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Cellular and Molecular Biology

Involvement of endogenous prostaglandin E_2 in tubular epithelial regeneration through inhibition of apoptosis and epithelial-mesenchymal transition in cisplatin-induced rat renal lesions

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Summary. In the kidney, prostaglandin (PG) E_2 is the main PG, playing important roles in maintaining homeostasis or development of pathological settings. Roles of PGE_2 in renal lesions remain to be clarified. The expression patterns of PGE_2 synthesis enzymes such as cyclooxygenase (COX)-1, COX-2 and microsomal PGE synthase (mPGES)-1, and PGE_2 receptors (EP2 and EP4) were examined in cisplatin-induced rat renal failure. The immunoexpressions for COX-1, mPGES-1 and EP4 receptor were increased exclusively in the affected renal tubules, but those of COX-2 and EP2 receptor were not detected; increased expression of COX-1 was confirmed at mRNA level. Using rat renal epithelial cell line (NRK-52E), the effects of PGE_2 on cell proliferation were investigated. The addition of PGE_2 or 11-deoxy-PGE_1 (EP4 receptor agonist) to NRK-52E increased the cell number, indicating the effects of PGE_2 via EP4 receptor. Furthermore, 11-deoxy-PGE_1-treated NRK-52E cells underwent the G_0/G_1 arrest and decreased apoptosis. NRK-52E treated with transforming growth factor (TGF)-ß1, an inducer of epithelial-mesenchymal transition (EMT), in the presence of 11-deoxy-PGE_1 decreased the mRNA expression of α-smooth muscle actin (a marker of myofibroblasts). Collectively, the present study shows that COX-1 plays more important roles than dose COX-2 in cisplatin-induced rat renal failure; the product, PGE_2, may regulate renal epithelial regeneration via EP4 receptor through inhibition of apoptosis and EMT.

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Introduction

Prostaglandin (PG), mainly PGE_2, plays important roles in renal hemodynamics, renin release and tubular sodium/water re-absorption (Breyer and Breyer, 2000; Kotnik et al., 2005). In pathological settings such as diabetic nephropathy, PGE_2 synthesis is elevated; the increased PGE_2 may influence cell proliferation, differentiation or apoptosis (Sanchez and Moreno, 2002; Moore et al., 2005; Zhang et al., 2006). Such functions may be related to regeneration after tubular epithelial cell injury in the kidney. However, the functional roles of PGE_2 in renal failure are not fully understood (Vukicevic et al., 2006).

Cisplatin (CDDP; *cis*-diamminedichloroplatinum) has been widely used as an anticancer drug in human clinic, but it is well known that the chemical has nephrotoxicity as a side effect (Hanigan and Devarajan, 2003). CDDP injection to rats causes renal tubular epithelial cell injury, especially in the lower straight part (P_3 segment) of the proximal tubules in the cortico-medullary junction, and, after injury, renal epithelial cells regenerate; however, when the basal lamina is completely damaged by injury, incomplete regeneration occurs (Yamate et al., 1995, 2000). Incompletely regenerating renal epithelial cells may undergo epithelial-mesenchymal transition (EMT) leading to interstitial fibrosis (Iwano et al., 2002). Cisplatin-induced renal lesions are useful for studies on the processes of renal tubular regeneration or the

pathogenesis of subsequent renal interstitial fibrosis (Yamate et al., 1995, 2000, 2005). In CDDP-induced renal lesions the expression patterns of PGE_2 synthesis-related enzymes remain to be investigated.

PGE_2 is synthesized from arachidonic acid through two-step reactions. The arachidonic acid is first converted to an unstable intermediate PGH_2 by one of two cyclooxygenase (COX) isoforms, COX-1 or COX-2. This rate-limiting step is followed by formation of PGE_2 via the prostaglandin E synthase (PGES) (Kotnik et al., 2005; Kudo and Murakami, 2005). The constitutive COX-1 is thought to be responsible for basal PG production for homeostasis in normal conditions, whereas COX-2 expression is primarily associated with inflammatory reactions (Bondesen et al., 2004). In the kidney, however, COX-2 is expressed constitutively, and seems to regulate various physiological conditions (Harris et al., 1994; Câmpean et al., 2003).

The activity of PGE_2 is carried out via four different receptor subtypes: EP1, EP2, EP3, and EP4. EP1 receptor is associated with calcium mobilization, EP2 and EP4 receptors with stimulation of adenylate cyclase (AC), and EP3 receptor with the inhibition of AC or with stimulation of phosphoinositol turnover (Breyer and Breyer, 2000). These EP receptors have been reported to be present in renal tissues (Breyer and Breyer, 2000; Vukicevic et al., 2006). Thus, it has been shown that EP receptor agonists or antagonists would be effective in amelioration of renal failure; EP1-selective antagonist is useful for the prevention of diabetic nephropathy in a streptozotocin-induced rat model (Makino et al., 2002), and EP4-selective agonist reduces renal lesions in a mercury chloride-induced rat renal failure model (Vukicevic et al., 2006).

In the present study, the expression patterns of main PGE_2 biosynthesis-related enzymes and EP receptors were examined in CDDP-induced rat renal failure. Because EP2 and EP4 receptors are related to the inhibition of fibroblast functions (Kolodsick et al., 2003), particularly, we focused on expressions of EP2 and EP4 receptors in association with renal fibrosis. Furthermore, the effects of PGE_2 and EP4 receptor on cell proliferation, apoptosis and EMT were investigated *in vitro* using a rat renal epithelial cell line (NRK-52E), to clarify the phenomena seen in CDDP-induced rat renal lesions.

Materials and methods

Animals and CDDP-induced renal lesions

The following experiments were in compliance with our institutional guideline for animal care. Fifty-two 6-week-old male F344/DuCrj rats (Charles River Japan, Hino, Shiga, Japan), weighing 125-150 g, were used after a one-week acclimatization period. They were housed in an animal room controlled at 22±3°C and with 12 hours-light-dark cycle, being allowed free access to a standard commercial diet (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water. CDDP (Nippon Kayaku Co. Ltd., Tokyo, Japan) was injected intraperitoneally into forty-four rats at a single dose of 6 mg/kg body weight. Four to eight animals were sacrificed each on days 1, 3, 5, 7, 9, 12 and 15 after CDDP dosing. The remaining eight rats were sacrificed on day 0, and served as controls.

Histopathology and immunohistochemistry

Renal tissues were fixed in 10% neutral buffered formalin and periodate-lysine-paraformaldehyde (PLP) fixatives. Formalin-fixed specimens were processed routinely and embedded in paraffin; PLP solution-fixed specimens were embedded in paraffin with the AMeX method (PLP-AMeX method) (Suzuki et al., 2000). Formalin-fixed, paraffin-embedded samples were cut at 3-4 μm thickness, and stained with hematoxylin-eosin (HE) for morphological observations.

For immunohistochemistry, specimens processed in the PLP-AMeX method were cut at 4-μm thickness, deparaffined with xylene, re-hydrated with graded ethanol, and washed in water. These sections were boiled in a micro-wave for 5 minutes for antigen retrieval, and then endogenous peroxidase was blocked using 3% H_2O_2 for 10 minutes at room temperature. Primary antibodies used were monoclonal anti-COX-1 (1:200) (Cayman Chemical Co., Ann Arbor, MI, USA), polyclonal anti-COX-2 (1:300) (Cayman Chemical), polyclonal anti-mPGES-1 (1:200) (Cayman Chemical), polyclonal anti-EP2 receptor (1:500) (Cayman Chemical.), and polyclonal anti-EP4 receptor (1:500) (Upstate Biotechnology Inc. Lake Placid, NY, USA). Tissue sections were incubated with the primary antibody overnight (12-14 hours) at 4°C. Thereafter, sections were washed three times with phosphate-buffered saline (PBS) and incubated for 45 minutes with the secondary antibody (Kit system; Histofine Simple Stain MAX PO (R) or (M); Nichirei Co., Tokyo, Japan). Positive reactions were visualized with 3,3'-diaminobenzidine (DAB). Sections were lightly counterstained with hematoxylin. Sections incubated with normal rat or mouse serum instead of the primary antibody served as negative controls.

