Genetic deficiency of Smad3 protects against murine ischemic acute kidney injury

Karl A. Nath,1* Anthony J. Croatt,1 Gina M. Warner,2 and Joseph P. Grande2*
1Division of Nephrology and Hypertension and 2Department of Pathology, Mayo Clinic, Rochester, Minnesota
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Nath KA, Croatt AJ, Warner GM, Grande JP. Genetic deficiency of Smad3 protects against murine ischemic acute kidney injury. Am J Physiol Renal Physiol 301: F436–F442, 2011. First published April 27, 2011; doi:10.1152/ajprenal.00162.2011.—TGF-β1 contributes to chronic kidney disease, at least in part, via Smad3. TGF-β1 is induced in the kidney following acute ischemia, and there is increasing evidence that TGF-β1 may protect against acute kidney injury. As there is a paucity of information regarding the functional significance of Smad3 in acute kidney injury, the present study explored this issue in a murine model of ischemic acute kidney injury in Smad3−/− and Smad3+/− mice. We demonstrate that, at 24 h after ischemia, Smad3 is significantly induced in Smad3+/− mice, whereas Smad3−/− mice fail to express this protein in the kidney in either the sham or posts ischemic groups. Compared with Smad3+/− mice, and 24 h following ischemia, Smad3−/− mice exhibited greater preservation of renal function as measured by blood urea nitrogen (BUN) and serum creatinine; less histological injury assessed by both semiquantitative and qualitative analyses; markedly suppressed renal expression of IL-6 and endothelin-1 mRNA (but comparable expression of MCP-1, TNF-α, and heme oxygenase-1 mRNA); and no increase in plasma IL-6 levels, the latter increasing approximately sixfold in posts ischemic Smad3+/− mice. We conclude that genetic deficiency of Smad3 confers structural and functional protection against acute ischemic injury to the kidney. We speculate that these effects may be mediated through suppression of IL-6 production. Finally, we suggest that upregulation of Smad3 after an ischemic insult may contribute to the increased risk for chronic kidney disease that occurs after acute renal ischemia.

IL-6; TGF-β1; heme oxygenase-1; chronic kidney disease

TRANSFORMING GROWTH FACTOR-β1 (TGF-β1) is a pleiotropic cytokine that affects many of the major pathobiological processes that contribute to tissue injury (4, 7, 8, 18, 30, 33, 40, 42, 49). Such effects of TGF-β1 are commonly cell specific and context dependent (18, 33); for example, depending upon the experimental conditions, TGF-β1 may promote processes that are either antiapoptotic or proapoptotic and may exert actions that are either proinflammatory or anti-inflammatory. Such diverse and, at times, seemingly contradictory effects of TGF-β1 probably reflect the myriad signaling systems recruited by TGF-β1, and these include 1) the Smad family of proteins, 2) the MAPK system, 3) phosphatidylinositol 3-kinase signaling, 4) small GTPases, 5) mammalian target of rapamycin, and 6) ILK (4, 7, 8, 18, 30, 33, 40, 42, 49). However, whatever the divergent, cell-specific effects of TGF-β1 on biological processes, one effect of TGF-β1 is firmly and consistently established irrespective of the involved tissues: the capacity of TGF-β1 to promote matrix expansion and fibrogenesis, processes in which the signaling intermediate, Smad3, is a fundamental participant. Indeed, TGF-β1 is uniformly regarded as a key contributor to the progression of chronic kidney disease, a contribution that reflects, at least in part, the signaling effects of Smad3 (4, 7, 8, 30, 40, 42, 49, 51).

