Leukemia Inhibitory Factor Is Involved in Tubular Regeneration after Experimental Acute Renal Failure

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Abstract. Leukemia inhibitory factor (LIF) is known to play a crucial role in the conversion of mesenchyme into epithelium during nephrogenesis. This study was carried out to test the hypothesis that LIF and LIF receptor (LIFR) are involved in the renal epithelial regeneration after acute renal failure. First, the authors investigated the spatiotemporal expression of LIF and LIFR in fetal and adult rat kidney. In developing kidney, LIF was expressed in the ureteric buds and LIFR was located in nephrogenic mesenchyme and the ureteric buds; in adult kidney, LIF and LIFR expression was confined to the collecting ducts. Next, the authors examined the expression of LIF and LIFR during the recovery phase after ischemia-reperfusion injury. Real-time PCR analysis revealed that LIF mRNA expression was significantly increased from day 1 to day 7 after reperfusion and that LIFR mRNA was upregulated from day 4 to day 14. Histologic analysis demonstrated that the increased expression of LIF mRNA and protein was most marked in the outer medulla, especially in the S3 segment of the proximal tubules. To elucidate the mitogenic role of LIF in the regeneration process, cultured rat renal epithelial (NRK 52E) cells were subjected to ATP depletion (an in vitro model of acute renal failure), and LIF expression was found to be enhanced during recovery after ATP depletion. Blockade of endogenous LIF with a neutralizing antibody significantly reduced the cell number and DNA synthesis during the recovery period. These results suggest that LIF participates in the regeneration process after tubular injury.

The mammalian kidney is susceptible to injury by ischemia and nephrotoxins, and recovery of normal renal function requires regeneration of damaged tubular epithelium. The process of regeneration after renal injury is characterized by a sequence of events that includes epithelial cell spreading, migration to cover exposed areas of the basement membrane, cell dedifferentiation and proliferation to restore cell number, and then differentiation (1,2). In many respects this nephrogenic repair process resembles the growth and maturation of nephrons during kidney development (3,4). Several genes critical for kidney development have been shown to be upregulated in the regeneration process after injury and participate in the regeneration. Pax-2 (5), Wnt-4 (6), and activin-A (7) have been shown to be re-expressed in the regenerating tubules after injury (8–11). These findings support the hypothesis that a cascade of developmental gene pathways is reactivated during tissue regeneration.

Leukemia inhibitory factor (LIF), a member of the interleukin-6 (IL-6) family, is a multifunctional cytokine originally identified as a proliferation inhibitor and differentiation in-
was then removed to allow reperfusion, and rats were killed after 1, 2, 4, 7, 14, and 30 d of reperfusion (n = 5 or 6 per group).

For histologic assessment, fetal and adult kidneys were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and cut serially into 5-μm-thick sections.

**Real-Time PCR**

Total RNA was isolated from kidney homogenates and cultured cells with the TRIzol Reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from total RNA by using random hexamers. Real-time PCR was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR amplification was performed using TaqMan PCR Universal Master Mix (Applied Biosystems). Thermal cycler conditions consisted of holding at 95°C for 2 min and 95°C for 10 min, followed by 30–40 cycles of 95°C for 15 s and 60°C for 1 min. The following oligonucleotide primers (50 nM) and probes (200 nM) were used: rat LIF (GenBank accession number AB010275): sense, 5'-TCATCGTGTGTTGGCAAGAAGTGC-3'; antisense, 5'-GTCATGTTGTTGGCAAGAAGTGC-3'; internal fluorescence-labeled MGB probe (FAM): 5'-CCAAACCTTGGTGAAGCTATGTGCGC-3'; rat LIF receptor (GenBank accession number D86345): sense, 5'-TCATCGTGTGTTGGCAAGAAGTGC-3'; antisense, 5'-TCTTGGGGTTCACTCAACCTTCA-3'; internal fluorescence-labeled MGB probe (FAM): 5'-TTCTGCGGTTCACTCAACCTTCA-3'. Gene expression of the target sequence was normalized in relation to expression of 18S ribosomal RNA as an endogenous control. Each sample was tested in duplicate. Results are expressed relative to data from pre-ischemic kidneys that were arbitrarily assigned a value of 1.

