Involvement of the Activin-Follistatin System in Tubular Regeneration after Renal Ischemia in Rats

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Abstract. This study was conducted to investigate the involvement of the activin-follistatin system in renal regeneration after ischemic injury. Expression of mRNA for the activin \(\beta_A\) subunit was not detected in normal kidneys but increased markedly after renal ischemia. Immunoreactive \(\beta_A\) subunit was detected in tubular cells of the outer medulla in ischemic but not normal kidneys. Expression of mRNA for follistatin, an antagonist of activin A, was abundant in tubular cells of the outer medulla in normal kidneys and decreased significantly after renal ischemia. For assessment of the role of the activin-follistatin system in renal regeneration after ischemic injury, recombinant follistatin was intravenously infused into rats with renal ischemia, at the time of reperfusion. Exogenous follistatin prevented the histologic changes induced by ischemic injury, reduced apoptosis in tubular cells, and accelerated tubular cell proliferation. Serum levels of creatinine and blood urea nitrogen were significantly lower in follistatin-treated rats. Conversely, intravenous administration of recombinant activin A inhibited tubular cell proliferation after ischemic injury. These results indicate that the activin-follistatin system participates in renal regeneration after ischemic injury. Follistatin administered intravenously accelerates renal regeneration after renal ischemia, presumably by blocking the actions of endogenous activin.

The kidney has a capacity to repair itself and recover its function after ischemia/reperfusion injuries by recapitulating the molecular and cellular events that are associated with nephrogenesis (1). During acute tubular necrosis induced by renal ischemia, normally quiescent cells undergo dedifferentiation and recover their potential to divide after enhancement of their DNA synthesis. After proliferation, the new cells differentiate to restore the functional integrity of the nephron (2). Many growth factors critical for kidney development (3,4) may be involved in kidney regeneration (5–14) and may play important roles in these processes as mitogens, motogens, and morphogens (15). Tubular cell regeneration is accelerated by the addition of various growth factors. For example, hepatocyte growth factor (HGF) is a growth factor with renotropic action (8), and administration of HGF promotes recovery from renal ischemia (9). Similarly, bone morphogenetic protein-7, insulin-like growth factor-1, and epidermal growth factor were demonstrated to be effective in promoting renal regeneration after ischemic injury (5,10,11). Heparin-binding epidermal growth factor (12), transforming growth factor-\(\beta\) (13), and platelet-derived growth factor (14) expression is up-regulated in injured kidneys and is also involved in renal regeneration after ischemia.

Activins are multifunctional cytokines that belong to the transforming growth factor-\(\beta\) superfamily and regulate the growth and differentiation of cells in various organs (16,17). The actions of activins are modified at several levels by various factors. The most important factor that modulates the actions of activins is an activin-binding protein, namely follistatin (18). This protein stoichiometrically binds to activins and blocks their action (18,19). Follistatin is expressed on the surface of the target cells of activins by binding to the extracellular matrix (20). Activins trapped by follistatin are internalized by endocytosis and subsequently degraded by proteolysis (21). The expression of follistatin is regulated by various factors, including activins themselves in many tissues (22–25). Hence, the activin-follistatin system is a complex regulatory system that controls diverse cellular functions, including growth and differentiation during development (23–25).

Follistatin is abundantly expressed in tubular cells of adult kidneys (26). However, the function of the activin-follistatin system in adult kidneys is not well understood. We recently demonstrated that activin A is an autocrine factor that is produced in tubular cells and tonically inhibits branching tubulogenesis (27). Activin A produced in tubular cells plays a vital role because HGF, which is a morphogen known to regulate tubulogenesis (28), induces branching tubulogenesis mainly by reducing the production of activin A (27). Activin A is expressed in fetal kidneys (29) and was also demonstrated to inhibit branching morphogenesis of ureteric buds in organ culture (30). Activin A may act as a negative regulator of branching morphogenesis during kidney development (27,30,31). These results raise the possibility that the activin-follistatin system, i.e., morphomodulating proteins involved in...
tubulogenesis during kidney development, may influence the migration, growth, and differentiation of tubular cells after renal ischemia. In this study, we examined this possibility. The results indicate that the production of activin A is upregulated in tubular cells after renal ischemia. Blockade of endogenous activin A action by administration of follistatin protects the kidney from ischemic renal injury by preventing apoptosis and promoting regeneration.