The pathobiological significance of TGF-β1 in acute kidney injury is increasingly of interest (2, 3, 14, 15, 26–28, 45). Early and marked upregulation of TGF-β1 and its receptors occurs following acute renal ischemia (2, 3, 14, 45), and, as shown by our prior studies, TGF-β1 is markedly induced in a sustained fashion following heme protein-induced renal injury (36). Studies in the rat model of acute renal ischemia demonstrate that antagonism of the actions of TGF-β1 inhibits the synthesis of extracellular matrix proteins, but fails to influence the course of renal function following such ischemic injury (14, 45). However, in other models of acute ischemic injury, a cytoprotective role for TGF-β1 has been demonstrated. In a murine model of acute renal ischemia, a deficiency in TGF-β1 exacerbates renal dysfunction and worsens renal histological injury (15), and in in vitro models inhibition of TGF-β1 exacerbates oxidant-induced necrosis of proximal tubular epithelial cells (27). Acquired resistance to renal ischemic injury in certain settings is also TGF-β1 dependent. For example, prior exposure to the anesthetic agent sevoflurane protects against renal damage following acute ischemic insults, and such protection by sevoflurane is impaired either by the administration of a neutralizing TGF-β1 antibody, or in TGF-β1−/− mice (26). In studies in vitro, a neutralizing TGF-β1 antibody vitiates the cytoprotection conferred by sevoflurane against hydrogen peroxide-induced injury to proximal tubular epithelial cells (27).

In light of evidence that TGF-β1 is protective in acute kidney injury, and the fundamental role of Smad3 in promoting the injurious effects of TGF-β1, at least in chronic kidney disease, the present study was undertaken to examine the role of Smad3 in ischemic acute kidney injury.

MATERIALS AND METHODS

Murine Smad3−/− model. Smad3+/+ and Smad3−/− mice employed for the present studies were generated from colonies of mice established from breeder stock obtained from the Jackson Laboratory (129-Smad3+/+Ij, stock no. 003451, Bar Harbor, ME), and maintained by mating Smad3−/− males with Smad3+/− females. Offspring were genotyped at the time of weaning using PCR to amplify the wild-type and mutant alleles of genomic DNA from tail samples. For studies of Smad3−/− and Smad3+/− mice, age-matched male mice from 10 to 24 wk were employed. Additionally, in other studies 14-wk-old male C57BL/6J mice (Jackson Laboratory) were employed. All experiments were performed in accordance with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.
Murine model of renal ischemia-reperfusion. This model of acute renal ischemia was induced in mice as described in detail by our prior studies (20, 21, 37, 39, 47). Briefly, after mice were anesthetized with pentobarbital (50 mg/kg ip), the renal pedicles were gently dissected and bilateral renal ischemia was induced with nontraumatic clamps (RS5426, Micro Aneurysm clip, straight, 10 mm, 125-g pressure; Roboz Surgical Instruments, Rockville, MD) through a midline abdominal incision. Two protocols of renal ischemia consisting of either 22.5 or 25 min were separately employed in Smad3+/+ and Smad3−/− mice so as to determine the consistency of the observed effect of the deficiency of Smad3 on different durations of renal ischemia. Sham procedures of the duration of the 25-min ischemia procedure included the abdominal incision but excluded renal pedicle dissection and clamping. For these studies, renal function was assessed by the measurement of serum creatinine and blood urea nitrogen (BUN) levels at 24 h after ischemia using a Creatinine Analyzer 2 and a BUN Analyzer 2 (Beckman Instruments, Fullerton, CA).

mRNA expression by quantitative real-time RT-PCR. For analysis of gene expression, total RNA was extracted from snap-frozen mouse renal tissues using the TRIzol method (Invitrogen, Carlsbad, CA) and subsequently further purified with an RNeasy Mini kit (Qiagen, Valencia, CA), according to each manufacturer’s protocol and as described in detail in our prior studies (39, 48). Three hundred nanograms of purified total RNA were used in 30-μl reverse transcription reactions (Transcriptor First Strand cDNA Synthesis kit, Roche Applied Science, Indianapolis, IN) employing random hexamers. The resulting cDNA was used in quantitative real-time PCR analysis as in our earlier study (39). Reactions were performed on an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) using TaqMan Mastermix reagent (part no. 432420, Applied Biosystems). Probes and primers obtained as assay sets (TaqMan Gene Expression Assays, Applied Biosystems) were employed in these reactions according to the manufacturer’s protocol. Parameters for quantitative PCR were as follows: 10 min at 95°C, followed by 40 cycles of amplification for 15 s at 95°C, and 1 min at 60°C. Expression of 18S rRNA was used for standardization of the expression of each target gene.

Western blot analysis. Western blot analysis was performed as described in our previous studies (39, 48). Briefly, proteins (150 μg) were separated on 10% Tris-HCl gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes. Primary antibodies for Smad3 (catalog no. 9523, Cell Signaling Technology, Danvers, MA) and β-actin (catalog no. 612657, BD Biosciences, San Jose, CA) were used in overnight incubations at 4°C. Horseradish peroxidase-conjugated secondary antibodies were then used, and bands were visualized using an enhanced chemiluminescence method.