Real-time reverse transcriptase-polymerase chain reactions (RT-PCR)

Total RNA was extracted from renal tissues using Trizol Reagent™ (Invitrogen Co., Carlsbad, CA, USA) and SV Total RNA Isolation System (Promega, Osaka, Japan). The RNA was reverse-transcribed to cDNA using SUPERSCRIPT First-Strand Synthesis SystemTM (Invitrogen). All PCR experiments were performed with SYBR Green Real-time PCR Master Mix (Toyobo). The amplification program consisted of 1 cycle at 95°C with a 1-minute hold followed by 45 cycles at 95°C with a 15-second hold, 65°C (62°C for COX-2) of annealing temperature with a 15-second hold, and 72°C with a 30-

second hold. Melting curve analysis to verify the accuracy of the PCR products followed amplification. The PCR-primer sequences were shown in Table 1. ß-actin was used as an internal control (Tsujino et al., 1997). Value was indicated as ΔΔCt value with comparative Ct method.

In vitro studies

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 Co., Tokyo, Japan) as the growth medium. The cultures were incubated at 37° in a humidified 5% CO_2 atmosphere, and cells were serially subcultured by treatment with a mixture of 0.1% trypsin and 0.02% ethylendiamine-tetraacetic acid in PBS.

Influences of PGE_2 on cellular growth of NRK-52E Cells

NRK-52E cells ($1x10^4$/ml) were seeded in 24 well plates (Corning Incorporated, Costar, NY, USA) and grown for 24 hours in the growth medium. After washing twice in PBS, the growth medium was changed to 1% FBS-supplemented DMEM and then the cells were incubated for 24 hours. After the incubation, PGE_2 (1, 10 or 100 nM, diluted in ethanol; Funakoshi Co. Ltd., Tokyo, Japan), forskolin (10 μM, diluted in ethanol; Sigma-Aldrich Co. St. Louis, MT, USA), H89 (a protein kinase A (PKA) inhibitor; 1 μM, diluted in ethanol; Biomol International, Plymouth meeting, PA, USA), 11-deoxy-PGE_1 (EP4 receptor agonist; 1, 10 or 100 nM, diluted in ethanol; Cayman Chemical) (Takeuchi et al., 2003) or vehicle (ethanol control) were added in the medium. After incubation for 48 hours, cells were trypsinized and collected by centrifugation at 1,500 rpm for 5 minutes and re-suspended in 1% FBS-supplemented DMEM. Cell viability was assessed by trypan blue (0.5% in PBS) dye exclusion, and the cell number was counted using a hemocytometer.

Flow cytometry analysis of cell cycle and quantitation of apoptosis

NRK-52E cells were treated with CDDP (25 μM) with or without 11-deoxy-PGE_1 (100nM) for 24 hours (Price et al., 2006). As a control, NRK-52E cells were incubated in the absence of CDDP and 11-deoxy-PGE_1. After the treatment, the cells were washed twice with PBS, trypsinized, and suspended in PBS containing 2% FBS. Then, the cells were rinsed and centrifuged at 1,500 rpm for 5 minutes. Finally, the cells were re-suspended in PBS containing 2% FBS. Cell concentrations were adjusted to $1x10^6$ cells/ml, and 1 ml of the cell suspension was centrifuged at 1,500 rpm for 5 minutes. Supernatant (0.9 ml) was removed, and cells were re-suspended in the remaining 0.1 ml fluid. Next, 1 ml of ethanol was added and the cells were fixed at 4°C for 1 hour. After the fixation, cells were centrifuged at 1,500 rpm for 5 minutes, suspended in the propidium iodide (PI) solution (50 μg/ml) (Invitrogen) containing RNase A (0.5 mg/ml; Roche Diagnostics Co. Ltd., Tokyo, Japan), and incubated for 30 minutes at room temperature (Lash et al., 2002). Samples were analyzed using a flow cytometer (FACSCalibur: BD Biosciences, Tokyo, Japan). PI staining enables us to estimate the sub-G_0/G_1 population as apoptotic cells and the distribution of cell cycle population. The percentage of apoptotic cells was measured by estimation of the sub-G_0/G_1 peak in the PI staining (Oyaizu et al., 2001).

RT-PCR analyses for α-SMA and E-cadherin in NRK-52E cells

NRK-52E cells were incubated with transforming growth factor (TGF)-ß1, a cytokine with fibrogenic properties (Fan et al., 1999; Zeisberg and Kalluri, 2004; Rhyu et al., 2005). After being seeded in 25 mm^2 flasks

Table 1. Sequences of primers used for the real-time or quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Name (Ref.)	Oligo.	Primer sequence
COX-1(Cheuk et al., 2000)	Forward	5'-TGGAGAAGTGCCAGCCCAACTCCC-3'
	Reverse	5'-GGGGCAGGTCTTGGTGTTGAGGCA-3'
COX-2 (Guo et al., 2006)	Forward	5'-TTTGTTGAGTCATTCACCAGACAGAT-3'
	Reverse	5'-ACGATGTGTAAGGTTTCAGGGAGAAG-3'
α-SMA (Morioka et al., 2004)	Forward	5'-CACGGCATCATCACCAACTG-3'
	Reverse	5'-ACGCGAAGCTCGTTATAGAAGG-3'
E-cadherin	Forward	5'-GAAGGCCTAAGCACAACAGC-3'
	Reverse	5'-CGGTGTACACAGCATTCCAC-3'
ß-actin (Tsujino et al., 1997)	Forward	5'-TAAAGACCTCTATGCCAACAC-3'
	Reverse	5'-CTCCTGCTTGCTGATCCACAT-3'

coated with collagen type I (BD Biosciences, Franklin Lakes, NJ, USA), cells grown to sub-confluence in the growth medium were incubated with 1% FBS-containing DMEM for 24 hours. After the incubation, 11-deoxy-PGE_1 (0 or 100 nM) was added to the culture in the presence of TGF-ß1 (10 ng/ml) (R&D Systems, Minneapolis, MN, USA). After 48-hour incubation, the cells were washed twice with PBS, and trypsinized; the suspended cells were centrifuged twice at 3,500 rpm for 5 minutes. Total RNA was extracted from the collected cells using SV Total RNA Isolation System, and the RNA was reverse-transcribed to cDNA as described above. cDNA was amplified by PCR with Go Taq Green Master Mix (Promega Corp., Madison, WI, USA) and each of specific primers for α-SMA, E-cadherin or ß-actin (internal control). The following conditions were used for the amplification: for α-SMA, 30 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 60 °C, and 30 seconds of synthesis at 72°C: for E-cadherin, 45 cycles of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 61°C, and 30 seconds of extension at 72°C. The oligonucleotides used are shown in Table 1. PCR products were subjected to electrophoresis in a 2.5% agarose gel. DNA was stained with ethidium bromide on the gel. The bands were semiquantitatively evaluated with image analysis software, relative to those of ß-actin (Tsujino et al., 1997). Value was indicated as ΔΔCt value with comparative Ct method.

