Nephronectin expression in nephrotoxic acute tubular necrosis

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Abstract

Background. Acute tubular necrosis (ATN) is characterized by an initiation phase, followed by an extension phase, and a maintenance and recovery phase, the latter of which involves increased regeneration of tubular cells. Nephronectin (NPNT), a ligand for α8β1 integrin, is expressed in the ureteric bud epithelium during kidney morphogenesis. However, little is known about the potential involvement of NPNT in the regeneration phase of ATN.

Methods. cDNA microarray, real-time polymerase chain reaction, in situ hybridization, immunohistochemistry, immuno-electron microscopy and immunoassay (for urine) were used to identify the time-course NPNT expression in a murine model of ATN.

Results. The gene transcript of NPNT was examined during a 14-day course of ATN by a cursory cDNA microarray analysis. Although NPNT was observed focally in normal renal tubular epithelium, it was greatly expressed in regenerating tubular cells during the maintenance and recovery phases of ATN. As early as day 1 following onset of ATN, NPNT was already present in the urine. Importantly, NPNT expression preceded proliferating cell nuclear antigen protein expression in regenerating renal tubular epithelial cells, as demonstrated by double immunohistochemistry.

Conclusion. The present study was the first to identify an enhanced expression of NPNT in regenerating tubular epithelium in an experimental model of ATN. NPNT may play a crucial role in the regenerating process of nephrotoxic ATN. Our data also suggest that NPNT may provide a useful tissue and urine biomarker for both the development and evolution of nephrotoxic acute renal injury.

Keywords: acute tubular necrosis; nephronectin; PCNA; regeneration; urine biomarker

Introduction

Acute tubular necrosis (ATN), which is pathologically characterized by destruction of the tubular epithelial cells and clinically identified by acute deterioration of renal function, is the most common cause of acute renal failure [1]. It can be divided into two main categories that include the ischaemic and nephrotoxic types [2]. ATN leads to the detachment and loss of large numbers of epithelial cells from the renal tubules [3,4]. However, cells that remain adherent to the basement membrane can dedifferentiate and spread to fill the gaps in the epithelium, restore cell polarity, and differentiate to reconstruct the normal architecture and function of the tubular epithelium [5,6]. The clinical course of ATN can be divided into initiation, extension, maintenance and recovery phases, although the course of the disease is highly variable [7,8]. The regenerating process is especially important because it involves recovery of kidney function, which depends on a sequence of events that include epithelial cell dedifferentiation [9] and regeneration of epithelial cells, followed by differentiation [10]. This re-epithelialization process involves various growth factors and cytokines produced locally by the tubular epithelial cells themselves (autocrine stimulation) or by inflammatory leucocytes in the neighbourhood of necrotic areas (paracrine stimulation). Growth factors are currently being explored as possible therapeutic agents to enhance re-epithelialization in ATN [11].

Although the morphological features of this process are well known, the molecular events that cause renal tubular regeneration after induction of ATN remain unclear. Several studies have demonstrated the presence of nephrogenic proteins, such as paired box gene 2 [12–14], neural cell adhesion molecule [9], bone morphogen protein 7 [15,16] and Noggin [17],...
during the regeneration phase. However, there are many proteins potentially involved in kidney development that have not been studied during the regeneration process. Using an ischaemia-induced ATN model, Villanueva et al. [17] observed that some nephrogenic proteins are transiently re-expressed in regenerating proximal tubular epithelial cells, after they had been expressed during the nephrogenic process of kidney development.

The cDNA microarray technology offers the advantages of monitoring global gene expression patterns and provides a powerful approach to the molecular dissection of cells and tissues through the simultaneous determination of the expression levels of thousands of genes [10].

Nephronectin (NPNT) protein, an α8β1 integrin ligand, is a recently discovered extracellular matrix protein associated with the Wolffian duct and the ureteric bud, which are epithelial structures that have well-defined roles in kidney development [18–20].

