Expression and Function of Ets-1 during Experimental Acute Renal Failure in Rats

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Abstract. The Ets family of transcription factors is defined by a conserved DNA-binding Ets domain that forms a winged helix-turn-helix structure motif. The Ets family is involved in a diverse array of biologic functions, including cellular growth, migration, and differentiation. The hypothesis in this study was that Ets-1 is re-expressed during regeneration after acute renal failure (ARF) and plays a key role in the transcriptional regulation of cyclin D1 and the cell cycle progression in renal tubular cells. For clarifying the significance of Ets-1 in ARF, a rat ARF model in vivo and LLC-PK1 cells as an in vitro model were used. After the left rat renal artery was clamped for 1 h, the whole kidney homogenate was examined and total RNA was extracted at 6, 12, 24, 48, and 72 h after reperfusion by Western blot analysis and real-time reverse transcription–PCR. Ets-1 mRNA and protein expression were strongly increased at 6 to 24 h after the ischemia, respectively. The expression of hypoxia-inducible factor-1α was increased dramatically as early as 6 h after ischemia-reperfusion and decreased at 48 and 72 h after ischemia-reperfusion. In the immunohistologic examination, Ets-1 was expressed in the proximal tubules and coexpressed with proliferating cell nuclear antigen (PCNA). Furthermore, overexpression of Ets-1 promoted the cell cycle and increased the promoter activity and protein expression of cyclin D1 in LLC-PK1 cells. Ets-1 promoter activity increased between 3 and 6 h in hypoxia, and hypoxia also induced changes in the Ets-1 protein level in LLC-PK1 cells. The Ets-1 induction by hypoxia was abolished by the transfection of dominant-negative hypoxia-inducible factor-1α. A gel shift assay demonstrated that Ets-1 binds to the Ets-1 binding site of the cyclin D1 promoter in the ischemia-reperfusion condition. Overexpression of Ets-1 did not significantly change the caspase 3 activity or the value of cell death ELISA in LLC-PK1 cells. Taken together, these data suggest that Ets-1 plays a key role in the cell-cycle progression of renal tubules in ARF. The Ets-1 pathway may regulate the transcription of cyclin D1 and control the regeneration of renal tubules in ARF.

Ischemic acute renal failure (ARF) is the most common form of ARF in the adult population. The molecular basis of the events that lead to tubular regeneration after ARF is not understood (1–3). An understanding of the mechanisms that lead to renal cell proliferation and regeneration will be necessary for the exploration of novel therapeutic strategies for the treatment of ischemic ARF. It has been suggested that regeneration processes may recapitulate developmental processes to restore organ or tissue function (4, 5). Adult tubular epithelial cells have a potent ability to regenerate after cellular damage. After ischemic renal damage, normally quiescent cells undergo de-differentiation and acquire the ability to proliferate after their DNA synthesis is enhanced (6, 7). In ARF, the regulation of cyclin and the cyclin-dependent kinase (CDK) inhibitor have been reported (8, 9). The restriction point of the G1-to-S phase is determined by the activities of cyclin D1, cyclin A, cyclin E, and CDK (10, 11). Cyclin D1 and cyclin A play key roles in the G1-S regulation of renal tubular epithelial cells (12).

The Ets family of transcription factors is defined by a conserved DNA-binding Ets domain that forms a winged helix-turn-helix structure motif (13, 14). This family of transcription factors is involved in a diverse array of biologic functions, including cellular growth, migration, and differentiation (14–16). Ets-1 is the first member discovered in the Ets family of transcription factors. The expression of Ets-1 has been detected in various cells, and the roles of the Ets-1 gene expressed in mesodermal lineage cells, such as fibroblasts and endothelial cells, has drawn wide attention in the fields of embryogenesis and angiogenesis (17–20). During morphogenesis, Ets-1 expression occurs in vascular structures and branching tissues, including kidneys (18, 21, 22). In adult tissues, the levels of Ets-1 expression are much lower than in embryonic tissues, including the kidneys (18). Ets-1 expression has been reported in mesangial cells and to increase in the glomeruli and inter-

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titation in rat crescentic glomerulonephritis (23, 24). However, there have been no reports concerning Ets-1 expression during ARF. Recently, we and another group reported that the developmental genes Wnt-4 and Pax2 are expressed during ischemic acute renal injury and that Wnt-4 expression promotes the proliferation of renal tubular cells (25, 26). These data suggested that some developmental genes are re-expressed during the recovery phase of ARF. A recent paper demonstrated that hypoxia induced Ets-1 expression via the activity of hypoxia-inducible factor-1 (HIF-1) in endothelial cells (27). Thus, there is a possibility that Ets-1, which is expressed during renal development, may play roles in ischemic renal failure.