**In Situ Hybridization**

A cDNA for rat LIF was generated by reverse transcription-PCR with the following primers: sense, 5'-TGAGCAATGGCTGACTCAT-3'; antisense, 5'-GACACAGGGCACATCCCAT-3'. The amplified PCR fragment was initially ligated to PCR II vector (Invitrogen) and digoxigenin (DIG)–labeled antisense, and sense cRNA probes were synthesized with T7 or SP6 RNA polymerases (DIG RNA labeling kit SP6/T7; Roche Diagnostics, Indianapolis, IN). Tissue sections were deparaffinized, permeabilized with proteinase K, and refixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). The prehybridization and hybridization steps were carried out at 65°C for 2 h and 12 h, respectively. The hybridization buffer was composed of 50% formamide, 5 × SSC, 5 × Denhardt's solution, 250 μg/ml Baker's yeast RNA, 500 μg/ml salmon sperm DNA, and cRNA probes. After post-hybridization washing, sections were incubated with anti-digoxigenin antiserum conjugated to alkaline phosphatase, and histochemical detection was performed using a 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate mixture (Roche Diagnostics). The same procedure performed with the sense cRNA probe served as a negative control.

**Immunohistochemical Staining**

After deparaffinization, sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to remove endogenous peroxidase activity. They were then blocked in normal horse serum and incubated at 4°C overnight with the primary antibodies: anti-mouse LIF antibody (R&D Systems, Minneapolis, MN), anti-human LIFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-human aquaporin-1 (AQP-1) antibody (Alomone Labs, Jerusalem, Israel). Next, the sections were incubated with biotinylated secondary antibody and incubated with avidin-biotin horseradish peroxidase complex (Vectorstain Elite ABC kit; Vector Laboratories, Burlingame, CA). The sections were visualized with a Vector DAB peroxidase substrate kit (Vector Laboratories) and were counterstained with hematoxylin. As a negative control, the primary antibody was replaced with normal horse serum, and no positive immunostaining was observed.

For 5-bromo-2'-deoxyuridine (BrdU) staining, rats were intraperitoneally injected with BrdU 2 h before sacrifice. Formalin-fixed paraffin sections were subjected to BrdU staining by using a Cell Proliferation Kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions. In brief, after deparaffinization, sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to remove endogenous peroxidase activity. Tissue sections were then placed in citrate buffer and heated for 10 min in a microwave oven; after incubating them with anti-BrdU antibody, the sections were incubated with HRP-conjugated IgG and developed with a Vector DAB peroxidase substrate kit.

Double immunohistochemistry was performed to detect LIF expression in combination with the proximal tubular marker AQP-1 (17,18). The first immunohistochemical staining was performed with a Vector VIP substrate kit (Vector Laboratories) to obtain a purple reaction product, and the sections were then washed and blocked with an avidin-biotin blocking kit (Vector Laboratories). The second immunostaining was performed with a Vector SG substrate kit (Vector Laboratories) to obtain a blue reaction product.

**Cell Culture**

A normal rat kidney epithelial-derived cell line, NRK 52E (19), was grown in Dulbecco modified essential medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin, in a humidified 5% CO₂/95% air environment at 37°C.

**ATP Depletion Protocol**

ATP depletion of NRK 52E cells was achieved by means of previously described protocols with modifications (20,21). A confluent monolayer of NRK 52E cells was incubated in PBS with 1.5 mM CaCl₂, 2 mM MgCl₂, and 1 μM antimycin A (Sigma, St. Louis, MO) for 60 min. During the recovery phase after the 1-h injury period, cells were repleted with ATP by incubation with a regular growth medium. Cellular ATP levels were determined with a luciferase-based assay kit (Sigma).