Evaluation and statistical analyses

Cells showing a distinct positive reaction for COX-2 immunohistochemistry were counted in five randomly selected areas at a magnification of x400 in the distal tubules, and the macula densa with the positive cells to COX-2 was counted per 20 glomeruli. Data obtained in these immunohistochemical examinations and by the real-time RT-PCR methods were presented as mean ± standard deviation (S.D.). Difference between controls and CDDP-injected groups were evaluated by the analysis of variance (ANOVA). The immunoreactivities for COX-1, mPGES-1 and EP4 receptor were assessed semiquantitatively; -, no change; ±, faintly positive staining; 1+, moderately positive staining; 2+, more clearly evident staining; 3+, markedly positive staining. Data obtained in the cell count, flow cytometer, and semiquantitative RT-PCR in *in vitro* studies was presented as mean ± S.D. Differences between control and treated cells were evaluated by the ANOVA. Values of $P<0.05$ were considered significant.

Results

CDDP-induced Renal Lesions In Vivo

Histopathological Findings

In control kidneys, no histopathological finding was seen (Fig. 1A). On day 1, the renal tubules, particularly the proximal tubules in the cortico-medullary junction, showed nuclear alterations, such as dispersed, segmented or condensed heterochromatin; some epithelial cells underwent apoptosis, demonstrable by the terminal deoxyribonucleotide-mediated dUTP nick and labeling method (data not shown). On day 3, epithelial cells in the affected renal tubules were desquamated into tubular lumina; some cells had swollen cytoplasm and enlarged nucleus with dispersed chromatin, indicating necrosis. On day 5, renal epithelial cells with such nuclear and cytoplasmic changes were more apparent (Fig. 1B), with extension of these lesions towards the cortex. In addition to these changes, on day 7, slight dilation of the affected tubules lined by flattened or cuboidal epithelial cells with basophilic cytoplasm (indicating regenerating epithelial cells) was observed, being accompanied with infiltration of mononuclear cells around the tubules and within their lumina (Fig. 1C). On days 9, 12 (Fig. 1D) and 15, renal tubules with variously-sized lumina were seen in the cortico-medullary junction; these tubules were rimmed by flattened, polygonal or cuboidal regenerating epithelial cells; some epithelial cells had a large nucleus with irregularly dispersed chromatin. In the interstitium around the affected renal tubules, fibrotic areas consisting of spindle-shaped myofibroblastic cells and mononuclear cells began to be seen (Fig. 1D), indicating development of fibrosis.

Immunohistochemistry for COX-1, COX-2 and mPGES-1, as well as EP2 and EP4 receptors

As shown in Table 2, in control kidneys, COX-1 immunoexpression was seen in the distal tubules of the cortex and cortico-medullary junction (Fig. 2A) (+). On days 3 to 9, the affected proximal tubules in the cortico-medullary junction showed greater reactivity (2+), and the reactivity was more prominent (3+) on days 12 and 15, especially in the regenerating tubular epithelial cells (Fig. 2B).

In control kidneys, the macula densa cells showed a positive reaction to COX-2, and renal epithelial cells of the distal tubules located mainly in the cortex occasionally reacted to COX-2 (Fig. 3A). In CDDP-injected rats, the number of COX-2-positive epithelial cells in the distal tubules was significantly decreased on

Table 2. Immunoexpression for cyclooxygenase (COX)-1 in the cortico-medullary junction.

	Days after CDDP injection							
	0	1	3	5	7	9	12	15
Expression	+	+	2+	2+	2+	2+	3+	3+

The degrees for COX-1 were assessed semiquantitatively as follows: +: moderately positive staining; 2+: more clearly evident staining; 3+: marked staining. CDDP, cisplatin.

days 3 to 7 (Fig. 3B), and it recovered to control levels on days 12 and 15 (Fig. 3C). The number of COX-2-positive cells in the macula densa also began to be significantly decreased on day 1; on day 3 onwards, the decreased number became more prominent and the number did not recover until day 15 (Fig. 3D). COX-2 immunoexpression was not detected in the affected proximal renal tubules.

In control kidneys, an immunoreaction for mPGES-1 was seen in renal epithelial cells to various degrees (Table 3); the reactivity was faint in the proximal tubules in the cortex (±) (Fig. 4A) and was not seen in the cortico-medullary junction (-) (Fig. 4C), whereas it was stronger in the thin limbs of loop of Henle and collecting ducts in the medulla (2+) (Fig. 4E). In CDDP-injected

Table 3. Immunoexpression for microsomal prostaglandin (PG) synthase (mPGES)-1.

Site	Days after CDDP injection							
	0	1	3	5	7	9	12	15
Cortex	±	±	+	+	2+	2+	2+	2+
Cortico-medullary junction	-	-	-	-	±	+	+	+
Medulla	2+	2+	2+	2+	+	+	+	+

The degrees for mPGES-1 were assessed semiquantitatively as follows: -: negative staining; ±: faintly positive staining; +: moderately positive staining; 2+: clearly evident staining.

