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ORIGINAL INVESTIGATION

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Cisplatin-induced toxicity in immortalized renal cell lines established from transgenic mice harboring temperature sensitive SV40 large T-antigen gene

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Abstract We established renal cell lines from definite nephron segments which were microdissected from kidneys of transgenic C57BL/6 mice, harboring the large T-antigen gene of temperature-sensitive mutant simian virus 40, pSVtsA58(ori-). Cell culture was under a humidified atmosphere of 5% CO_2 in air, on collagen-coated dishes, and in RITC80-7 medium with 5% fetal bovine serum, 10 μg/ml transferrin, 1 μg/ml insulin, 10 ng/ml recombinant human EGF, penicillin and streptomycin. Cell line which kept contact inhibition character was established from each segment. Cells derived from distal tubule, cortical and outer medullary collecting duct possessed their cyclic AMP response to arginine-vasopressin, like their original nephron segment. On the other hand, cells derived from terminal proximal tubules (S3 segment) formed a cobblestone-like confluent monolayer, and did not respond to arginine-vasopressin like their fresh segments. Since cisplatin, a well-known nephrotoxic substance, damages proximal tubules (especially S3) rather than collecting ducts, we assayed cell number, protein content, and ATP content of cultured S3 cells at various times after addition of 0.2 mM cisplatin. Decrease of cell number, total protein content and total ATP content of culture cells occurred after 10 h incubation with 0.2 mM cisplatin. The 50% lethal dose (LD_{50}) of cisplatin in S3 cells was 4×10^{-5} M after 20 h incubation and 8.5×10^{-6} M after 40 h incubation. Outer medullary collecting duct (OMCD) cells were damaged 30% maximally after 20 h incubation with cisplatin, and LD_{50} in them became 2.5×10^{-5} M after 40 h incubation. We could show that the LD_{50} of cisplatin in the OMCD cell line was three times higher than that in the S3 cell line. Thus, these cell lines are the first in the kidney to definite the segmental origin and to maintain some differentiated unique functions. They are valuable for studies on intrarenal site-specific actions and possible mechanisms of action of pharmacological and toxic substances.

Key words Cisplatin · Nephrotoxicity · Simian Virus 40 Large T antigen · Transgenic mouse · Renal tubule cell

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Introduction

Cisplatin [*cis*-diamminedichloroplatinum (II): CDDP] is one of the most frequently used agents in the treatment of cancer, and it is essential in the treatment of germ-cell cancer. The use of this drug has been shown to cause dose-related acute renal failure (ARF) both in human clinical use (Hardaker et al. 1974; Dentino et al. 1978; Davis et al. 1980; Tirelli et al. 1985; Macleod et al. 1988) and in animal studies. In rats, a number of morphologic studies have been reported that emphasize cisplatin-induced ARF involving selective damage to S3 of the proximal tubule located in the outer strip of outer medulla (Ward and Fauvie 1976; Dobyan et al. 1980; Choie et al. 1981; Jones et al. 1985). The major damage in humans has been observed in more distal parts of the proximal tubule or in the distal nephron segments (Gonzales-Vitale et al. 1977).

Immediately after administration of cisplatin in dogs, the renal blood flow and glomerular filtration rate remained unchanged, whereas a significant decrease was observed in both the fractional and absolute proximal reabsorption rates of sodium and water. Cisplatin-induced nephrotoxicity seems to be initiated by an acute proximal tubular impairment that precedes alterations in renal hemodynamics. At 48–72 h after cisplatin administration, depressed renal function can be attributed to the impairment of proximal as well as distal tubular reabsorptive capacities associated with increased vascular resistance. The polyuria occurring at this time appears to be due to the impaired reabsorption

rate in the distal nephron segments, which affects the concentration mechanism (Daugaard et al. 1986, 1988).

To study the cellular mechanism of cisplatin-induced nephrotoxicity, cultured renal cells are necessary. Primary culture of renal proximal tubule cell (Tay et al. 1988), and cell lines which express characters of proximal tubule, LLC-PK1 (Williams 1989), or NRK-52E (Fukuishi and Gemba 1989) have been used as materials. We established immortalized renal tubular cell lines from transgenic mice harboring large T-antigen gene from temperature sensitive (ts) mutant of simian virus 40, tsA58. Established cell lines have definite origins of nephron segments, because they were derived from microdissected nephron segments. This study describes cellular toxicity of cisplatin to the cells from S3 segment, the most vulnerable segment to cisplatin. We have also compared the toxicity of cisplatin in S3 cells with that in OMCD cells, which were derived from the segment located in the same outer medullary layer as the S3 segment.