**Effect of LIF Antibody on Cell Proliferation**

NRK 52E cells were subcultured in 24-well plates for cell counting, or in 96-well plates for measurement of BrdU incorporation, and subjected to ATP depletion for 1 h as described above. After the injury, cells were repleted with ATP by incubation with recovery medium containing affinity-purified anti-human LIF antibody (Genzyme, Cambridge, MA) at the concentration indicated, or with normal goat IgG as a negative control. The number of living cells was counted with a Cell Counting Kit-8 (Wako Pure Chemicals, Osaka, Japan) after the 48-h recovery phase (n = 4). DNA synthesis was assessed by an enzyme-linked immunosorbent assay for BrdU (Roche Diagnostics) after the 24-h recovery phase (n = 6).

**Statistical Analyses**

Statistical significance was evaluated by one-way ANOVA with a Fisher’s post-hoc test. Statistical significance was defined as P < 0.05.
Results

Localization of LIF and Its Receptor in Developing and Adult Rat Kidneys

The spatiotemporal expression of LIF and LIFR during kidney development was investigated by an immunohistochemical technique. At embryonic day 15 (E15), LIF expression level was very low (Figure 1A). Weak LIF immunoreactivity was observed in the ureteric buds, whereas LIFR immunoreactivity was observed in the mesenchymal cells surrounding the ureteric buds (Figure 1B). At E17, LIF immunostaining was noted in derivatives of the ureteric buds, whereas no immunostaining was detected in the mesenchymal cells (Figure 1C). In the nephrogenic outer cortex, LIF was expressed in the tips of the branches of the ureteric buds (Figure 1D); in the medulla, intense staining was found in the major branches of the ureteric buds and collecting ducts (Figure 1E). At E17, LIFR was expressed ubiquitously in mesenchyme, and the most intense signal was observed in the condensing mesenchymal cells surrounding the tips of the ureteric buds in the nephrogenic zone (Figure 1F). In addition to the mesenchymal cells, the derivatives of the ureteric buds, including ureteric bud branch tips and collecting ducts, expressed LIFR, a pattern that resembled the distribution of LIF protein observed. The ureteric buds differentiated into collecting ducts, as nephrogenesis progressed. In neonatal kidneys, LIF was expressed predominantly in mature and immature collecting ducts (Figure 1G). LIFR was also expressed in mature and immature collecting ducts, whereas LIFR immunoreactivity was still evident in mesenchymal cells in the nephrogenic cortex (Figure 1H).

We also localized LIF and LIFR protein in adult kidneys. Cytoplasmic immunostaining for LIF was observed mainly in the collecting ducts (Figure 2, A to C), and, as shown in Figure 2B, no LIF immunoreactivity was detected in the S3 segment of the proximal tubules in the outer medulla. In mature kidneys, LIFR immunoreactivity was also predominantly detected in the collecting ducts, coinciding with the distribution of LIF protein (Figure 2, E to G). No immunostaining was observed in sections incubated with normal horse serum (Figure 2, D and H).

Expression of LIF and LIFR in the Rat Kidney after Ischemia/Reperfusion Injury

To investigate whether LIF is involved in renal tubular regeneration, we first used the real-time PCR method to quantify changes in the expression of LIF and LIFR mRNA after ischemia/reperfusion (I/R) injury.

In post-ischemic kidneys, LIF mRNA expression had increased 14.3-fold on day 1 after reperfusion, compared with its expression in pre-ischemic kidneys (Figure 3A). The increase in LIF mRNA persisted until day 7, after which gradually returned to the basal level by day 14. Interestingly, the increase in LIFR mRNA occurred later than the increase in LIF mRNA (Figure 3B). The LIFR mRNA level began to increase on day 4 and persisted until day 14, before returning to baseline by day 30.

The LIF mRNA expression in the kidneys was localized by means of in situ hybridization. In normal kidneys, LIF mRNA appeared to be mainly present in the collecting ducts (data not shown). At 1 d after the renal I/R injury, the hybridization signal for LIF mRNA was progressively increased, consistent with the results of the real-time PCR analysis (Figure 4, A and B). The most prominent increase in signal for LIF mRNA was noted in the tubular epithelial cells in the outer medulla, which is the site most vulnerable to ischemic injury. No positive signal was obtained with the sense cRNA probe (Figure 4C).

