

Short Communication

Relationship of Cell Proliferating Marker Expressions with PGE₂ Receptors in Regenerating Rat Renal Tubules after Cisplatin Injection

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Abstract: Cisplatin, an anticancer drug, is well known to have nephrotoxicity as an adverse effect. We investigated the expressions of cell cycle markers and prostaglandin E₂ (PGE₂) receptors (EP) in the affected renal tubules in rats injected with a single dose (6 mg/kg body weight) of cisplatin. On days 1–3 after dosing, the affected renal epithelial cells were almost desquamated, showing necrosis. On day 5 onwards, the renal tubules were rimmed by flattened or cuboidal epithelial cells with basophilic cytoplasm; BrdU-immunopositive cells began to significantly increase, indicating regeneration. Simultaneously, TUNEL-positive apoptotic cells were also seen. On days 1–5, cyclin D1-immunopositive cells were decreased with an increased expression in p21 mRNA, indicating G₁ arrest in the cell cycle. The affected renal epithelial cells began to react to EP4 receptor, but not to EP2 receptor. Some EP4 receptor-reacting epithelial cells gave a positive reaction to BrdU or cyclin D1. Collectively, the affected renal tubules underwent various alterations such as necrosis, apoptosis, regeneration and G₁ arrest; the aspects might be influenced by endogenous PGE₂ through EP4 receptor. (*J Toxicol Pathol* 2010; 23: 271–275)

Key words: cisplatin-induced renal failure, regeneration, cell cycle, PGE₂ receptor

Cis-diamminedichloroplatinum (CDDP; cisplatin) has been widely used as an anticancer drug; it is well known to have nephrotoxicity¹. The primary injury resulting from CDDP occurs in the proximal renal tubules in the corticomedullary junction. Previously, we have shown that rats treated with a high dose of CDDP are useful for studies on the pathogenesis of renal tubular alterations after injury². The affected epithelial cells have the capacity to regenerate; however, incomplete regeneration occurs when the basal lamina is completely damaged. The pathological alterations of the affected renal tubules in CDDP-treated rats remain to be investigated.

Recently, it has been shown that endogenous prostaglandin (PG) plays an important role in the pathophysiology of the kidneys³. PGE₂, the main type of PG produced in the kidney, has been reported to be associated with renal hemodynamics and water and salt excretion^{3,4}. PGE₂ also influences cell proliferation and differentiation mainly through PG receptor (EP) 2 or EP4^{5–7}. In the present study, we investigated the epithelial alterations in CDDP-treated rat kidneys

using cell cycle markers and pursued the relationship between the expressions of cell cycle markers and EP receptors.

The present animal experiments were conducted according to the authors' institutional guidelines for animal care. Thirty-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. Of them, 28 rats were injected with CDDP (Nippon Kayaku Co., Ltd., Tokyo, Japan) at a single dose of 6 mg/kg body weight intraperitoneally, and four animals were sacrificed each on days 1, 3, 5, 7, 9, 12 and 15 after CDDP dosing. The remaining four rats were injected with saline (control) and sacrificed on day 0. All animals received intraperitoneal injection of 5'-bromo-2'-deoxyuridine (BrdU, 50 mg/kg body weight) one hour before sacrifice.

The renal tissues were fixed in 10% neutral buffered formalin or periodate-lysine-paraformaldehyde (PLP) fixatives. Formalin-fixed specimens were processed routinely for morphological evaluation. PLP solution-fixed specimens were embedded in paraffin by the AMeX method (PLP-AMeX method)⁸. The specimens made by the PLP-AMeX method were stained immunohistochemically with monoclonal antibodies to cyclin D1 (1:200; Upstate Biotechnology Inc., Lake Placid, NY, USA) and BrdU (1:100;

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DAKO Denmark A/S, Glostrup, Denmark), as well as polyclonal antibodies to EP2 receptor (1:500; Cayman Chemical Co., Ann Arbor, MI, USA) and EP4 receptor (1:500; Upstate Biotechnology Inc., Woburn, MA, USA). Tissue sections were incubated with the primary antibody overnight (12–14 hr) at 4°C. Thereafter, sections were incubated for 45 min with the secondary antibody (Histofine Simple Stain MAX PO; Nichirei Corporation, Tokyo, Japan). Positive reactions were visualized with 3, 3'-diaminobenzidine (DAB). Sections were lightly counterstained with hematoxylin.