Materials and Methods

Experimental Protocols

Male Wistar rats weighing 200 to 230 g were obtained from the Imai Animal Co. (Saitama, Japan). Under anesthesia induced with sodium pentobarbital (30 mg/kg body wt), the abdominal cavity was exposed via a midline incision. Renal ischemia was induced by clamping both renal arteries for 45 min, using a nontraumatic vascular clamp. After removal of the clamp to allow reperfusion for the indicated periods, rats were euthanized and the kidneys were removed. The right kidney was fresh-frozen for RNA extraction, and the left kidney was fixed in 4% paraformaldehyde for routine paraffin embedding and sectioning for histologic analysis. Reperfusion was assessed by visual examination of the kidneys, which recovered their usual color within 30 s. Sham operations were performed in a similar manner, except for clamping of the renal arteries. Blood samples were obtained at the time of death, and serum samples were maintained at −20°C until measurements. Blood urea nitrogen (BUN) levels, serum creatinine levels, and electrolyte concentrations were measured with a multiparametric autoanalyzer (model 7050; Hitachi, Tokyo, Japan). For analysis of the efficacy of exogenous follistatin or activin A after ischemic renal injury, the indicated dose of recombinant human (rh)-follistatin or rh-activin A, dissolved in 0.5 ml of physiologic saline solution, was administered via the tail vein at the time of reperfusion. Renal functions, histologic changes, and the degree of DNA synthesis and apoptosis in tubular cells were analyzed. Control animals received the same volume of saline solution alone. Compared with no treatment, treatment with physiologic saline solution did not affect the outcomes of the experiments performed (data not shown). The experimental design is presented in Figure 1. rh-Follistatin and rh-activin A were generously provided by Dr. Ito of the Central Research Laboratory, Ajimonoto Inc. (Kawasaki, Japan).

RNA Extraction and Northern Blot Analyses

Total RNA was extracted from kidney homogenates with the TRIzol reagent (Life Technologies BRL, Grand Island, NY). Northern blot analyses were performed as described previously (27), using cDNA for rat follistatin (provided by Dr. S. Shimasaki, Salk Institute, La Jolla, CA) and cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech, Palo Alto, CA). The membranes were subjected to autoradiography and analyzed using a Fuji BAS 2000 (Fuji Photo Film, Tokyo, Japan). For quantification of the relative follistatin mRNA content, the intensities of the autoradiographic signals for follistatin and GAPDH were quantified and expressed in arbitrary density units. The follistatin/GAPDH ratio (based on integrated signals) was determined for each sample.