Our hypothesis in this study is that Ets-1 is re-expressed during regeneration after ARF and plays a key role in the transcriptional regulation of cyclin D1 and in the cell-cycle progression in renal tubular cells. To test this hypothesis, we examined the expression pattern of Ets-1 during the recovery phase of an ischemia-reperfusion kidney. Our data demonstrate that Ets-1 is upregulated in the early phase of an ischemia-reperfusion kidney and that the overexpression of Ets-1, using an adenovirus, regulates the transcription of cyclin D1 and cell-cycle progression in renal tubular cells.

Materials and Methods

Cell Culture and Exposure to Hypoxia

LLC-PK1 cells, originally purchased from American Type Culture Collection (Rockville, MD), were grown in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% heat-inactivated FCS (Life Technologies). Cells were cultured at 37°C in 20% O2 and 5% CO2 (referred to as the normoxic condition). For the hypoxia experiments, cells were placed in a hypoxic chamber (Bellow Glass, Vineland, NJ) that contained 0% O2 and 5% CO2, which was maintained at 37°C.

Plasmid Constructs

The Ets-1 reporter construct used for the luciferase assays contained a mouse Ets-1 promoter (−2.1 kbp) cloned upstream of the luciferase gene (28). The cyclin D1 reporter construct used for the luciferase assays contained a human cyclin D1 promoter from residues −944 to 139 cloned upstream of the luciferase gene (gift of Dr. M. Eilers Zentrum für Molekularbiologie Heidelberg, Heidelberg, Germany) (29). Expression vectors coding for HIF-1α and dominant-negative (dn) HIF-1α were prepared as described previously (30, 31) using the following primers: forward 5′-GGAAGACAACGGCGG-GCAC-3′ and reverse 5′-GGAGCTGTGAATGTGCTGTGATCT-3′, and dnHIF-1α forward 5′-CCGCTGAGACCATGCGAAGGCA- and reverse 5′-GGGTTACCTCACTTATCA-3′. An open reading frame coding for the dnHIF-1α is a 1.1-kbp fragment whose product lacks both the DNA binding and transactivation domain. In both cases, RNA out of a C57BL mouse kidney was used as a template. The PCR fragment was subcloned into pcDNA3.1 (Invitrogen, San Diego, CA) and was processed to deleteoxy-DNA sequencing.

Transient Transfection and Luciferase Assay

LLC-PK1 cells were transfected by the electroporation method with plasmid DNA (10 μg). Data are representative of at least four independent experiments performed in duplicate and are expressed as an n-fold increase in the luciferase activity calculated relative to the indicated level of Ets-1 promoter activity. Normalization was achieved by co-transfecting a β-galactosidase reporter construct, as described previously (12). Luciferase and β-galactosidase activities were measured according to the Promega (Madison, WI) protocol.

Recombinant Adenoviruses

Replication-defective, recombinant adenoviruses encoding human Ets-1 (Adets-1) and a control adenovirus (Adnull) were prepared as described previously (32). Replication-defective, recombinant adenoviruses encoding the rat dominant-negative Akt (AdAktDN) were also prepared as described previously (33). In this adenovirus, both Thr308 and Ser473 of the rat Akt1 were replaced by alanine (33).

Induction of ARF

Male Sprague-Dawley rats (Saitama Experimental Animal Supply, Saitama, Japan) that weighed 150 to 200 g were anesthetized intra-peritoneally with sodium pentobarbital (30 mg/kg) at surgery. The left renal artery was occluded with Sugita aneurysm clips (Mizuho Ikagyo, Tokyo, Japan) for 60 min. The clamps were removed after 60 min; the incisions were closed; and the rats were killed at 0, 6, 12, 24, 48, and 72 h (n = 5). The left kidney was removed quickly and processed for histologic evaluation, protein extraction, or RNA extraction. Age- and weight-matched Sprague-Dawley rats also received sham operations in a similar manner, except for clamping of the renal arteries at 6, 12, and 24 h (n = 5).