Double immunohistochemical stainings were performed using antibodies for EP4 receptor and cyclin D1. At the first reaction, sections were incubated with the anti-EP4 receptor primary antibody. After visualization with DAB (brown in color), sections were washed with water and then incubated with the anti-cyclin D1 primary antibody overnight at 4°C at the second reaction. The sections were then incubated with EnVision-alkaline phosphatase (DAKO). The positive reactions at the second reaction were visualized as red with a Fuchsin substrate (DAKO). To identify renal epithelial cells coexpressing EP4 receptor and BrdU (S phase in the cell cycle), immunohistochemical stainings with antibodies for EP4 receptor and BrdU were performed using serially cut sections.

For detection of DNA fragmentation for apoptotic cells, the standard *in-situ* TdT-mediated dUTP-biotin Nick End Labeling (TUNEL: ApopTag[®] Peroxidase In Situ Apoptosis Detection Kit; Chemicon International Inc, Temecula, CA, USA) method was used according to the manufacturer's instructions.

The number of cells reacting to cyclin D1, BrdU or TUNEL was counted in five randomly selected areas at a magnification of $\times 400$ in the cortico-medullary junction. The immunoreactivities for the EP2 and EP4 receptors were assessed semiquantitatively as follows: –, no change; \pm , faintly positive staining; 1+, moderately positive staining; 2+, more clearly evident staining; and 3+, markedly positive staining.

Total RNA was extracted from renal tissues with Trizol Reagent[™] (Invitrogen Corp., Carlsbad, CA, USA) and a SV Total RNA Isolation System (Promega, Osaka, Japan). The RNA was reverse transcribed to cDNA using a SuperScript First-Strand Synthesis System[™] (Invitrogen). All PCR experiments were performed with SYBR Green Real-time PCR Master Mix (Toyobo Co., Ltd., Life Science Department, Osaka, Japan)⁹. The amplification program consisted of 1 cycle at 95°C with a 1-min hold followed by 45 cycles at 95°C with a 15-sec hold, annealing at 60°C for p21 (one of the cyclin-dependent kinase inhibitors) and at 62°C for cyclin D1 with a 15-sec hold and 72°C with a 30-sec hold. Melting curve analysis to verify the accuracy of the PCR products followed amplification. The PCR primer sequences for cyclin D1 were 5'-TGGAGCCCTGAA-GAAGAG-3' (forward) and 5'-AAGTGCCTTGTGCGG-TAGC-3' (reverse). The sequences for p21 were 5'-CAAAGTATGCCGTCGTCTGTT-3' (forward) and 5'-GCTGGTCTGCCTCCGTTTTC-3' (reverse). The relative