Materials and methods

Biochemical materials

Collagenase (type I), bovine serum albumin (BSA), human transferrin, forskolin (FK), arginine-vasopressin (AVP), rat parathyroid hormone 1–84 (rPTH), 3-isobuthyl 1-methyl xanthine (IBMX), adenosine triphosphate (ATP), and methylene blue were obtained from Sigma (St Louis, Mo.). RITC80-7 medium was from Kyokuto Pharmaceutical Industrial (Tokyo, Japan). Insulin was from Shimizu Seiyaku (Shizuoka, Japan). Recombinant human epidermal growth factor (rhEGF) was from Wakunaga Seiyaku (Tokyo, Japan). Fetal bovine serum (FBS), penicillin and streptomycin solution, and trypsin solution were from Life Technologies (Gaithersburg, Md.). Creatinine phosphate and creatinine kinase were from Boehringer Mannheim (Mannheim, Germany). Cyclic AMP radioimmunoassay (RIA) kit was from Yamasa (Chiba, Japan). Cisplatin and all other chemicals were of the analytical grade from Wako Pure Chemical Industries (Osaka, Japan).

Establishment of immortalized cell lines

We used transgenic mice harboring the large T-antigen gene of temperature-sensitive SV40, pSVtsA58, which were produced by the same method of Yanai et al. (1991a, b). Tissue preparation and tubule microdissection techniques used in this study have been previously described (Morel et al. 1976; Nonoguchi et al. 1985). Briefly, each mouse, weighing 15–18 g, was anesthetized with an intraperitoneal injection of pentobarbital sodium solution (0.05 mg/g body weight). Both kidneys were perfused through a fine needle inserted into the abdominal aorta with 5 ml sterile modified Handks' solution (pH 7.4) containing 0.1% BSA, followed by 5 ml sterile modified Handks' solution (pH 7.4) containing 0.1% BSA and 0.1% collagenase. Both kidneys were excised and sliced with sterile razor blade, and digested at 37 °C for 30 min with the same solution containing collagenase as that for perfusion. After incubation, the slices were thoroughly rinsed in Handks' solution (pH 7.4) and the tubules were dissected manually in Handks' solution (pH 7.4), utilizing a stereomicroscope. The nephron segments were identified as reported previously (Morel et al. 1976). The early proximal tubule (S1) was identified by its attachment to a glomerulus. The second portion of the proximal tubule (S2) was the transitional part of convolution to the straight portion, which was dissected from the medullary ray of the outer cortex. The late proximal tubule (S3) was identified by its attachment to the thin descending limb of Henle's loop. Medullary and cortical thick ascending limbs, of Henle's (MTAL and CTAL) were dissected from the inner strip of the outer medulla and the medullary ray of the cortex, respectively. The distal tubule, including connecting tubule (DT), was identified by its attachment to a glomerulus. The cortical collecting duct (CCD) and OMCD were dissected from the medullary ray of the cortex and the inner strip of outer medulla, respectively. Each nephron segment was transferred to a well of a 24-well culture plate (MS-0024K, Sumitomo Bakelite, Japan).

Microdissected glomeruli and tubules were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C on day, and incubated at 33 °C after day 2. Basal medium was RITC80-7 supplemented with 5% FBS, 10 μg/ml transferrin, 1 μg/ml insulin, 10 ng/ml rhEGF, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin. When cells became confluent after 5–7 days, cells were subcultured 1:3 with phosphate buffered saline (PBS) containing 0.05% trypsin (w/v) and 1 mM EDTA.

Cell growth evaluation

Cells (1.5 × 105) derived from S3 segment were inoculated in each well of four-well culture plates (Nunc, Naperville, Ill.), and cultured at 33 °C until the day of assay. In some plates, the medium was replaced with serum-free medium on day 10.

For phase-contrast microscopic observations, 0.5 ml PBS containing calcium and magnesium was poured on the culture after discarding the culture medium. Monolayers were observed under a phase-contrast microscope (diaphoto TMD300, Nikon, Tokyo, Japan) at magnification of 100–400× and photographed with Fuji Neopan SS film (Fuji Film, Tokyo Japan).

After microscopic observation, the cultured cells were rinsed with 1 mM EDTA-PBS deprived of calcium and magnesium after discarding the PBS. A 0.5-ml aliquot of EDTA-PBS containing 0.05% trypsin was poured in two wells for numeration of the cells released by trypsinization. The cells were incubated at 37 °C for 5 min and suspended in 25 ml buffer for cell counting (Isoton III, Japan Sci., Japan), and they were counted with an electronic cell counter (Industrial D, Coulter, UK). The thoroughness of trypsinization was checked by examination of culture wells following trypsinization using phase contrast microscopy. Complete trypsinization was achieved for all wells.