The LIF and LIFR proteins in the I/R-injured kidneys were precisely localized by an immunohistochemical technique. Whereas LIF protein was expressed in the collecting ducts in normal kidneys, increasingly prominent expression of LIF protein was found in the outer medulla beginning 24 h after the ischemic injury (Figure 5A). Immunostaining was particularly evident in the S3 segment of the proximal tubules, where no LIF immunoreactivity was detected under normal conditions. At higher magnification, immunoreactivity was seen in both the detached and the attached cells in the injured tubules (Figure 5B). Double-staining was performed to detect LIF expression in combination with aquaporin-1 (AQP-1), which is known to be expressed in proximal tubules, especially in the S3 segment (17,18). The results of double-staining showed that most tubules staining for LIF expressed AQP-1, however, some AQP-1–negative tubules contained LIF-positive cells (Figure 5C). This suggests that the LIF protein was predominantly localized in the S3 segments of the proximal tubules of ischemic kidneys and to some extent in the distal tubules in their outer medulla.

We then examined the relationship between LIF expression and tubular cell proliferation after I/R injury. Cell proliferation was assessed by incorporation of BrdU injected into the rats 2 h before sacrifice. BrdU-positive cells were rarely observed in normal kidney. In ischemic kidney, BrdU-positive cells were observed predominantly in the outer medulla, where damage to the tubule cells was most obvious. Double immunohistochemical staining revealed that most BrdU-positive cells were present in tubules expressing LIF (Figure 5D).

LIFR was also predominantly localized in the proximal...
tubular cells of the outer medulla in the I/R-injured kidneys, coinciding with the distribution of LIF protein (Figure 5E).

Expression of LIF mRNA in an In Vitro Model of Ischemic Renal Injury

An in vitro model of ischemic renal injury in cultured rat renal epithelial cell line NRK 52E (19) was used to further elucidate whether LIF is involved in renal tubular regeneration. The in vitro model of ischemic renal injury was created by inducing ATP depletion by producing chemical anoxia with the mitochondrial inhibitor antimycin A. When monolayers of NRK 52E cells were exposed to 1 µM antimycin A, cellular levels of ATP rapidly declined to 2.1% of the control values within 30 min (Figure 6). This effect could be reversed by removing the antimycin A and restoring the regular medium. The ATP level recovered to 64.0% of the control values 3 h after ATP repletion.

The change in LIF mRNA expression during the recovery period was quantified by using the real-time PCR method (Figure 7). The LIF mRNA level began to increase on 6 h and persisted until 12 h after ATP depletion and then gradually decreased to baseline (Figure 7).

Role of LIF in Tubular Regeneration after ATP Depletion

To investigate the mitogenic effect of LIF on renal tubular cells, we assessed the effect of anti-LIF neutralizing antibody on recovery from the antimycin A-induced injury. NRK 52E cells were injured by exposure to antimycin A for 1 h and then incubated in a recovery medium containing anti-LIF neutralizing antibody. The number of surviving cells was determined 48 h after ATP depletion (Figure 8A). Blocking endogenous LIF protein with 1.0 µg/ml of anti-LIF antibody induced a significant reduction in cell number at cell number at 48 h during the recovery phase. DNA synthesis was also measured as incorporation of BrdU. In agreement with the results for cell number, 1.0 µg/ml of anti-LIF antibody lowered DNA synthesis at 24 h during recovery (Figure 8B). No significant change was observed in NRK 52E cells incubated with normal goat IgG (data not shown). To determine whether the mitogenic effect of endogenous LIF was specific to the post-injury period, we performed similar studies with uninjured NRK 52E cells. In contrast to the results in the post-injury period, anti-LIF antibody had no effect on cell number or DNA synthesis in uninjured NRK 52E cells (Figure 9).

Discussion

It has been suggested that regeneration processes may recapitulate developmental paradigms to restore organ or tissue function (3,4). Several genes including Pax-2, Wnt-4, and activin-A, which play a pivotal role in nephrogenesis, have been shown to be re-expressed in the kidney in the regeneration process following injury (8–11). We focused our attention on LIF, since it has been shown to play an important role in renal development (16,22). This study demonstrates for the first time that LIF participates in kidney regeneration after ischemic injury.

