Effect of Notch activation on the regenerative response to acute renal failure

Sandeep Gupta,1 Shunan Li,¹ Md. J. Abedin,¹ Lawrence Wang,¹ Eric Schneider,¹ Behzad Najafian,² and Mark Rosenberg¹

¹Department of Medicine and Stem Cell Institute, and ²Departments of Laboratory Medicine and Pathology, and Pediatrics, University of Minnesota, Minneapolis, Minnesota

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Gupta S, Li S, Abedin MJ, Wang L, Schneider E, Najafian B, Rosenberg M. Effect of Notch activation on the regenerative response to acute renal failure. Am J Physiol Renal Physiol 298: F209–F215, 2010. First published October 14, 2009; doi:10.1152/ajprenal.00451.2009.—Episodes of acute renal failure (ARF) are not always fully reversible and may lead to chronic disease, due in part to an inadequate regenerative response. The Notch signaling pathway is involved in determining cell fate during development, and tissue maintenance and repair in adult organs. The purpose of this study was to examine the role of the Notch pathway in renal regeneration following ARF. Kidney injury, induced by ischemia-reperfusion, resulted in early activation of the Notch pathway, as evidenced by increased expression of Notch1 and Notch2 intracellular domain (cleaved Notch). The effect of exogenous administration of the Notch ligand Delta-like-4 (DLL4) on recovery from ARF was then studied. Rats were pretreated by intraperitoneal injection of DLL4 or vehicle control. Two days following the last DLL4 dose, ARF was induced by bilateral renal artery clamping for 45 min followed by reperfusion. The severity of renal injury was similar in DLL4 and control rats. Renal recovery was facilitated by DLL4 treatment, as evidenced by faster return of serum creatinine to baseline by 48 h in DLL4-treated rats as against 5 days in vehicle-treated control rats. Cell proliferation was higher in the DLL4-treated group. In conclusion, activation of the Notch pathway plays a role in kidney regeneration following ARF and may represent a potential novel therapeutic option for regenerating the injured kidney.

Delta-like-4; acute kidney injury; cellular proliferation; ischemia-reperfusion

EPISODES OF ACUTE RENAL FAILURE (ARF) are associated with increased short-term complications, significant morbidity, and may contribute to long-term loss of kidney function (2, 25). Structural changes can develop in the kidney following ischemic injury including permanent damage to peritubular capillaries and interstitial fibrosis (4, 45). Part of the reason for these structural changes and for the poor outcome following ARF may be related to an inadequate regenerative response. Currently, there is no effective clinical therapy for enhancing renal repair.

Following injury, the kidney undergoes a regenerative response, leading to recovery of renal function. The cell source for regenerating kidneys is poorly understood with the strongest evidence supporting a role for less injured tubular cells (27). Recapitulating developmental paradigms, these cells dedifferentiate, proliferate, and eventually reline denuded tubules, restoring the structural and functional integrity of the kidney (49, 59). Tissue-specific stem cells have been found in many organs, and there is emerging evidence supporting the presence of stem cells in the adult kidney of organisms ranging from Drosophila to mammals (19, 20, 22, 37, 43, 53). The role stem cells play in kidney regeneration remains an important area of investigation.

The Notch pathway is an evolutionarily conserved intercellular signaling pathway responsible for the control of cell fate and tissue morphogenesis during development. In adult tissue, the Notch pathway is involved in tissue maintenance and repair (58). The Notch family consists of four different single-pass transmembrane receptors, Notch1, 2, 3, and 4, and five different ligands expressed on the surface of neighboring cells (14). The ligands are Delta-like-1, 3, 4 (DLL1, 3, 4), Jagged-1, and Jagged-2. A key event that results in activation of Notch signaling following ligand binding is cleavage of the Notch intracellular domain (cleaved Notch, NICD) by γ-secretase. Cleaved Notch translocates to the nucleus, where it directly affects gene expression. Generation of cleaved Notch and expression of target genes have been used as markers of activation of the Notch pathway. Biological effects following Notch activation can be tissue and ligand specific and depend on which Notch receptor is activated and what target genes are expressed. Target genes for Notch include Hairy/enhancer of split (HES) and HES-related protein (HERP), which encode basic helix-loop-helix (bHLH) transcription factors, leading to expression of downstream genes that regulate cell fate.