From the other two wells from which EDTA-PBS solution was aspirated and for protein measurements replaced with 1 ml Lowry's solution C, which had been prepared by mixing together solution A (2% Na_2CO_3, w/v) and solution B (1% sodium-tartrate and 0.5% CuSO4, w/v) in the ratio 50:1. The plates were then incubated for 30 min at room temperature. The reaction was developed by mixing directly in a test tube 0.2 ml distilled water, 1 ml of the solution C in which the sample was dissolved, and 0.1 ml 1 N Folin-Ciocalteu's phenol reagent. The test tubes were kept for 30 min in darkness at room temperature. Absorbance was measured at 500 nm on a spectrophotometer (DU650, Beckman, UK), and the obtained values were compared with those of a BSA standard curve.

Determination of cAMP production

Cells derived from S1 segment, DT, CCD and OMCD were incubated for 3 days at 33 °C to make cell monolayers in 96-well collagen-coated culture plate. For permeabilization of the cells, 10 μl of preincubation solution (8 mM TRIS-HCl, 1 mM $MgCl_2$, 0.25 mM EDTA, 0.1% BSA) with or without 5×10^{-6} M AVP was added on the culture after discarding the medium and incubated for 30 min on ice. During incubation, the plates were frozen on dry ice and thawed repeatedly three times. After hypoosmotic shock and freeze-thawing, 40 μl of incubation solution (100 mM TRIS-HCl, 1 mM IBMX, 0.25 mM EDTA, 0.5 mM ATP, 20 mM creatinine phosphate, and 1 mg/ml creatinine kinase) was added and incubated for 15 min at 37 °C. The reaction was terminated by addition of 50 μl 0.3 N HCl and centrifuged for 15 min at 3000 *g*. Cyclic AMP productions were determined on the supernatants, using the RIA kit (Torikai and Imai 1979).

For cAMP measurements of intact nephron segments, male C57BL/6j mice (Saitama Experimental Laboratories, Saitama,

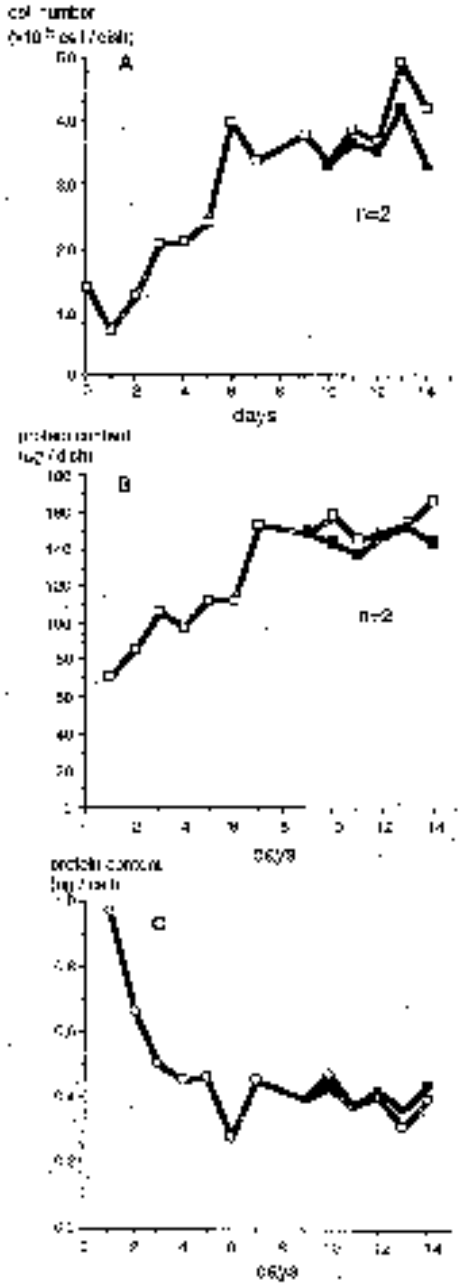

Fig. 1 A–C Growth curve of the S3 cells. **A** Time course of the cell number, **B** time course of total protein content, and **C** time course of cellular protein content. The cells were cultured in medium containing 5% FBS (❑) and in serum free medium (■). Points in the figure represent the average of cell numbers and protein contents in two wells

Japan), weighing 20–30 g, were killed by cervical dislocation. The nephron segments which had been prepared by collagenase digestion and microdissection of the kidneys as described previously were transferred into 45 µl pre-incubation solution (Hank's buffer solution containing 1 mM IBMX and 0.5 mM Ca^{2+}). After pre-incubation for 5 min at 37 °C, the reactions were started by addition of 5 µl incubation solution with or without 1×10^{-5} M AVP. Then, they were incubated for 5 min at 37 °C, and the reaction was terminated by addition of 50 µl 10% trichloroacetic acid (v/v). Assays were performed on ether-extracted supernatants, using the RIA kit.

