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Priming of Glomerular Mesangial Cells by Activated Macrophages Causes Blunted Responses to Proinflammatory Stimuli1

Kunihiro Hayakawa,* Yiman Meng,‡ Nobuhiko Hiramatsu,* Ayumi Kasai,* Kozue Yamauchi,* Jian Yao,* and Masanori Kitamura2*

Macrophage-mesangial cell interaction plays a crucial role in the pathogenesis of glomerulonephritis. Activated macrophages trigger mesangial cells to express an array of inflammation-associated genes via activation of NF-κB and AP-1. However, this inflammatory response is often transient and subsides spontaneously. We found that mesangial cells activated by bystander macrophages showed blunted responses of NF-κB to subsequent macrophage exposure. It was associated with sustained levels of IκBα, but not IκBβ. The tolerance observed was reversible and reproduced by conditioned media from activated macrophages (macrophage-conditioned medium (MφCM)). In vivo priming of mesangial cells by activated glomerular macrophages also caused the tolerance of mesangial cells. The macrophage-derived tolerance inducers were heat-labile, and multiple molecules were involved. Among inflammatory cytokines produced by macrophages, TNF-α and IL-1β were able to induce mesangial cell tolerance dose-dependently. The mesangial cell tolerance was also observed in activation of the MAPK-AP-1 pathway; i.e., phosphorylation of ERK, JNK, and p38 MAPK by macrophages was blunted when the cells were pre-exposed to MφCM. Induction of c-fos and c-jun was also abrogated in mesangial cells pre-exposed to MφCM, and the suppression was attenuated by blockade of MAPK activation during the first exposure to MφCM. These data elucidated that mesangial cells, once exposed to macrophages, become insensitive to subsequent activation by macrophages and proinflammatory stimuli. This self defense of glomerular cells may play a role in the resolution of macrophage-mediated, acute glomerulonephritis. The Journal of Immunology, 2006, 176: 2529–2537.

Infiltration of leukocytes, especially monocytes/macrophages, plays a decisive role in the development of glomerulonephritis (1, 2). Under pathologic conditions, activated macrophages secrete a variety of inflammatory mediators and stimulate resident cells toward activation (3). Once activated, resident cells express chemokines and accelerate accumulation of monocytes/macrophages, leading to progression of glomerular injury (4). Depletion of macrophages or inhibition of macrophage infiltration attenuates glomerular injury, suggesting the crucial role of macrophages in the progression of glomerulonephritis (5–7).

We previously reported that, under the acute inflammation, renal glomeruli became insensitive to inflammatory stimuli (8–10). For example, in glomeruli isolated from the regeneration phase of acute glomerulonephritis, induction of inflammation-associated genes was blunted in vitro (8–10). When activated macrophages were adoptively transferred into the nephritic glomeruli, the induction of chemokines and metalloproteinases in resident cells was suppressed ex vivo, compared with the induction that occurred in normal glomeruli (8, 9). We hypothesized that, after inflammatory insults, glomeruli may acquire potential for protecting themselves from further activation and injury (10). Currently, however, molecular mechanisms involved in this phenomenon are not fully elucidated.

In addition to the in vitro and ex vivo evidence for the tolerance of glomerular cells, described above, other in vivo evidence may support our hypothesis. In an acute model of anti-Thy 1 glomerulonephritis induced by a mAb 1-22-3, accumulation of macrophages is observed within 24 h, peaks at 1 wk, and decreases within 2 wk. However, when inflammation is induced again 2 wk after the first administration with the Ab, infiltration of macrophages is abrogated (11). To date, mechanisms involved in this blunted response of macrophages in the “two shot model” are unknown. Because accumulation of macrophages in glomeruli is regulated by resident cells via expression of chemokines (4), we speculated that, once activated, glomerular cells may acquire tolerance against subsequent exposure to inflammatory stimuli, resulting in attenuated production of chemokines.

NF-κB plays a crucial role in the expression of chemokines in glomerular cells (12–14). For example, exposure of rat mesangial cells to inflammatory cytokines induces rapid activation NF-κB and consequent induction of MCP-1 (12). In human proliferative glomerulonephritis, chemokines including IFN-γ-inducible protein of 10 kDa and monokine induced by IFN-γ are produced by glomerular cells, and the production of these chemokines in glomerular mesangial cells is mediated by NF-κB (13). In Shiga toxin-2-induced microangiopathy, expression of MCP-1 and IL-8 in glomerular endothelial cells is also NF-κB-dependent (14). Based on these previous observations, we hypothesized that activation of NF-κB could be blunted in glomerular cells pre-exposed to proinflammatory stimuli produced by macrophages.
In addition to NF-κB, expression of chemokines in glomerular cells may also be regulated by AP-1. For example, in some cell types, expression of IL-8 and MCP-1 is regulated by both NF-κB and AP-1 (15, 16). Activation of AP-1 is observed in glomerular cells during inflammation (17, 18), and it is associated with proliferation and apoptosis of glomerular cells (10). The transacting potential of AP-1 depends on its induction and phosphorylation by the MAPK family. For example, expression of c-fos is regulated by ternary complex factors whose activity is regulated by ERK, p38 MAPK, and JNK. Expression of c-jun is regulated by c-Jun and ATF-2 that are phosphorylated by JNK and/or p38 MAPK. Post-translational activation of AP-1 is also regulated by MAPK-mediated phosphorylation (19). We speculated that activation of the MAPK-AP-1 pathway, as well as NF-κB, could also be blunted in glomerular cells pre-exposed to inflammatory stimuli produced by macrophages.