The rationale for examining the Notch pathway in renal regeneration includes the role this pathway plays in the regenerative response to injury in adult tissues such as kidney, liver, bone marrow, skin, muscle, intestine, heart, trachea, and pancreas (1, 16, 17, 24, 30, 31, 36, 42, 48, 54). In addition, Notch signaling is involved in self-renewal and cell-fate commitment of tissue stem cells (14, 21). Infusion of the Notch ligand DLL4 expands the stem cell population in the adult brain and improves function following ischemic brain injury by enhancing the regenerative response (3). Notch pathway genes are expressed in the developing and adult kidney, and Notch activation occurs in the adult kidney following injury (5, 7, 9, 11–13, 18, 33, 38–41, 46, 52, 56, 57). Side population (SP) cells are defined by their ability to extrude Hoechst 33342 dye, a characteristic shared by stem cells isolated from numerous adult organs. These cells have been isolated from the kidney and represent a potential progenitor cell pool (7). Many Notch-related genes are expressed in kidney SP cells including Notch1, Notch2, and Notch3 receptors; Jagged-1 and Jagged-2 ligands; and Notch transcriptional regulators and target genes, suggesting a potential role for Notch signaling in the responses of this important cell population. Potential mechanisms whereby Notch activation enhances tissue regeneration have included expansion of the progenitor cell population, regulation of the differentiation status of cells, and enhanced proliferation (58).
The purpose of this study was to test the hypothesis that Notch activation can enhance the regenerative response following ARF. The strategy was to test the effects of exogenously administered Notch ligand DLL4 on the course of acute kidney injury in an established rat model of ARF. DLL4 was chosen as the ligand to test based on its known biological effects and our preliminary data demonstrating expression in the adult kidney and the failure of kidney injury to induce its expression (52). We hypothesize that lack of upregulation of DLL4 expression following kidney injury is a rate-limiting step in kidney regeneration can be improved by exogenous administration of DLL4.

MATERIALS AND METHODS

Experimental protocol. All animal work was approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Male Sprague-Dawley (SD) rats, weighing between 150 and 175 g, were used for all studies. Initial studies were performed examining expression of Notch genes following ischemia-reperfusion injury induced by bilateral renal artery clamping for 45 min under Nembutal (50 mg/kg body wt) anesthesia. Rats were killed before injury (control), and 1 h, 1 day, and 2 days following reperfusion (n = 3/group). Kidneys were harvested for Western blot and immunohistochemical studies. Rats were then randomly assigned into four groups: DLL4 pretreatment with and without ischemia-reperfusion injury; and control with and without ischemia-reperfusion injury (n = 5/group). Starting 3 days before kidney injury, DLL4-treated groups received four intraperitoneal injections of 6.25 μg of recombinant mouse DLL4 (catalog no. 1389-D4, R&D Systems, Minneapolis, MN) dissolved in PBS/0.1% FBS for a total dose of 25 μg. The DLL4 in this preparation is the extracellular domain of mouse DLL4 that has a calculated molecular mass of 55.6 kDa. The company measured the activity of this DLL4 preparation by its ability to bind Notch1 in a functional ELISA. Ischemia-reperfusion injury was induced in the appropriate groups as described above. Serum creatinine was measured before surgery and 24 and 48 h after reperfusion, at which time the rats were killed and the kidneys were harvested for the studies described below.

A second experiment was performed to examine the time course of recovery after ischemia-reperfusion injury. Two groups of rats were studied. One group received DLL4 as described above followed by ischemia-reperfusion injury (n = 6). A control group was studied that did not receive DLL4 but underwent ischemia-reperfusion injury (n = 5). Serum creatinine was measured before injury and daily for 5 days.