Isolation of Kidney Tissue and Histologic Examination

Rats were anesthetized with pentobarbital at indicated times after the ischemic event. Their kidneys were perfused with sterile PBS. The left kidney was excised quickly, frozen in liquid nitrogen, and homogenized in the SDS sample buffer described later.

For immunohistochemical studies, renal tissues were fixed in formalin overnight and then dehydrated and embedded in paraffin. Thin sections were examined with periodic acid-Schiff staining as described previously (34). Immunohistochemical staining was performed by a streptavidin and biotin technique using an anti-Ets-1-specific antibody (Santa Cruz Biochemical; N-276, sc-111), anti-proliferating cell nuclear antigen (PCNA)-specific antibody (Santa Cruz Biochemical; PC-10, sc-56), and anti-aquaporin-1-specific antibody (Santa Cruz Biochemical; B-11, sc-25287) as markers for proximal tubules, as described previously (35–37).

Western Blot Analysis

Homogenized total renal tissue or LLC-PK1 cells were lysed in an SDS sample buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, and 0.1 mM sodium orthovanadate) at 4°C (38). Protein was transferred to a nitrocellulose membrane and probed with the anti–Ets-1–specific antibody. The primary antibodies were detected using horseradish peroxidase–conjugated rabbit anti-mouse IgG and visualized by the Amersham ECL system (Amer sham, Arlington Heights, IL).

Electrophoretic Mobility Shift Assay

Nuclear extracts from the renal cortex were prepared as described previously (39). The extracts (10 μg) underwent a reaction in a premixed incubation buffer (Gel Shift Assay Kit; Promega) with the γ,32P-end-labeled ets-1 binding site of a cyclin D1 promoter-lesion (5′-GATCTGAGACCGTTATCGA-3′) for 30 min at 25°C. To establish the specificity of the reaction, we performed competition...
assays with 100-fold excess of unlabeled ets-1 binding oligonucleotides (heterologous competitor DNA). To perform supershift assay, we added 5 μg of anti–Ets-1 antibody (Santa Cruz; N-276, cs-111) to the nuclear extracts, incubated extracts for 1 h at 4°C, and performed gel shift assay. After the reaction, the samples were analyzed on a 6% nondenaturing polyacrylamide gel. The gel was dried, and the protein-DNA complexes were visualized by autoradiography.

Real-Time Quantitative PCR
We performed a reverse transcription–PCR (RT-PCR) reaction for Ets-1 from RNA extracted from the ischemia-reperfusion kidneys. Total RNA was harvested from renal tissue using TRI-REAGENT (Life Technologies) (40). One microgram of total RNA samples was used for the RT-PCR as follows. The real-time quantitative PCR method was used to detect accurately the changes in Ets-1 gene copies. Total RNA was harvested from renal tissue. Rat Ets-1 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA were amplified. The primers for rat Ets-1 were 5'-GCCCCACCTCATCA-CAGAGT-3' (upper) and 5'-GTGGTAAGATGCTGGCTG-3' (lower) (23). The predominant cDNA amplification product was predicted to be 296 bp in length. The RT-PCR of GAPDH served as positive controls. The primers for rat GAPDH were 5'-TCCCTCAAA-GATGTCAGCAA-3' (upper) and 5'-AGATCCACCAAGGATA-CATT-3' (lower) (41). The predominant cDNA amplification product was predicted to be 309 bp in length. PCR products were detected and quantified in real time using the LightCycler Real-Time PCR (Roche Molecular Biochemicals, Tokyo, Japan) as described previously (42,43). A three-step PCR was performed for 35 cycles. Denaturation was performed at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s. The PCR products of Ets-1 and GAPDH were subcloned to the TA cloning vector (Promega, Biotec, Madison, WI) as described previously (40). The plasmids that contained Ets-1 cDNA and GAPDH cDNA were used to make standard curves of quantitative PCR.

Cell Proliferation Analysis by [3H]Thymidine Incorporation
After transfection, the LLC-PK1 cells were plated in 24-well plates and incubated in a medium without FCS for 20 h. For the last 4 h, the cells were pulsed with 1 μCi [3H]thymidine (Amersham). After the incubation, the cells were redissolved in 0.5 M NaOH and counted in an Aquasol-2 scintillation cocktail (NEN Research Products, Boston, MA) (44).