We hypothesized that NPNT may play a crucial role in initiating the regenerating process in ATN. We showed that NPNT was highly and widely expressed in regenerating tubular cells during the maintenance and recovery phases of ATN. Moreover, it was expressed in these cells much earlier than proliferating cell nuclear antigen (PCNA), a well-known biomarker for cell regeneration. The damaged tubular epithelial cells appeared to release NPNT during the early phase of nephrotoxic ATN. Therefore, screening for this protein in either renal tissue or urine may be helpful in the early diagnosis of this renal condition.

Subjects and methods

ATN model and experimental protocol

Female 8-week-old C57BL/6 mice, purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan), were used to establish a uranyl nitrate-induced ATN model, as described previously [21]. After collection of control samples, all mice received a single tail vein injection of uranyl nitrate [(UO2(NO3)2]·6H2O, 100 μg in 100 μl of 5% NaHCO3), and the animals were then sacrificed at day 0 or 3, 7 or 14 days later. Blood and urine were collected for clinical evaluation and measurement of NPNT protein levels, and the kidneys were removed for molecular and histopathology studies. In the latter, scoring of renal tubular necrosis and regeneration was performed as described previously [21]. Briefly, for evaluation of necrosis, 100 intersections were examined in each kidney by light microscopy and a score from 0 to 3 was given for each tubular profile involving an intersection: 0, normal histology; 1, flattened epithelial cells with hyperchromatic nuclei and mitotic figures with up to one-third of tubular profiles showing hyperchromatic nuclei and mitotic figures; 2, same as score 1, but more than one-third and less than two-thirds of tubular profiles showing nuclear loss; and 3, more than two-thirds of tubular profiles showing nuclear loss. The total score for each kidney was calculated by addition of all 100 scores, with a maximum score of 300.

Tubular regeneration was defined as flattened epithelial cells with hyperchromatic nuclei and mitotic figures. One hundred intersections were examined for each kidney and a score from 0 to 3 was given for each tubular profile involving an intersection: 0, normal histology; 1, flattened epithelial cells with hyperchromatic nuclei and mitotic figures with up to one-third of tubular profiles showing hyperchromatic nuclei and mitotic figures; 2, same as score 1, but more than one-third and less than two-thirds of tubular profiles showing hyperchromatic nuclei and mitotic figures; and 3, more than two-thirds of tubular profiles showing hyperchromatic nuclei and mitotic figures. The total score for each kidney was calculated by addition of all 100 scores, with a maximum score of 300. Renal function was evaluated by measuring serum levels of creatinine (Cr) and blood urea nitrogen (BUN) using a Fuji DRI-CHEM 3030 (Fuji Photo Film Co. Ltd., Tokyo, Japan). A cursory cDNA microarray analysis was performed on renal cortical tissues.

cDNA microarray analysis

Total RNA was extracted from renal cortical tissue using Trizol reagent (Invitrogen Corporation, CA, USA), then 40 μg of control (day 0) total RNA and 60 μg of test total RNA were separately annealed to oligo-dT; the control sample was then reverse transcribed in the presence of Cy5-labelled dUTP and the test sample in the presence of Cy3-labelled dUTP. After concentration, the Cy5- and Cy3-labelled cDNA probes were mixed with hybridization solution (1 μl of poly(dA)60–6mer) (8 mg/ml), 1 μl of yeast tRNA (4 mg/ml), 10 μl of mouse Cot-1 DNA (1 mg/ml), 1 μl of 50× Denhardt solution (Sigma-Aldrich, MO, USA), and 6 μl of 20× SSC, and samples were then heat denatured at 95°C for 10 min, stored in ice for 2 min, mixed with 0.5 μl of 10% w/v SDS, applied to a standard cDNA microarray slide, and sealed under a coverslip, as described previously [22]. The slide was placed in a humidified chamber, incubated at 65°C overnight and then washed at room temperature with 2× SSC—0.05% w/v SDS for 2 min and with 0.06× SSC for 2 min. The slide was dried and scanned with a GenePix 4000A array reader (Axon Instruments, CA, USA).

The intensity of the scanning image was quantified using SCANALYZE, a freeware program available from Stanford University (http://rana.lbl.gov/EisenSoftware.htm), and the data (intensity of signal) for gene expression for day 0 sample was divided by those for days 3, 7 and 14 to determine from the Cy3/Cy5 ratio, and analysed using the GENE CLUSTER program [6].

