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貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料： **研 究 報 告 書**


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2．研 究 テ ー マ

軽度の腎障害から回復したラットにおける酢酸ウラニウム再投与時の抵抗性獲得

3．成 果 の 概 要（100字程度）

軽度障害から回復したラットでは2度目の腎毒性用量酢酸ウラニウムにより尿細管細胞障害のピークが早期かつ軽度となった。又、HGF/c-Met経路の発現亢進を介する尿細管細胞の増殖促進が、抵抗性獲得と回復促進に因子している可能性がある。

4．研 究 業 績

(1) 学会における発表 無 ・ ㊒（学会名・演題）

Acceleration of tubular cell proliferation as a possible mechanism of acquired resistance in uranyl acetate-induced acute renal failure in rats.
Yuan Sun, Yoshihide Fujigaki, Masanori Sakakima, Takayuki Tsuji, Akira Hishida
第５１回日本腎臓学会学術総会

(2) 発表した論文 ㊀ ・ 有（雑誌名・題名）

－日中医学協会助成事業－

# 軽度の腎障害から回復したラットにおける酢酸ウラニウム再投与時の抵抗性獲得

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## Abstract

**Background:** Animals recovered from acute renal failure (ARF) induced by nephrotoxic substances are resistant to subsequent insult by the same substance (acquired resistance). However, animals recovered from mild proximal tubular (PT) injury without renal dysfunction acquire similar resistance remains unknown.

**Methods:** Renal function and fate of PT cells 2 weeks after recovery from 0.2 mg/kg of uranyl acetate (UA) insult (low dose to induce mild PT injury) was examined in response to 4 mg/kg of UA insult (nephrotoxic dose to induce severe PT injury). To examine the contribution of accelerated PT cell proliferation on recovery from ARF, cell cycle was inhibited by colchicine and factors affecting cell survival or proliferation were investigated.

**Results:** Rats recovered from mild PT injury gained partial resistance with reduced renal dysfunction in response to the second UA. This resistance was associated with accelerated proliferation (BrdU-positivity) and upregulated hepatocyte growth factor (HGF)/c-Met axis compared with vehicle treatment in the first insult. Colchicine inhibited PT cell proliferation, reduced the upregulated cyclin D1 and phospho-Rb, totally abolishing accelerated recovery. Unexpectedly, rats recovered from mild PT injury showed earlier peak of PT damage (necrosis and apoptosis) but less severity of peak damage in the proximal three-quarter of S3.

**Conclusions:** Rats that recovered from mild PT injury showed partial acquired resistance to nephrotoxic dose of UA with accelerated PT cell injury and proliferation. Cell cycle progression of PT cells with upregulated HGF/c-Met axis may contribute to the accelerated recovery from ARF.

**Key Words** acquired resistance, acute renal failure, hepatocyte growth factor, proliferation, uranyl acetate

## Introduction

Animals recovered from acute renal failure (ARF) are resistant to subsequent insults with nephrotoxins, including uranyl acetate (UA), and this phenomenon is termed acquired resistance to ARF [1]. A series of studies on UA-induced ARF in our laboratory suggested that renal tubular cells that recover from ARF may become resistant to a rechallenge injury with UA [2-4]. However, whether animals recovered from mild proximal tubular (PT) injury without renal dysfunction acquire similar resistance remains unknown.

We previously reported that rats with ARF induced by 2 or 5 mg/kg of UA (nephrotoxic dose of UA) exhibited significant increase in Scr and almost complete depletion of tubules in the proximal three-quarter of the S3 segment of the nephron. On the other hand, 0.25 or 0.5 mg/kg of UA (low dose of UA) induced focal PT cells depletion mainly in the proximal three-quarter of S3 segment without significant increase in Scr [5]. It is clear that kidneys recovered from apparent ARF induced by nephrotoxic dose of UA can gain resistance to subsequent insult [4]. However, it remains unknown whether kidneys recovered from mild PT injury by low dose of UA can also gain similar resistance to nephrotoxic dose of UA.

## Materials and Methods

*Experiment 1*

Male Sprague-Dawley rats were divided into four groups: Group 1 received i.v. injection of saline, followed by another saline injection 14 days after the first insult. Groups 2, 3 or 4 received i.v. injection of saline, 0.2 mg/kg of UA (low dose of UA) or 4 mg/kg of UA (nephrotoxic dose of UA), respectively, followed by a second insult of 4 mg/kg of UA 14 days after the first insult. At 0, 3, 5, and 7 days after the second insult, three rats in each group were served for histological examination of PT cell injury and proliferation and for measurement of Scr level.