Serum IL-6 quantitation by ELISA. Serum concentrations of IL-6 protein were measured using a commercially available ELISA set (OptEIA, catalog no. 555240, BD Biosciences) according to the manufacturer’s assay instructions.

Histological analysis. Histological analysis was performed on 5-μm hematoxylin- and eosin-stained sections prepared from formalin-fixed, paraffin-embedded renal tissues from Smad3+/+ and Smad3−/− mice 24 h after bilateral renal ischemia or sham operation. Blinded semiquantitative evaluation of necrosis was performed by assessing the percentage of tubules demonstrating any evidence of epithelial cell necrosis, and the degree of extension of necrosis into the cortex, determined by the mean percentage of the distance from the corticomедullary junction to the surface of the kidney in which necrosis was present.

Statistics. Data are expressed as means ± SE. Data for Smad3−/− and Smad3+/+ mice for a given condition were compared using Student’s t-test for parametric data and the Mann-Whitney test for nonparametric data. Results were considered significant at P < 0.05.

RESULTS

Effect of Smad3 deficiency on renal ischemia. At 3 h, Smad3 expression by Western blot analysis was unaltered in response to renal ischemia compared with sham ischemia in wild-type mice (data not shown). However, at a later time point, 24 h, there was a marked and significantly increased expression (~3-fold) of Smad3 in Smad3−/− mice subjected to renal ischemia (Fig. 1). Western blot analysis also confirmed that Smad3−/− mice failed to express Smad3 in the kidney after either sham ischemia or renal ischemia, thereby confirming the deficiency of this protein in this mutant strain (Fig. 2). Effect of Smad3 deficiency on renal function. Renal functional studies were undertaken in two separate protocols, employing different durations of ischemia that lead to duration-dependent, renal dysfunction in wild-type mice. As demonstrated, Smad3−/− mice exhibited substantial preservation in renal function in response to renal ischemia as reflected by
BUN (Fig. 3) and serum creatinine (Fig. 4) 24 h after renal ischemia.

**Effect of Smad3 deficiency on renal histological injury.** Along with this preservation of renal function in Smad3<sup>−/−</sup> mice following renal ischemia, there was substantial reduction in renal histological injury as reflected by less acute tubular necrosis, and less intratubular sloughing and cast formation in Smad3<sup>−/−</sup> mice following renal ischemia (Fig. 5). Semiquantitative analyses confirmed such qualitative analyses: scores for the severity of acute tubular necrosis and the extent to which tubular necrosis extended from the corticomedullary junction into the cortex were both significantly lower in Smad3<sup>−/−</sup> mice following renal ischemia either 22.5 or 25 min in duration (Fig. 6).

**Effect of Smad3 deficiency on renal gene expression.** In an attempt to determine the basis for this effect of Smad3 deficiency, we assessed the expression of cytokines that contribute to ischemic acute kidney injury. As previously described, expression of IL-6 mRNA is markedly increased following ischemia, and this effect was greatly attenuated in Smad3<sup>−/−</sup> mice compared with Smad3<sup>+/+</sup> mice following renal ischemia (Fig. 7). While not as striking, expression of endothelin-1 was also significantly reduced in Smad3<sup>−/−</sup> mice compared with Smad3<sup>+/+</sup> mice following renal ischemia (Fig. 8). Interestingly, a similar pattern was found with TGF-β1 (Fig. 9). Other cytokines that are well established as contributors to ischemic acute kidney injury, specifically, MCP-1 and TNF-α, were not significantly altered by Smad3 deficiency (Table 1). In addition to pathways that promote renal injury, we also examined a pathway that can protect against renal injury, namely, heme oxygenase (HO)-1 (19, 35): HO-1 mRNA expression was comparable induced, thus demonstrating that this was unlikely to be a mechanism contributing to the protective effects of Smad3 deficiency (Table 1).

**Effect of Smad3 deficiency on plasma IL-6 levels.** We also measured plasma levels of IL-6, since systemic levels of cytokines are a determinant of the outcome from acute kidney injury. Following ischemia, IL-6 was markedly increased at 24 h in Smad3<sup>+/+</sup> mice, and such elevation in IL-6 after ischemia did not occur in Smad3<sup>−/−</sup> mice (Fig. 10).