Gene expression profiling was performed on renal tissue at days 3, 7 and 14. The Cy3/Cy5 ratios of the gene spots on the microarray assay were calculated to correlate their temporal changes to the clinical progression of ATN. Using cluster analysis, we identified seventeen overexpressed genes (Table 1), and divided these into three clusters according the profile of their changes in expression during the progression of ATN (Table 1). Cluster 0 consisted of transcripts showing the highest expression on day 7 (middle phase) or day 14 (recovery phase), cluster 1 included those showing highest expression on day 3 (early phase), high expression on day 7, and a great decline in expression on day 14, and cluster 2 included those showing highest expression on day 3, with a great decline in expression on days 7 and 14. As described in our recent article [21], annexin A2 and S100A6 have already been identified as biomarkers of
The primers used were: NPNT: 5'-CACGGAAGGCCAT- GCCAGTGA-3', 5'-AAAGGAACTGGGAGTGT- TC

The fluorescence threshold value was calculated using the iQ system (BioRad) according to manufacturer instructions.

Quantification was performed using the BIO-RAD iCycler System. Real-time polymerase chain reaction (PCR) was used to study the expression of NPNT. In the present study, we focused on NPNT.

**Real-time polymerase chain reaction (PCR)**

Total RNA was extracted from the renal cortex using Trizol reagent (Invitrogen). For the first strand cDNA synthesis, 1.5 μg of the total RNA was used in a single-round reverse transcription reaction. All real-time PCR reactions were performed in 25 μl mixture containing 1 μl of a 1:10 dilution of the cDNA preparation, 2× SYBR Green Supermix (BioRad, CA, USA), and 0.2 μM of each primer. The primers used were: NPNT: 5'-GGGAAAGGGACA AAGAAGATAG-3', 5'-AAGGAAACTCGGAGTGT- TC TG-3'; GAPDH: 5'-TCCGCCCCCTTCTGCGATG-3', 5'-CAGGGAAGGCCAT- GCCAGTGA-3'. Real-time quantification was performed using the BIO-RAD iCycler iQ system (BioRad) according to manufacturer instructions. The fluorescence threshold value was calculated using the iCycle iQ system software. Amplifications were normalized to GAPDH using the 2^(-ΔΔCT) method.

**In situ hybridization (ISH)**

ISH was performed as described previously [22]. In brief, 3 μm paraffin sections were mounted on charged glass slides and stored in airtight boxes at 70 °C. A riboprobe plasmid was generated using a pGEMT-EASY vector (Promega, WI, USA) containing a cDNA insert coding for mouse NPNT to produce an antisense dig-labelled mRNA probe for ISH, which was diluted 100-fold in a hybridization buffer, as described previously [20]. After deparaffinization, the kidney sections were digested for 20 min at room temperature with 20 μg/ml of proteinase K (Sigma, MO, USA) in phosphate-buffer saline (PBS), pH 7.4. After acetylation, 25–50 μl of hybridization mixture (2 mmol/l ethylenediaminetetraacetic acid, 20 mmol/l Tris, pH 7.5, 0.6 mol/l NaCl, 2× Denhardt’s solution, 20% dextran sulfate, 0.1 mg/ml tRNA and 0.2 mol/l DTT) was placed on each section, which was then covered with a siliconized glass coverslip. Hybridization was performed in moist chambers at 42 °C for 16 h, then the coverslips were removed and slides washed in SSC buffer, pH 7.0, rinsed in maleic buffer, pH 7.5, for 1 min at room temperature, blocked with 1x blocking buffer (Roche, IN, USA) for 60 min at room temperature, then incubated with the anti-daiogenin antiserum conjugated to alkaline phosphatase (1:200, v/v) in a blocking buffer at room temperature for 2 h. They were then washed with maleic buffer, pH 7.5, and developed with NBT/BCIP solution in the dark.