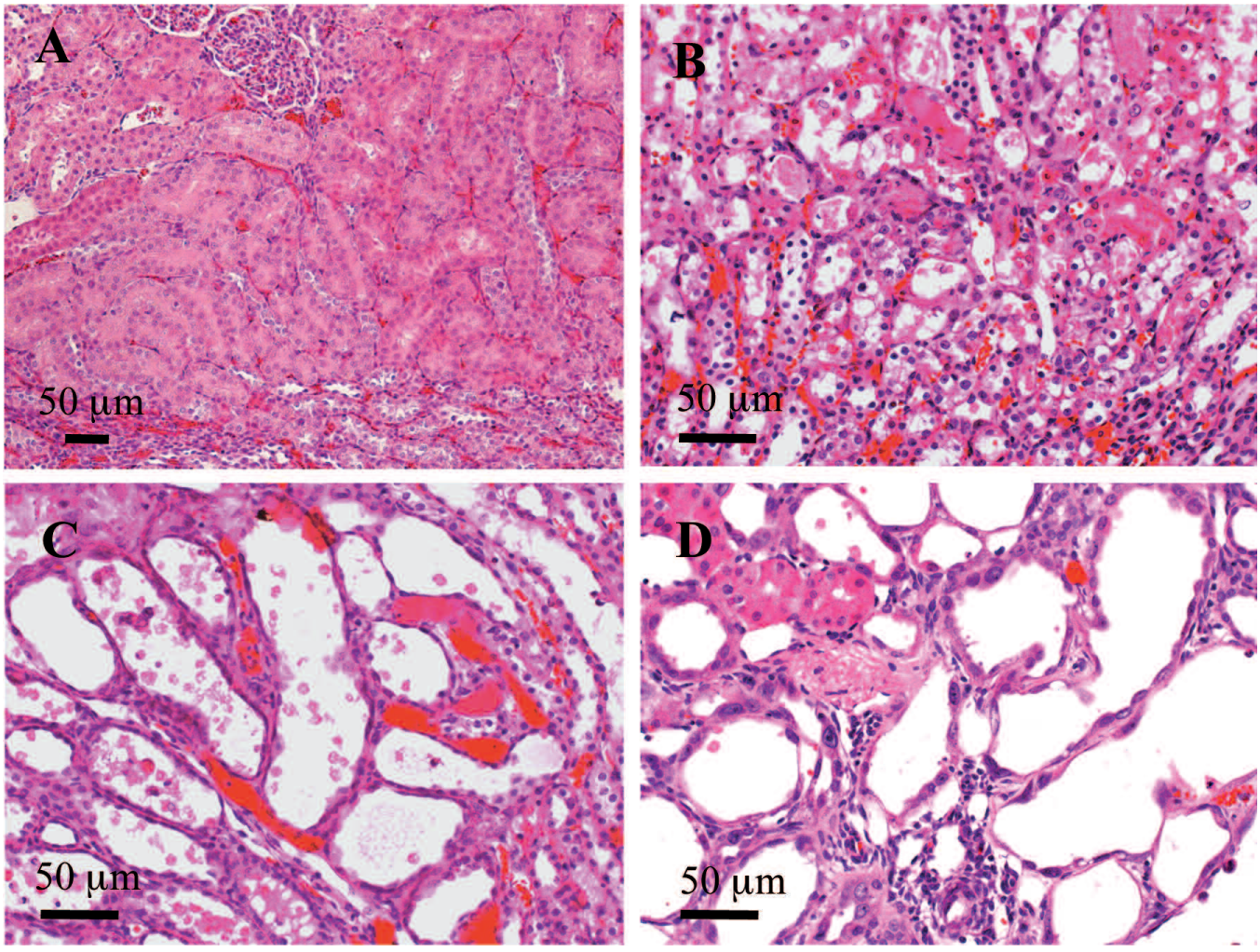

Fig. 1. Histopathological findings of kidneys of control **(A)** and cisplatin (CDDP)-injected rats (**B-D**). **A.** Cortico-medullary junction of control rat. Proximal tubules in the cortico-medullary junction show normal histology. **B.** Day 5 after CDDP dosing; epithelial cells in the cortico-medullary junction desquamate into tubular lumina, and some epithelial cells have a condensed hyperchromatic nucleus. **C.** Day 7; slight dilation of the affected renal tubules lined by flattened or cuboidal epithelial cells is observed, and desquamated epithelial cells are seen in the lumina. **D.** Day 12; renal tubules with variously sized lumina are seen; some epithelial cells have a large nucleus with irregular dispersed chromatin; spindle-shaped myofibroblastic cells and mononuclear cells are seen around the affected tubules. HE stain.

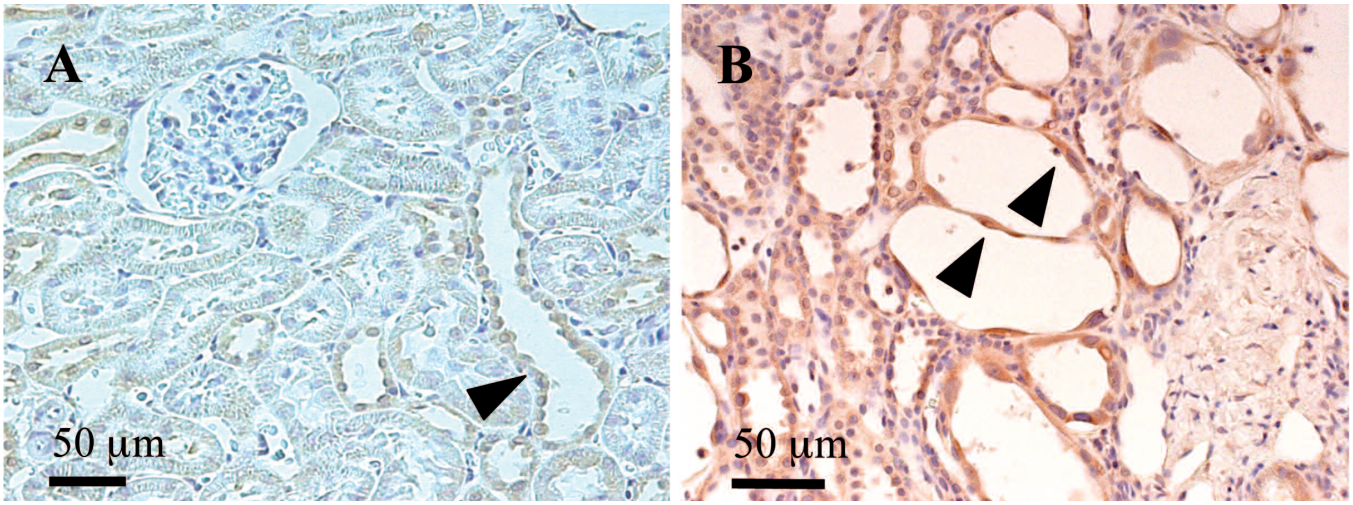

Fig. 2. Immunohistochemistry for cyclooxygenase (COX)-1 in kidneys of control **(A)** and CDDP-injected rat **(B). A.** Control; epithelial cells in the distal renal tubules in the cortex react to COX-1 (arrowheads). **B.** Day 12 after CDDP dosing; epithelial cells in the affected proximal renal tubules, especially in the regenerating epithelial cells in the cortico-medullary junction give a strong reaction to COX-1 (arrowheads). Immunohistochemical staining, counterstained with hematoxylin.