Reverse Transcription-PCR

Total RNA was isolated from whole kidneys with the TRIzol reagent (Life Technologies BRL). First-strand cDNA was made from total RNA using Superscript Preamplification System (Life Technologies BRL) according to the manufacturer’s instructions. Contaminating genomic DNA was removed with RNase-free DNase. Five micrograms of DNase-treated RNA was incubated with 1 μl of oligo(dT) at 70°C for 10 min. Two microliters of 10× PCR buffer, 1 μl of dithiothreitol (0.1 M), 2 μl of dNTP mixture (10 mM), and 2 μl of MgCl2 (25 mM) were added to each reaction. After incubation for 5 min at 42°C, 1 μl of reverse transcriptase was added. Samples were incubated at 42°C for 50 min and then at 70°C for 15 min. RNase H (1 μl) was added to each reaction, and samples were incubated at 37°C for 20 min. PCR was performed as indicated by the manufacturer (Perkin-Elmer, Norwalk, CT), with the following primers: rat βA subunit: sense, 5'-GGACCCTACTTCAAGCCAGAGATG-3'; antisense, 5'-TCTCAAAATGATGCTCTTTCTTG-3'; rat activin type II receptor: sense, 5'-GGTCTATGAGCAGGGGAAGATGAC-3'; antisense, 5'-ACATT-TTCGCCCTGGCCAGC-3'; rat activin type I receptor: sense, 5'-AGATGGAAGTCACACAGCCCAC-3'; antisense, 5'-CAACACTGCTGCTCTTCTCTCTG-3'; rat GAPDH: sense, 5'-
CATGACCACAGTCCATGCCATC-3'; antisense, 5'-CACCCTGTT-
GCTGTAG- CCATATTC-3'. Reactions included 5
mM of PCR
buffer, 2
mM of MgCl₂ (50 mM), 1
mM of dNTP mixture, 1
mM of 3'-primer, 1
mM of 5'-primer, 0.5
mM of Taq polymerase, and 1
mM of cDNA. Samples were incubated at 94°C for 5 min, followed by the
indicated numbers of cycles of 30 s at 94°C, 30 s at 58°C, and 90 s at
72°C, with final extension at 72°C for 10 min, in a Perkin-Elmer DNA
thermal cycler. PCR used 30 cycles for the
βA subunit and activin type
I and II receptors and 18 cycles for GAPDH. The levels of transcrip-
tion of the GAPDH “housekeeping” gene were found to be similar at
all time points examined, enabling analysis of the relative levels of
expression of the desired genes. Reactions without cDNA were used
as negative controls. Rat hepatocyte cDNA was used as a positive
control in each experiment. Reactions were repeated at least twice.

Measurement of DNA Synthesis
DNA synthesis in renal tubular cells was measured using bromode-
oxuridine (BrdU) (32). At the indicated times after reperfusion,
BrdU (100 mg/kg), an analogue of thymidine, was injected intraperi-
toneally into every animal used in the study. After 1 h, rats were euthanized, and the kidneys were removed and fixed with 4% form-
alddehyde for 24 h. Sections were immunostained using a cell prolifer-
ation kit (Amersham, Tokyo, Japan), as described previously (33).
The number of BrdU-positive cells was counted in five randomly
selected fields of the outer medulla, using a light microscope at ×400
magnification. The proliferation index was measured in five sections
per rat, and the average of the five determinations was calculated. The
results were indicated as the numbers of BrdU-positive cells per
square micrometer.

In Situ Hybridization
The cRNA probe was transcribed from a pBluescript SK(+) vector
containing an approximately 845-bp PstI/XbaI fragment derived from
rat follistatin cDNA (provided by Dr. S. Shimasaki, Salk Institute).
Linearized plasmids were used for in vitro transcription of digoxige-
nin-11-UTP-labeled antisense and sense riboprobes with SP6 and T7
RNA polymerase, respectively, according to the instructions provided
by the manufacturer (Boehringer Mannheim, Mannheim, Germany).
Eight-micrometer sections were mounted on poly-L-lysine-coated
slides. After digestion with 5
mg/ml proteinase K at room temperature
for 30 min, sections were postfixed in 0.4% paraformaldehyde at 4°C
for 20 min and incubated overnight at 50°C with hybridization buffer
containing 1
mg/ml digoxigenin-labeled cRNA. The buffer contained
50% formamide, 10 mM Tris-HCl (pH 7.5), 600 mM NaCl, 1 mM
ethylenediaminetetraacetate, 0.25% sodium dodecyl sulfate, 1
3 Den-
hardt’s solution, 200 µg/ml yeast tRNA, and 10% dextran sulfate.
After hybridization, sections were washed in 2× SSC/50% formamide
at 58°C for 30 min, incubated in 1 µg/ml digoxigenin-labeled cRNA
solution at 37°C for 30 min, and then washed once in 2× SSC and twice in 0.2× SSC
at 50°C, for 20 min each time. Sections were then incubated in a 1:500

Figure 3. Induction of activin A in tubular cells after renal ischemia.
The localization of activin A was examined by immunohistochemical
analysis using an anti-human activin A antibody, as described in the
Materials and Methods section. Paraffin sections from normal (A) and
ischemic (B) kidneys 24 h after reperfusion are presented. Arrow-
heads indicate brown staining for activin A. Magnification, ×400.