**DISCUSSION**

Our data, in aggregate, demonstrate that Smad3 is induced in the kidney following acute renal ischemia and that genetic deficiency of Smad3 leads to greater preservation of renal function, less histological injury, reduced renal expression of specific cytokines, and less systemic inflammation following acute renal ischemia. Based on these findings, we conclude that induction of Smad3 is a maladaptive response that contributes to the functional and structural damage attendant upon acute renal ischemia.

In an attempt to uncover mechanisms that may underlie the reduced ischemic injury incurred in Smad3<sup>−/−</sup> mice, we evaluated the expression of a number of cytokines that contribute to acute kidney injury following ischemia. The most dramatic effect was noted in the renal expression of IL-6, the latter markedly lower in Smad3<sup>−/−</sup> mice compared with Smad3<sup>+/+</sup> mice following ischemia. These findings are of interest in that multiple lines of evidence attest to the injurious effects of IL-6 following an ischemic insult. For example, the administration of a neutralizing IL-6 antibody leads to greater preservation of renal function and less histological injury following acute renal ischemic injury (22, 38); IL-6<sup>−/−</sup> mice evince less renal dysfunction and histological injury in response to an acute ischemic insult (22, 38); HO-1<sup>−/−</sup> mice, compared with HO-1<sup>+/+</sup> mice, exhibit an exaggerated induction of IL-6, increased renal dysfunction, and increased mortality following renal ischemia (47), while the administration of a neutralizing IL-6 antibody attenuates such renal dysfunction and mortality observed in HO-1<sup>−/−</sup> mice following ischemia (47). Clinical observations support the pathogenetic significance of increased IL-6 production as increased urinary excretion of IL-6 is a predictor for human acute kidney injury (9, 25). Our finding that IL-6 mRNA was markedly induced in the ischemic kidney, and that such expression of IL-6 was drastically reduced in the ischemic kidney in Smad3<sup>−/−</sup> mice, raises the possibility that the protection conferred by Smad3 deficiency may reflect reduced production of IL-6. In this regard, observations in other cell types demonstrate that the induction of IL-6 by TGF-β1 critically requires Smad3 (1, 13).
Renal ischemia provokes a systemic inflammatory response, and among the recognized and prominent cytokines so produced is IL-6. Such a systemic response is implicated in the adverse distant effects of renal ischemia such as lung injury (23). Clinical observations in acutely ill patients demonstrate that plasma IL-6 levels predict ventilator dependency, severity of acute kidney injury, and mortality that occur in this patient population (6, 29, 44). Our data demonstrate that the marked elevation in plasma IL-6 levels observed following ischemia in wild-type mice did not occur in Smad3\(^{-/-}\) mice. Thus the suppressive effect of the deficiency of Smad3 on IL-6 occurs not only regionally in the kidney but also in the systemic circulation.

It is possible that the protective effect of Smad3 deficiency we observed may reflect decreased production of other cytokines that are recognized as contributors to acute ischemic injury; in this regard, substantial evidence indicates that renal injury following acute ischemia can reflect increased renal expression of endothelin-1, MCP-1, and TNF-\(\alpha\) (10–12, 17, 24, 31, 34, 50). Our data demonstrate that Smad3\(^{-/-}\) mice compared with Smad3\(^{+/+}\) mice, in response to ischemia, had a blunted expression of ET-1 mRNA, and thus such reduced expression in ET-1 may be relevant to the reduced renal injury in Smad3\(^{-/-}\) mice observed after ischemia. These findings are consistent with observations that cellular induction of ET-1 by TGF-\(\beta\) requires Smad3 (5, 41).

The finding that MCP-1 and TNF-\(\alpha\) expression was comparably increased in the kidney in Smad3\(^{+/+}\) and Smad3\(^{-/-}\) mice is of interest for at least two reasons. First, it is unlikely that the adverse effects of Smad3 in acute renal ischemia are mediated through these cytokines; second, the lack of alteration in expression of these cytokines indicates that the reduction in IL-6 and ET-1 was not a nonspecific, generalized reductive effect on cytokine expression attendant upon the deficiency of Smad3.