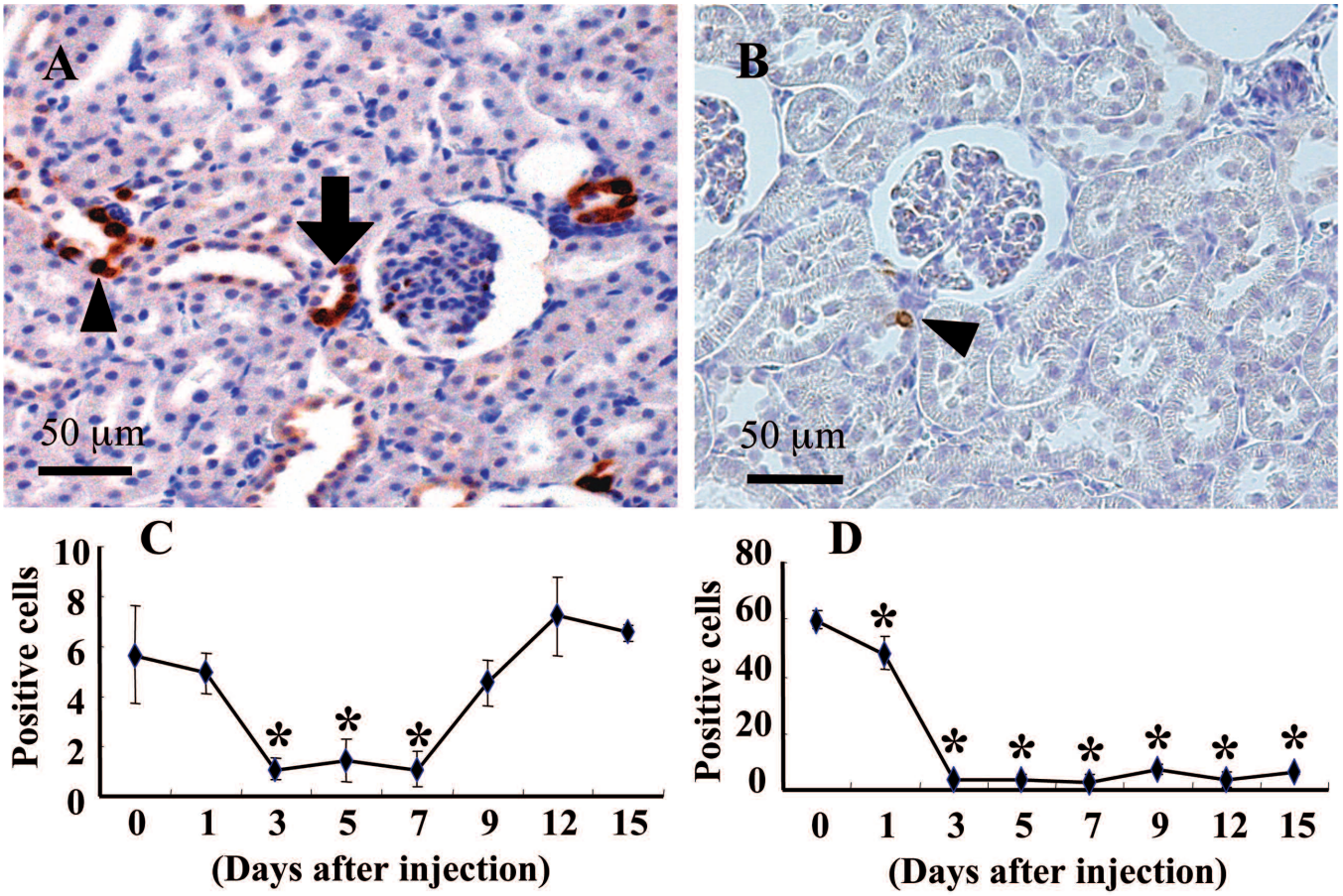

Fig. 3. Immunohistochemistry for COX-2 in kidneys of control **(A)** and CDDP-injected rats **(B)**, and the kinetic of COX-2-positive cell number in the distal renal tubules (x 400 field) **(C)** or in macula densa (per 20 glomeruli) **(D)**. **A.** Control; COX-2-positive reactions are seen in the epithelial cells of the distal renal tubules (arrowhead) and macula densa (arrow). **B.** Day 5 after doing; in contrast to control, the number of cells reacting to COX-2 is smaller (arrowhead), indicating decreased number of the positive cells in CDDP-injected rats. **C.** The COX-2-positive cell number is significantly decreased on days 3, 5 and 7. **D.** The number of COX-2-positive cells in the macula densa remains significantly decreased until day 15. **A and B**, immunohistochemical staining, counterstained with hematoxylin. **C and D**, *, significantly different from day 0 (controls) at $P<0.05$.

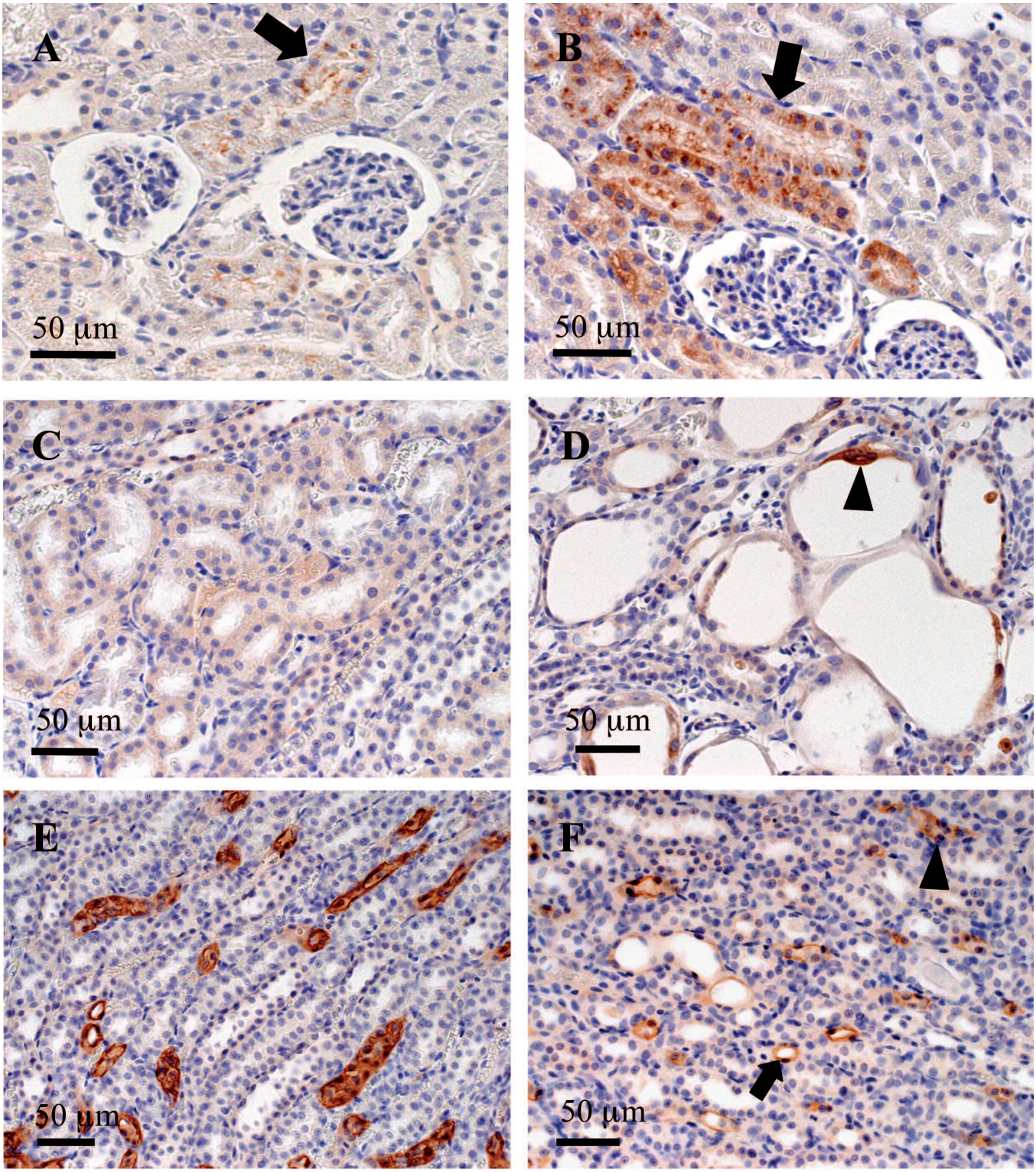

Fig. 4. Immunohistochemistry for microsomal prostaglandin E synthase (mPGES)-1 in kidneys of control (**A, C, E**) and CDDP-injected (**B, D, F**) rats. **A.** Control; a faint positive reaction to mPGES-1 is seen in epithelial cells (arrow) of the proximal tubules in the cortex. **B.** Day 9 after doing; a strong reaction to mPGES-1 is seen in epithelial cells (arrow) in the proximal tubules in the cortex. **C.** Control; no positive reaction to mPGES-1 is seen in the cortico-medullary junction. **D.** Day 9; a marked reaction to mPGES-1 is seen in epithelial cells (arrow) in the affected renal tubules in the cortico-medullary junction. **E.** Control; there are positive reactions to mPGES-1 in the thin limbs of loop of Henle (arrow) and collecting ducts (arrowhead) in the medulla. **F.** Day 9; the reactivity to mPGES-1 is decreased in the thin limbs of loop of Henle (arrow) and collecting ducts (arrowhead) in the medulla, as compared with Figure 4F. Immunohistochemical staining, counterstained with hematoxylin.

rats, the mPGES-1 reactivity in the proximal tubules in the cortex became stronger (Fig. 4B) on days 3 to 15 (+ - 2+) (Table 3); in addition, epithelial cells in the dilated proximal renal tubules in the affected cortico-medullary junction showed a clearly positive reaction to mPGES-1 on days 7 to 15 (± - +) (Fig. 4D, Table 3). The intensity of positive reaction seen in the thin limbs of loop of Henle and collecting ducts in the medulla was decreased on days 7 to 15 (+) (Fig. 4F, Table 3).

