MCP-1 in explanted glomeruli is dependent not only on MAP kinases but also on NF-κB. Indeed, Western blot analysis revealed that protein levels of IκBα and IκBβ, inverse indicators for NF-κB activation, were markedly downregulated in glomeruli during ex vivo incubation (Fig. 2B). Furthermore, the binding activity of the p65 NF-κB subunit to the κB consensus sequence was significantly increased in explanted glomeruli (Fig. 2C), confirming activation of NF-κB in glomerular cells.

To examine whether NF-κB-activating factors are indeed produced by explanted glomeruli, we used a reporter mesangial cell SM/NFκB-SEAP5 that produces SEAP under the control of κB enhancer elements. Our previous data showed that this reporter clone secreted SEAP abundantly in response to stimulators of NF-κB including IL-1 and TNF-α, but not to other unrelated triggers such as phorbol ester, forskolin, and platelet-derived growth factor (23). Isolated normal rat glomeruli were seeded onto confluent cultures of reporter cells and incubated for 24 h. After the incubation, glomeruli were removed, and expression of SEAP was examined by Northern blot analysis.

Fig. 1. Spontaneous expression of monocyte chemoattractant protein-1 (MCP-1) in explanted glomeruli via autocrine/paracrine factors. A: isolated normal rat glomeruli were immediately stored at −80°C [incubation (−)] or incubated ex vivo at 37°C for 6 h [incubation (+)] and subjected to Northern blot analysis of MCP-1. B: SM43 rat mesangial cells seeded into 12-well plates (2 × 10⁵ cells/well) were coincubated with (+) or without (−) isolated glomeruli (G; 5000 glomeruli·well⁻¹·ml⁻¹) for 24 h. After incubation, glomeruli were removed, and mesangial cells were subjected to Northern blot analysis. C: SM43 cells were exposed to medium conditioned by isolated normal rat glomeruli (GCM) for 6 h and subjected to Northern blot analysis. Expression of GAPDH is shown at the bottom as a loading control.

Fig. 2. Evidence for activation of NF-κB in explanted glomeruli. A: SM43 cells were exposed to GCM for 6 h in the absence (−) or presence (+) of an inhibitor of NF-κB, MG132 (25 μM), and subjected to Northern blot analysis of MCP-1. B: isolated glomeruli were immediately stored at −80°C [incubation (−)] or incubated ex vivo at 37°C for 24 h [incubation (+)] and subjected to Western blot analysis of IκBα and IκBβ. The level of β-actin is shown at the bottom as a loading control. C: isolated glomeruli were kept on ice [incubation (−)] or incubated ex vivo at 37°C for 3 h [incubation (+)] and subjected to an ELISA-based assay for the DNA binding activity of the NF-κB p65 subunit. *Statistically significant difference (P < 0.05).
As shown in Fig. 3A, substantial induction of SEAP mRNA was observed in the reporter cells cocultured with isolated glomeruli. Consistently, the culture medium of SM/NFkB-SEAP5 cells exposed to glomeruli showed higher levels of SEAP [13,419 ± 316 relative light units (RLU), means ± SE, P < 0.05] compared with that of the cells without exposure to glomeruli (2,456 ± 53 RLU) (Fig. 3B). The culture medium of isolated glomeruli alone did not show any SEAP activity (data not shown). This result was further confirmed by using crossfeeding. Confluent SM/NFkB-SEAP5 cells were exposed to GCM for 6 h and subjected to Northern blot analysis of SEAP expression. Consistent with the result shown in Fig. 3A, GCM markedly induced expression of SEAP mRNA in reporter cells (Fig. 4A). It was associated with upregulation of SEAP activity in the cells exposed to GCM (31,318 ± 1,587 RLU in GCM-treated cells vs. 2,891 ± 106 RLU in untreated cells, P < 0.05) (Fig. 4B). Consistent with the result shown in Fig. 2A, the induction of SEAP mRNA and SEAP activity by GCM was abrogated by the treatment with MG132 (Fig. 4, A and B).

To further confirm the findings described above, we attempted to monitor intraglomerular activators of NF-kB using a technique for adoptive cell transfer (15, 18). For this purpose, SM/NFkB-SEAP5 cells were transferred into normal rat glomeruli via renal artery injection. After the cell transfer, glomeruli were isolated, incubated ex vivo for 24 h, and subjected to Northern blot analysis and SEAP assay. Before incubation, expression of SEAP mRNA was not detectable in the glomeruli. However, after ex vivo incubation, substantial induction of SEAP mRNA was observed (Fig. 5A). Consistently, SEAP activity was markedly induced in the culture medium of cells-transferred glomeruli [cell (+); 27,268 ± 4,292 RLU], but not in that of unmodified glomeruli [cell (−); 1,352 ± 89 RLU], after ex vivo incubation (Fig. 5B). The activation of NF-kB observed was not caused by the cell transfer per se but resulted from ex vivo incubation. It is because, in contrast to reporter cell transfer into nephritic glomeruli, serum levels of SEAP were never increased significantly in vivo after the transfer of SM/NFkB-SEAP5 cells into normal glomeruli, as we recently reported (24).