**Immunohistochemistry (IHC)**

IHC was performed on formaldehyde-fixed and paraffin-embedded tissues or cells using the avidin-biotin immunoperoxidase method, as described previously [21], with minor modification. For double staining procedures, rabbit anti-PCNA (Santa Cruz, CA, USA) and rabbit anti-NPNT (1:100 dilution in PBS) (Trans Genic Inc., Kumamoto, Japan) antibodies were used. Scoring of NPNT and PCNA protein levels were performed under light microscopy. Twenty randomly selected cortical fields were examined in each section as described previously [21].

**Immunoelectron microscopic study**

Samples were fixed in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in PBS, pH 7.4, and prepared routinely for electron microscopy with final embedding in LR White resin, as described previously [23]. Rabbit anti-NPNT antibody (1:200 dilution in PBS, pH 7.4) and gold-labelled secondary antibody (1:40 dilution in PBS) (British Biocell International, Cardiff, UK) were used, as described previously [21]. The presence and localization of the 10 nm gold particles were examined under an electron microscope (Zeiss EM 902, Germany).
Western blot analysis for urinary NPNT

Each renal cortex tissue or urine sample (10 μl) was run on a 10% non-reducing SDS-PAGE gel with (tissue) or without (urine) prior boiling of the sample. Previously described preliminary tests for determining the optimal electrophoresis parameters demonstrated the crucial importance of these conditions [22]. The proteins were electro-blotted onto a nitrocellulose membrane, which was incubated for 1 h at room temperature in 20 ml of blocking buffer [Tris-buffered saline (TBS), pH 7.6, containing 5% skimmed milk], washed three times in TBS containing 1% Tween 20 (TBST), and incubated overnight at 4°C with rabbit anti-NPNT antibody (Trans Genic) in a blocking buffer. After three washes with TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Pierce, IL, USA) in blocking buffer, washed three times with TBST, and membrane-bound antibody as detected by incubation with Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, MA, USA) and captured on X-ray film. Urine levels of Cr were evaluated by Fuji DRI-CHEM 3030 (Fuji Photo Film Co. Ltd.). The data are presented as ratios of the density of target protein to urine Cr concentration.

Culture of renal epithelial cells

A mouse kidney epithelial cell line (TCMK-1, CCL-139) was obtained from American Type Culture Collection, and maintained in minimum essential medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 5% foetal bovine serum (FBS), in a humidified atmosphere of 5% CO₂ in air at 37°C. Cell cultures were free of mycoplasma.

Methyl thiazoleterazolium (MTT) assay in renal epithelial cells

Cultured cells (1 x 10⁴/well) were plated in 96-well plates. Subsequently, cells were arrested in 0% FBS medium for 16 h, then incubated with different concentrations of FBS (0%, 5%, or 10%) for 24, 48, 72 or 96 h. MTT (5 mg/ml, Sigma) was added (20 μl/well) and incubated for 3 h at 37°C. Then, dimethyl-sulforide (Merck, Darmstadt, Germany) was added (150 μl/well) and allowed to react for 15 min. The absorbance at 540 nm was determined using an ELISA plate reader (Bio-Tek, MA, USA). The arithmetic mean OD of six wells for each experimental point was used to express cell proliferation levels.

Identification of NPNT and PCNA in renal epithelial cells

For assessing the protein expressions of NPNT or PCNA in cultured renal epithelial cells, 5 x 10⁵ cells were arrested in 0% FBS medium for 16 h, then incubated with different concentrations of FBS (0%, 5%, or 10%) for 24, 48, 72 or 96 h. Cultured cells were then fixed with 10% formaldehyde for 18 h. IHC with NPNT or PCNA in the cultured epithelial cells was performed as described earlier.

Statistical analysis

The data are expressed as means ± SD. Comparisons between groups were made using unpaired Student’s t-tests.

Results

Clinical and pathological features of the uranyl nitrate-induced ATN model

Levels of serum Cr and BUN were significantly increased on day 3 after administration of uranyl nitrate compared to day 0 (Cr 1.46 ± 0.36 mg/dl, BUN 105 ± 23 mg/dl compared to Cr 0.31 ± 0.08 mg/dl, BUN 30 ± 4 mg/dl; both P < 0.01) (Figure 1). Thereafter, Cr and BUN levels gradually declined, but did not return to the normal range until day 14 (Cr 0.32 ± 0.18, BUN 39 ± 10 mg/dl) (Figure 1).