*Experiment 2*

Rats were divided into five groups: Group I received i.v. injection of 4 mg/kg of UA at 14 days after saline injection as vehicle. Groups II and III received 4 mg/kg of UA at 14 days after 0.2 mg/kg of UA injection, followed by intraperitoneal injection of saline or 1 mg/kg of colchicine at 6 hours before 3 days after the second insult, respectively. Groups IV and V received saline at 14 days after saline or 0.2 mg/kg of UA, respectively, followed by intraperitoneal injection of 1 mg/kg of colchicine at 6 hours before 3 days after the second insult. For determination of cells at the S-phase, all rats were injected intraperitoneally with 40 mg/kg of bromodeoxyuridine (BrdU) one hour before sacrifice. Three to five rats in each group were sacrificed just before and at 1, 2, 3, 5 and 7 days after the second insult.

*Histological examination*

For evaluation of tubular damage, sections were stained with periodic acid Schiff (PAS) reagent. Standardized avidin-biotin-complex technique was applied as described previously [5] to detect antigens by using the antibodies of Ki67 and BrdU. Apoptosis was assessed using the terminal uridine nick-end labeling (TUNEL) technique as described previously [6]. The number of necrotic tubules, Ki67-positive, BrdU-positive and TUNEL-positive PT cells were counted in 20 randomly selected fields of OSOM at × 400 magnification.

*HGF measurement*

The HGF concentration in OSOM of the kidneys was determined using an enzyme immunoassay kit specific for rat HGF based on the protocol provided by the manufacturer.

*Western blot*

OSOM of the kidneys were dissolved at 4°C in lysis buffer in the presence of protease inhibitor cocktail. Equal amounts of proteins were loaded for SDS-PAGE gel electrophoresis as described previously [6]. Blots were probed with the primary antibodies of Cyclin-D1 and phosphorylated retinoblastoma at 4°C overnight. β-actin was used as an internal control.

*Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR)*

Total RNA was extracted from the OSOM of the kidneys using ISOGEN. The mRNA samples were reversibly transcribed into cDNA using a First Strand cDNA Synthesis Kit for RT-PCR. Real time RT-PCR was performed in a Light Cycler. Data were analyzed using Light Cycler software, and normalized by GAPDH mRNA expression.

*Statistical analyses*

All values are expressed as mean ± SD. Differences between groups were examined for statistical significance using analysis of variance followed by least significant difference. A $P$ value less than 0.05 denoted the presence of a statistically significant difference.

## Results

*Experiment 1-Renal injury by rechallenge insult with nephrotoxic UA*

Following the second insult, Scr and the number of necrotic tubules at day 5 were significantly lower in group 3 than those in group 2. Unexpectedly, Scr was significantly higher at day 3 in group 3 than in group 2 (Fig. 1). The number of Ki67-positive PT cells in group 3 were significantly higher than in group 2 at days 3 and 5 (Fig. 1B-c).

*Experiment 2*

*1) Early changes in rechallenge injury with UA*

In group II, Scr level was significantly higher at days 1, 2 and 3 than in group I (Fig. 1A). Significantly larger numbers of necrotic tubules and TUNEL-positive PT cells were noted at day 2 in group II than in group I (Fig. 1B and 1C).

*2) Proliferation and cell cycle progression of PT cells in response to rechallenge injury with UA*

The number of BrdU-positive PT cells in S3 were significantly greater at days 3 and 5 in group II than in group I (Fig. 2D). Western blot analysis showed significantly higher levels of cyclin D1 protein (early G1 phase marker) in OSOM at day 5 in group II than in group I, and of phospho-Rb protein (G1 to S phase marker) at days 3 and 5 in group II than in group I (Fig. 3).

*3) Colchicine inhibits cell cycle progression*

Colchicine treatment in group III inhibited the number of BrdU-positive PT cells and protein levels of cyclin D1 and phospho-Rb at day 5 compared with group II (Fig. 2D and 3). Colchicine treatment in group III induced increases in Scr level, number of proximal tubules and TUNEL-positive PT cells at days 5 and 7 than in group II (Fig. 2A-C).

*4) HGF/c-Met axis and other growth factors*

HGF protein level in OSOM was significantly higher at day 0 in groups II and III than in group I (Fig. 4A). HGF mRNA and c-Met mRNA in OSOM were significantly higher at day 2 in groups II and III, compared with group I (Fig. 4B and 4C). The expression levels of IGF-1, EGF and VEGF mRNAs in OSOM were not significantly higher in groups II and III than in group I at anytime (Fig. 4D-G).

## Discussion

In this study, we demonstrated that rats recovered from mild PT injury induced by low dose of UA showed less functional injury and acquired partial resistance to rechallenge injury with nephrotoxic dose of UA, with earlier peak of PT damage but less severity of peak damage and accelerated PT proliferation compared with animals treated with vehicle alone followed by nephrotoxic dose of UA.