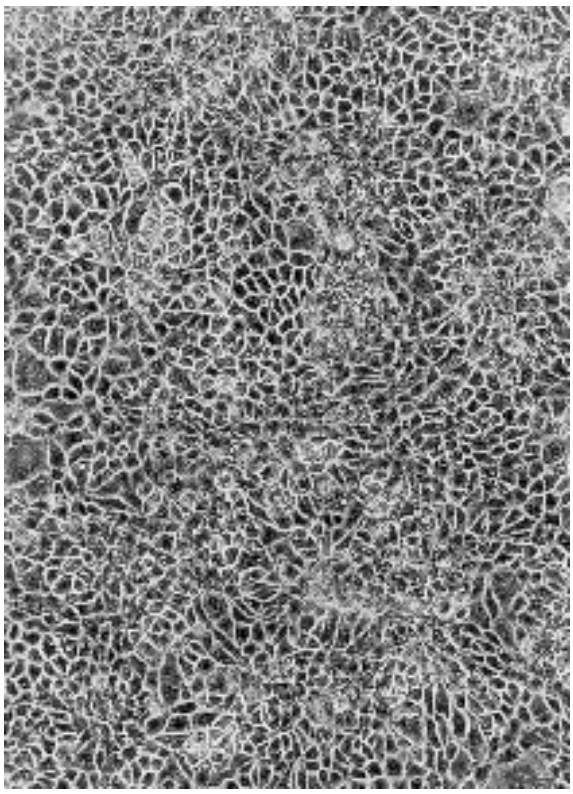

Fig. 2 Phase-contrast micrograph of a cell line established from S3 segment. Confluent appearance on day 3 after subcloning. × 100

With cells derived from S3 segments, the assay was performed on non-permeabilized cell. The intact cells were incubated for 3 days at 33 °C to make cell monolayers on collagen membranes (CM-24, Koken, Japan). After discarding the medium and rinsing with PBS, 180 µl and 450 µl of incubating solutions, the same ones used at cAMP measurements of nephron segments, were poured on and under the membrane, respectively, and incubated for 5 min at 37 °C. The reactions were started by addition of 20 µl and 50 µl of incubating solutions with or without agonist, 5×10^{-4} M FK, 1×10^{-5} M AVP, and 1×10^{-6} M rPTH. They were incubated for 5 min at 37 °C and terminated by addition of 700 µl 10% trichloroacetic acid (v/v). Assays were performed on the ether-extracted supernatants, using the RIA kit.

Exposure of cisplatin to the cells

The 40–60th passage cells derived from S3 and OMCD were used. Cells (10^3–10^4 per well) were inoculated in 96-well culture plates and each well was filled with 200 µl of RITC80-7 medium supplemented with 5% FBS, 10 µg/ml transferrin, 1 µg/ml insulin, 10 ng/ml rhEGF, and 100 U/ml penicillin G sodium and 100 µg/ml streptomycin.

For studies of the time-course of cisplatin toxicity, 100 µl of new medium was added to the all wells of two 96-well culture plates after removal of old medium at the start of the experiment. They were cultured in 95% air, 5% CO_2 at 33 °C and during the experiment, another 100 µl of medium with 4×10^{-4} M cisplatin was added in the experimental groups as well as controls without cisplatin for the different exposure times.

Twenty-four hours later, the cultured cells were washed three times with PBS after discarding the medium. The cells for cell number assay were fixed with 10% (v/v) formalin in PBS for 10 min and washed three times with 0.01 M borate buffer, pH 8.4 before the plates were stored at room temperature until staining. The cells were stained with a solution of 1% methylene blue (w/v, in the borate buffer) for 10 min, and rinsed with the same buffer and dried thoroughly. To each culture

vessel, 100 µl of 0.1 N hydrochloric acid was then added. Absorbance of the obtained blue acid solution was measured on a microplate reader (MTP-12, Corona, Japan) at 610 nm and 550 nm (Pelletier et al. 1988).

To wells for protein assay 40 µl distilled water were added and 200 µl of Lowry's C solution, after 10 min at room temperature, an aliquot of the samples (120 µl) and 10 µl diluted Folin-Ciocalteu's phenol reagent were mixed and kept in the dark for 30 min at room temperature. Absorbance was measured at 610 nm and 550 nm on a microplate reader. Obtained values were compared with those given by a BSA scale (Lowry et al. 1951).

For the ATP assay 100 µl of 10%TCA-4 mM EDTA solution was added to the cells. Aliquots of 5 µl were mixed with 320 µl 0.5 mM EDTA/0.1 M TRIS-acetate buffer (pH 7.75), and 80 µl of ATP monitoring reagent (LKB1243-200) and the light intensity was measured on a Luminometer (LKB-Wallac 1251 Luminometer, Finland) (Uchida and Endou 1988).

For the study of dose-dependent effects of cisplatin on cell survival, 200 µl of medium containing various concentrations of cisplatin, zero or 2×10^{-4}–7.8×10^{-7} M was added to the 96-well culture plates. Then, the cells were cultured in 95% air, 5% CO_2 at 33 °C for 20 h or 40 h. After the culture, the protein assay was performed on the cells as described above for the study of the time-course of cisplatin toxicity.