Microscopic examination of the renal tissues showed focal necrosis of the renal tubules with cell swelling, brush border loss and nuclear condensation on day 3 (Figure 2B), followed on day 7 by a more diffuse and intense pattern and regeneration of the renal tubules, as indicated by flattened epithelial cells with hyperchromatic nuclei (Figure 2C). On day 14, the histological alterations were mild and had been mainly replaced by morphologically normal renal tubules and a normal background of renal architecture (Figure 2D).

Scoring of necrosis and regeneration
showed that necrosis peaked at day 3 and regenerated at day 7.

**Quantitative measurement of NPNT mRNA levels in the renal cortex**

To assess changes in NPNT mRNA levels, real-time PCR analysis was performed on total RNA isolated from the renal cortex at various times after administration of uranyl nitrate (Figure 3). The results showed that NPNT mRNA was detectable at day 0 and that its levels increased approximately 2-fold ($P < 0.05$) at day 3 and 5-fold ($P < 0.01$) at day 7, then declined by day 14 ($P < 0.005$). The time-course of the changes in NPNT mRNA levels correlated well with the initiation, maintenance, and recovery phases of the ATN model.

Expression of NPNT mRNA (Figure 4A–D) was seen in the renal tubules of the renal cortex by ISH on day 3 and persisted throughout the recovery phase until day 14. Consistent with this, NPNT protein (Figure 4E–H) was expressed in the renal tubules of the renal cortex from days 3 through 14, with a peak on day 7, as shown by IHC. To confirm the intracellular localization of NPNT, immuno-electron microscopy was performed on renal samples taken at day 7 and showed an intracytoplasmic distribution of the protein in the regenerating tubular epithelial cells (Figure 5).

Expression of NPNT protein precedes that of PCNA in regenerating renal tubular epithelial cells

ATN is characterized by both the loss of epithelial cell polarity and the subsequent regeneration and proliferation response. To characterize the expression pattern of NPNT protein during the development and evolution of this ATN model, we performed double IHC for both NPNT and PCNA (a well-known biomarker for cell regeneration that was used here as a positive control for tubular cell regeneration) on renal tissues obtained on days 0, 3, 7 or 14. The results showed that NPNT was expressed at very low levels (in about 15% of renal tubular epithelial cells) on day 0 (Figure 6A, E). On day 3, over 40% of tubular epithelial cells expressed NPNT, while less than 20% of renal tubular epithelial cells expressed PCNA. Although the expressions of NPNT and PCNA were increased in the regenerating renal tubules on day 3, as many as 60% of renal tubular epithelial cells expressing NPNT did not express PCNA during this time point (Figure 6B, E). On day 7, the expressions of both NPNT and PCNA in the tubular epithelium increased...
dramatically, and almost all NPNT-positive epithelial cells (>90%) expressed PCNA (Figure 6C, E). By day 14, the expressions of both NPNT and PCNA were down-regulated and the percentage of cells co-expressing the proteins was also reduced in the tubular epithelial cells (Figure 6D and E). These data suggest that NPNT is a sensitive biomarker for renal tubular epithelial cell regeneration and that it appears much earlier than PCNA in this ATN model.

**NPNT protein leaks into the urine immediately after induction of ATN**

We determined whether NPNT protein could be detected in the urine, to test its utility as an early noninvasive biomarker of ATN. The urine samples, which were not boiled, were subjected to 10% non-reducing SDS-PAGE and Western blot analysis for NPNT. Cr concentrations were used to normalize the levels of NPNT. As shown in Figure 7, urinary NPNT was detected as early as day 1 and reached a peak at day 2. After the early phase of renal injury, urinary NPNT levels gradually declined, suggesting that NPNT leaked from damaged tubular epithelial cells, mainly during the early phase of experimental ATN.