Using cultured mesangial cells, we aimed in the present study to examine our hypothesis. We tested whether mesangial cells pre-exposed to macrophages become “tolerant” to subsequent inflammatory stimuli, and if so, what kind of mechanisms are involved in the “self-defense” mechanism. Our results evidenced for the first time that activated macrophages have an ability to induce tolerance of resident cells via secretion of multiple paracrine factors and that proinflammatory cytokines may be potential tolerance inducers produced by activated macrophages.

Materials and Methods

Cells

Reporter rat mesangial cells SM/NF-B-SEAP5 were established as described previously (20). This reporter clone secretes secreted alkaline phosphatase (SEAP)3 under the control of the NF-κB enhancer elements (20). The mesangial cell phenotype of SM/NF-B-SEAP5 cells was confirmed by positive staining for α-smooth muscle actin and Thy 1.1. Parental SM43 rat mesangial cells do not exhibit any SEAP activity. The normal alveolar macrophage cell line NR8383 derived from a Sprague-Dawley rat was a gift from Dr. S. Hirano (National Institute for Environmental Studies, Tsukuba, Japan). NR8383 cells also do not show any SEAP activity under both unstimulated and stimulated conditions. Cells were maintained in DMEM/F-12 (Invitrogen Life Technologies) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 1–10% FBS. Medium containing 1% FBS was generally used for studies.

Coculture

NR8383 macrophages were activated with LPS (1 μg/ml; Escherichia coli 0111, B4; Sigma-Aldrich) for 6 h. After washing twice, the stimulated macrophages (1 × 106 to 1 × 107 cells/well) were seeded onto confluent cultures of SM/NF-B-SEAP5 cells (1 × 105 cells/well) in 24-well plates. After 24 h, cells and media were collected and subjected to Northern blot analysis and SEAP assay, respectively, as described later. To examine whether macrophage-pre-exposed mesangial cells become tolerant to subsequent exposure to macrophages, reporter mesangial cells were first cocultured with activated macrophages (pretreated with LPS for 6 h) for 24 h and then treated with G418 (500 μg/ml) to kill macrophages selectively. Of note, SM/NF-B-SEAP5 cells express neo and are resistant to G418 (20). After 3 days, activated macrophages were again added to the macrophage-pre-exposed SM/NF-B-SEAP5 cells and incubated for an additional 24 h. The cells and media were collected and subjected to Northern blot analysis and SEAP assay.

Cross-feeding

Macrophage-conditioned medium (MdCM) was prepared as described previously (21). In brief, NR8383 macrophage (5 × 106 cells) in medium containing containing 10% FBS were stimulated with LPS (1 μg/ml) for 6 h. After washing twice, the macrophages were incubated in 10 ml of medium containing 1% FBS for 24 h. The conditioned media were filtered through 0.45-μm filters to remove macrophages completely and stored at −20°C until use. Cross-feeding studies using MdCM were performed as follows. Confluent SM/NF-B-SEAP5 reporter mesangial cells were exposed to MdCM or control medium for indicated time periods. After the exposure, cells and media were collected and used for SEAP assay and Northern blot analysis, Western blot analysis, and MAPK assays. To examine whether MdCM induces tolerance of mesangial cells, SM/NF-B-SEAP5 cells were pretreated with MdCM for 24 h, incubated in 1% FBS for 3 days without MdCM, and then re-exposed to MdCM for another 24 h. For Northern blot analysis, cells were pretreated with MdCM for 6 h and then re-exposed to MdCM for 45–60 min. In some experiments, SM/NF-B-SEAP5 cells were pretreated with MdCM in the presence of the selective MAPK inhibitor, PD98059 (inhibitor of ERK, 50 μM; Calbiochem-Novabiochem) or SB203580 (inhibitor of p38 MAPK: 25 μM, Calbiochem-Novabiochem) and subjected to the second exposure to MdCM. After the exposure, cells and media were collected and subjected to analyses. In some experiments, MdCM was: 1) heated at 80°C for 30 min, or 2) fractionated with molecular cutoff membranes (Microcon Centrifuge Filters YM-30 and YM-50; Nihon Millipore), and their ability to induce mesangial cell tolerance was retested. To examine whether the tolerance of mesangial cells induced by MdCM was reversible, reporter cells were treated by MdCM for 24 h, incubated without MdCM for 3, 10, and 17 days, and subjected to SEAP assay to evaluate NF-κB activity.

In vitro priming of mesangial cells with inflammatory stimuli

Confluent SM/NF-B-SEAP5 cells were pretreated with TNF-α (human recombinant, 0.08–50 ng/ml; Genzyme), IL-1β (human recombinant, 0.04–10 ng/ml; Genzyme), or LPS (1 μg/ml) for 24 h. After the treatment, the cells were washed and incubated in 1% FBS for 3 days and then re-exposed to TNF-α (10 ng/ml), IL-1β (1 ng/ml), LPS (1 μg/ml), or MdCM for 24 h. The media were collected and subjected to SEAP assay.