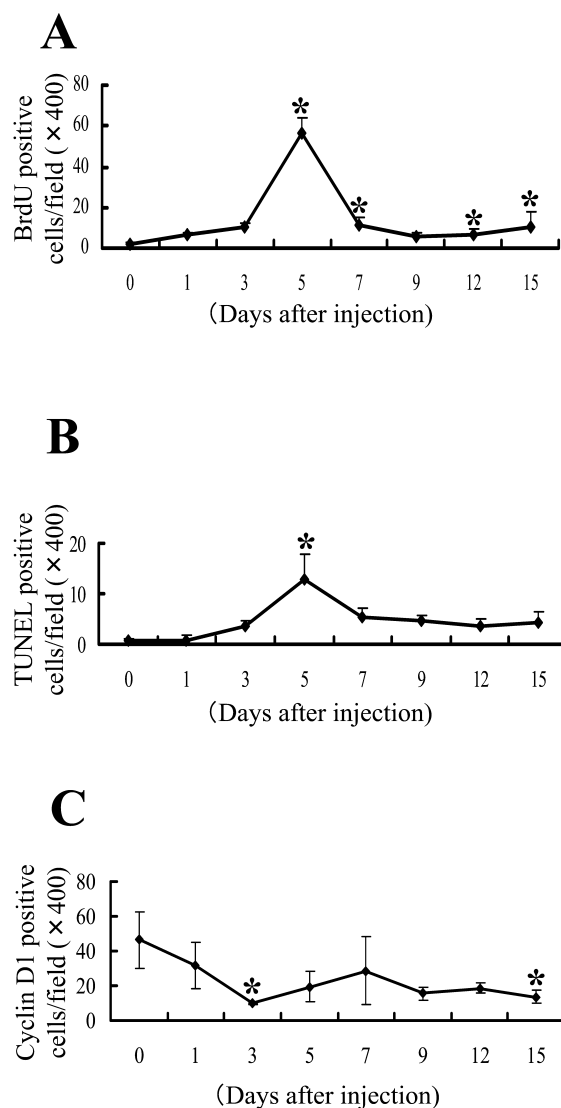


Fig. 1. The kinetics of positive cells to 5'-bromo-2'-deoxyuridine (BrdU) (A), the standard *in-situ* TdT-mediated dUTP-biotin Nick End Labeling (TUNEL) method (B) and cyclin D1 (C). The symbol and whisker represent the mean and standard deviation, respectively. A: The number of BrdU-positive cells gradually increases from day 1, peaking on day 5; on days 5, 7, 12 and 15, the number shows a significant increase. B: The number of apoptotic cells, demonstrable by the TUNEL method, exhibits a significant transient increase on day 5. C: The cyclin D1-positive cell number begins to decrease from day 1 and remains decreased until day 15; on days 3 and 15, the number shows a significant decrease. * $P < 0.05$ compared with the controls (day 0).

expression values were normalized to the expression value of β -actin¹⁰. Values were calculated by the comparative Ct method. Data are presented as means \pm standard deviation (SD). Values of $P < 0.05$ were considered significant. Differences between the control and CDDP-injected groups were evaluated by analysis of variance (ANOVA).

Histopathologically, renal tubules in the cortico-medullary junction, mainly in the P₃ segment of the proximal