Figure 3. Expression of mRNA for LIF (A) and LIFR (B) in rat kidneys after ischemia/reperfusion (I/R) injury. Total RNA was extracted from kidneys at the indicated times after reperfusion. Quantification of mRNA was determined by real-time PCR. Expression was normalized to an endogenous control 18S ribosomal RNA (rRNA) and is shown as ratio to value of day 0. (A) LIF mRNA expression after I/R injury. (B) LIFR mRNA expression after I/R injury. Values represent means ± SEM for five or six animals. * P < 0.05 versus day 0.

Figure 2. Immunohistochemical localization of LIF (A to C) and LIFR (E to G) in normal adult rat kidneys. Paraffin sections from the cortex (A, D, E and H), outer medulla (B and F), and papilla (C and G) of normal adult kidneys are presented. Immunostaining of both proteins were mainly detected in the collecting ducts in the adult rat kidney. When using normal horse serum instead of the anti-LIF antibody (D) or normal horse serum instead of the anti-LIFR antibody (H), no positive signal was observed. Magnification: ×200.
The metanephros, which becomes the permanent kidney, arises from two mesodermal derivatives: the ureteric bud and the metanephric mesenchyme. Once the ureteric bud grows out from the Wolffian duct and encounters the mesenchyme, a series of reciprocal inductive events takes place that results in the ureteric bud growing and branching to form the collecting ducts, and the metanephric mesenchyme condensing and forming the tubules and the glomeruli (23). LIF secreted by the ureteric buds plays an important role in the conversion of the mesenchyme into epithelium (16,22); however, the spatiotemporal expression patterns of LIF and its receptor have not been fully elucidated. Accordingly, we first investigated the localization of LIF and LIFR in fetal and adult rat kidney.

In developing rat kidney, expression of LIF and LIFR was observed in the derivatives of the ureteric buds on E17 and thereafter. As nephrogenesis progressed, the ureteric buds differentiated into collecting ducts, which consistently expressed LIF and LIFR even after nephrogenesis had been completed. Only LIFR was expressed in the mesenchyme, with no expression of LIF in mesenchymal cells being detected in either fetal or adult kidney. LIFR expression in the mesenchymal cells was already apparent on E15, and it was sustained until the neonatal kidney.

The nephrogenic zone of the embryonic kidney is where primitive tubules and glomeruli are generated. In the nephrogenic zone, the tips of ureteric buds were observed to express LIF and LIFR, whereas the surrounding mesenchymal cells expressed LIFR. Presumably, secreted LIF binds to the LIFR located in the ureteric buds in an autocrine fashion and to the LIFR located in mesenchyme in a paracrine fashion. The LIF-LIFR system is speculated to play a pivotal role in nephrogenesis, and that would be consistent with the results reported by other investigators (16,22). In the adult kidney, expression of LIF and LIFR was most often localized in the collecting ducts. These changes in distribution suggest a different role of LIF/LIFR in the embryonic and adult kidney.

LIF is a pleiotropic cytokine that is particularly involved in growth and development. In the kidney, it is known to play an important role in nephrogenesis. In the adult, LIF has been shown to be involved in a variety of acute and chronic inflammations. LIF has been identified within the urine of renal allograft recipients during episodes of acute rejection (24). The LIF expression level in cultured mesangial cells has been shown to be increased by cytokines (25). Glomerulonephritis induces LIF in the glomeruli, and administration of exogenous LIF ameliorates glomerulonephritis (26). The function of LIF in the kidney under physiologic conditions is largely unknown, although there is a solitary report by Tomida M et al. (27) on the action of LIF on renal tubule cells. They found that LIF inhibits the development of Na\(^+\)-dependent hexose transport in LLC-PK1 cell, which was isolated from pig kidney and is thought to have the characteristics of proximal tubule cells. In our study, LIF was found to be predominantly expressed in the collecting duct cells. The findings of Tomida et al. (27) and our own suggest that LIF may be involved in ion transport in renal collecting ducts.