No positive reaction for EP2 receptor was seen in control kidneys (Fig. 5A). In CDDP-injected rats, epithelial cells of a few collecting ducts in the medulla or papilla showed a positive reaction for EP2 receptor (Fig. 5B), whereas no reaction was seen in the affected renal tubules of the cortico-medullary junction. In control kidneys, although epithelial cells of the distal renal tubules in the cortico-medullary junction (+) and collecting ducts in the medulla (2+) reacted to EP4 receptor (Fig. 5C, Table 4), epithelial cells of the proximal renal tubules in the cortico-medullary junction did not show a positive reaction. In CDDP-injected rats, the positive reaction to EP4 receptor began to be seen in the affected, dilated renal tubular epithelial cells on days 7 to 15 (2+) (Fig. 5D, Table 4).

mRNA Expressions of COX-1 and COX-2

COX-1 mRNA expression in CDDP-injected rats was significantly increased on days 3 and 5 (Fig. 6A). In contrast, in CDDP-injected rats, COX-2 mRNA

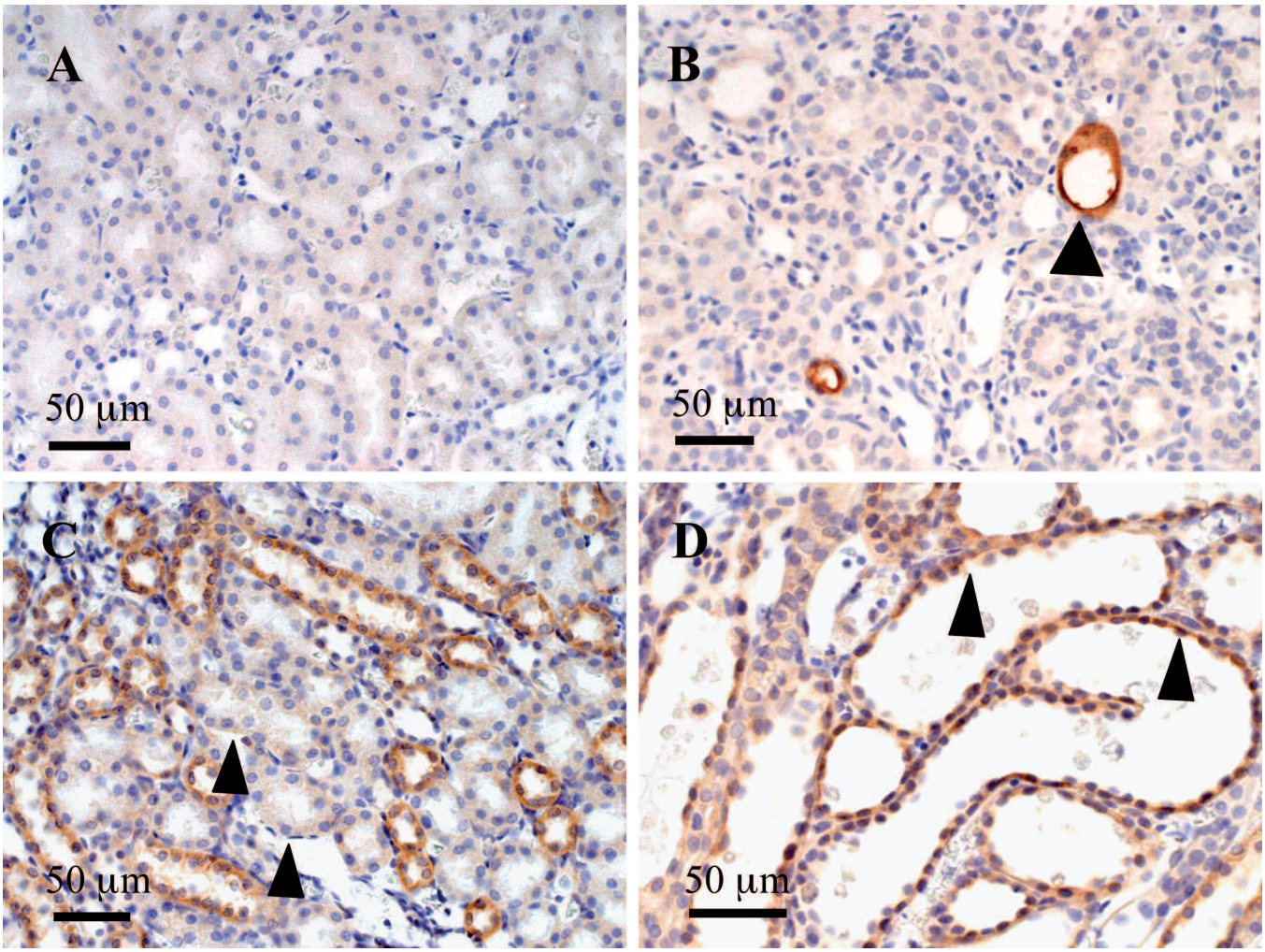

Fig. 5. Immunohistochemistry for PGE_2 receptors (EP2 and EP4 receptors) in kidneys of control (**A, C**) and CDDP-injected (**B, D**) rats. **A.** Control; no positive reaction to EP2 receptor is seen in the medulla. **B.** Day 9; there are a few epithelial cells (arrowhead) reacting to EP2 receptor in the collecting ducts in the medulla. **C.** Control; EP4 receptor-positive cells are seen in the distal tubules in the cortico-medullary junction, whereas epithelial cells of the proximal tubules are negative to EP4 receptor (arrowheads). **D.** Day 7; epithelial cells in the affected, dilated renal tubules react strongly to EP4 receptor (arrowheads) in the cortico-medullary junction. Immunohistochemical staining, counterstained with hematoxylin.

expression was markedly decreased with a significant change on days 3, 5, and 15 (Fig. 6B). Expression patterns for both COX-1 and COX-2 mRNAs generally agreed with those of immunohistochemical stainings.

In vitro studies

Influences of PGE_2 through Cyclic Adenosine Monophosphate (cAMP) Dependent Pathway on Cell Growth of NRK-52E Cells

The addition of PGE_2 to NRK-52E cells significantly increased the cell number at 10 and 100 nM (Fig. 7A). The addition of H89, an inhibitor of PKA, to PGE_2-incubated NRK-52E cells significantly abolished the effects of PGE_2 (Fig. 7B). The treatment with forskolin, which activates AC, resulted in a significantly increased number of NRK-52E cells (Fig. 7C). NRK-52E cells incubated with 11-deoxy-PGE_1 (1, 10 or 100 nM), an agonist of EP4 receptor, showed a dose-dependent, significant increase in the cell number (Fig. 7D).

Effects of 11-deoxy-PGE_1 on apoptosis and cell cycle of NRK-52E cells

The CDDP (25 μM) treatment increased apoptosis in NRK-52E cells, and significantly decreased G_0/G_1 phase population (Fig. 8A,B). These alterations induced by CDDP treatment were inhibited by the addition of 11-deoxy-PGE_1 (Fig. 8A,B). There was no difference in the

Table 4. Immunoexpression for PGE_2 receptor (EP)4.

Site	Days after CDDP injection							
	0	1	3	5	7	9	12	15
Cortico-medullary junction	+	+	+	+	2+	2+	2+	2+
Medulla	2+	2+	2+	2+	+	+	+	+

The degrees for EP4 receptor were assessed semiquantitatively as follows: –, negative staining; ±, faintly positive staining; +, moderately positive staining, 2+, clearly evident staining.

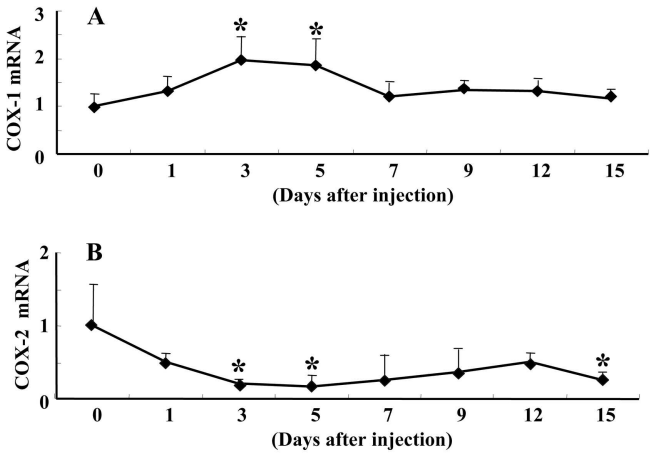

Fig. 6. mRNA expressions of COX-1 and COX-2 in control (on day 0) and CDDP-injected rats (on days 1-15) by the real-time RT-PCR method. **A.** Expression level of COX-1 mRNA is significantly increased on days 3 and 5. **B.** Expression level of COX-2 mRNA is significantly decreased on days 3, 5, and 15. *, significantly different from day 0 (controls) at P<0.05.