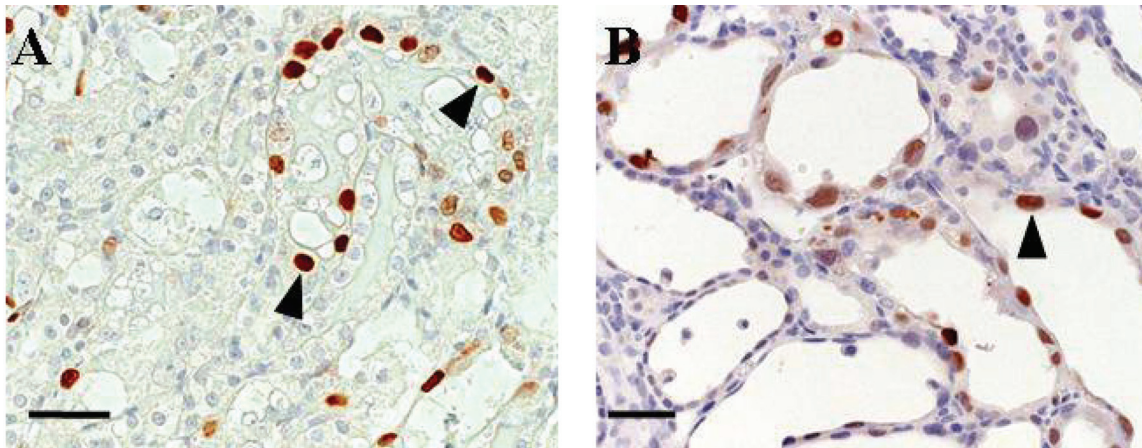


Fig. 2. BrdU and cyclin D1-immunopositive cells in CDDP-treated rat kidneys. A: BrdU stain on day 5; arrowheads indicate BrdU-positive cells in cuboidal epithelial cells of the affected renal tubules. B: Cyclin D1 stain on day 9; arrowhead indicates the positive reaction in the dilated tubules. Immunohistochemical staining counterstained with hematoxylin. Bar=50 μ m.

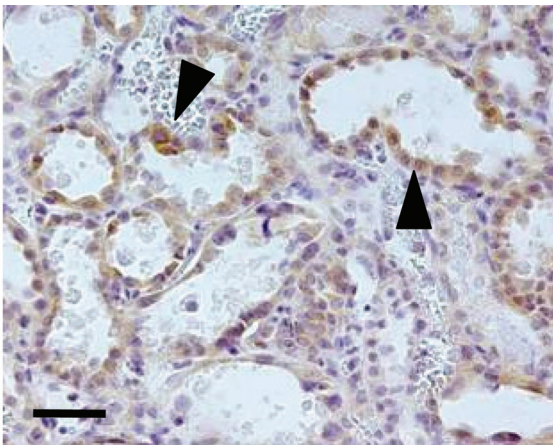


Fig. 4. Immunohistochemistry for EP4 receptor in the kidneys of CDDP-injected rats. On day 7, the epithelial cells in the affected renal tubules react strongly to EP4 receptor (arrowheads) in the cortico-medullary junction. Immunohistochemical staining counterstained with hematoxylin. Bar=50 μ m.

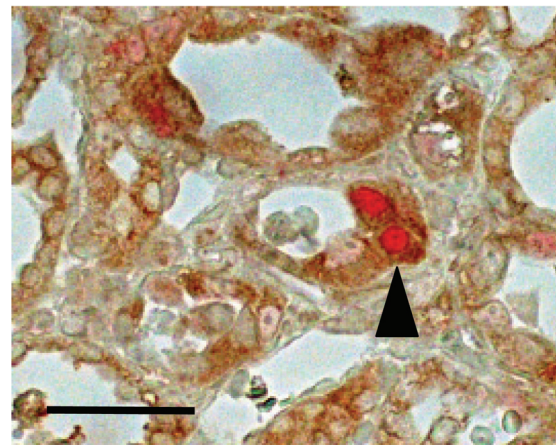


Fig. 5. Double immunostaining for cyclin D1 and EP4 receptor from renal samples on day 9. Cyclin D1 is visualized with fuchsin (red) and EP4 receptor is stained with DAB (brown). Some cyclin D1 positive cells are also positive for EP4 receptor antibody (arrowhead). Immunohistochemical staining counterstained with hematoxylin. Bar=50 μ m.