Figure 4. Localization of activin receptors in normal kidneys. The localization of type II (A through C) and type IIB (D and E) activin receptors
was examined by immunohistochemical analysis, as described in the Materials and Methods section. Paraffin sections from the cortex (A, D,
and F), outer medulla (B), and inner medulla (C and E) of normal kidneys are presented. A section reacted with normal goat serum is shown
in F. Magnification, ×400.
diluted solution of polyclonal sheep anti-digoxigenin Fab antibody conjugated with alkaline phosphatase, before washing and detection of the label with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Nick-End-Labeling

For identification of nuclei with DNA strand breaks at the cellular level, the terminal deoxynucleotidyl transferase-mediated dUTP-nick-end-labeling (TUNEL) method (34) was performed, using an apoptosis in situ detection kit (Wako, Tokyo, Japan). Quantification of TUNEL-positive cells was performed by counting positive nuclei in tubular cells from five randomly selected fields of the outer medulla, with a light microscope at ×400 magnification. Apoptosis was measured as a percentage of total tubular cells in five sections per rat kidney, and the average of the five determinations was used as an apoptotic index.

Immunohistochemical Analyses

The localization of activin A was examined immunohistochemically with an avidin-biotin coupling immunoperoxidase technique, using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the instructions provided by the manufacturer. Briefly, the paraffin-embedded sections (4 μm) were deparaffinized and rehydrated in a routine manner. After inactivation of endogenous peroxidase with 1% metaperiodic acid in phosphate-buffered saline (PBS) for 10 min at room temperature, sections were preincubated with normal goat serum for 60 min. The sections were then incubated with a polyclonal rabbit anti-activin A antibody for 2 h, washed with PBS, and reacted with a biotinylated goat anti-rabbit IgG for 1 h. After washing with PBS, sections were reacted with Vectastain Elite ABC reagent. The antibody was detected with diaminobenzidine tetrahydrochloride in PBS, and the sections were counterstained with hematoxylin. For immunohistochemical controls, the primary antibody was replaced with 5% normal goat serum in PBS, which did not demonstrate positive staining, thus confirming specificity. The anti-human activin A antibody used in this study recognizes dimers as well as monomers of the rat βA subunit (35).

The localization of type II and type IIB activin receptors was examined in a similar manner. Polyclonal rabbit anti-activin type II

Figure 5. Changes in the expression of mRNA for follistatin after ischemic renal injury. (A) Changes in follistatin mRNA expression after ischemic renal injury. Rat kidneys were isolated from ischemia-treated or sham-operated rats at the indicated times after reperfusion. Total RNA (20 μg) was subjected to Northern blotting, using cDNA probes for follistatin and GAPDH. Representative results from three separate experiments are shown. (B) Quantification of follistatin/GAPDH levels after ischemic renal injury. Values are mean ± SEM of three separate experiments and are expressed in arbitrary density units.

Figure 6. Localization of follistatin mRNA in normal and ischemic kidneys. In situ hybridization with a digoxigenin-labeled riboprobe was performed using sections from the cortex (A), outer medulla (B, D, and F), and inner medulla (C) of normal kidneys and the outer medulla of ischemic kidneys (E) 48 h after reperfusion. (A through E) Hybridization with an antisense probe. (F) Hybridization with a sense probe. Magnifications: ×400 in A, B, C, and F; ×1000 in D and E.
receptor and anti-activin type IIB receptor antibodies were generously provided by Dr. K. Miyazono (Cancer Institute, Tokyo, Japan).