In vivo priming of mesangial cells

Acute anti-Thy 1 glomerulonephritis was induced in rats (Sprague-Dawley rats; 250–300 g body weight) by a mAb 1-22-3 (22), as described previously (23). In this experimental model, accumulation of macrophages in glomeruli is observed within 24 h (11). Twenty-four hours after the Ab injection, SM/NF-B-SEAP5 cells (1 × 106 cells) were trypsinized and injected into the nephritic rat kidneys, as described previously (23). In this experimental setting, 90% of glomeruli are transferred with the reporter mesangial cells (24). Three days later, glomeruli were isolated from the cell-injected kidneys and cultured for 1–2 wk in the presence of 15% FBS. Then the outgrowing cells were treated with G418 (500 μg/ml) for up to 5 wk, and responses of the recovered reporter cells to MdCM were compared with SM/NF-B-SEAP5 cells without in vivo priming.

Northern blot analysis

Total RNA was extracted by a single-step method, and Northern blot analysis was performed as described previously (25). Radiolabeled cDNAs for SEAP (BD Biosciences), IxBo (26), TNF-α (27), IL-1β (28), c-Fos (29), and c-Jun (30) were used as probes. Expression of GAPDH was used as a loading control.

SEAP assay

Activity of SEAP in culture media was evaluated by a chemiluminescent assay and Northern blot analysis, Western blot analysis, and MAPK assays. To examine whether MdCM induces tolerance of mesangial cells, SM/NF-B-SEAP5 cells were pretreated with MdCM for 24 h, incubated in 1% FBS for 3 days without MdCM, and then re-exposed to MdCM for another 24 h. For Northern blot analysis, cells were pretreated with MdCM for 6 h and then re-exposed to MdCM for 45–60 min. In some experiments, SM/NF-B-SEAP5 cells were pretreated with MdCM in the presence of the selective MAPK inhibitor, PD98059 (inhibitor of ERK, 50 μM; Calbiochem-Novabiochem) or SB203580 (inhibitor of p38 MAPK: 25 μM, Calbiochem-Novabiochem) and subjected to the second exposure to MdCM. After the exposure, cells and media were collected and subjected to analyses. In some experiments, MdCM was: 1) heated at 80°C for 30 min, or 2) fractionated with molecular cutoff membranes (Microcon Centrifuge Filters YM-30 and YM-50; Nihon Millipore), and their ability to induce mesangial cell tolerance was retested. To examine whether the tolerance of mesangial cells induced by MdCM was reversible, reporter cells were treated by MdCM for 24 h, incubated without MdCM for 3, 10, and 17 days, and subjected to SEAP assay to evaluate NF-κB activity.

Western blot analysis

Extracted proteins were separated by 10% SDS-polyacrylamide gels and electrotransferred onto membranes. Western blot analysis was performed by the ECL system (Amersham Biosciences), as described previously (32). In brief, 5 μl of culture media were mixed with 15 μl of 1× dilution buffer and incubated at 65°C for 30 min. After the incubation, the samples were mixed with 20 μl of assay buffer containing 1-homoarginine, left at room temperature for 5 min and added with 20 μl of chemiluminescent enhancer containing 1.25 mM CSPD chemiluminescent substrate. After incubation in the dark for 30 min, the samples were subjected to assays using a luminometer (Gene Light L5; Microtech Nition). All assays were performed in quadruplicate.

3 Abbreviations used in this paper: SEAP, secreted alkaline phosphatase; MdCM, macrophage-conditioned medium; MKP-1, MAPK phosphatase 1; RLU, relative light unit.
MAPK assay

Phosphorylation of ERKs, JNK, and p38 MAPK was evaluated by Western blot analyses as described previously (33) using the PhosphoPlus p44/42 MAP Kinase (Thr202/Tyr204) Ab kit, the PhosphoPlus SAPK/JNK (Thr183/Tyr185) Ab kit, and the PhosphoPlus p38 MAP Kinase (Thr180/Tyr182) Ab kit (Cell Signaling Technology) following protocols provided by the manufacturer.

Statistical analysis

Assays were performed in quadruplicate. Data were expressed as means ± SE. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data in different groups. A value of $p < 0.05$ was considered to be a statistically significant difference.

Results

Activation of NF-κB in mesangial cells exposed to macrophages

Activated macrophages produce an array of molecules that affect activity of NF-κB positively (e.g., IL-1β and TNF-α) or negatively (e.g., NO) (34). Using reporter cells that express SEAP under the control of the κB enhancer element, we first examined activity of NF-κB in mesangial cells exposed to macrophages. Reporter mesangial cells SM/NF-κB-SEAP5 were cocultured with activated macrophages for 24 h, and expression of SEAP mRNA was examined by Northern blot analysis. Before exposure to macrophages, the reporter cells exhibited low levels of basal SEAP mRNA. When these cells were exposed to activated macrophages, expression of SEAP was markedly induced (Fig. 1A). Similarly, chemiluminescent assay on culture media showed an increase in the level of SEAP activity (761,285 ± 33,111 relative light unit (RLU) vs 12,374 ± 751 RLU, before exposure; means ± SE, $p < 0.05$) (Fig. 1B). Time-lapse experiments revealed that significant induction of SEAP was observed within 4 h, and activity of SEAP increased continuously for at least 24 h (Fig. 1C). These results suggested that NF-κB was substantially activated in mesangial cells after exposure to activated macrophages.