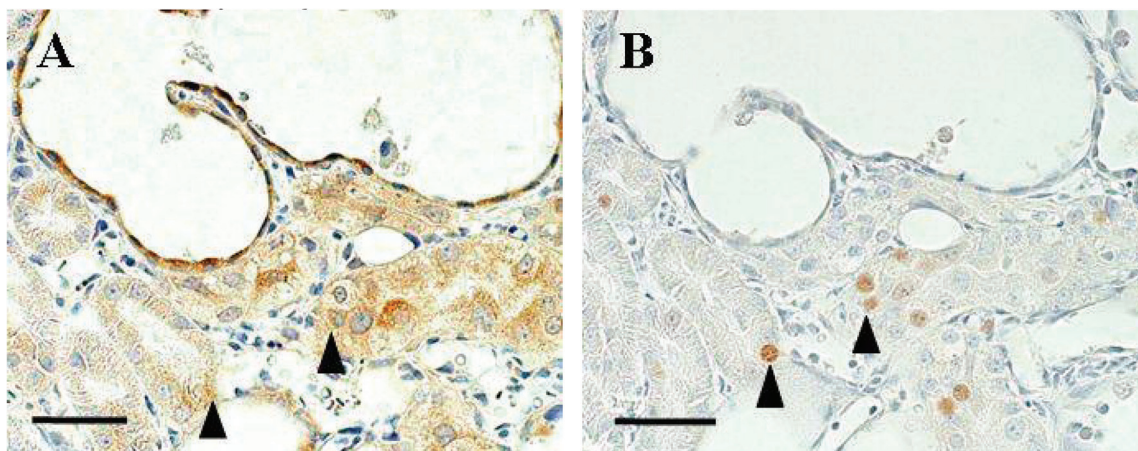


Fig. 6. Serial sections of immunostainings for EP4 receptor (A) and BrdU (B) from renal samples on day 9. A: Immunoreaction for EP4 receptor in flattened or cuboidal epithelial cells of the affected tubules. B: Immunoreaction for BrdU in cuboidal epithelial cells of the regenerating renal tubules. Arrowheads indicate representative cells positive for both EP4 receptor and BrdU. Immunohistochemical staining counterstained with hematoxylin. Bar=50 μ m.

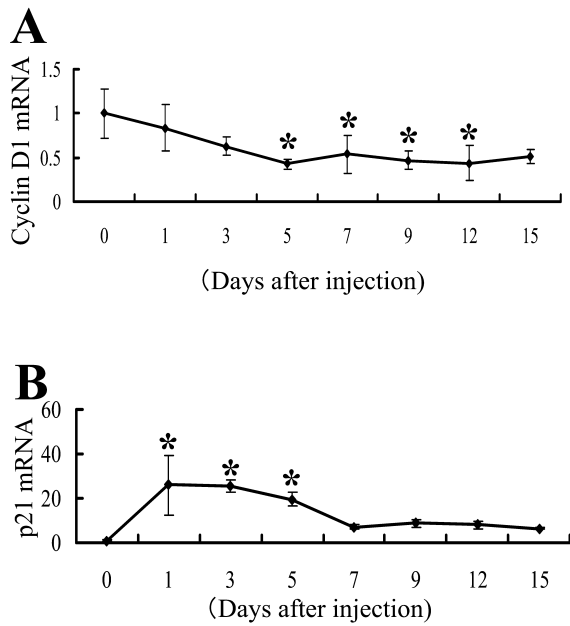


Fig. 3. mRNA expressions of cyclin D1 (A) and p21 (B) by the real-time RT-PCR method. The symbol and whisker represent the mean and standard deviation, respectively. A: The expression of cyclin D1 mRNA significantly decreases on days 5 to 12. B: The expression of p21 mRNA significantly increases on days 1 to 5. * $P < 0.05$ compared with the controls (day 0).

tubules, were injured by CDDP. On days 1 and 3, desquamation of damaged epithelial cells into the tubular lumina was seen. In addition to the necrotic changes, on days 5 and 7, the affected renal tubules were variously dilated and contained desquamated epithelial cells. On days 7, 9, 12 and 15, the affected renal tubules were rimmed by flattened, polygonal or cuboidal epithelial cells that appeared to be regenerating epithelial cells; interstitial fibrosis developed around the affected tubules. These findings agreed with those in a previous report².