Next, we tested the hypothesis that LIF is involved in the
Figure 5. Immunohistochemical localization for LIF and LIF receptor protein in ischemia/reperfusion-injured kidneys 24 h after reperfusion. (A and B) Immunohistochemical localization for LIF protein (brown staining) in the outer medulla of ischemic kidneys. The dramatic increase in LIF immunostaining in injured epithelial tubular cells, especially of S3 segment of the proximal tubules. Detached cells and attached cells in the tubules were stained. (C) Double immunohistochemistry for LIF and aquaporin-1 (AQP-1), marker of proximal tubules. Note that LIF (purple staining) was co-localized with AQP-1 (blue staining). (D) Double immunohistochemistry for LIF and BrdU. BrdU were injected with rat 2 h before sacrifice. Most BrdU staining (brown nuclear staining) was found in proximal tubules expressing LIF (purple cytoplasmic staining). (E) Immunohistochemical localization for LIF receptor (brown staining) in the outer medulla of ischemic kidneys. The expression pattern is similar to that of LIF. Magnifications: \( \times 100 \) in A; \( \times 400 \) in B–D; \( \times 200 \) in E.
process of kidney regeneration after injury by investigating expression of LIF and LIFR during the recovery phase from injury by real-time PCR, in situ hybridization, and immunohistochemistry in a model of acute renal failure created by inducing ischemia. Our first finding in the ischemic kidneys was that the increase in LIF mRNA expression began on day 1 after the injury and was sustained for 7 d, whereas the increase in LIFR mRNA expression was observed several days later. Many genes have been reported to be upregulated after tubular injury. Egr-1 (28), c-fos (28), c-myc (29), and the genes encoding heat shock protein-70 (30) and parathyroid hormone–related protein (PTHrP) (31) have been shown to be upregulated in the very early phase after injury. Their upregulation was detected as early as within a few hours and returned to baseline within 1 d. These are considered the early response genes to renal injury. The increase in LIF mRNA expression persisted for several days, and these genes are thought to be responsible for tubular regeneration. The upregulation of LIF was observed on 1 d after the ischemic insult and persisted for 7 d. Its time course suggests that LIF participates in the regeneration process. Interestingly, there was a time lag between the gene upregulation of LIF and LIFR, and there is a possibility that LIF itself or some other molecules in the downstream of LIF signaling may stimulate the transcription of LIFR.

We did not perfuse the kidney to remove the blood before total RNA isolation. The possibility of contamination by RNA from blood cells cannot be ruled out, however, any contribution by blood cells to the increase in LIF mRNA is thought to be minimal. The increase in LIF expression is mainly attributable to the regenerating epithelial tubular cells, and this is supported by two observations. First, immunohistochemistry in situ hybridization studies showed the most prominent staining of LIF in the epithelial cells. Second, in vitro study in the absence of blood cells demonstrated upregulation of LIF mRNA.

Our second observation in the ischemic kidneys was that the increase in expression of LIF and LIFR was predominantly in the outer medulla, especially in the S3 segment of the proximal tubules, where LIF or LIFR is not usually expressed. The outer medulla is also the most prominent site of ischemia-induced acute tubular necrosis and subsequent DNA synthesis (29,37). In our study, most of the cells that stained with BrdU were found in LIF/LIFR-positive tubules. Interestingly, LIF and LIFR were expressed in the detached cells as well as the attached cells of S3 segment of the proximal tubules, and in previous studies, c-Met (34), galectin-3 (35), TGF-β1 (36), and PDGF (32) were shown to be expressed strongly in the detached cells of S3 proximal tubules. By the same analogy as in the speculations about these genes, the increase in LIF staining in the detached cells presumably reflects an unsuccessful protective response to injury before the cells’ detachment and necrosis.