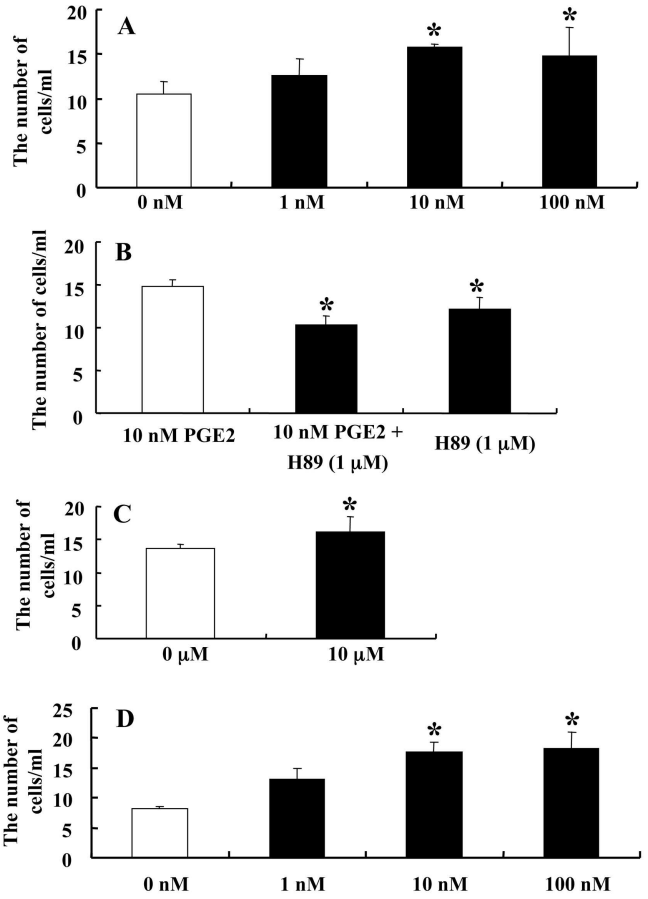

Fig. 7. Effects of PGE_2 (**A**), H89 (**B**), forskolin (**C**) and 11-deoxy-PGE_1 (**D**) on NRK-52E cells. **A.** The addition of PGE_2 (0, 1, 10 or 100 nM) to NRK-52E cells significantly increases the cell number at 10 and 100 nM. **B.** The addition of H89 (1μM), an inhibitor of protein kinase A, to NRK-52E cells in the presence and absence of PGE_2 (10 nM) significantly abolishes the effects of added PGE_2 or endogenous PGE_2, respectively. **C.** The treatment with forskolin (0 or 10 μM), which activates adenylate cyclase, results in significantly increased number of NRK-52E cells. **D.** NRK-52E cells incubated with 11-deoxy-PGE_1 (0, 1, 10 or 100 nM), an agonist of EP4 receptor, show a dose-dependent, significant increase in the cell number. *, significantly different from controls (**A, C, D**) or PGE_2 (**B**) at P<0.05.

cell population at S or G_2/M phase.

There are two potential mechanisms for the increased cell number; stimulation of cell proliferation or inhibition of cell death (Aoudjit et al., 2006). The present findings suggested that the mechanism for the increased cell number in NRK-52 cells treated with 11-deoxy-PGE_1 might be due to the inhibition of cell death.

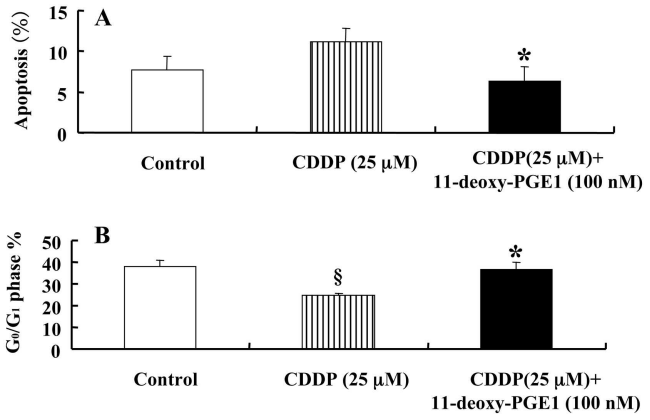

Fig. 8. Effects of 11-deoxy-PGE_1 on apoptosis **(A)** and cell cycle at G_0/G_1 phase **(B)** of NRK-52E cells treated with CDDP (25 μM) by flow cytometry analysis. **A.** The treatment of 11-deoxy-PGE_1 (0 (control) or 100 nM) significantly decreases apoptosis in CDDP (25μM) -treated NRK-52E cells. **B.** The treatment of 11-deoxy-PGE_1 (0 (control) or 100nM) significantly increases cell population at G_0/G_1 phase in CDDP (25 μM)-treated NRK-52E cells. NRK-52E cells incubated without CDDP or 11-deoxy-PGE_1 served as controls. §and *, significantly different from controls and CDDP treatment, respectively, at $P<0.05$.

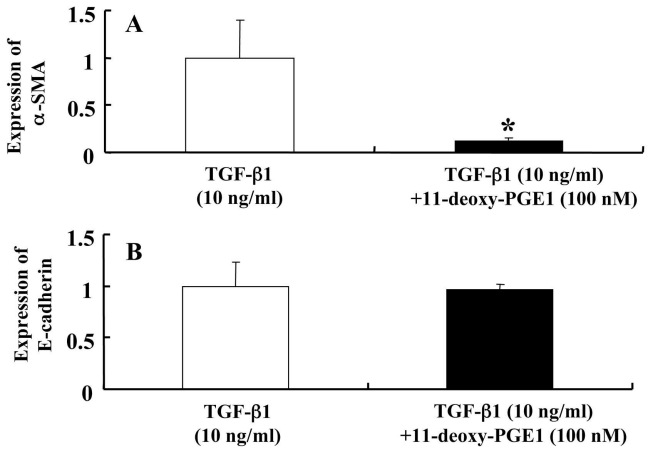

Fig. 9. Effects of 11-deoxy-PGE_1 (0 or 100 nM) on transforming growth factor-ß1 (TGF-ß1; 10 ng/ml)-treated NRK-52E cells. mRNA expressions of α-smooth muscle actin (α-SMA; a marker for myofibroblasts) **(A)** and E-cadherin (a marker for epithelial cells) **(B)** were evaluated by the RT-PCR methods. **A.** The addition of 11-deoxy-PGE_1 to TGF-ß1-treated NRK-52E cells significantly inhibits the α-SMA mRNA expression. **B.** E-cadherin mRNA expression is not changed in TGF-ß1-treated NRK-52E cells, regardless of the addition of 11-deoxy-PGE_1. *, significantly different from TGF-ß1-treated NRK-52 cells at $P<0.05$.