**Histologic Analyses**

Sections were cut at 4 μm and stained with periodic acid-Schiff stain. Sections were microscopically examined for lesions 1, 2, 3, and 5 d after reperfusion. The changes observed were limited to the outer medulla, where tubular damage is most obvious, and were graded as follows: 0, normal; 1, areas of tubular dilation, necrosis, hemorrhage, and cell desquamation involving <20% of the fields; 2, similar changes involving >20% but <40% of the fields; 3, similar changes involving >40% but <60% of the fields; 4, similar changes involving >60% of the fields. Five sections per rat were used for analysis. The results were expressed as mean ± SEM for each experimental group.

**Statistical Analysis**

The significance of differences between means was compared by t test. *P < 0.05 was considered significant.

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**Results**

**Changes in the Expression of Activin and Activin Receptors after Ischemic Renal Injury**

To examine whether activin A is involved in renal regeneration, we analyzed changes in the expression of mRNA for the βA subunit of activin after ischemic renal injury by using reverse transcription-PCR. As shown in Figure 2, levels of βA subunit mRNA, which was virtually absent in normal and sham-operated kidneys, were upregulated 12 h after ischemic renal injury and remained elevated for approximately 96 h. The localization of activin A was studied by immunohistochemical analysis using an anti-activin A antibody. Immunoreactive activin A was not observed in normal kidneys (Figure 3A) or sham-operated kidneys (data not shown), an observation consistent with the reverse transcription-PCR results. In contrast, immunoreactive activin A was detected in tubular cells in the outer medulla of ischemic kidneys (Figure 3B). No positive cells were observed in sections from ischemic kidneys reacted with normal rabbit serum (data not shown). The biologic effects of activin A are mediated by heteromeric receptor complexes consisting of two different types of receptor, i.e., type I and II activin receptors (24). We examined changes in the expression of activin receptors. Type I and II activin receptor expression was detected but was not altered after ischemic renal injury (Figure 2). The localization of type II and IIB activin receptors in normal and ischemic kidneys was examined by immunohistochemical analyses (Figure 4). Type II activin receptor immunoreactivity was observed ubiquitously in tubular cells (including proximal and distal tubules) and collecting ducts in normal kidneys (Figure 4, A to C). Type IIB activin receptor immunoreactivity was also observed, in a pattern similar to that for type II activin receptors, in normal kidneys (Figure 4, D and E). No positive cells were observed in sections from normal kidneys reacted with normal goat serum (Figure 4F). However, distribution of these receptors was not altered by renal ischemia (data not shown).

**Changes in the Expression of Follistatin after Ischemic Renal Injury**

We then examined changes in the expression of follistatin mRNA by using Northern blotting (Figure 5). Consistent with previous reports (26), follistatin mRNA was abundantly expressed in normal kidneys. Sham operations did not affect follistatin mRNA expression levels. However, ischemia/reperfusion injury transiently decreased follistatin mRNA expression in the kidneys (Figure 5A). The relative levels of follistatin mRNA in ischemic kidneys were dramatically reduced at 24 and 48 h and returned to basal levels 72 h after reperfusion (Figure 5B).

Next, we examined the localization of follistatin mRNA in normal, sham-operated, and ischemic kidneys by *in situ* hybridization (Figure 6). In normal kidneys, hybridization signals were observed mainly in the inner stripe of the outer medulla, without any detectable hybridization in the cortex or inner medulla (Figure 6, A to C). Follistatin mRNA was distributed in tubular cells (Figure 6D). No staining was observed in any
cells of the glomeruli (Figure 6A). Hybridization signals in ischemic kidneys were considerably reduced, compared with normal kidneys (Figure 6E). Hybridization with a control sense probe demonstrated no positive signals (Figure 6F).