Although LIF expression is restricted to the ureteric bud and...
its derivatives, the major site of action of LIF is the mesenchyme. LIF is excreted by the ureteric bud and induces a mesenchymal to epithelial conversion via binding to its receptor located in the mesenchyme. Thus, LIF is important to the development of metanephric mesenchyme-derived structures. In view of this, the involvement of LIF in regeneration of the injured proximal tubules, which are derived from the mesenchyme, is consistent with the functional role of LIF in developing kidney. However, we do not know why LIF is expressed in bud-derived structures during development and re-expressed in mesenchyme-derived structures in disease. The expression pattern of galectin-3 is similar to that of LIF. Galectin-3 is

Figure 8. The effect of anti-LIF neutralizing antibody on recovery from ATP depletion in NRK 52E cells. The injured cells were allowed to recover in regular growth medium containing 0, 0.1, 0.2, or 1.0 μg/ml of anti-LIF antibody. (A) Cell number on day 2 (n = 4) and (B) BrdU incorporation on day 1 (n = 6) after ATP depletion were decreased by 1.0 μg/ml of anti-LIF antibody. The values are indicated as percentage of the value without anti-LIF antibody, and means ± SEM. * P < 0.05 versus the value without anti-LIF antibody.

Figure 9. The effect of anti-LIF neutralizing antibody on proliferation in NRK 52E cells. The non-injured cells were incubated in regular growth medium containing 0, 0.1, 0.2, or 1.0 μg/ml of anti-LIF antibody. (A) Cell number on day 2 (n = 4) or (B) BrdU incorporation on day 1 (n = 6) were not changed. The values are indicated as percentage of the value without anti-LIF antibody and means ± SEM. NS, no significant difference.
expressed in ureteric bud-derived structures in fetal and adult kidney and re-expressed in injured proximal tubule cells (35,38). One hypothesis to explain the re-expression in cells of different origin in disease is that the regenerating proximal tubule cells are dedifferentiated and have different characteristics.

LIF is a member of the IL-6 family, which also includes IL-6, IL-11, oncostatin M, cardiothrophin-1, and CNTF. IL-6 family members share the same intracellular signaling system, gp130/JAK/STAT (39). To our knowledge, there have been no reports on involvement of LIF in epithelial cell regeneration. However, two members of the IL-6 family, IL-6 and CNTF, have been shown to participate in renal tubule cell regeneration after kidney injury. Administration of IL-6 stimulates tubule regeneration after glycerol-induced acute renal failure (40), and CNTF has been shown to be involved in renal tubule regeneration after ischemia-reperfusion injury (33). These observations suggest the importance of the role of the IL-6 family in renal tubule cell regeneration. LIF itself has been shown to prompt the regeneration processes after damage to other cells and organs, including neurons (41), the liver (42), heart (43), and muscle (44). In this study, we showed that LIF expression is upregulated after an ischemic insult and that its expression is localized in BrdU-positive, proliferating proximal tubules. These findings provide evidence in support of our hypothesis that LIF participates in renal tubule regeneration after renal injury.

To clarify the role of LIF in the regeneration of tubular cells, we utilized the reversible in vitro model of ATP depletion in NRK 52E cells. The cell injury caused by ATP depletion is thought to mimic the effect of ischemia in vivo, and the recovery phase after ATP depletion is thought to reproduce the regeneration (45). With this in mind, we used anti-LIF neutralizing antibody to examine the role of LIF in the mitogenic response during the recovery phase from injury. The blocking of endogenous LIF with anti-LIF neutralizing antibody significantly reduced cell number and DNA synthesis after recovery from ATP depletion, and the mitogenic effect of endogenous LIF appeared to be specific to the post-injury period, since anti-LIF antibody had no effect on non-injured NRE 52E cells. Tubular cell proliferation is the hallmark of early regeneration after ischemic renal injury (29), and the results of the in vitro study provide strong support for the hypothesis that LIF plays a pivotal role in renal epithelial regeneration after injury.

In conclusion, we have demonstrated that LIF expression in the kidney is transiently upregulated after an ischemic insult. The greatest increase in LIF occurred in the damaged proximal tubules in the outer medulla, and LIF protein was co-localized with the proliferation marker BrdU. The blockade of endogenous LIF also reduced the regeneration after in vitro injury. On the basis of these findings as well as our observations in the developing kidney, we concluded that the LIF/LIFR axis is reactivated during renal regeneration after I/R injury and that it may recapitulate the developmental process to restore organ or tissue function.

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References