The number of BrdU-positive cells was higher at appearance and peaked earlier in group II than in group I, indicating accelerated proliferation of PT cells in group II. In the process of cell proliferation, the cell is required to pass a number of checkpoints. Cyclin D1 acts as a growth factor sensor in G1 phase [7] and cyclin D1-CKD4 complex acts as the Rb phosphorylator [8]. Phosphorylation of Rb disrupts complexes with E2Fs, allowing for cell cycle progression into S phase [8]. In the present study, the expression levels of cyclin D1 and phospho-Rb at days 5 and 3, respectively, were higher in group II than in group I, probably contributing to the accelerated PT cell proliferation in rats pretreated with low dose of UA.

To examine whether accelerated proliferation of PT cells observed in rats pretreated with low dose of UA contributes to accelerated recovery from ARF, we inhibited cell cycle progression by colchicine [9]. Korrapati et al. reported successful inhibition of PT cell proliferation by colchicine in mouse model of ARF and concluded that stimulated cell division and renal tissue repair by the priming dose of DCVC [9], were critical mechanisms that allow sustained compensatory tissue repair and survival of mice. In the present study, kidneys of group III treated with colchicine tended to show reduced phospho-Rb at day 3 and significant reduction at day 5 after the second insult, resulting in not only partial inhibition of PT cell proliferation but also total abolishment of accelerated cellular and functional recovery from ARF. Our findings suggest that accelerated PT cell proliferation in rats pretreated with low dose of UA is essential for accelerated recovery from ARF.

Among possible cytoprotective growth factors for PT cells [10], only HGF mRNA in OSOM of the kidneys was significantly upregulated after the second insult in the present study. In the kidney, HGF plays an important role in tubular regeneration and in the antiapoptotic response of tubular epithelial cells through its receptor c-Met [11]. In the present study, HGF mRNA and c-Met mRNA in OSOM was significantly higher day 2 in group II but not in group I, suggesting that HGF could act earlier on PT cells with upregulated c-Met in group II than in group I. Upregulation of renal HGF/c-Met axis may

contribute to inhibition of PT apoptosis and to acceleration of PT proliferation. Since protein levels and their receptors were not examined for other possible cytoprotective and/or renotropic growth factors, such as IGF-1, EGF, VEGF and BMP-7, contribution of these molecules to accelerated recovery from ARF in group II remains to be clarified.

In the clinical setting, many patients are treated with various drugs at the same time and in series. The results of the present study showed that kidneys with subclinical damage may be vulnerable to the next nephrotoxic agent and may develop renal damage much earlier than we expect. In addition, the current study demonstrated that previous subclincal damage attenuates the peak level of renal damage in rechallenge injury. Therefore, one may underestimate the toxic dose of drugs and administer the same or larger dose of drugs in occasions when the patients were not resistant to that drug. The results of this study emphasize the need for a closer assessment of renal function when we use nephrotoxic drugs in series.

In summary, kidneys recovered from mild PT injury gained acquired resistance in response to nephrotoxic dose of UA. Furthermore, PT cells mainly in the distal area of S3 showed accelerated proliferation with dedifferentiated phenotype as increased regenerative response to rechallenge injury by UA under upregulated renal HGF/c-Met axis, contributing to accelerated recovery from ARF.

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## Figure Legends

**Figure 1.** Renal injury and proliferation of proximal tubular cells before and after the second insult in Experiment 1. Serial changes in serum creatinine level (A). Morphometric analysis of the number of necrotic proximal tubules (B) and number of Ki67-positive proximal tubular cells (C) in the outer stripe of the outer medulla OSOM. V, vehicle; 4 mg, 4 mg/kg of uranyl

acetate; 0.2 mg, 0.2 mg/kg of uranyl acetate. #$P$ <0.05, ##$P$ <0.01 *v.s.* day 0; *$P$ <0.05, * $P$ <0.01.

**Figure 2.** Serial changes in serum creatinine level (A), morphometric analysis of the number of necrotic proximal tubules (B), number of TUNEL-positive proximal tubular cells (C) and number of BrdU-positive proximal tubular cells (D) in OSOM in Experiment 2. Abbreviations are the same as figure 1. CLC, colchicine. #$P$ <0.05, ##$P$ <0.01 *v.s.* day 0; *$P$ <0.05, **$P$ <0.01.

**Figure 3.** Western blot of protein from OSOM of kidneys of groups I, II and III with antibodies for cyclin D1, phospho-Rb and β-actin (A) and relative abundance of cyclin D1 (B) and phospho-Rb (C) to β-actin. Western blot and densitometric analyses were carried out in triplicate. Abbreviations are the same as figure 2. #$P$ <0.05, ##$P$ <0.01 *v.s.* day 0; *$P$ <0.05, **$P$ <0.01.