Immunohistochemically, the number of cells reacting to BrdU increased significantly on days 5–15, peaking on day 5 (Figs. 1A, 2A). BrdU immunopositive cells were observed mainly in cuboidal cells of the affected renal tubules. Simultaneously, the TUNEL-positive apoptotic cell number exhibited a significant transient increase on day 5 (Fig. 1B). TUNEL-positive cells were observed in epithelial cells of the dilated tubules containing desquamated epithelial cells. On the other hand, the cyclin D1-positive cell number tended to be decreased in the CDDP-treated kidneys on days 1–15 (Fig. 1C); although the number of positive cells was decreased, stronger expression compared with the controls was observed after CDDP injection, especially from day 7 onwards; the expression was observed exclusively in flattened or cuboidal epithelial cells of the dilated renal tubules, which appeared to be regenerating cells after desquamation (Fig. 2B). A similar tendency to decrease was confirmed by the real-time RT-PCR (Fig. 3A). The p21 mRNA analysis

showed a significant increase on days 1–5 after CDDP injection (Fig. 3B).

No positive reaction to EP2 receptor was seen in the control kidneys (–). Although epithelial cells of some renal tubules in the medulla and papilla showed a positive reaction to EP2 receptor (\pm or +), no reaction to EP2 receptor was observed in the affected tubules of the CDDP-injected rats (–). In the control kidneys, epithelial cells of the proximal renal tubules did not show any positive reaction to EP4 receptor (–); interestingly, in the CDDP-injected rats, a positive reaction to EP4 receptor began to be seen in flattened or cuboidal epithelial cells of the affected renal tubules on day 7, and positive reactions were observed on subsequent days (2+ or 3+; Fig. 4).

The double immunohistochemical staining revealed that there were EP4 receptor-positive cells expressing cyclin D1 (Fig. 5). On serial sections, some renal epithelial cells coexpressing BrdU and EP4 receptor were identified (Fig. 6, arrowheads).

Cyclin D1 is a regulator acting in the G₁ phase¹². The loss of cyclin D1 expression and the increase of p21 expression have been considered to be a feature of cell cycle arrest^{12,13}. Since CDDP causes DNA damage^{12,14}, which has been considered one of the causes of cell cycle arrest, the changes of p21 and cyclin D1 expressions might be due to the toxic effect of CDDP. On the other hand, there were cells reacting strongly to cyclin D1 in the affected renal tubules, particularly from day 7 onwards; these cells were characterized morphologically by flattened or cuboidal epithelial cells with basophilic cytoplasm, indicating regeneration. Since cyclin D1 plays a role in exit from the quiescence period¹⁵, the cells observed to be expressing cyclin D1 strongly on day 7 onwards might reentry the cell cycle, an important step for regeneration¹⁶. More interestingly, TUNEL-positive apoptotic cells were also significantly increased on day 5. It has been reported that G₁ arrest is a stage in which the fate of affected cells, proliferation or apoptosis can be determined¹⁴. The simultaneous presence of BrdU- and TUNEL-positive cells on day 5 might imply that there was a critical point between regeneration and apoptosis in the affected renal tubules; the mechanisms remain to be investigated in the future. Collectively, it was considered that the affected renal epithelial cells underwent various pathological alterations such as necrosis, apoptosis, active regeneration and G₁ arrest.

Endogenous PGE₂ plays important roles in cellular proliferation and differentiation in injured renal tubules^{7,17–19}. These functions act through elevation of cAMP⁷. In the present study, no immunoreaction to EP2 receptor was seen in the affected renal tubules in the CDDP-injected rats. On the other hand, EP4 receptor immunoreaction began to be seen in the affected renal tubules. Because some cells reacting to EP4 receptor showed a positive reaction to BrdU or cyclin D1, EP4 receptor expression might be involved in cellular alterations of the affected renal tubules.

In conclusion, the present study showed that CDDP-injured renal epithelial cells underwent various pathological

alterations. Endogenous PGE₂ might be attributable to such aspects through upregulated EP4 receptor expression. Because of these complicated renal tubular changes, the regeneration of epithelial cells might be incomplete, presumably leading to progressive interstitial fibrosis in CDDP-injured kidneys². It would be interesting to pursue the relationship between renal alteration and interstitial fibrosis.

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