**Effect of rh-Follistatin on Renal Function after Ischemic Renal Injury**

To assess the role of endogenous activin A in renal regeneration, we administered rh-follistatin (25 μg/kg) or saline solution to rats with ischemic renal injuries. First, we evaluated parameters of renal function (Figure 7). In control (saline-treated) rats, the serum creatinine levels peaked 12 h after reperfusion and rh-follistatin significantly reduced the peak creatinine concentration (Figure 7A). The serum BUN levels peaked 24 h after reperfusion and returned to normal levels 72 h after reperfusion. Peak BUN levels were significantly lower for rh-follistatin-treated rats than for control rats (Figure 7B). Electrolyte concentrations were not affected by rh-follistatin administration (data not shown). No significant differences in body weights between the control and rh-follistatin-treated groups were observed at the indicated times (Figure 7C).
Effect of rh-Follistatin on Histologic Changes Induced by Renal Ischemia

We next examined whether exogenous follistatin affects the histologic changes induced by ischemia. Control (saline-treated) kidneys exhibited signs of congestion, hemorrhage, tubular dilation, cast formation, and tubules plugged with desquamated epithelial cells after ischemic renal injury (Figure 8, A and B). In contrast, rh-follistatin-treated kidneys exhibited no cast formation and fewer tubules were dilated (Figure 8D). Apoptotic tubular cells with highly condensed nuclear chromatin were also observed in control kidneys (Figure 8C) but not in rh-follistatin-treated kidneys. Neutrophil infiltration was observed in both control kidneys and rh-follistatin-treated kidneys (data not shown). Semiquantitative analysis demonstrated that the damaged tubular areas in rh-follistatin-treated kidneys were smaller than those in control kidneys (Figure 8E).

Effect of rh-Follistatin on Apoptosis Induced by Ischemic Renal Injury

We next examined the effect of rh-follistatin on renal ischemia-induced apoptosis by using the TUNEL method (Figure 9). TUNEL-positive cells were not observed in normal or sham-operated kidneys (Figure 9, A and B). In contrast, TUNEL-positive cells were predominantly localized in the outer medulla of control (saline-treated) kidneys after ischemic renal injury (Figure 9C); only a few positive cells were observed in the cortex and inner medulla (data not shown). Consistent with light-microscopic examination results, the

**Figure 9.** Apoptotic tubular cells in kidneys treated with saline solution or rh-follistatin after ischemic renal injury. (A through D) rh-Follistatin (25 μg/kg) (D) or saline solution (C) was injected at the time of reperfusion, via the tail vein. The kidneys were removed 48 h after the injection. Tissue sections from normal (A) and sham-operated (B) kidneys were also used for experiments. The TUNEL method was performed as described in the Materials and Methods section. (E) Quantification of TUNEL-positive cells was performed by counting the number of TUNEL-positive tubular cells in randomly selected fields of the outer medulla of saline- or rh-follistatin-treated kidneys, and results are expressed as percentages of total tubular cells. Values are mean ± SEM (n = 6). *, P < 0.01 versus ischemia treated with saline solution. Magnification, ×1000 in A through D.
number of TUNEL-positive cells in rh-follistatin-treated kidneys was markedly lower than that in control kidneys (Figure 9D). Reduction of the number of TUNEL-positive cells by exogenous follistatin was observed 24 and 48 h after reperfusion (Figure 9E).

Effect of rh-Follistatin on DNA Synthesis in Tubular Cells after Ischemic Renal Injury

Proliferation of tubular cells is the hallmark of early regeneration after ischemic renal injury (1). To investigate the effect of exogenous rh-follistatin on cell proliferation after ischemic renal injury, we measured DNA synthesis in renal tubular epithelial cells (Figure 10). In normal (Figure 10A) and sham-operated (data not shown) kidneys, BrdU-positive cells were rarely observed. Twenty-four hours after ischemic renal injury, BrdU-positive cells were observed predominantly in the outer medulla of control (saline-treated) kidneys (Figure 10B), where damage to the tubular cells was most obvious, but not in other regions (data not shown). In contrast, the number of BrdU-positive cells in rh-follistatin-treated kidneys was significantly greater than that in control kidneys (Figure 10, C and D). Quantitative analysis demonstrated that intravenous infusion of rh-follistatin enhanced renal tubular epithelial cell proliferation (Figure 11). Enhancement of DNA synthesis by rh-follistatin was observed at concentrations of ≥15 µg/kg.