Caspase 3 Assays
A Caspase 3 Fluorometric Protease Assay Kit (MBL, Tokyo, Japan) was used for the measurement of caspase 3 activities as described previously (33). In brief, the cells were plated in six-well dishes and infected with adenoviruses. The cell lysates were then incubated with the same amounts of reaction buffer and a 50-mM DEVD-AFC substrate for 2 h at 37°C. Fluorescence was monitored with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Cell Death ELISA
Histone-associated DNA fragments were quantified by ELISA (Boehringer Mannheim). All cells from each well were collected by trypsinization and pipetting, then pelleted (800 rpm, 5 min), lysed, and subjected to ELISA capture according to the manufacturer’s protocol (33). Cytosolic proteins were collected using a cell lysis buffer and centrifuged according to the manufacturer’s protocol. The nucleus formed into a pellet, and the cytoplasmic fraction became the supernatant. These supernatants were collected for the ELISA assay. Each experiment was carried out in triplicate and repeated in at least five independent experiments.

Statistical Analyses
The results are given as means ± SEM. The differences were tested using a two-way ANOVA followed by the Scheffe test for multiple comparisons. Two groups were compared by the unpaired t test. P < 0.05 was considered significant.

Results
Western Blot Analysis of the Protein Expression of Ets-1 and HIF-1α after Ischemic Renal Failure
The left renal artery was clamped for 60 min and the left kidney was excised at 0, 6, 12, 24, 48, and 72 h after reperfusion. Western blot analysis was used to detect the protein levels of Ets-1 and actin. The expression of Ets-1 was weak in the control kidney (0 h) and sham-operated kidneys (6 and 12 h; Figure 1). The expression of Ets-1 was dramatically increased at 6 to 24 h after ischemia-reperfusion (Figure 1A). The up-regulation of Ets-1 protein expression was temporary, with the intensity of the Ets-1 band decreasing at 48 and 72 h after ischemia-reperfusion. The protein levels of Ets-1 exhibited no changes after 6 to 12 h in the sham-operated rats (the data of 24 h are not shown). Western blot analysis was used to detect the protein levels of HIF-1α and actin. The expression of HIF-1α was weak in the control kidney (0 h) and sham-operated kidneys (6 and 12 h; Figure 1). The expression of HIF-1α was dramatically increased as early as 6 h after ischemia-reperfusion (Figure 1A). The up-regulation of HIF-1α protein expression was temporary, with the intensity of the

![Figure 1](https://example.com/figure1.png)
Ets-1 band decreasing at 48 and 72 h after ischemia-reperfusion. We performed an immunoblot for actin as a loading marker, and there were no significant changes in actin during ischemia-reperfusion. The protein levels of HIF-1α exhibited no changes after 6 to 12 h in the sham-operated rats (the data of 24 h are not shown).

**Immunohistochemical Examination of Ets-1 in ARF**

Next, we performed immunohistologic studies on Ets-1 in ARF (Figures 2 and 3). In a low-power view examination, Ets-1 expression was observed in cortical renal tubules at 12 h after ischemia-reperfusion (Figure 2A). We used the anti-aquaporin-1 antibody as a marker of proximal tubules (35–37). As shown in Figure 2, A and B, the expression of Ets-1 is co-localized with aquaporin-1 in the low-power view examination in continuous sections. Conversely, only a slight Ets-1 expression is observed in the renal cortex form control rats (Figure 2E), whereas the expression of aquaporin-1 is clearly observed in the renal cortex form control rats (Figure 2F). From these results, Ets-1 was expressed mainly in the proximal tubules of the renal cortex 12 h after ischemia-reperfusion (Figure 2). In the renal medulla, Ets-1 could not be detected in either the ischemia-reperfusion kidney (Figure 2C) or the control kidney (data not shown). In a higher-power view, Ets-1 staining was observed in the nucleus of proximal tubular cells (Figure 3A). As shown in Figure 3B, the expression of Ets-1 is co-localized with aquaporin-1 in continuous sections. To examine the specificity of the antibody, we used a blocking peptide (sc-111p; Santa Cruz Biotechnology). The nuclear signal diminished in the presence of the blocking peptide in the cortex of ischemia-reperfusion kidney (Figures 2G and 3C).