Blunted response of NF-κB in macrophage-pre-exposed mesangial cells

To examine whether macrophage-pre-exposed mesangial cells become more sensitive or tolerant to subsequent exposure to macrophages, reporter mesangial cells were first cocultured with the same number of activated macrophages for 24 h and then treated with G418 to kill macrophages selectively. After 3 days, activated macrophages were again added to the macrophage-pre-exposed mesangial cells and cocultured for 24 h. Northern blot analysis...
showed that expression of SEAP was markedly induced in mesangial cells after the first exposure to activated macrophages (→−+). This induction was abrogated in mesangial cells pre-exposed to macrophages (→−++) (Fig. 2A). Consistently, SEAP assay on culture media revealed that NF-κB activity was induced in mesangial cells after the first exposure to macrophages (584,070 ± 51,397 RLU), and this induction was reduced to ~31% after the second exposure (183,532 ± 12,702 RLU, p < 0.05) (Fig. 2B). Of note, the basal activity of NF-κB in the macrophage-pre-exposed cells without the second exposure (→−) was 151,959 ± 11,264 RLU and was not significantly different from the value of macrophage-pre-exposed cells with the second exposure to macrophages (→−++). In this study, however, coculture of mesangial cells with the same number of macrophages (1:1 ratio) may not appropriately mimic in vivo situations. We estimated that a physiologically relevant ratio of macrophages to resident glomerular cells is ~1:10. Using this ratio, we have repeated the coculture study. As shown in Fig. 2C, even in this experimental setting, significant tolerance of NF-κB was similarly observed in macrophage-pre-exposed mesangial cells (21,037 ± 2,056 RLU vs 41,705 ± 2,495 RLU in cells without pre-exposure, p < 0.05). These results suggested that mesangial cells, once exposed to activated macrophages, showed blunted activation of NF-κB in response to subsequent macrophage exposure.

**Induction of mesangial cell tolerance by macrophage-derived soluble factors**

The induction of mesangial cell tolerance by activated macrophages may be caused by direct cell-to-cell contact (35) or cross-talk via macrophage-derived paracrine factors (36, 37). Alternatively, once activated, mesangial cells may produce macrophage-deactivating factors that suppress the effector function of macrophages (21, 38, 39). The mesangial cell-derived deactivators could cause “pseudotolerance” in the coculture studies. To investigate mechanisms involved in the induction of mesangial cell tolerance by macrophages, we conducted cross-feeding experiments. MφCM was prepared by using macrophages prestimulated with LPS, as described in Materials and Methods. Reporter mesangial cells were pretreated with MφCM for 24 h, incubated in 1% FBS for 3 days without MφCM, and then re-exposed to MφCM for an

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**FIGURE 3.** Induction of mesangial cell tolerance by macrophage-derived soluble factors. Media conditioned by activated Mφ (MφCM) were prepared as described in Materials and Methods and used for studies. A. SM/NFκB-SEAP5 cells were treated with (+) or without (−) MφCM for 24 h and subsequently cultured in the absence of MφCM for 3 days. The cells were then treated with (+) or without (−) MφCM for 24 h, and culture media were subjected to SEAP assay. B. MφCM was heated at 80°C for 30 min, and its potential to induce tolerance was retested. C. MφCM was fractioned into >50- and >30-kDa molecular masses, and the potential of individual fractions to induce tolerance was retested. Right graph shows activation of NF-κB by individual fractions. Whole, unfracated MφCM. *, Statistically significant differences (p < 0.05). D. SM/NFκB-SEAP5 cells were pretreated with or without LPS (1 μg/ml) for 24 h and subsequently cultured in the absence of LPS for 3 days. The cells were then treated with or without LPS or MφCM for 24 h, and culture media were subjected to SEAP assay. NS, not statistically significant.
additional 24 h. As shown in Fig. 3A, after the exposure to MφCM, activity of NF-κB was markedly induced in mesangial cells (340,745 ± 18,936 RLU vs 3,681 ± 192 RLU in untreated cells). This induction was significantly suppressed in mesangial cells pretreated with MφCM (182,949 ± 10,396 RLU, p < 0.05). This result indicated that the induction of mesangial cell tolerance by macrophages required neither direct cell-to-cell contact nor mesangial cell-derived deactivators of macrophages and that macrophage-derived paracrine factors were responsible for the induction of the mesangial cell tolerance.