Analysis of the results

All data were expressed as means tandard error (SE). The differences between treated and untreated cells were evaluated statistically by the unpaired Student's *t*-test. Differences with a *p*-value of < 0.05 were considered significant.

Results

Cell characteristics

Cell lines have been established from each microdissected nephron segment. Figures 1A and 1B show the growth curve of S3 cells given as cell number and protein content, respectively. In this experiment, cell culture was started with 1.4×10^5 cells/2.0 cm² dish in basal medium supplemented with 5% FBS and came into stationary phase on day 6 of experiment. In the logarithmic phase, the apparent doubling time was 2.32 days (Fig. 1B), and the cellular protein content decreased (Fig. 1C). In the stationary phase, the cell number was around 4.0×10^5 cells/2.0 cm² dish and the cellular protein content was 0.4 ng protein/cell. The average of protein content was 154.7 µg/2.0 cm² dish between days 9 and 14. When cells were cultured in serum-free medium after day 9 of the experiment, no differences in cell numbers and protein content were observed compared to cells cultured in serum-supplemented medium. Thus, in the stationary phase, these S3 cells survived in serum-free medium.

Figure 2 shows the phase-contrast microscopic appearance of a cell layer derived from S3 segment. The S3 cells formed a cobblestone-like confluent monolayer and had kept contact inhibition character.

Figure 3A and 3B show cAMP production stimulated by 10^{-6} M AVP in cell lines and nephron segments, respectively. Cells were permeabilized by three freeze-thaw cycles and incubated for 15 min at 37 °C. Nephron segments were not permeabilized and incubated for 5 min. Cells derived from proximal tubule (S1 segment) did respond weakly to AVP, whereas freshly microdissected S1 segment did not. Cells derived from DT, CCD and OMCD responded qualitatively similar to AVP as the original nephron segments.

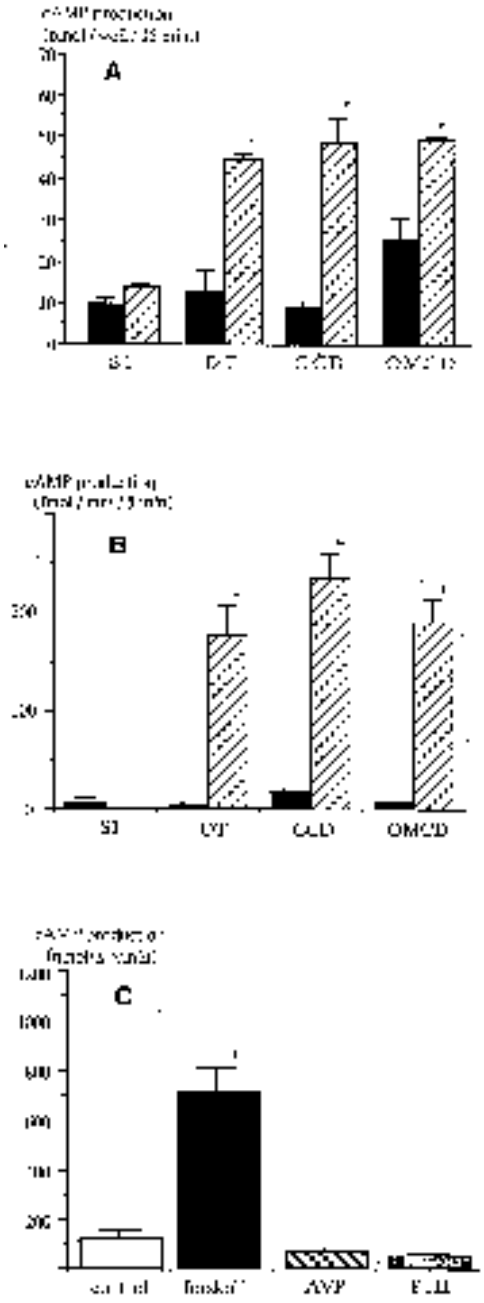

Fig. 3 **A** cAMP production in established cell lines derived from various nephron segments; S1, DT, CCD, and OMCD. Permeabilized cells were incubated for 15 min at 37 °C with 1.0×10^{-6} M arginine-vasopressin (*hatched bars*) or vehicle (*closed bar*). **B** cAMP production in freshly isolated nephron segments, S1, DT, CCD, and OMCD. Intact nephron segments were incubated for 15 min at 37 °C with 1.0×10^{-6} M arginine-vasopressin (*hatched columns*) or vehicle (*closed columns*). **C** cAMP production in a S3 cell line. Intact cells were stimulated by 1.0×10^{-5} M FK (*closed column*), 1.0×10^{-7} M AVP (*hatched column*), and 1.0×10^{-8} M rPTH (*shaded column*) (mean EM, $n = 3$, * $P < 0.05$)