To co-localize Ets-1 with dividing cells, we examined Ets-1 staining at 24 h after ischemia-reperfusion (Figure 4). Ets-1 expression was observed at the proximal tubules of the cortex (Figure 4, A and C). PCNA staining was also observed at the proximal tubules of the cortex (Figure 4, B and D). The higher power view of Ets-1 staining also revealed co-localization of Ets-1 and PCNA in the same tubules at 24 h after ischemia-reperfusion in continuous sections (Figure 4, C and D). Conversely, only a slight Ets-1 expression is observed in the renal cortex from control rats (Figure 4E). No PCNA-positive tubules were observed from control rats (Figure 4F). To examine the specificity of the antibody, we used a blocking peptide (sc-9857p; Santa Cruz Biotechnology). The nuclear signal diminished in the presence of the blocking peptide in the cortex of ischemia-reperfusion kidney (Figure 4G).

**Real-Time PCR**

Quantification of the Ets-1 mRNA transcript using the real-time quantitative PCR method revealed 8.0-fold (6 h), 3.8-fold (12 h), and 2.2-fold (24 h) increases in Ets-1 mRNA levels compared with the 0-h value (Figure 5). The linear curve between the cDNA amount and PCR product was observed in the same range utilized by using the Ets-1 cDNA plasmid. The signals of Ets-1 were not significantly changed in the sham-operated rat kidneys. The GAPDH signal was not significantly changed by ischemia-reperfusion (Figure 5A).

**Hypoxia Stimulates Ets-1 Expression and Promoter Activity via HIF-1α in LLC-PK1 Cells**

To examine whether hypoxia-reperfusion stimulates Ets-1 expression and promoter activity in renal tubular cells, we used a hypoxic culture system with LLC-PK1 cells. We exposed LLC-PK1 cells to hypoxia for 3, 6, and 6 h and examined the promoter activity of Ets-1 and the expression of Ets-1 protein by Western blot analysis. In the
promoter assay experiments, the Ets-1 promoter-luciferase plasmid was transfected in LLC-PK1 cells 48 h before hypoxia. As shown in Figure 6B, Ets-1 promoter activity was increased 7.1-fold at 3 h, 9.1-fold at 6 h, and 12.1-fold at 6 h + reoxygenation 6 h. Hypoxia also induced changes in the Ets-1 protein level in LLC-PK1 cells (Figure 6A). There were no significant changes in actin during hypoxia/reoxygenation.

To test whether the upregulation of Ets-1 is dependent of HIF-1α, we examined Ets-1 protein expression and promoter activity in HIF-1α–transfected LLC-PK1 cells and dnHIF-1α–transfected LLC-PK1 cells. The hypoxic inducibility of the Ets-1 promoter assay in HIF-1α–overexpressing and dnHIF-1α–overexpressing cells was 17.8- and 1.5-fold, respectively, as compared with 9.1-fold in control LLC-PK1 cells at 6 h (Figure 6D). The Western blot analysis demonstrated that transfection of dnHIF-1α reduced the increment of Ets-1 protein by hypoxia (Figure 6C). These results indicated that the upregulation of Ets-1 by hypoxia is dependent on HIF-1α.

Electrophoretic Mobility Shift Assay

To examine the induction of cyclin D1 expression via the ets-1 binding site, we performed an electrophoretic mobility shift assay using nuclear extracts from a rat renal cortex. Figure 7 shows that the nuclear extract from the ischemia-reperfusion rat renal cortex gave rise to a protein-DNA complex (Figure 7, lane 2). However, we did not detect protein-DNA complex band in control rat renal cortex (Figure 7, lane 3). The band was completed using 100-fold excess of the unlabeled oligonucleotides (heterologous competitor DNA; Figure 7, lane 4). When nuclear extracts from the ischemia-reperfusion rat renal cortex were preincubated with anti–Ets-1 antibody, the super-
shift of the band was observed (Figure 7, lane 6). These results confirm that Ets-1 binds to the ets-1 binding site of the cyclin D1 promoter in the ischemia-reperfusion condition.