To characterize the active entity responsible for the induction of mesangial cell tolerance, heat sensitivity of the macrophage-derived, paracrine factor(s) was tested. MφCM was first heated at 80°C for 30 min, and reporter mesangial cells were pretreated with the heated MφCM or untreated MφCM for 24 h. After incubation in 1% FBS for 3 days, the cells were exposed to intact MφCM for 24 h, and activity of NF-κB was evaluated. In contrast to intact MφCM, heated MφCM did not induce tolerance of mesangial cells (Fig. 3B). That is, pretreatment with MφCM significantly reduced activation of NF-κB by MφCM (349,642 ± 21,896 RLU). To further characterize the active entity, MφCM was fractioned into molecular masses <50 and <30 kDa using cutoff membranes, and the tolerance-inducing potential of individual fractions was restested. As shown in Fig. 3C (left), all fractions significantly induced mesangial cell tolerance. However, the suppressive potential of the fraction <30 kDa (159,820 ± 3,329 RLU) was significantly less than that of the fraction <50 kDa (159,820 ± 3,329 RLU). Similarly, the potential of the fraction <50 kDa was significantly less than that of unfraccionated whole MφCM (118,141 ± 4,728 RLU). This result suggested that multiple molecules with molecular masses >50 kDa, 30–50 kDa, and <30 kDa were involved in the induction of mesangial cell tolerance by macrophages. Interestingly, NF-κB activators released from macrophages are mainly in the fraction >50 kDa (Fig. 3C, right). It indicated that some inducers of mesangial cell tolerance are distinct from NF-κB activators.

For the preparation of MφCM, macrophages were pretreated with 1 μg/ml LPS. The remaining LPS in MφCM could induce tolerance of mesangial cells. To exclude this possibility, we examined effects of LPS on the induction of tolerance in mesangial cells. Reporter mesangial cells were pretreated with 1 μg/ml LPS for 24 h and, after a 3-day incubation without LPS, re-exposed to LPS or MφCM. As shown in Fig. 3D, although LPS markedly induced activation of NF-κB, it did not induce significant tolerance of mesangial cells to LPS or MφCM. These results excluded the possibility that the induction of tolerance by MφCM was due to the remaining LPS.

Potential of macrophage-derived cytokines in the induction of mesangial cell tolerance

NR8383 macrophages stimulated by LPS produce biologically active proinflammatory cytokines including TNF-α and IL-1β (40). Indeed, NR8383 macrophages stimulated with LPS exhibited abundant expression of TNF-α and IL-1β in our experimental setting (Fig. 4A). To examine whether macrophage-derived cytokines have the potential to induce tolerance of mesangial cells, reporter cells were pretreated with serial concentrations of TNF-α and IL-1β for 24 h, and after 3 days, re-exposed to these cytokines for 24 h. As shown in Fig. 4B, TNF-α induced tolerance of mesangial cells in a dose-dependent manner. Significant induction of tolerance was observed at concentrations ≥0.4 ng/ml. Of note, maximum activation of NF-κB in mesangial cells was achieved by TNF-α at concentrations >50 ng/ml (our unpublished data), and relatively low concentrations were found to be sufficient to induce tolerance. The induction of tolerance by TNF-α was observed regardless of secondary stimuli, i.e., pretreatment with TNF-α depressed activation of NF-κB not only by TNF-α but also by IL-1β, LPS, and MφCM (data not shown). In contrast, low to moderate dosages (~5 ng/ml) of IL-1β did not significantly induce mesangial cell tolerance (Fig. 4C). Only a high dose of IL-1β (10 ng/ml) modestly induced tolerance of mesangial cells without affecting cell viability. Of note, maximum activation of NF-κB in mesangial cells was achieved by IL-1β at concentrations ≥0.2 ng/ml (our unpublished observation), and relatively high concentrations of IL-1β were required for the induction of tolerance.

Reversibility of the macrophage-primed tolerance

To examine whether the tolerance of mesangial cells induced by macrophages is reversible, reporter cells were pretreated by

FIGURE 4. Induction of mesangial cell tolerance by proinflammatory cytokines. A, Expression of TNF-α and IL-1β in macrophages stimulated with LPS. NR8383 macrophages were treated with LPS (1 μg/ml) for 6 h, and expression of TNF-α and IL-1β was examined by Northern blot analysis. Expression of GAPDH is shown at the bottom as a loading control. B and C, Dose-dependent induction of tolerance by cytokines. SM/NF-κB-SEAP5 cells were pretreated with TNF-α (0.08–50 ng/ml) or IL-1β (0.04–10 ng/ml) for 24 h. After the treatment, the cells were incubated for 3 days in the absence of cytokines and re-exposed to TNF-α (B) or IL-1β (C) for 24 h, respectively. The media were collected and subjected to SEAP assay. *, Statistically significant differences (p < 0.05).
MΦCM for 24 h, incubated without MΦCM for 3, 10, and 17 days, and re-exposed to MΦCM. Three days after the pre-exposure to MΦCM, activation of NF-κB by MΦCM was reduced to 49.5 ± 6.7% of the response in the cells without pre-exposure (100 ± 4.5%; p < 0.05). However, the response was recovered to 92.3 ± 4.4% after 10 days and to 92.9 ± 6.2% after 17 days (both values were not statistically different) (Fig. 5).