Identification of NF-kB activators produced by explanted glomeruli. To identify NF-kB activators produced by explanted glomeruli, molecular fractionation of GCM was performed using cut-off membranes. After centrifugation, the filtered GCM (molecular wt <50 kDa) was collected, and its effect on NF-kB was retested. As shown in Fig. 6A, the active entity present in whole GCM was not contained in the fraction with a molecular weight <50 kDa. This result indicated that the glomerulus-derived factors responsible for activation of NF-kB are relatively large molecules. IL-1β and TNF-α are well-known NF-kB activators that may be produced by glomerular cells. The molecular weight of IL-1β is <50 kDa (17 kDa), whereas that of TNF-α is >50 kDa (50–150 kDa). A previous report indicated that TNF-α may be spontaneously released from isolated normal glomeruli (9). To examine whether TNF-α is one of glomerulus-derived factors responsible for activation of NF-kB, GCM were prepared in the

Fig. 3. Activation of NF-kB in reporter mesangial cells cocultured with isolated glomeruli. A: SM/NFkB-SEAP5 reporter mesangial cells seeded into 12-well plates (2 × 10⁵ cells/well) were cocultured with (+) or without (−) isolated glomeruli (G; 5,000 glomeruli·well⁻¹·ml⁻¹) for 24 h. After incubation, glomeruli were removed, and reporter cells were subjected to Northern blot analysis of secreted alkaline phosphatase (SEAP). B: SM/NFkB-SEAP5 cells were seeded into 96-well plates (2 × 10⁵ cells/well) and incubated with (+) or without (−) isolated glomeruli (1,000 glomeruli·200 μl⁻¹·well⁻¹) for 24 h. After incubation, culture media were subjected to chemiluminescent assay to evaluate SEAP activity. Assays were performed in quadruplicate. RLU, relative light units. Values are means ± SE *Statistically significant difference (P < 0.05).

Fig. 4. Activation of NF-kB in reporter mesangial cells exposed to media conditioned by isolated glomeruli. SM/NFkB-SEAP5 cells were exposed to undiluted GCM for 24 h in the absence (−) or presence (+) of MG132 (25 μM). After incubation, the cells and culture media were subjected to Northern blot analysis (A) and chemiluminescent assay (B), respectively. Values are means ± SE. *Statistically significant difference (P < 0.05).
We speculated that MAP kinase inhibition abrogated the induction of MCP-1. It is known that, in some signaling cascades, MAP kinases are located upstream of NF-κB. Although our previous data suggested that the spontaneous expression of MCP-1 is largely dependent on MAP kinases, the current results showed that inhibition of MAP kinases (11) attenuated by PD098059 to 22,083 ± 1,087 RLU (P < 0.05). The suppressive effects of PD098059 and SB203850 were not due to nonspecific effects of these agents on reporter cells, because treatment of SM/SV-SEAP28 cells with these drugs did not affect SEAP activity (Fig. 7B).

There are two possibilities that may explain the decrease in NF-κB activation by PD098059 and SB203850. One possibility is that production of NF-κB-activating factors by isolated glomeruli is dependent on MAP kinases. To examine these possibilities, cross-feeding experiments were performed. SM/NFkB-SEAP5 cells were exposed to GCM in the presence or absence of PD098059 and SB203850, and the activity of SEAP in culture media was examined. As shown in Fig. 7C, the inhibitory effects of PD098059 and SB203850 observed in Fig. 7A were not reproduced in this experimental setting. This result suggested that MAP kinases were, at least in part, involved in the production of NF-κB activators by isolated glomeruli and that activation of NF-κB in mesangial cells by the glomerulus-
derived factors was MAP kinase independent. Our current hypothesis is illustrated in Fig. 8.

**DISCUSSION**

We previously reported that, in explanted normal glomeruli, MAP kinases are spontaneously activated, leading to expression of AP-1-dependent genes. In the present report, we have demonstrated that the NF-κB pathway, another crucial signaling pathway that regulates a diverse range of cell function, was also induced in isolated glomeruli without external stimulation. The activation of NF-κB was associated with downregulation of both IκBα and IκBβ proteins and upregulation of DNA binding activity of the NF-κB subunit to the κB consensus sequence. Our data also showed that the spontaneous activation of NF-κB was mediated by autocrine/paracrine factors produced by glomeruli and that MAP kinases were involved in the production of the NF-κB stimulating factors.