**Co-expression of NPNT and PCNA in cultured renal epithelial cells**

As shown in Figure 8A, MTT assay revealed a significant increase in proliferation of cultured epithelial cells in higher concentrations (5% or 10%) of FBS compared to the serum free condition (each, \( P < 0.005 \)). Moreover, IHC demonstrated that the enhanced expression of NPNT protein (Figure 8B) coincided with that of PCNA, the biomarker for cell regeneration/proliferation (Figure 8C) in the cultured epithelial cells subjected to high concentration FBS, although only few of the cultured epithelial cells showed positive staining for either NPNT or PCNA by IHC (Figure 8D and E).

**Discussion**

In a nephrotoxic ATN murine model, we performed a cursory scanning analysis of global gene expression in cortical renal tissue. Changes in the levels of multiple gene transcripts were noted during the 14-day course of ATN. Most of the up-regulated genes belonged to groups that are important in normal cell structure maintenance, extracellular matrix, regulating calcium levels, cell division/differentiation, and tissue repair.
NPNT was further examined for a potential function in these events because it has been shown to play a crucial role during early metanephric kidney development [18,19]. Although NPNT was present in only some normal renal tubular epithelial cells, it was highly expressed in regenerating tubular cells during the maintenance and recovery phases of ATN. Specifically, NPNT expression was markedly increased in renal tissues as early as day 3, and it was present in the urine as early as day 1. NPNT expression was closely linked to the regenerating process of renal tubular epithelial cells, including the repopulation of tubular cells and recovery from acute renal failure. In addition, damaged tubular epithelial cells tended to release NPNT during the early phase of nephrotoxicity in this ATN model.

The process of kidney injury and repair during ATN follows many of the stages seen during development, such as the processes of dedifferentiation and regeneration of epithelial cells, followed by differentiation [24]. Among these, the regeneration process may play a crucial role in restoring organ or tissue function [25, 26]. Importantly, our double IHC findings showed that the majority of PCNA-positive cells also expressed NPNT as early as day 3, but only a small fraction of NPNT-expressing cells expressed PCNA (Figure 6). During the initial recovery phase (day 7), NPNT expression increased dramatically in the kidneys, and most of the tubular epithelial cells co-expressed NPNT and PCNA, indicating that they were actively proliferating. In the late recovery phase (day 14), the expression of both proteins declined and only a small percentage of tubular epithelial cells expressed both PCNA and NPNT. In addition, the close temporal relationship between the impairment of kidney function and NPNT expression in the regenerating tubular epithelium suggests a functional link for NPNT. Furthermore, NPNT, which is a ligand for α8β1 integrin, may play a role in an autocrine feedback loop mediated by α8β1 integrin by regulating the time-restricted hyper-proliferating state of the renal tubules. After kidney injury, the normal local expression of NPNT in some of the epithelial cells changed to a wide-spread expression in the regenerating renal tubules. Our data support the idea that NPNT is involved in both kidney morphogenesis and function during ATN.

Although our in vitro experiment using cultured renal epithelial cells showed co-expression of NPNT and PCNA in the cells (Figure 8), these data do not...
provide evidence of a direct contribution of NPNT in renal epithelial cell proliferation. However, when combined with the data from the nephrotoxic ATN model showing that NPNT expression was closely linked to the regenerating process in renal tubular epithelial cells, the in vitro data appear to indicate that NPNT is involved in regeneration or proliferation of renal tubular cells during this renal disorder.

NPNT was detected in the urine as early as day 1 and its levels peaked at day 2 (Figure 6). Urinary excretion of NPNT may provide an early sign of renal tubular epithelial cell damage, and detection of urinary NPNT may prove to be a widely applicable non-invasive clinical tool for the very early diagnosis of ATN.

In conclusion, NPNT may play a role in renal tubular epithelial cell regeneration during the initial recovery process following nephrotoxic ATN and may prove useful as a tissue and urine biomarker for both the development and evolution of nephrotoxic renal injury. The development of a sensitive urine test for NPNT may be helpful in monitoring the potential nephrotoxicity of pharmaceutical agents. Whether NPNT plays a role in re-epithelialization in human renal conditions warrants further investigation.

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Conflict of interest statement. None declared.

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