**Induction of tolerance by in vivo priming of mesangial cells by glomerular macrophages**

As described, mesangial cells pre-exposed to activated macrophages in vitro become tolerant against subsequent stimulation by macrophages. We examined whether mesangial cells primed by macrophages within an in vivo inflammatory milieu also acquire tolerance to macrophages. For this purpose, we used a technique for "in vivo priming" of mesangial cells. Acute anti-Thy 1 glomerulonephritis was induced in rats by a mAb 1-22-3. In this experimental model, accumulation of macrophages in glomeruli is observed within 24 h and lasts for 1 wk (11). Twenty-four hours after the Ab injection, reporter mesangial cells were trypsinized and injected into nephritic left kidneys via the renal artery. Three days later, glomeruli were isolated from cell-injected left kidneys and cultured for 1–2 wk. Then the outgrowing cells were treated with G418 for 1 wk to eliminate intrinsic, G418-sensitive cells, and the response of the recovered, G418-resistant reporter cells to MΦCM was compared with that of unprimed reporter cells. In the reporter cells without priming, activation of NF-κB was increased by 78.2–± 7.0-fold after the exposure to MΦCM (Fig. 6A, left). In contrast, the reporter cells recovered from macrophage-accumulating, nephritic glomeruli showed blunted activation of NF-κB in response to MΦCM (14.3–± 2.0-fold; Fig. 6A, right). This blunted response was found to be reversible; i.e., during the course of culture, activation of NF-κB by MΦCM was gradually recovered, and after 5 wk, the magnitude of the response was comparable to that of unprimed reporter cells (99.2 ± 2.4% vs 100 ± 3.6%, NS) (Fig. 6B).

**Mechanisms involved in the mesangial cell tolerance**

IkBα and IkBβ are known to be endogenous inhibitors of NF-κB. Under unstimulated conditions, NF-κB locates in the cytoplasm as complexes with IkBs. When cells are stimulated, IkB kinases phosphorylate IkBs and cause rapid degradation of IkBs by the proteasome pathway, allowing for translocation of NF-κB into the nucleus and its binding to the κB sites. To investigate mechanisms involved in the macrophage priming of mesangial cell tolerance, the levels of IkBα and IkBβ proteins were examined. After the exposure to MΦCM, the level of IkBβ, but not IkBα, was down-regulated within 1 h and recovered to the basal levels within 2 days (Fig. 7A). Based on these results, reporter cells were pretreated with or without MΦCM for 24 h, incubated in 1% FBS for 3 days without MΦCM, and then re-exposed to MΦCM for 3–6 h. Western blot analysis revealed that, after the second exposure to MΦCM, the level of IkBβ was transiently up-regulated in MΦCM-primed cells, but not in the cells without priming (3 h). After 6 h, the level of IkBβ in MΦCM-primed cells was still significantly higher than that in unprimed cells (Fig. 7B). This result indicated a possibility that attenuated degradation or increased production of IkBβ may be an upstream event responsible for the mesangial cell tolerance induced by macrophages.

**Blunted response of AP-1 in macrophage-primed mesangial cells**

In addition to NF-κB, AP-1 is another important transcription factor that regulates inflammatory processes. We examined expression of AP-1 components, c-fos and c-jun, in macrophage-primed mesangial cells. Mesangial cells were pretreated with MΦCM for 6 h and re-exposed to MΦCM for 1 h. Northern blot analysis revealed that MΦCM markedly induced expression of c-fos and c-jun in unprimed cells. This induction was abrogated in MΦCM-pretreated

**FIGURE 5.** Reversibility of the macrophage-primed tolerance. SM/NFsB-SEAP5 cells were pretreated by MΦCM for 24 h, incubated without MΦCM for 3, 10, and 17 days, and re-exposed to MΦCM for 24 h. Culture media were subjected to SEAP assay. Data are shown as relative percentages against the response of the cells without pretreatment. * Statistical significant difference (p < 0.05). NS, Not statistically significant.

**FIGURE 6.** Induction of tolerance by in vivo priming of mesangial cells by glomerular macrophages. A, Acute anti-Thy 1 glomerulonephritis was induced in rats by a mAb 1-22-3. Twenty-four hours after the Ab injection, SM/NFsB-SEAP5 cells were injected into nephritic left kidneys via the renal artery. Three days later, glomeruli were isolated from cell-injected left kidneys and cultured for 1–2 wk. Then the outgrowing cells were treated with G418 for 1 wk to eliminate intrinsic, G418-sensitive cells, and the response of the recovered, G418-resistant reporter cells to MΦCM (right, in vivo priming (+)) were compared with that of unprimed SM/NFsB-SEAP5 cells (left, in vivo priming (−)). B, Responses of recovered SM/NFsB-SEAP5 cells were examined after culturing for 1, 2, 3, and 5 wk. * Statistical significant differences (p < 0.05). NS, not statistically significant.
mesangial cells (Fig. 8). Interestingly, in MøCM-primed mesangial cells, expression levels of both c-fos and c-jun were down-regulated further by the exposure to MøCM.