Histology and immunohistochemistry. Kidneys from rats were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with periodic acid-Schiff (PAS). The histological evaluations were done by a single pathologist (B. Najafian) in a blinded manner. The scoring system for acute tubular epithelial cell injury was based on a scale of 0–3 as proposed by Chatterjee et al. (8) with some modifications. Nonoverlapping digital images of the cortex from subcapsular to corticomedullary junction, including the medullary rays and the outer stripe of the outer medulla, were obtained in four perpendicular directions. Only tubules with their entire profile visible in the image were scored. Images at each direction were classified as superficial (1/2) or deep (1/2). The scoring system was as follows: 0 = normal histology; 1 = tubular cell swelling, loss of brush border, apoptosis, or nuclear loss (necrosis) in up to one-third of tubular cross section; 2 = same as for score 1, except for nuclear loss present in greater than one-third and less than two-thirds of tubular cross section; and 3 = greater than two-thirds of tubular cross section shows nuclear loss (necrosis).

The average scores for superficial and deep cortex were calculated separately (comparable numbers to those of Chatterjee’s can be obtained by multiplying our scores by 100). The total score is the average of all tubular scores.

For immunohistochemistry, sections were deparaffinized and then rehydrated in ethanol. Antigen retrieval was performed by placing sections in antigen-unmasking solution (99.5°C) for 30 min (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was quenched with Peroxo-blocks (Invitrogen, Carlsbad, CA) for 2.5 min. Nonspecific protein binding was blocked with Histoblock solution (3% BAS, 20 mM MgCl2, 0.3% Tween 20, 5% feta bovine serum in PBS) for 1 h at room temperature. After blocking, tissue sections were incubated at 4°C overnight with the following primary antibodies: anti-cleaved Notch1 (1:200, Cell Signaling Technology, Danvers, MA) and anti-Ki-67 (ready to use; Thermo Scientific, Fremont, CA).

Fig. 1. Activation of Notch following acute renal failure. Ischemia-reperfusion injury was induced in rats by bilateral renal artery clamping for 45 min. Kidneys were harvested at 1 h, 1 day, and 2 days following reperfusion, and Western blots for cleaved Notch1 (Notch1 intracellular domain) or cleaved Notch2 (Notch2 intracellular domain) were examined. A: Western blot probed with the Val1744 rabbit anti-human cleaved Notch1 antibody (Cell Signaling). No signal was detected. Actin was used as a loading control. B: Western blot probed with the 100–402–407 rabbit anti-human cleaved Notch1 antibody (Rockland) demonstrating increased expression of cleaved Notch1 with a peak at 1 h postischemia. Actin was used as a loading control. C: Western blot probed with the 100–402–406 rabbit anti-human cleaved Notch2 antibody (Rockland) demonstrating increased expression of cleaved Notch2 with a peak at 1 h postischemia. Actin was used as a loading control.
For Ki-67, endogenous biotin activity was blocked with an Avidin/Biotin blocking kit (Vector Laboratories); diaminobenzidine (DAB) substrate (Vector Laboratories) was used for color reaction, and sections were counterstained with hematoxylin/eosin (Vector Laboratories). For cleaved Notch1 staining, a Tyramide Signaling Amplification kit (TSA-cyanine 3 kit, PerkinElmer, Shelton, CT) was used to detect the signal. 4,6-Diamidino-2-phenylindole was used to counterstain nuclei. Ki-67 was used as a marker of proliferation as it is expressed during all phases of the cell cycle except for G0. For calculation of the proliferation index, the total number of Ki-67-stained nuclei and the total number of nuclei were counted in 10 randomly selected fields at ×20 magnification by a single observer. Separate counts were done for the outer half and inner half of the cortex. The proliferation index was calculated as the number of positively stained nuclei over total nuclei multiplied by 100.