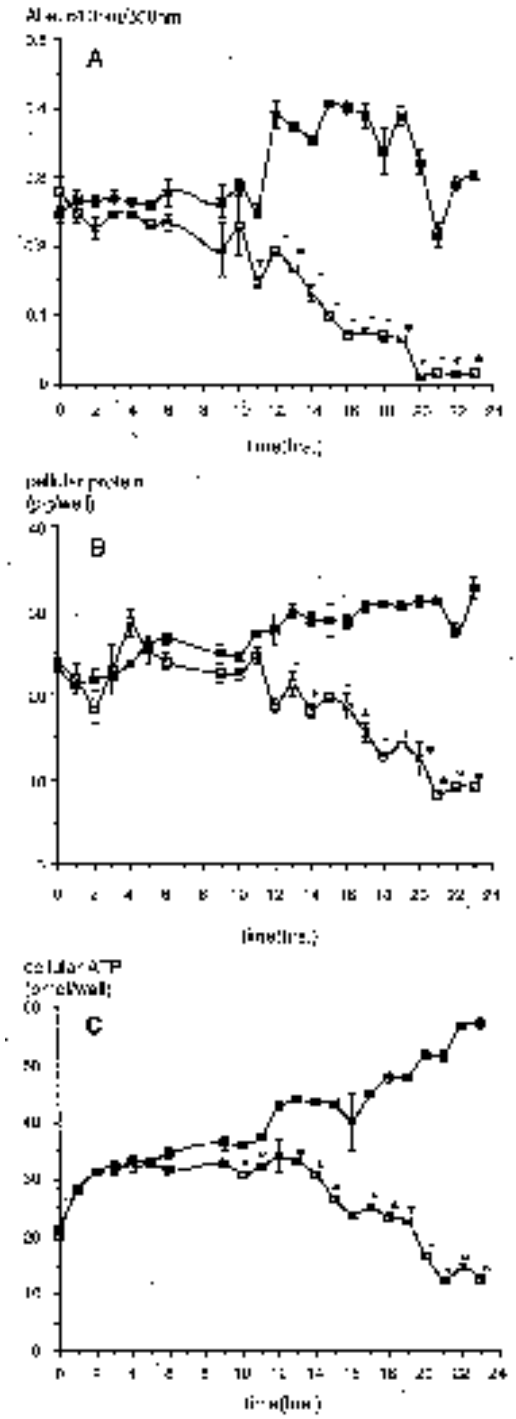

Fig. 4 A–C Time course of cisplatin toxicity to the S3 cell line. **A** Nucleic acid content assayed by methylene blue method, **B** protein content, and **C** ATP content in the wells treated with 0.2 mM cisplatin (❍) and in the control wells of S3 cells (●) (mean EM, n = 3, * $P < 0.01$)

Figure 3C shows cAMP production stimulated by 10^{-5} M FK, 10^{-7} M AVP, and 10^{-8} M rPTH in non-permeabilized S3 cells. The cell layer on collagen membrane was incubated for 5 min at 37 °C. According to their positive response to FK, the S3 cells had an adenylate cyclase activity, although they did not respond to AVP and PTH.

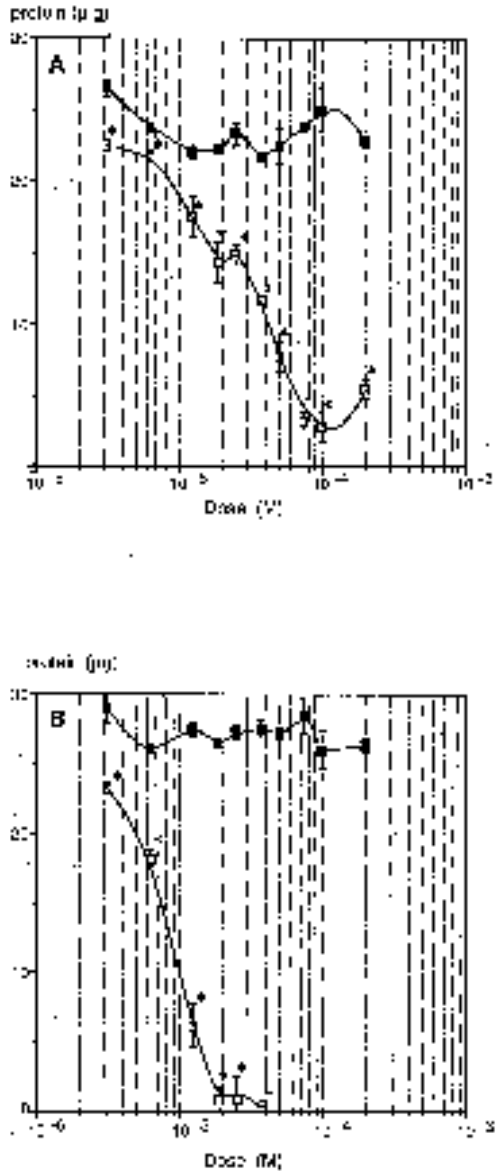

Fig. 5 Cisplatin toxicity to the total protein content of the S3 cell line after 20 h exposure **A**, and after 40 h exposure **B**. Cells were incubated with cisplatin (■) or without cisplatin (❑) (mean EM, n = 3, * $P < 0.05$)