![Figure 10](image)

Figure 10. DNA synthesis in renal tubular cells in the kidneys of rats treated with saline solution or rh-follistatin after ischemic renal injury. rh-Follistatin (25 µg/kg) or saline solution was injected at the time of reperfusion, via the tail vein. The kidneys were removed 24 h after the injection. DNA synthesis was assessed by BrdU staining, as described in the Materials and Methods section. Tissue sections from normal (A) and ischemic kidneys treated with saline solution (B) or rh-follistatin (C and D) were used for experiments. Magnifications: ×400 in A, B, and C; ×1000 in D.

![Figure 11](image)

Figure 11. Quantitative analysis of DNA synthesis in renal tubular cells after ischemic renal injury. Saline solution, rh-activin A (12.5 µg/kg), or rh-follistatin (5, 15, or 25 µg/kg) was administered to rats with ischemic renal injury, at the time of reperfusion. In separate experiments, rh-follistatin (15 µg/kg) was administered to normal rats. After 24 h, the kidneys were removed and used for experiments. DNA synthesis was assessed by BrdU staining, as described in the Materials and Methods section. BrdU-labeled cells were counted in randomly selected fields of the outer medulla in each kidney, and results are expressed as the number per square micrometer (proliferation index). Values are mean ± SEM (n = 5 to 10). *, P < 0.01 versus ischemia treated with saline solution. **, P < 0.001 versus ischemia treated with saline solution.
Effect of rh-Activin A on DNA Synthesis in Tubular Cells after Ischemic Renal Injury

To further assess the role of the activin-follistatin system in renal regeneration, we administered rh-activin A (12.5 μg/kg) to rats with ischemic renal injuries. Exogenously administered activin A did not affect renal function after ischemic injury (data not shown) but inhibited DNA synthesis in tubular cells. The number of BrdU-positive cells in ischemic kidneys treated with rh-activin A was significantly lower than that in ischemic kidneys treated with saline solution (Figure 11).

Effect of rh-Follistatin on DNA Synthesis in Tubular Cells in Intact Kidneys

To elucidate the mechanism of DNA synthesis enhancement by exogenous follistatin, we administered rh-follistatin to normal rats and examined whether tubular cell proliferation in intact kidneys was induced by rh-follistatin administration. Exogenously administered follistatin did not induce tubular cell proliferation (Figure 11) or affect tubular structure or renal function (data not shown).

Discussion

In this study, we investigated changes in the activin-follistatin system during kidney repair and regeneration. As in other tissues (36), the expression of activin and follistatin in the kidneys changed significantly after ischemic injury. The expression of activin A was not detected in normal kidneys, whereas activin A mRNA and protein expression was augmented after renal ischemia. Histologically, activin A immunoreactivity was induced in tubular cells. Because the expression of activin receptors was detected but was not altered after renal ischemia, it is expected that the action of activin A becomes dominant in the kidneys after ischemic injury. In addition, follistatin mRNA expression was markedly reduced after renal ischemia. Because follistatin is an inhibitor of activin action (18), these results indicate that the upregulated activin A in tubular cells is functionally active after renal ischemia. The significance of the induction of activin A expression in acute renal failure caused by renal ischemia is unclear. However, results obtained with the administration of exogenous activin A to ischemic kidneys provide some insights into the role of activin A under these conditions. As demonstrated in Figure 11, exogenous activin A significantly reduced the number of BrdU-positive cells after renal ischemia. Activin A also inhibits DNA synthesis in the proximal tubular cell line LLC-PK1 in vitro (Maeshima A, Kojima I, unpublished observations). These observations are corroborated by previous reports that activin A acts as a growth inhibitor in various types of cells (23–25,37). Therefore, it is likely that endogenous activin A tonically inhibits the regeneration of renal tubular cells. Consistent with this hypothesis, follistatin administration markedly increased the number of BrdU-positive cells after renal ischemia. Furthermore, follistatin attenuated histologic changes in tubular cells and improved renal function. Exogenously administered follistatin did not induce tubular cell proliferation in intact kidneys (Figure 11). These results suggest that the acceleration of renal regeneration induced by exogenous follistatin after ischemic injury results from the inactivation of upregulated endogenous activin A and not from the mitogenic effects of follistatin itself.