**Mechanisms involved in the blunted response of AP-1**

Expression of c-fos is regulated by ternary complex factors whose activity is regulated by ERK, p38 MAPK, and JNK. Expression of c-jun is also regulated by c-Jun and ATF-2 that are phosphorylated by JNK and/or p38 MAPK (19). To elucidate upstream events involved in the macrophage-primed tolerance of mesangial cells, responses of MAPKs were examined. Mesangial cells were pre-treated with MøCM for 6 h and re-exposed to MøCM for 0.5–1 h. Western blot analyses revealed that all three MAPKs were phosphorylated by MøCM in unprimed cells, whereas the activation was abolished in MøCM-primed mesangial cells (Fig. 9A). Fig. 9B shows MAPK responses to MøCM 24 h after the first exposure. In contrast to the NF-κB tolerance that sustained for at least 3 days, the blunted responses of ERK and JNK recovered within 24 h. In contrast, the blunted response of p38 MAPK was still observed even 24 h after the first exposure to MøCM.

We examined whether the activation of MAPKs during the first exposure to MøCM was involved in the induction of tolerance for AP-1. Mesangial cells were pretreated with MøCM for 6 h in the absence or presence of a selective inhibitor of ERK (PD098059) or a selective inhibitor of p38 MAPK (SB203580) and subjected to the second exposure to MøCM for 45–60 min without MAPK inhibitors. As shown in Fig. 10, the blunted responses of c-fos and c-jun in MøCM-pre-exposed mesangial cells were reversed by the treatment with either PD098059 or SB203580. This result suggested that the initial activation of the MAPK pathways plays a crucial role in the acquisition of tolerance for AP-1 in macrophage-exposed mesangial cells.

Activity of MAPKs is regulated by the family of inducible dual-specificity phosphatases (41), and MKP-1 is the prototypic member of the family (42). MKP-1 is induced through various mechanisms including the MAPK pathways per se and selectively binds and inactivates MAPKs including ERK1/2, JNK1/2, and p38 MAPK (43). To further investigate upstream events involved in the macrophage-primed tolerance of mesangial cells, we examined the level of MKP-1. Mesangial cells were stimulated with MøCM for up to 6 h and subjected to Western blot analysis. The result showed that the level of MKP-1 protein was not induced by the treatment with either PD098059 or SB203580 (data not shown). These results indicated that, although induction of MKP-1 is an important mechanism for the self-defense of mesangial cells against oxidative stress (33), this molecule may not be involved in the induction of tolerance by activated macrophages.

**FIGURE 7.** Kinetics of IκB proteins in mesangial cells after the treatment with MøCM. A, SM/NFκB-SEAP5 cells were treated with MøCM for indicated time periods, and levels of IκBα and IκBβ proteins were examined by Western blot analysis. The level of β-actin is shown at the bottom as a loading control. B, SM/NFκB-SEAP5 cells were pre-treated with (+) or without (−) MøCM for 24 h. After incubation for 3 days without MøCM, the cells were re-exposed to MøCM for 3–6 h, and the level of IκBβ was examined by Western blot analysis.

**FIGURE 8.** Blunted response of AP-1 in macrophage-primed mesangial cells. SM/NFκB-SEAP5 cells were pre-treated with (+) or without (−) MøCM for 6 h and re-exposed to MøCM for 1 h. Expression of c-fos and c-jun was examined by Northern blot analysis. Expression of GAPDH is shown at the bottom as a loading control.

**FIGURE 9.** Blunted activation of MAPKs in macrophage-primed mesangial cells. SM/NFκB-SEAP5 cells were pre-treated with (+) or without (−) MøCM for 6 h (A) or 24 h (B) and re-exposed to MøCM for 0.5–1 h. Activity of ERK, JNK, and p38 MAPK (p38) was evaluated by Western blot analysis using phosphospecific Abs. Total protein levels of ERK, JNK, and p38 MAPK are shown at the bottom as loading controls (“Protein”).
Discussion
Induction of tolerance in local tissues and organs in response to environmental stresses is well-known under certain pathophysiologic situations. The best-known example is “ischemic tolerance (preconditioning),” in which a brief period of ischemia results in the resistance of tissues to subsequent, severe ischemia (44). Another example of tissue defense is “thermotolerance.” In various tissues and cultured cells, exposure to thermal stress induces a set of stress proteins, so-called heat shock proteins, thereby affording tolerance against subsequent insults (45). Some tolerance may also occur under inflammatory situations. A typical example is “endoxin tolerance” (46). The endoxin tolerance is defined as a reduced capacity of the host (in vivo) or cultured cells (in vivo) to respond to LPS following a first exposure to this stimulus. Mechanisms involved in the endoxin tolerance have been investigated by a number of investigators, and roles of some cell surface molecules, cytoplasmic signaling molecules, and nuclear factors have been elucidated (46). However, the majority of the previous studies focused on the tolerance of monocytes/macrophages or other leukocytes. Little is known about tolerance of nonleukocyte lineages under inflammatory situations. Furthermore, little has been reported about induction of tolerance by inflammatory stimuli other than LPS, and induction of tolerance of resident cells by macrophages has never been reported. In the present investigation, we demonstrated that activated macrophages have an ability to induce tolerance of mesangial cells. This effect was reversible and mediated by heat-labile, multiple mediators with molecular masses >50, 30–50, and <30 kDa. We found that inflammatory cytokines including TNF-α and IL-1β may be possible candidates responsible for the induction of mesangial cell tolerance. For example, priming of mesangial cells with TNF-α induced blunted responses of NF-κB to different inflammatory stimuli including TNF-α, IL-1β, LPS, and MφCM. Activation of NF-κB per se may not be required for the induction of mesangial cell tolerance because: 1) in MφCM, some tolerance inducers were present in the fractions with little or no NF-κB-activating potential (Fig. 3C), 2) concentrations of IL-1β that activated NF-κB fully did not induce tolerance (Fig. 4C), and 3) LPS markedly activated NF-κB, whereas it did not cause induction of tolerance (Fig. 3D). Currently, mechanisms involved in the tolerance of mesangial cells have not been fully elucidated, but our current data revealed selective involvement of IxB proteins, the endogenous inhibitors of NF-κB. We found that exposure to MφCM caused selective down-regulation of IxBβ in mesangial cells and that the down-regulation of IxBβ was attenuated in the cells pre-exposed to MφCM. This result indicated that decreased degradation or increased production of selective IxB may be an upstream event responsible for the mesangial cell tolerance induced by macrophages.