Time-course of cisplatin toxicity in a S3 cell line

We assayed cell number (Fig. 4A), total protein content (Fig. 4B) and total ATP content (Fig. 4C) of cultured S3 cells which were exposed to 0.2 mM cisplatin or vehicle for various times. The protein contents of the control group were 23.6 μg/0.32 cm² well at 0 h and 32.8 μg/0.32 cm² well at 23 h, as shown in Fig. 4B. This corresponds to 147.5 and 205.0 μg/2.0 cm², respectively, and these values are comparable with the stationary phase of Fig. 1B. The ATP

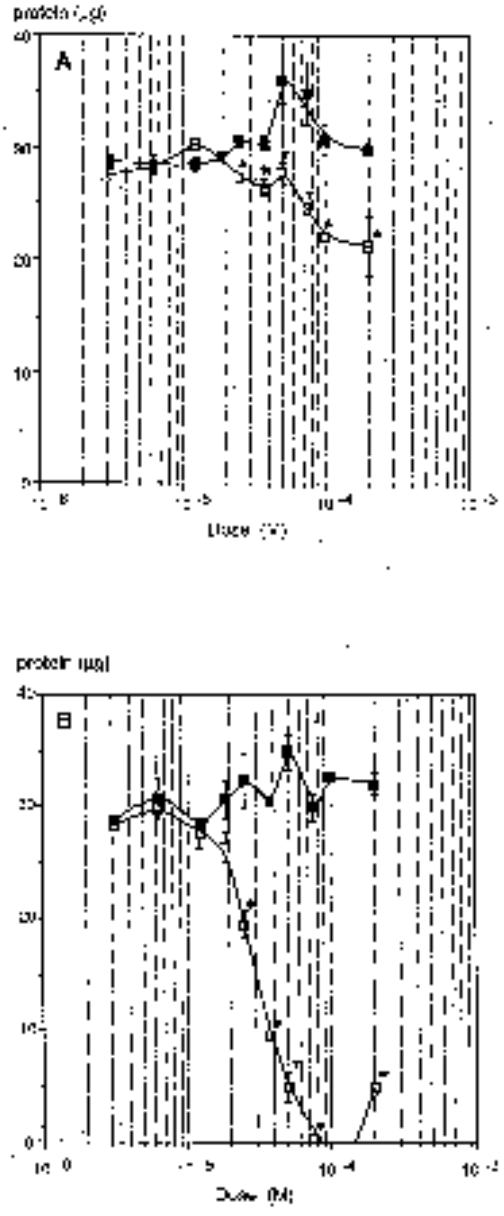

Fig. 6 Cisplatin toxicity to the total protein content of the OMCD cell line after 20 h exposure **A**, and after 40 h exposure **B**. Cells were incubated with cisplatin (■) or without cisplatin (❑) (mean EM, $n = 3$, * $P < 0.05$)