Western blot analysis. Kidneys were lysed in Tissue Protein Extraction Reagent (Pierce, Rockford, IL) containing protease and phosphatase inhibitors (Pierce) at 40°C for 15 min. Lysates were centrifuged at 10,000 g for 15 min at 4°C, supernatants were removed, and protein concentration was measured by BCA assay (Pierce). Protein (50 μg) was loaded and run on 12% SDS-PAGE gels under reducing conditions (Pierce); transferred onto nitrocellulose membranes (Amersham, Aylesbury, UK); blocked with 5% dry milk in TBS/0.5% Tween 20 (TBST) for 1 h; washed with TBST; and incubated with the following antibodies diluted in TBST: anti-cleaved Notch1 (1:500 dilution; no. 100-401-407; Rockland, Gilbertsville, PA); anti-cleaved Notch1 (1:1,000 dilution; Val1744 antibody no. 2421; Cell Signaling Technology); anti-Notch2 (1:400 dilution; no. 100-401-406, Rockland); anti-DLL4 antibody (1:1,000 dilution; no. NB600–892; Novus Biologicals, Littleton, CO); or anti-actin (1:2,500; Calbiochem). Blots were washed, and incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:2,000; Amersham); washed; and detected with ECL (GE Healthcare). Actin antibody was used on the same blot as a loading control.

Creatinine assay. The rat tail was punctured using 18-gauge sterile needles. The rat blood samples were collected with microhematocrit capillary tubes (Fisher, Pittsburgh, PA), centrifuged, and stored at −80°C. Serum creatinine was measured using an enzymatic assay (Creatinine assay kits; Biovision, Mountain View, CA).

Statistical analysis. Results are presented as means ± SE. An unpaired t-test was used for two-group comparisons. Kruskal-Wallis ANOVA was used for multiple group comparisons with the Mann-Whitney U-test performed to identify intergroup differences. P < 0.05 was considered statistically significant.

RESULTS

Activation of Notch signaling following kidney injury. To determine whether ARF induced by ischemia-reperfusion led to activation of the Notch pathway, we examined expression of cleaved Notch1 and Notch2 by Western blotting at various time points following kidney injury. The first anti-cleaved Notch1 antibody we used was a polyclonal antibody produced by immunizing rabbits with a synthetic human peptide corresponding to the sequence at the Val1744 cleavage site of human Notch1 (Val1744 antibody no. 2421; Cell Signaling Technology). No signal could be detected with this antibody by Western blotting despite multiple attempts at protocol optimization (Fig. 1A), a finding consistent with studies by Kobayashi et al. (30) in a similar rat model of renal ischemia-reperfusion injury. However, when we probed with a different antibody for human cleaved Notch1 (100-401-407; Rockland), a robust signal was obtained, and as can be seen in the Western blot in Fig. 1B, marked induction of cleaved Notch1 was observed that peaked at 1 h following reperfusion and remained elevated on day 1, before returning to baseline levels on day 2. Supporting this finding was the increased expression of cleaved Notch1 when examined by immunohistochemistry (Val1744 antibody no. 2421) 24 h following reperfusion (Fig. 2). We also demonstrated induction of cleaved Notch2 following ischemia-reperfusion injury (antibody no. 100-401-406; Rockland) consistent with previous published results (30) (Fig. 1C). Thus acute kidney injury resulted in activation of both Notch1 and Notch2 signaling. We next examined expression of DLL4 in control rat kidney and following ischemia-reperfusion injury (Fig. 3). Western blot data demonstrated baseline expression of DLL4 but no induction following kidney injury.

Effects of DLL4 administration on ARF. Rats were pretreated for 24 h with the Notch ligand DLL4 or vehicle control 3 days before ischemia-reperfusion injury (total dose 25 μg ip; see MATERIALS AND METHODS). ARF was induced by bilateral renal
artery clamping for 45 min followed by reperfusion. Additional controls were DLL4- or vehicle-treated rats that did not undergo ischemia-reperfusion injury. As can be seen in Fig. 4, DLL4 treatment did not alter serum creatinine from the baseline in animals without kidney injury. Similar peak elevation in serum creatinine was seen at 24 h in both the DLL4- and vehicle-treated rats ($P$ not significant). Creatinine returned to baseline at 48 h in the DLL4-treated rats but remained elevated in the rats that were not treated with DLL4 (Fig. 4). Therefore, DLL4 pretreatment did not alter the severity of kidney injury; however, it facilitated a faster recovery following ARF.