Apoptosis triggered by ischemia potentially contributes to the development of postischemic tubular cell death (38). A previous study demonstrated a biphasic pattern of tubular cell apoptosis after renal ischemia, suggesting that apoptosis observed in the early phase after renal ischemia is mainly attributable to the ischemic injury and that late-phase apoptosis is attributable to the programmed removal of tubular cells for control of excessive proliferation (39). In this study, the expression of activin A was observed 12 h after reperfusion, which preceded the appearance of TUNEL-positive tubular cells. Exogenous follistatin reduced the number of TUNEL-positive cells 24 and 48 h after reperfusion but not thereafter (Figure 9E). This result suggests that, after renal ischemia, upregulated endogenous activin A may be involved in the induction of early-phase apoptosis of tubular cells.

Inflammation is also considered to be one of the most important causes of tissue injury in organs subjected to ischemia. A series of recent studies suggested a novel role for activin in inflammation and repair processes in various organs (36,40–42). For example, increased expression of activin was observed in various types of inflammatory processes, including cutaneous wound repair and inflammatory arthropathies. The level of activin expression was correlated with the degree of inflammation in inflammatory bowel disease. Strong expression of activin A was induced in vitro by proinflammatory cytokines such as interleukin-1 and tumor necrosis factor-α, which are known to be released from macrophages and stromal cells at the sites of tissue injury and inflammation (43). Therefore, proinflammatory cytokines are possible inducers of activin expression in this model. With respect to the mechanism of action of activin, the release of activin into the circulation precedes the release of proinflammatory cytokines after lipopolysaccharide treatment, suggesting a proinflammatory action of activin (41). In contrast, activin A produces anti-inflammatory effects by blocking the action of interleukin-6 (42). The action of activin in inflammatory processes probably depends on the type of cell or tissue. It was recently reported that inhibition of ischemia-induced apoptosis prevents inflammation and subsequent tissue injury (44). Our results demonstrated that rh-follistatin reduced the number of apoptotic cells (Figure 9). Therefore, it is possible that rh-follistatin exerts an anti-inflammatory action. Although we could not observe rh-follistatin modulation of neutrophil infiltration in this study, we cannot completely exclude the possibility that endogenous activin regulates the degree of inflammation after renal ischemia, including myeloperoxidase activity, the degree of monocyte/macrophage infiltration, and the tissue levels of proinflammatory cytokines. This issue remains to be addressed.

Our results demonstrate for the first time that blockade of the action of endogenous growth inhibitors is an alternative to the infusion of growth factors for acceleration of renal regeneration. Follistatin probably potentiates multiple types of endogenous growth factors involved in renal regeneration. Follistatin may also enhance the effects of any exogenous growth factors.
The advantage of using follistatin is that it accumulates in the kidneys after intravenous administration (45). In the kidneys, follistatin binds to heparan sulfate proteoglycan (20) and remains in the extracellular matrices for several days (45). The stability of follistatin may be beneficial for the treatment of acute renal failure after a variety of insults.

In summary, the activin-follistatin system is altered in kidneys after ischemic injury. Endogenous activin A may function to promote apoptosis and inhibit regeneration of renal tubular cells. Blockade of the actions of activin with the administration of follistatin accelerates recovery from ischemic renal injuries. Follistatin has therapeutic potential for the prevention and treatment of tubular damage leading to acute renal failure.

Acknowledgments
This study was supported by a grant-in-aid from the Ministry of Education, Sports, Science, and Culture of Japan. We thank Mayumi Odagiri for technical secretarial assistance.

References