In addition to the blunted response of NF-κB, we found that induction of AP-1 was also suppressed in macrophage-pre-exposed mesangial cells. MAPKs were identified as upstream targets for the mesangial cell tolerance. That is, activation of ERK, JNK, and p38 MAPK involved in the induction and activation of AP-1 components was abrogated in mesangial cells pre-exposed to macrophages. Interestingly, the blunted responses of AP-1 components in MφCM-pre-exposed mesangial cells were significantly recovered by the treatment with MAPK inhibitors during the first exposure to MφCM. This result suggested that the initial activation of MAPKs by macrophages plays an essential role in the acquisition of tolerance for AP-1.

Activity of MAPKs is down-regulated by the family of inducible dual-specificity phosphatases, and MKP-1 is the prototypic member of the family that is involved in glomerular pathophysiology. However, in mesangial cells, the level of MKP-1 protein was not induced by the treatment with MφCM, and re-exposure of macrophage-primed cells to MφCM did not affect the level of MKP-1. These results excluded a possibility that MKP-1 was involved in the blunted response of the MAPK-AP-1 pathway in macrophage-exposed mesangial cells.

In this report, we used MφCM derived from LPS-activated macrophages. LPS contaminated in the MφCM could cause the tolerance of mesangial cells. However, for the preparation of MφCM, LPS-stimulated macrophages were washed repeatedly, and the level of remaining LPS should be very low. Furthermore, pretreatment with LPS did not induce significant tolerance of mesangial cells to MφCM or LPS (Fig. 3D), excluding this possibility.

During the recovery from acute glomerulonephritis, activated NF-κB and AP-1 in glomerular cells must be subdued. From this viewpoint, acquisition of tolerance against macrophages and macrophage-derived factors is advantageous for glomeruli to recover from acute inflammation. NF-κB plays an important role in the survival of various cells, including mesangial cells (47, 48). Blunted activation of NF-κB may lead to glomerular cell apoptosis that is required for clearance of excessive mesangial cells during the resolution of mesangial proliferative glomerulonephritis (49). Similarly, lack of AP-1 activation may lead to suppression of glomerular cell proliferation (50). The attenuated activation of NF-κB and AP-1 in macrophage-primed mesangial cells can also cause attenuated expression of chemokines, leading to reduction in the number of macrophage. Previous reports showed that, in acute anti-Thy 1 glomerulonephritis, accumulation of macrophages was observed within 24 h, peaked at 1 wk and decreased within 2 wk. However, when inflammation was induced again 2 wk after the first injection of the Ab, infiltration of macrophages was not evident (11). Our current findings, together with the previous data, support an idea that, under inflammatory situations, glomerular cells become tolerant to activated macrophages and proinflammatory stimuli, leading to blunted activation of NF-κB and AP-1. This self-defense mechanism of glomerular cells may contribute to the resolution of macrophage-mediated, acute glomerulonephritis. Under certain inflammatory situations, macrophages are thus involved not only in activation of resident cells but also in the induction of tolerance in resident cells against subsequent inflammatory stimuli.

We described the light side of the macrophage-primed, mesangial cell tolerance in glomerulonephritis, but its possible dark side should also be considered. In the chronic model of anti-Thy 1 glomerulonephritis, progressive glomerulosclerosis is observed in parallel with attenuated accumulation of macrophages (11). Blunted activation of NF-κB may cause excessive apoptosis of glomerular cells as well as failure in removal of excessive extracellular matrix by matrix metalloproteinases (51). The tolerance of glomerular cells might lead to glomerulosclerosis.

In summary, the present data elucidated that mesangial cells, once exposed to macrophages, become insensitive to subsequent inflammatory stimuli. To our knowledge, this is the first study to demonstrate: 1) the potential of macrophages to prime resident cells for acquiring tolerance, and 2) the paradoxical role of proinflammatory cytokines in the induction of tolerance against inflammatory stimuli. Further investigation will be required to determine whether the macrophage-induced tolerance of resident cells is also observed in other cell types, in other tissues, and under other pathophysiological situations.

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References