To confirm these finding in a separate group of rats, and to provide a longer follow-up during recovery, the experiment depicted in Fig. 5 was performed. The time course of serum creatinine was followed for 5 days following 45 min of ischemia induced by bilateral renal artery clamping in rats pretreated with DLL4 or vehicle control as described above. Consistent with the findings in Fig. 4, a similar degree of injury was induced in DLL4 and control rats as evidenced by the peak serum creatinine on day 1, but recovery was faster throughout the 5 days of follow-up in the DLL4-treated rats.

To determine whether DLL4 pretreatment altered the severity of histological injury despite similar peak serum creatinine levels, kidneys from the rats depicted in Fig. 4 were harvested at 48 h, and a detailed histological analysis was performed as described in MATERIALS AND METHODS. Ischemia-reperfusion injury resulted in significant injury primarily in the deep cortex and superficial medulla. The total injury score was higher with DLL4 pretreatment (Table 1).

As activation of the Notch pathway can result in cell proliferation, the effect of DLL4 pretreatment on cell proliferation was studied in kidneys harvested 48 h following injury from rats depicted in Fig. 4. Ischemia-reperfusion injury significantly increased cell proliferation in both the outer and inner cortex compared with uninjured rats (Fig. 6). DLL4 treatment resulted in a higher proliferation index compared with vehicle-treated rats following injury, a finding that may underlie the faster recovery seen in this group.

**DISCUSSION**

The major finding of our study was the beneficial effect of the Notch ligand DLL4 on renal functional recovery following acute kidney injury. We demonstrated a consistent beneficial effect of DLL4 in a reproducible model of ARF in two different experimental groups, one followed for 2 days and one for 5 days. At each time point after 24 h, kidney function was better in the DLL4 groups compared with control. Peak serum creatinine was not different in DLL4 vs. the control group although there was a trend toward reduced creatinine with DLL4 treatment. This finding supports similar degrees of kidney injury between groups. In fact, histological assessment demonstrated more severe tubular changes in the DLL4 group. The enhanced proliferation of tubular cells in the DLL4-treated
Table 1. Histological assessment of kidney injury

<table>
<thead>
<tr>
<th>Group</th>
<th>Superficial Cortex</th>
<th>Deep Cortex/Superficial Medulla</th>
<th>Total Injury Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DLL4/no I-R</td>
<td>0.644±0.280</td>
<td>0.449±0.485</td>
<td>0.546±0.546</td>
</tr>
<tr>
<td>No DLL4/I-R</td>
<td>0.479±0.334</td>
<td>1.572±0.540*</td>
<td>1.008±0.278*</td>
</tr>
<tr>
<td>DLL4/no I-R</td>
<td>0.508±0.289</td>
<td>0.402±0.436</td>
<td>0.449±0.356</td>
</tr>
<tr>
<td>DLL4/I-R</td>
<td>1.035±0.350</td>
<td>2.153±0.278*</td>
<td>1.604±0.351†</td>
</tr>
</tbody>
</table>

Values are means ± SD. DLL-4, Delta-like-4; I-R, ischemia-reperfusion. The scoring system was based on a scale of 0–3 as proposed by Chatterjee et al. (8) with some modifications (see MATERIALS AND METHODS). The total score is the average of all tubular scores in the superficial and deep cortex. *P < 0.05 vs. no I-R controls. †P < 0.05 vs. no DLL4/I-R.

...group is a potential mechanism of improved recovery following ARF. Thus Notch activation represents a new strategy to influence the course of ARF.

