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Involvement of neutrophil gelatinase-associated lipocalin and osteopontin in renal tubular regeneration and interstitial fibrosis after cisplatin-induced renal failure

Emi Kashiwagi[a,b], Yutaka Tonomura[a], Chiaki Kondo[a], Koichi Masuno[a], Kae Fujisawa[a], Noriko Tsuchiya[a], Shuuichi Matsushima[a], Mikinori Torii[a], Nobuo Takasu[a], Takeshi Izawa[b], Mitsuru Kuwamura[b], Jyoji Yamate[b,*]

[a] Developmental Research Laboratories, Shionogi & Co., Ltd., 3-1-1 Futaba-cho, Toyonaka, Osaka 561-0825, Japan
[b] Laboratories of Veterinary Pathology, Life and Environmental Sciences, Osaka Prefecture University, Rinkuu Ourai Kita 1-58, Izumisano-shi, Osaka 598-8531, Japan

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ABSTRACT

The kidney has a capacity to recover from ischemic or toxic insults that result in cell death, and timely tissue repair of affected renal tubules may arrest progression of injury, leading to regression of injury and paving the way for recovery. To investigate the roles of neutrophil gelatinase-associated lipocalin (NGAL/*lcn2*) and osteopontin (OPN/*spp1*) during renal regeneration, the expression patterns of NGAL and OPN in the cisplatin-induced rat renal failure model were examined. NGAL expression was increased from day 1 after injection; it was seen mainly in the completely regenerating proximal tubules of the cortico-medullary junction on days 3–35; however, the expression was not seen in abnormally dilated or atrophied renal tubules surrounded by fibrotic lesions. On the other hand, OPN expression was increased from day 5 and the increased expression developed exclusively in the abnormal renal tubules. NGAL expression level well correlated with the proliferating activity in the regenerating renal epithelial cells, whereas OPN significantly correlated with the α-smooth muscle actin-positive myofibroblast appearance, expression of transforming growth factor (TGF)-β1, and the number of CD68-positive macrophages. Interestingly, rat renal epithelial cell line (NRK-52E) treated with TGF-β1 decreased NGAL expression, but increased OPN expression in a dose-dependent manner. Because increases of TGF-β1, myofibroblasts and macrophages contribute to progressive interstitial renal fibrosis, OPN may be involved in the pathogenesis of fibrosis; on the contrary, NGAL may play a role in tubular regeneration after injury. Expression analysis of NGAL and OPN would be useful to investigate the tubule damage in renal-toxicity.

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1. Introduction

In contrast to the heart or brain, the kidney has a capacity to recover from ischemic or toxic insults that result in cell death (Bonventre, 2003; Lieberthal, 2009). When the kidney recovers from injury, the surviving renal epithelial cells undergo the regulated process including epithelial cell spreading and migration to cover the exposed areas of the basement membrane, cell dedifferentiation, and proliferation to restore cell number, followed by differentiation (Bonventre, 2003; Lieberthal, 2009; Dankers et al., 2011). However, when the basal lamina is damaged, incomplete regeneration occurs. Incomplete regeneration gives rise to interstitial fibrosis around the affected renal tubules probably through epithelial-mesenchymal transition (EMT) (Yamate et al., 1995, 2000), ultimately culminating in chronic kidney disease (CKD) (Ferguson et al., 2008; Bonventre et al., 2010). Whereas timely tissue repair of affected renal tubules may arrest progression of injury, resulting in regression of injury and paving the way for recovery.

Neutrophil gelatinase-associated lipocalin (NGAL/*lcn2*) was initially found as a 25-kDa protein bound to gelatinase in specific granules of neutrophils; thereafter, NGAL has been reported to be expressed in epithelial cells in lesions of inflammation and malignancy (Vaidya et al., 2008). Osteopontin (OPN/*spp1*), known as a 44 kDa bone phosphoprotein, is synthesized in bone and various epithelial tissues (Vaidya et al., 2008; Xie et al., 2001). OPN has diverse functions such as bone morphogenesis and tumorigenesis and accumulation/maintenance of inflammatory macrophages

* Corresponding author. Tel.: +81 072 4635334; fax: +81 072 4635346.
E-mail address: yamate@vet.osakafu-u.ac.jp (J. Yamate).

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(Vaidya et al., 2008). It has been reported that the expressions of NGAL and OPN are rapidly up-regulated after renal injury (Ferguson et al., 2008; Mishra et al., 2004; Mori and Nakao, 2007; Vaidya et al., 2008). However, the pathological roles of NGAL and OPN in the regenerating process after renal injury remain not fully understood.

To shed some light behind the pathogenesis of renal regeneration, we investigated the expression pattern of NGAL and OPN in cisplatin (*cis*-dichlorodiammineplatinum, CDDP)-induced tubular injury in rats. CDDP is an anticancer drug with nephrotoxicity as a side effect (Zhang et al., 2009), which causes dose limitation in clinical usage. It has been experimentally known that CDDP injection to rats causes degeneration and necrosis in renal epithelial cells, especially in the S3 proximal tubules.

The present study shows that NGAL may attribute to favorable regeneration to renal epithelial cells, whereas OPN may oppose the contribution of NGAL; that is, OPN could be related to interstitial fibrosis.