Cell Proliferation and Cyclin D1 Expression by the Overexpression of Ets-1 in LLC-PK1 Cells

We first examined the effects of the overexpression of Ets-1, using an adenovirus, on the cell proliferation of LLC-PK1 cells by \[^{3}H\]thymidine uptake. Figure 8A shows the effects of Ets-1 on \[^{3}H\]thymidine uptake. Overexpression of Ets-1 stimulated the \[^{3}H\]thymidine uptake to 225% dose-dependently. Overexpression of Adnull (control adenovirus) did not significantly change the \[^{3}H\]thymidine uptake in LLC-PK1 cells. We next examined the role of Ets-1 in the regulation of cyclin D1 promoter activity and protein expression. We performed a transient transfection with the cyclin D1-luciferase reporter gene and the \(\beta\)-galactosidase expression vector and then infected it with Adets-1 or Adnull. When Ets-1 was overexpressed, cyclin D1 promoter activity increased significantly, by 3.6-fold, in LLC-PK1 cells. In the case of transfection of Adnull, there were no significant changes in cyclin D1 promoter activity or protein expression (Figure 8B). When Adets-1 was transfected, a higher level of cyclin D1 protein expression could be detected when compared with Adnull (Figure 8B).

Apoptotic Changes by the Overexpression of Ets-1 in LLC-PK1 Cells

In vascular endothelial cells, overexpression of Ets-1 causes apoptotic changes (30). Thus, we examined the effects of the overexpression of Ets-1 using an adenovirus on apoptotic changes of LLC-PK1 cells by Western blot analysis. We used AdAktDN as a positive control. Overexpression of AdAktDN induced changes in the Ets-1 protein level in LLC-PK1 cells by Western blot analysis. (A) Hypoxia induced changes in the Ets-1 protein level in LLC-PK1 cells by Western blot analysis. (B) Ets-1 promoter activity was increased 7.1-fold at 3 h, 9.1-fold at 6 h, and 12.2-fold at 6 h reoxygenation. (C and D) To test whether the upregulation of Ets-1 is dependent on HIF-1\(\alpha\), we examined Ets-1 protein expression and promoter activity in HIF-1\(\alpha\)-transfected LLC-PK1 cells and dnHIF-1\(\alpha\)-transfected LLC-PK1 cells; \(n = 5\), mean \pm SEM; \(*P < 0.05\) versus control.

Discussion

In the present study, we demonstrated that Ets-1 is upregulated in proximal tubules in the recovery phase of ARF, that
hypoxia causes the transcriptional stimulation of Ets-1 in LLC-PK1 cells via HIF-1α, and that the overexpression of Ets-1 stimulates [3H]thymidine uptake and cyclin D1 transcription in renal tubular cells. Recovery from ARF requires the replacement of damaged cells with new cells that restore tubule epithelial integrity. Regeneration processes are characterized by the proliferation of dedifferentiated cells and subsequent redifferentiation of the daughter cells into the required cell phenotype. A similar phenomenon can also be observed during embryogenesis. Therefore, it was postulated that regeneration processes may repeat parts of the genetic program that serve during organogenesis to reestablish proper tissue function after damage (5,45). Recently, we reported that the developmental gene Wnt-4 is expressed in ischemic acute renal injuries and that Wnt-4 expression promotes the proliferation of renal tubular cells (26). This article suggested that some developmental genes are re-expressed during recovery of ARF. To confirm this hypothesis, we examined the expression patterns and function of Ets-1 in an ischemic acute renal model and in renal tubular cells.

In this study, we first demonstrated that Ets-1 expression is upregulated in the early phase of ischemic ARF. The Ets-1 expression was localized exclusively in the proximal tubule at the site of tubule regeneration where PCNA is expressed. These results suggest that Ets-1 protein may induce the transformation of regenerative renal tubular cells. During development, Ets-1 expression occurs in vascular structures and branching tissues, including the kidneys (18). In the adult kidney, the levels of Ets-1 expression are much lower than in the embryonic kidney (18,22). Thus, our data suggest that the cells that express Ets-1 after ischemic injury have characteristics of embryonic renal cells, such as in the mesenchymal-to-epithelial progression and proliferation.