**Figure 4.** Serial changes of HGF protein content in OSOM of the kidneys (A) before and after the second insult in groups I, II and III. Real-time RT-PCR analysis of HGF (B), c-Met (C), IGF-1 (D), EGF (E), VEGF (F) and BMP-7 (G) mRNA expressions in OSOM before and after the second insult in groups I, II and III. Abbreviations are the same as figure 2. #$P$ <0.05, ##$P$ <0.01 *v.s.* day 0; *$P$ <0.05, **$P$ <0.01.

Figure 1

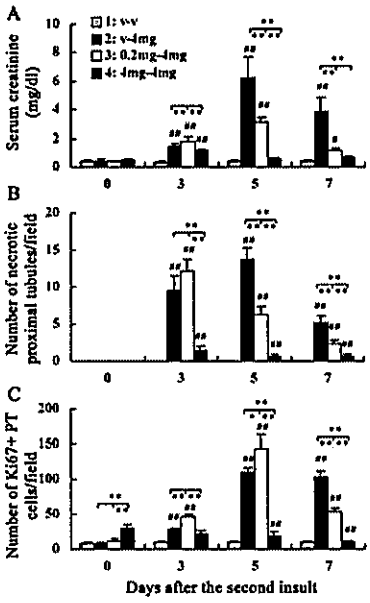



Figure 2

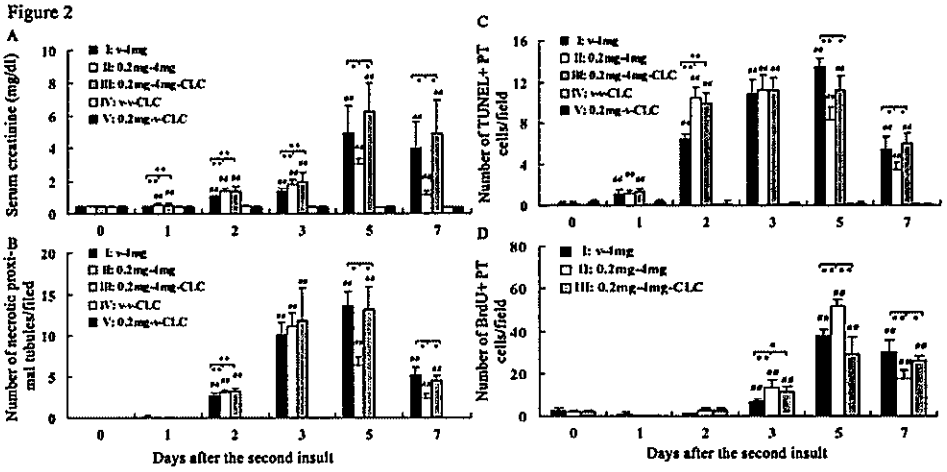



Figure 3

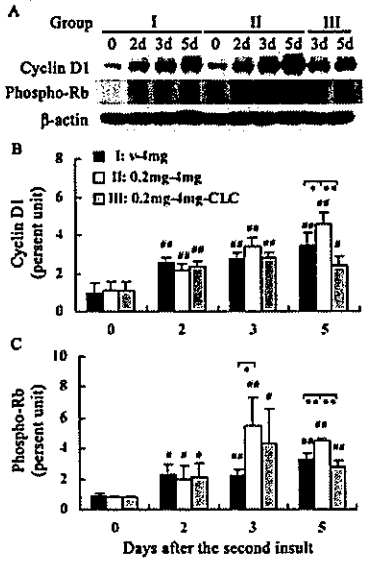
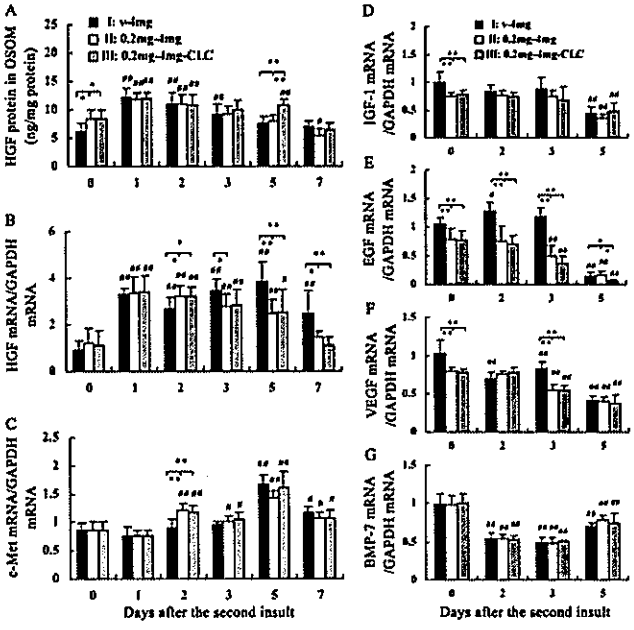



Figure 4




注：本研究は、2008 年 6 月 1 日＜第 51 回日本腎臓学会学術総会＞にポスター発表。

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