Activation of the endogenous Notch pathway in the kidney occurs following ARF. In our study, we demonstrated increased expression of cleaved Notch1 and cleaved Notch2 as early as 1 h following reperfusion after 45 min of ischemia. Our findings are consistent with studies by Kobayashi et al. (30) in a similar model of ARF with a few exceptions. These investigators showed increased mRNA and protein expression of Delta-1, cleaved Notch2, and the target gene HES-1 following unilateral ischemia induced by 60 min of clamping the left renal artery. Expression was localized to injured proximal tubules. In their study, cleaved Notch1 was minimally detected under basal conditions or following injury. However, this may be a problem with the antibody used. When we probed Western blots with the Val1744 anti-cleaved Notch1 antibody used by Kobayashi et al., we did not detect a signal (Fig. 1A). Using the cleaved Notch-1 antibody from Rockland (no. 100-401-407), we detected a robust signal for cleaved Notch1 with increased expression seen as early as 1 h following injury. These results were confirmed by immunohistochemistry using the Val1744 antibody. Thus this antibody is suitable for immunohistochemistry, but not for detection of cleaved Notch1 by Western blotting in the adult rat kidney. Therefore, both Notch1 and Notch2 are activated in the kidney following ARF. This finding is of particular importance since DLL4 interacts with Notch1 and Notch4 (52). Also, Notch1 is preferentially induced in other organs during regeneration and remodeling (16, 17, 31, 42, 48, 54). We were able to detect DLL4 protein in control and injured kidneys although no upregulation occurred with injury, which is similar to the findings of Kobayashi et al. (30), where DLL4 expression was examined by RT-PCR. The failure to induce endogenous DLL4 was part of the rationale for studying effects of exogenously administered DLL4 on recovery following ARF.

The Notch pathway plays an important role in cell fate determinations during kidney development. For example, inhibition of γ-secretase, and therefore Notch signaling, decreases the number of glomerular and proximal tubular cells formed in cultured metanephric kidneys (12). Similar findings were seen in presenilin knockout mice, a functional equivalent to γ-secretase inhibition (13, 18, 57). More specific genetic analysis reveals that Notch2 but not Notch1 is required for proximal and distal cell fate decisions and nephron patterning (11, 34, 35). A deficiency of Notch2 leads to an absence of podocytes and proximal tubules. Also, haploinsufficiency of the Notch ligand jagged-1 can lead to the development of Alagille syndrome, which includes defects in kidney formation (47). The specific role of different Notch receptors or ligands in response to kidney injury is not known.

Hypoxia has been implicated as a factor promoting epithelial-to-mesenchymal transition (EMT) in tumor cells, resulting in increased migration, invasiveness, and metastatic potential (11, 15). Similar to its role in hypoxia-induced EMT in tumor cells (23, 50, 51), the Notch pathway could play an essential role in EMT in the adult kidney following hypoxic injury (59). Therefore, therapy with DLL4 could promote EMT in the injured kidney and facilitate kidney regeneration by accelerating the dedifferentiation component of the injury response. Alternatively, DLL4 may act to enhance cellular redifferentiation following dedifferentiation and proliferation of injured tubular cells. A role for Notch signaling would fit with a common theme in which kidney regeneration recapitulates molecular events occurring during development.

Notch signaling has been classically viewed as requiring cell-cell contact between ligand and receptor. However, a role for soluble Notch ligands has been increasingly recognized (32). Soluble Notch ligands are found in Caenorhabditis elegans and Drosophila, and Notch ligands can be cleaved from the cell surface by γ-secretase (6, 10, 29, 55). Activation of Notch receptors can occur in vitro following incubation of a variety of cells with soluble Notch ligands, although these ligands have been shown to be antagonists of Notch signaling in some settings (26, 28, 32). The biological effects of soluble and membrane-bound Notch ligands may also differ. For example, in hematopoietic stem cells (HSC) membrane-bound...
DISCLOSURES
No conflicts of interest are declared by the authors.

REFERENCES


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DLL4 decreases HSC proliferation and maintains cells in an undifferentiated state whereas soluble DLL4 stimulates proliferation and differentiation of HSC (32). The signaling pathways may also be different between membrane-bound and soluble Notch ligands (32). Further support for a biological role of soluble DLL4 comes from in vivo studies of the central nervous system (CNS) where intraventricular injection of DLL4 is biologically active, resulting in an increase in CNS progenitor cells and enhanced functional recovery following CNS injury (3, 44). The pathway linking intraperitoneal injection of DLL4 to kidney regeneration is uncertain and is currently under investigation in our laboratory.

In summary, activation of Notch signaling occurs following ARF as evidenced by increased expression of cleaved Notch1 and Notch2. Administration of the Notch ligand DLL4 resulted in faster recovery following ARF in association with enhanced renal tubular cell proliferation. These findings support a role of the Notch pathway in kidney regeneration although the mechanism remains to be determined. Therapeutic targeting of Notch signaling is a potential strategy for enhancing renal repair.