Previous reports demonstrated that, in some signaling cascades, activation of MAP kinase pathways may precede activation of NF-κB. For example, Lee et al. (19) showed that MAP kinase/ERK kinase kinase 1, an upstream kinase in the MAP kinase pathway, phosphorylated the IκB kinase complex containing IκKα and IκKβ. Zhao and Lee (38) also demonstrated that MAP kinase family members MKK2 and MKK3 activated IκKα and IκKβ and thereby mediated activation of NF-κB. Because our previous and present data showed that both MAP kinases and NF-κB are required for the spontaneous expression of MCP-1, we speculated that activation of MAP kinases may be an upstream event involved in the activation of NF-κB in glomerular cells. Indeed, in cultured mesangial cells, exposure to isolated glomeruli induced activation of NF-κB, and this induction was abrogated by MAP kinase inhibitors, as shown in this report. However, our hypothesis was found to be incorrect because induction of NF-κB activity by GCM was unaffected by either PD098059 or SB203850. These results indicated a two-step mechanism involved in the spontaneous activation of NF-κB in isolated glomeruli (Fig. 8). First, in explanted glomeruli, MAP kinases are activated, and AP-1-dependent genes are subsequently induced. Second, AP-1-dependent soluble factors are produced and trigger glomerular cells toward activation of NF-κB in an autocrine and/or paracrine fashion. We identified that one candidate responsible for the activation of NF-κB was TNF-α. The following evidence further supports our conclusion. 1) The molecular weight of the active entity produced by isolated normal rat glomeruli was >50 kDa (Fig. 6A). Normally, active TNF-α is a homotrimer with its molecular weight >50 kDa. 2) TNF-α is an AP-1-dependent gene, and the 5′-regulatory region of the TNF-α gene contains binding motifs for AP-1 (20, 33). 3) TNF-α is a strong activator of NF-κB in glomerular cells including rat mesangial cells (23). 4) A previous report showed that TNF-α protein was spontaneously released from isolated normal rat glomeruli (9).
GCM prepared in the presence of TACE inhibitors was less effective for the activation of NF-κB in mesangial cells. Of course, however, we cannot exclude a possibility that other signaling pathways are also involved in the production of NF-κB activators by glomerular cells, because suppressive effects of PD098059 and SB203850 were only partial (Fig. 7A).

Glomerular cell types responsible for production of NF-κB activators as well as for expression of NF-κB-dependent genes in explanted glomeruli are currently unknown. We speculate that endothelial cells and/or mesangial cells, but not podocytes, should be responsible for the spontaneous activation of NF-κB. It is because, as we previously reported, podocytes (but not endothelial and mesangial cells) rapidly and progressively undergo apoptosis during ex vivo incubation of glomeruli (10). Furthermore is the fact that induction of MCP-1 in explanted glomeruli is observed even in mesangial cell-ablated glomeruli (11), indicating crucial involvement of glomerular endothelial cells. Further investigation will be required to examine this possibility.

During this study, we found some discrepancy between the results of cross-feeding and the data of coculture. Dramatic induction of MCP-1 mRNA and SEAP mRNA was observed in reporter mesangial cells when they were exposed to GCM, whereas induction of those was modest when the cells were cocultured with isolated glomeruli. One possible reason is that NF-κB activators released from glomeruli were enriched in GCM, and concentrations of those were higher in GCM than in media of cocultures. Another possible reason may be that some NF-κB inhibitors with short half-lives could be produced by glomerular cells and offset the effect of NF-κB activators in coculture, but not in cross-feeding. For example, nitric oxide (NO) is known to be an inhibitor of NF-κB with a short half-life (4, 5) and produced by glomerular cells by a NF-κB-dependent mechanism (2, 26). NO produced by glomerular cells might be involved in the modest activation of NF-κB in mesangial cells cocultured with glomeruli.

The magnitude of SEAP induction in reporter cells following exposure to isolated glomeruli was somewhat different from experiment to experiment. For example, in Fig. 7A, the induction of SEAP was higher than the value shown in Fig. 3B (30,000 vs. 14,000 RLU), although the identical experimental protocol was used. Similarly, although the same protocol was used for preparation of GCM, some lots of GCM caused modest damage of reporter cells possibly because glomerulus-derived factors were excessively enriched. In such situations, diluted, nontoxic concentrations of GCM were used for studies. The different magnitude of reporter cell responses from study to study could be due to some differences in the quality and activity of GCM or in the quantity and quality of isolated glomeruli used for individual experiments.

What is the trigger for spontaneous upregulation of MAP kinases that precedes NF-κB activation? Some environmental factors including alteration in oxygen tension, reduced supply of nutrients, impaired removal of metabolic products, and/or lack of hemodynamic forces could contribute to the activation of MAP kinases in culture. The contribution of blood components (e.g., leukocytes, platelets, and plasma/serum growth factors) is unlikely, because the induction of MCP-1 was similarly observed in blood-free glomeruli and even under serum-free culture conditions (11).

The MAP kinase pathway and the NF-κB pathway regulate expression of a wide range of genes involved in various biological and pathophysiological processes. We propose that the peculiar biochemical properties of isolated glomeruli demonstrated here should be considered carefully in the design of experiments and interpretation of data when ex vivo incubation of glomeruli is used for investigation.
REFERENCES