We observed that renal induction of TGF-\(\beta\)1 mRNA was substantially reduced in Smad3-deficient mice following ischemia. To the best of our knowledge, we are unaware of
has been shown that Smad2 offsets the fibrogenic actions of TGF-β1 is pleiotropic in its actions and engages diverse signaling molecules. Even within the Smad family, constituent Smad3 in the ischemic kidney: in response to ischemia, Smad7, a negative regulator of TGF-β1 signaling (32). TGF-β1 also engages Smad7, which is a robust inducer of HO-1 (16), a cytoprotective molecule that reduces acute kidney injury incurred by ischemia, heme proteins, nephrotoxins, and endotoxins (19, 35, 43). Presumably, the salutary effects of TGF-β1 in ischemic renal injury reflect the preferential elicitation or the preponderating effects of cytoprotective rather than injurious signaling pathways. Our findings delineate that the effect of Smad3-deficient mice is uncertain, we speculate the basis for this reduced TGF-β1 mRNA expression in stressed Smad3-deficient mice is that of promoting, rather than protecting against, acute ischemic renal injury.

A novel and important investigative line has recently demonstrated that prior exposure to the volatile anesthetic sevoflurane confers protection against acute ischemic renal injury (26–28). These studies demonstrate involvement of TGF-β1/Smad3 signaling (32). TGF-β1 also engages Smad7, which is a robust inducer of HO-1 (16), a cytoprotective molecule that reduces acute kidney injury incurred by ischemia, heme proteins, nephrotoxins, and endotoxins (19, 35, 43). Presumably, the salutary effects of TGF-β1 in ischemic renal injury reflect the preferential elicitation or the preponderating effects of cytoprotective rather than injurious signaling pathways. Our findings delineate that the effect of Smad3-dependent pathways is that of promoting, rather than protecting against, acute ischemic renal injury.

Fig. 7. Renal expression of IL-6 mRNA 24 h after IR for either 22.5 or 25 min in duration and after Sham in Smad3+/+ and Smad3−/− mice. IL-6 mRNA expression was determined by quantitative real-time RT-PCR and standardized for 18S rRNA; n = 7–8/group. *P < 0.05, Smad3−/− vs. Smad3+/+ mice subjected to respective IR duration.

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In summary, to the best of our knowledge, we provide the first demonstration that induction of Smad3 occurs following acute ischemic insults, and such induction of Smad3 represents a maladaptive response that promotes acute isch-
emic injury. We suggest that the adverse effects of Smad3 reflect the upregulation of IL-6, which is known to occur in the ischemic kidney. The adverse effect of Smad3 in acute kidney injury described in the current study is notable in that other established mediators of chronic kidney disease [for example, TGF-β1 and hypoxia-inducible factor-1α (46)] are protective rather than contributory to acute ischemic injury. We also speculate that such upregulation of Smad3 may exert adverse long-term consequences. In our prior studies we described a sequential, triphasic response in the kidney subjected to repeated exposure to an acute insult: initial sensitivity, acquired resistance, and then chronic inflammation (36). In light of the established role of Smad3 in chronic inflammation and matrix expansion, we speculate that up-regulation of Smad3 may be germane to the risk of chronic kidney disease that ensues after acute ischemia.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

16. Hill-Kapturczak N, Truong L, Thamilselvan V, Visner GA, Nick HS, Agarwal A. Smad7-dependent regulation of heme oxygenase-1 by trans-

Table 1. mRNA expression assessed by quantitative real-time RT-PCR

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<th>Smad3+/+</th>
<th>Smad3+/-</th>
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<td>11.9 ± 3.7</td>
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<td>5.8 ± 0.7</td>
<td>62.5 ± 5.5</td>
<td>49.6 ± 6.7</td>
<td>55.6 ± 7.1</td>
<td>53.0 ± 6.1</td>
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<tr>
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<td>8.1 ± 1.8</td>
<td>63.0 ± 6.0</td>
<td>53.4 ± 6.7</td>
<td>51.8 ± 4.9</td>
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*Values are means ± SE and are the results of relative quantification performed against a standard curve constructed for each mRNA target, normalized for expression of 18S rRNA and expressed in arbitrary units; n = 7–8 for all groups. HO-1, heme oxygenase-1.