Effects of 11-deoxy-PGE_1 on the mRNA Expression of α-SMA and E-cadherin of NRK-52E Cells Treated with TGF-ß1

TGF-ß1-treated NRK-52E cells increased α-SMA mRNA expression and decreased E-cadherin mRNA expression (data not shown), indicating loss of epithelial phenotype and acquisition of myofibroblastic phenotype. The α-SMA mRNA expression was significantly inhibited by the addition of 11-deoxy-PGE_1 (Fig. 9A), whereas E-cadherin mRNA expression was not changed in TGF-ß1-treated NRK-52E cells in the presence of 11-deoxy-PGE_1 (Fig. 9B).

Discussion

In control kidneys, immunoexpressions for COX-1 and COX-2 were seen in the distal renal tubules (for COX-1 and COX-2) or macula densa (for COX-2), indicating constitutive expressions of these factors in the kidney (Câmpean et al., 2003). CDDP-induced rat renal lesions were characterized by epithelial necrosis/apoptosis and subsequent regeneration, as well as interstitial fibrosis in the cortico-medullary junction; the histopathological findings in the present study were in agreement with those described previously (Yamate et al., 1995, 2000). In CDDP-induced renal lesions, COX-1-immunoreaction appeared stronger in the affected renal tubules and the degree, particularly in regenerating epithelial cells, was increased with the observation period; furthermore, along with the increased immunoreaction, increased mRNA level of COX-1 was confirmed in the early stages after injection. On the other hand, COX-2 immunoexpression seen in the distal tubules and macula densa were decreased after injection, and COX-2 mRNA expression also remained decreased until day 15. It has been reported that COX-1 deficient mice showed morphological abnormality of the renal tubule, whereas COX-2-deficient mice underwent abnormalities characterized by overabundance of immature glomeruli and dysplastic tubules (Langenbach et al., 1995; Hirose et al., 1998). COX-1 and COX-2 play important roles in the genesis of renal tubules and in the development of both glomeruli and renal tubules, respectively. Renal tubular regeneration has been considered to repeat the course of renal tubulo-genesis (Lin et al., 2005). In the CDDP-induced renal failure, increased expression of COX-1 might be related to the regeneration of the proximal renal tubules after injury.

Generally, COX-2 expression is increased in correlation with inflammatory reactions after injury and in fact, increased COX-2 expression has been reported in rat renal failure models such as 5/6 renal ablation, passive Heymann nephritis and unilateral ureteral obstruction (Blume et al., 1999; Sanchez et al., 1999; Harding, 2004). The reason why the COX-2 expression

was decreased in the present CDDP-induced rat renal failure was unknown. TGF-ß1 is known to decrease COX-2 formation (Jones and Budsberg, 2000). Because TGF-ß1 has been reported to be increased in fibrotic kidneys (Yamate et al., 2004), the increased expression of TGF-ß1 might be related in part to the decreased expression of COX-2. As another possibility, the CDDP-specific pathophysiological conditions, in which PG production is decreased (Safirstein and Deray, 1998), may be involved in the down-regulated COX-2 expression in CDDP-treated rats. The present study at least showed that expression patterns of COX-1 and COX-2 in CDDP-induced rat renal failure were different from those in other rat renal failure models.

Arachidonic acid is converted to PGH_2 by either COX-1 or COX-2, followed by formation of PGE_2 via PGES (Kotnik et al., 2005; Kudo and Murakami, 2005). In CDDP-induced rat renal failure, immunoexpressions for COX-1 and mPGES-2 became much greater in the affected renal tubules, whereas COX-2 immunoreaction was not detected in the affected cortico-medullary junction. These findings show that PGE_2 produced via COX-1 rather than COX-2 plays important roles in the development of CDDP-induced renal pathological changes.

Epithelial regeneration in the proximal renal tubules of the affected cortico-medullary junction and subsequent development of myofibroblasts are responsible for the progression of CDDP-induced renal failure; the myofibroblasts may be formed by the EMT (Iwano et al., 2002; Yamate et al., 2005). Epithelial cells in the affected renal tubules showed a strong reaction to EP4, but not to EP2. The dual immunohistochemistry revealed that some affected epithelial cells positive to EP4 receptor also reacted to cyclin D1 or 5'-bromo-2'-deoxyuridine (BrdU) (data not shown), indicating proliferating activity of the affected renal cells. Taken together, EP4 receptor expression might be related to regeneration of tubular epithelial cells after injury in CDDP-induced rat renal failure.

Dedifferentiation and proliferation are important aspects in the regeneration of renal tubular epithelial cells (Maeshima et al., 2003; Zeisberg and Kalluri, 2004; Lin et al., 2005). The activity of PGE_2 is carried out partly via EP4 receptor and the receptor is associated with stimulation of AC (Breyer and Breyer, 2000). The present *in vitro* studies indicated that the addition of PGE_2 to NRK-52E cells increased the cell number and that the increased number occurred through the cAMP-PKA pathway. The treatment of NRK-52E cells with 11-deoxy-PGE_1 showed the same effects as PGE_2, indicating that PGE_2 is capable of increasing the cell number of NRK-52E cells via EP4 receptor. These *in vitro* results are likely to reflect the epithelial aspects (epithelial regeneration after injury as mentioned above) seen in the affected renal tubules in CDDP-induced rat renal failure.

Flow cytometry analysis using the PI staining revealed that the addition of 11-deoxy-PGE_1 to CDDP-treated NRK-52E cells inhibited apoptosis and increased the G_0/G_1 cell population, indicating G_0/G_1 arrest. It has been considered that G_0/G_1 arrest is important to protect cells from effects of chemotherapeutic agents (Chen et al., 2000). The affected epithelial cells in CDDP-induced rat renal failure might have been arrested at the G_0/G_1 phase, which aspect might be regulated by activated PGE_2 in association with increased expressions of COX-1, mPGES-1 and EP4. Furthermore, because it has been reported that apoptosis plays an important role in the development of CDDP-induced renal failure (Zhou et al., 1999; Yamate et al., 2000; Sheikh-Hamad et al., 2004), PGE_2 might ameliorate CDDP-induced renal failure through inhibition of apoptosis.

Cells arrested at the G_0/G_1 phase are not only associated with cell protection, but also with differentiation (Massagué, 2004). The addition of 11-deoxy-PGE_1 to TGF-ß1-incubated NRK-52E cells caused the decrease in mRNA expression of α-SMA, a representative marker of myofibroblasts which might be developed by EMT (Rhyu et al., 2005). On the other hand, the mRNA expression of E-cadherin, an epithelial marker of EMT (Yamashita et al., 2005), did not alter in PGE_2-treated NRK-52E cells pre-incubated with TGF-ß1. These findings suggest that PGE_2 inhibits conversion of renal epithelial cells into myofibroblastic cells. PGE_2 produced in renal tubular epithelial lesions may be an inhibitor of EMT in which renal epithelial cells acquire a myofibroblastic nature leading to interstitial fibrosis (Zeisberg and Kalluri, 2004).

In conclusion, the present study has shown that COX-1 plays more important roles than does COX-2 in the tubular epithelial alterations in CDDP-induced rat renal failure; furthermore, PGE_2, activated by increased COX-1 and mPGES-1 expressions, effects epithelial regeneration via the up-regulated EP4 receptor in the affected renal tubules; additionally, the regulation of PGE_2 on epithelial regeneration involves the suppression of apoptosis and EMT. Collectively, it was demonstrated that endogenous PGE_2 participated in the complicated pathological processes of regeneration of tubular epithelial cells in CDDP-induced rat renal failure. Microenvironmental conditions (such as inflammatory cytokines and adhesion molecules) evoked via cell-cell or cell-matrix interaction is crucial for repair after tissue injury (Bonventre, 2003). It is interesting to pursue the relationship of these factors with PGE_2 production for the sake of explorations of therapeutic strategies for chronic renal insufficiency, because incomplete epithelial regeneration of renal tubules leads to EMT resulting in progressive interstitial fibrosis.

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