Our data also indicate for the first time that Ets-1 signaling contributes to the activation of cyclin D1 promoter and protein expression. Sequences that resemble the core motif (GGA) required for Ets protein binding are located within the proximal cyclin D1 promoter (16). Overexpression of Ets-2 activates the
nomenon caused by Ets-1 may be dependent on cell types or LLC-PK1 cells. This result suggests that the apoptotic phase change caspase 3 activity or the value of cell death ELISA in conditions, the overexpression of Adets-1 did not significantly change stimulated caspase 3 activity (A) and the value of cell death ELISA (B). Overexpression of AdAktDN caused apoptotic changes in LLC-PK1 cells; AdAdnull is used as a positive control. Overexpression of AdAktDN stimulated caspase 3 activity (A) and the value of cell death ELISA (B). Overexpression of Adets-1 did not significantly change stimulated caspase 3 activity (A) or the value of cell death ELISA (B) in LLC-PK1 cells; n = 5, mean ± SEM; *P < 0.001 versus control.

Figure 9. Apoptotic changes were not observed by the overexpression of Ets-1 in LLC-PK1 cells. We examined the effects of the overexpression of Ets-1 using an adenovirus on apoptotic changes of LLC-PK1 cells by caspase 3 activity and a cell death ELISA kit. AdAktDN was used as a positive control. Overexpression of AdAktDN stimulated caspase 3 activity (A) and the value of cell death ELISA (B). Overexpression of Adets-1 did not significantly change stimulated caspase 3 activity (A) or the value of cell death ELISA (B) in LLC-PK1 cells; n = 5, mean ± SEM; *P < 0.001 versus control.

cyclin D1 promoter through the proximal 22 bp (46). The Ets-1 pathway plays a key role in normal embryonic development and in malignant vascular formation (17–20). However, the functional role of the Ets-1 signaling pathway in renal tubular cells is not well known. Our data demonstrate that overexpression of Ets-1 increases cyclin D1 promoter activity, protein expression, and cell-cycle progression in renal epithelial cells. If this is the case, then the expression of Ets-1 in the recovery phase of ARF could be expected to promote cell-cycle progression after tubular injury. Our data also demonstrated that the Ets-1 binding site of the cyclin D1 promoter binds to the nuclear extract of ischemic renal tissue. Our ischemia-reperfusion model also demonstrated that Ets-1 was co-localized with PCNA, suggesting that Ets-1 might be a proliferative signal in regenerating renal tubules.

In vascular endothelial cells, overexpression of Ets-1 caused apoptotic changes (32). Apoptosis of renal tubular cells is observed during ARF (47,48). Thus, we hypothesized that upregulated Ets-1 may cause apoptotic changes in renal tubular cells. To demonstrate this, we examined the effects of the overexpression of Ets-1 on apoptotic changes in LLC-PK1 cells. AdAktDN was used as a positive control, and overexpression of AdAktDN caused apoptotic changes in LLC-PK1 cells as shown in Figure 6. However, in our experimental conditions, the overexpression of Adets-1 did not significantly change caspase 3 activity or the value of cell death ELISA in LLC-PK1 cells. This result suggests that the apoptotic phenomenon caused by Ets-1 may be dependent on cell types or tissues. In the case of renal tubular cells, Ets-1 does not seem to play a role in apoptosis in ARF.

We have yet to see which kinds of mechanisms induce the transient upregulation of Ets-1 after ischemia-reperfusion acute renal injury. A recent paper by Oikawa et al. (27) reported that hypoxia induced Ets-1 via the activity of HIF-1α. In their study, the Ets-1 promoter contained a hypoxia-responsive element-like sequence, and HIF-1α bound to it under the hypoxic condition. The expression of HIF-1α was dramatically increased as early as 6 h after ischemia-reperfusion (Figure 1C). The upregulation of HIF-1α protein expression was temporary; the intensity of the Ets-1 band decreased at 48 and 72 h after ischemia-reperfusion. The time course of HIF-1α exceeded the time course of Ets-1. The hypoxic inducibility of the Ets-1 promoter assay is inhibited by the overexpression of dnHIF-1α in LLC-PK1 cells. The Western blot analysis also demonstrated that transfection of dnHIF-1α reduced the increase of Ets-1 protein by hypoxia (Figure 6C). These results indicated that the upregulation of Ets-1 by hypoxia is dependent on HIF-1α. This evidence led to the hypothesis that ischemia causes hypoxia in the renal tubule, and the hypoxic condition induces HIF-1α, then HIF-1α activates Ets-1 transcription in renal tubular cells. It will be of interest to examine which kinds of signal cascades exist between ischemia and Ets-1 induction. Further studies will be necessary to gain a more precise understanding of the molecular mechanisms of renal recovery after ischemia-reperfusion injury.

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References