2. Materials and methods

2.1. In vivo study

2.1.1. Animals and CDDP-induced renal failure model

The animal study protocol was carefully reviewed by the Institutional Animal Care and Use Committee (IACUC). Forty eight 5-week-old male F344/DuCrj rats (Charles River Japan, Hino, Shiga, Japan), weighing 70–90 g, were used after a one-week acclimatization period. They were housed in an animal room controlled at 23 ± 3 °C, 45–65% humidity and with 12 h-light–dark cycle, and allowed free access to a standard commercial diet (CA-1, Clea Japan Inc., Tokyo, Japan) and water (sterilized city water *via* an automatic water supplying system). CDDP (Nippon Kayaku Co. Ltd., Tokyo, Japan) was injected intraperitoneally into 40 rats at a single dose of 6 mg/kg body weight. Four animals were sacrificed each on days 1, 3, 5, 7, 9, 12, 15, 20, 25 and 35 after CDDP dosing. The remaining eight rats were injected with equivalent volume of physiological saline and sacrificed on each day 0 (on first injection day) and 35, and served as controls.

At necropsy, blood was collected from the posterior vena cava under intraperitoneal pentobarbital anesthesia, and then put into a vacuum blood-collecting tube containing heparin sodium. Plasma was obtained by centrifugation at 1500 × *g* for 15 min at 4 °C, and plasma creatinine (Cre) and blood urea nitrogen (BUN) were measured using an automatic analyzer H7180 (Hitachi High-Technologies Co., Tokyo, Japan).

2.1.2. Histopathology and immunohistochemistry

Kidneys were removed and then fixed in 10% neutral buffered formalin or Methacarn. Fixed specimens were processed routinely and embedded in paraffin. Formalin-fixed, paraffin-embedded samples were cut at 3–4 μm thickness, and stained with hematoxylin & eosin (H&E) for morphological observations. Additionally, the formalin-fixed tissue sections were stained with the Azan–Mallory method for collagen deposition.

For immunohistochemistry, specimens were cut at 4 μm thickness, deparaffined with xylene, re-hydrated with graded ethanol, and washed in water. These sections were immersed in methanol containing 3% H_2O_2 to block endogenous peroxidase for 10 min at room temperature. Primary antibodies used were NGAL (1:300; Abcam, Cambridge, UK), OPN (1:3000; LSL Co. Ltd., Tokyo, Japan), proliferating cell nuclear antigen (PCNA) (1:100; Dako. Glostrup, Denmark), α-smooth muscle actin (α-SMA) (Dako) and CD68 (ED1; 1:150; Serotec Ltd., Oxford, UK). Tissue sections were incubated with the primary antibody for 60 min at room temperature. Thereafter, sections were washed three times with phosphate-buffered saline (PBS) and incubated for 30 min with the secondary antibody (Histofine Simple Stain, Nichirei Co., Tokyo, Japan). Positive reactions were visualized with 3,3′-diaminobenzidine (DAB). Sections were lightly counterstained with hematoxylin.

Double immunohistochemical staining for PCNA and NGAL or PCNA and OPN was performed with samples of day 7. Immunohistochemistry for NGAL or OPN was conducted as mentioned above. After visualization with DAB, specimens were treated with microwave for 5 min. Primary antibody for PCNA was applied and incubated for 60 min in the room temperature, and then histofine simple stain for alkaline phosphatase (Nichirei) was used with a chromogen new fuchsin (red in color).

2.1.3. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

The piece of kidneys was quickly removed following exsanguination. Then the samples were stored in RNAlater® at −80 °C until use. Kidney samples were then homogenized in QIAzol Lysis Reagent (Qiagen,Valencia, CA, USA) with a TissueLyser (Qiagen), and total RNA was extracted using an RNeasy® Mini Kit (Qiagen). The RNA concentration was determined using a Nano-Drop ND1000 spectrophotometer (Labtech International, East Sussex, UK). Total RNA (5 μg) was converted to cDNA by using the High Capacity cDNA Reverse Transcription Reagents (Applied Biosystems) for RT-PCR with random primer according to the manufacturer's instructions. The levels of various RNAs were evaluated using commercially available TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) with optimized primer and probe concentrations. The ABI assays used in this study were as follows: *lcn2* (Rn00590612_m1; NGAL), *spp1* Rn01449972_m1; OPN) and transforming growth factor (TGF)-β1 (Rn00572010_m1). Real-time PCR was performed with an ABI PRISM 7500 Fast System (Applied Biosystems, Weiterstadt, Germany) by using 1 μL of template cDNA and SYBR® Premix Ex Taq™ (Perfect Real Time; Takara, Shiga, Japan) as recommended in the manufacturer's protocol. Amplification conditions were 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The resulting cycle threshold (C_t) value was processed based on the comparative C_t method, where Gapdh was used as an endogenous reference gene to normalize the expression level of OPN, NGAL and TGF-β1 genes.

In the *in vitro* study, the primers and internal fluorescent TaqMan probes used were as follows for α-SMA, 5′-GACCCTGAAGTATCCGATAGAACA-3′ and 5′-CACGCGAAGCTCGTTATAGAAG-3′ (for primer); 5′-FAM-TGCCAGATCTTTTCC-TAMRA-3′ (for probe) and for E-cadherin, 5′-ACCGAGGGCATTCTGAAAACA-3′ and 5′-CACTGTCACGTGCAGAATGTACTG-3′ (for primer); 5′-FAM-TGCTTGGCCTCAAAATCCAAGCCCT-TAMRA-3′ (for probe).

2.2. In vitro studies

2.2.1. Cell line and cell culture

NRK-52E cells (Dainippon-Sumitomo Pharma Co., Esaka, Japan), which were established from normal rat proximal renal tubules (Lash et al., 2002), were used. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Nichirei) as the growth medium. The cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere, and cells were serially subcultured by treatment with a mixture of 0.1% trypsin and 0.02% ethylendiaminetetraacetic acid in PBS.

2.2.2. Real-time RT-PCR in NRK-52E cells

NRK-52E cells were incubated with TGF-β1, the major fibrogenic factor (Fan et al., 1999; Rhyu et al., 2005; Zeisberg and Kalluri,

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