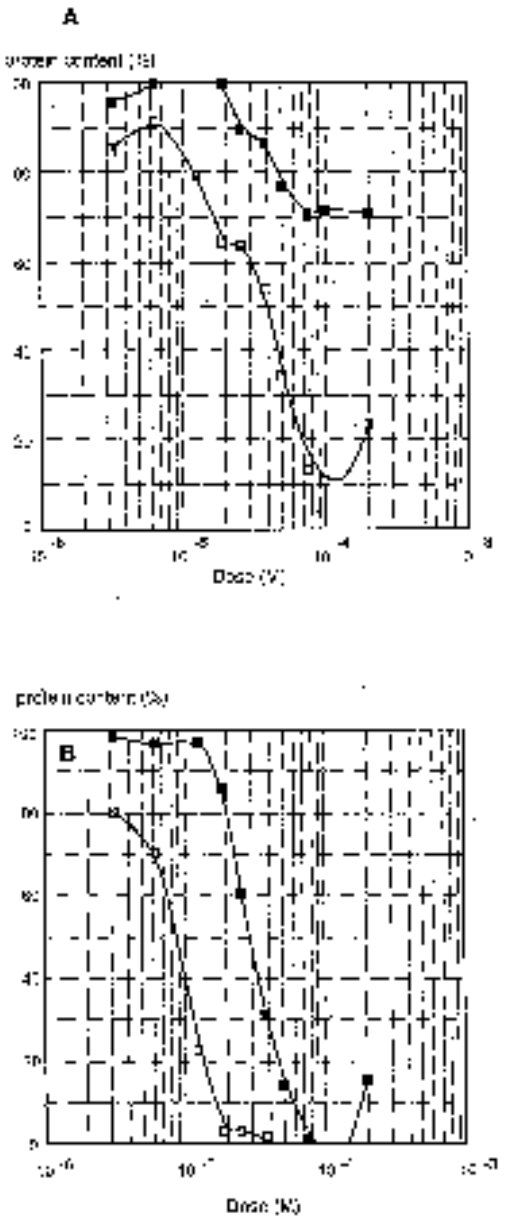

Fig. 7 Dose-survival relationship of the S3 cell line (❑) and OMCD cell line (■), after 20 h exposure of cisplatin (**A**) and after 40 h exposure of cisplatin (**B**)

contents of the control group were 21.6 pmol/0.32 cm² well at 0 h and 57.5 pmol/0.32 cm² well at 23 h (Fig. 4C). These values correspond to 0.915 and 2.055 pmol/μg protein, respectively. In this experiment, the control groups showed small increases in cell number (Fig. 4A) and protein content, 1.2 times and 1.4 times respectively, and their ATP content was elevated 2.7 times.

As shown in Fig. 4, the cell number, the protein content and the ATP content decreased upon treatment with 2×10^{-4} M (60 μg/ml) cisplatin. With regard to the differences between the cisplatin-treated group and the control group which also received fresh medium at the same time, there were statistically significant differences in the ATP content after 10 h exposure of cisplatin, in the cell number after 11 h exposure, and in the protein content after 13 h.

Dose-related toxicity of cisplatin in S3 or OMCD cell lines

To study the dose-dependency of cisplatin toxicity in two different nephron segment in vitro, total protein contents of S3 and OMCD cell layers were assayed after 20 and 40 h exposure to various concentrations of cisplatin. We used total protein content to judge survival of cells upon treatment with cisplatin, because of smaller deviation of protein

data. Cisplatin solutions were diluted to make media which have final concentrations of cisplatin, such as 200, 100, 75, 50, 37.5, 25, 18.25, 12.5, 6.25 and 3.13 μM.

Figures 5 and 6 are dose-effect curves obtained with the cell lines derived from S3 segments and OMCD segments, respectively. The protein contents in the control group of S3 cells in the stationary phase showed 23.38 μg/0.32 cm^2 well at 20 h and 27.16 μg/0.32 cm^2 well at 40 h. OMCD cells also seemed to reach a stationary phase because of their confluent appearance (not shown) and since no increase in their protein contents was observed (30.64 μg/0.32 cm^2 well at 20 h and 31.05 μg/0.32 cm^2 well at 40 h).

The protein contents of S3 cell cultures were significantly decreased even by 3×10^{-6} M (0.9 μg/ml) cisplatin after 20 h exposure (Fig. 5A). In contrast, OMCD cell cultures showed similar decreases of protein contents only at high doses, over 2.5×10^{-5} M (7.5 μg/ml) cisplatin, and after 40 h exposure (Fig. 6B).

The 50% lethal dose (LD_{50}) for cisplatin in S3 cells was about 4×10^{-5} M (12 μg/ml) after 20 h incubation (Fig. 7A) and 8.5×10^{-6} M (2.55 μg/ml) after 40 h incubation (Fig. 7B). OMCD cells were damaged up to 30% maximally after 20 h (Fig. 7A), and after 40 h, the LD_{50} observed in the range of 2.9×10^{-5} M or 8.7 μg/ml (Fig. 7B). Thus, the LD_{50} cisplatin in cultured OMCD cells was three times higher than in S3 cells.

Discussion

The purpose of this study was the introduction of new cell lines derived from transgenic mice harboring the temperature-sensitive SV40 large T antigen gene, and the application of these cells to renal toxicology.

Cultured cells established to study renal function such as LLC-PK1, OK, MDCK, or A6 cell lines have raised controversy about the precise origins of these cell lines. On the other hand, primary cultures of nephron segments can neither be subcultured nor cloned because of their limited life span and dedifferentiation after several passages.

These problems can sometimes be overcome with oncogene-transformed cell lines. Thick ascending limb cells (Scott et al. 1986) or rabbit proximal and distal tubule cells (Vandewalle et al. 1989) have been established by in vitro transfection of simian virus 40 (SV40). Glomerular cell lines (Mackay et al. 1987), Cortical collecting duct cells (Stoos et al. 1991) and inner medullary collecting duct cells (Rauchman et al. 1993) were derived from transgenic mice harboring early region of the SV40, Tg(SV40E)Bri7. These transformed cells were reported to exhibit differentiated functions of original nephron segments.

We used transgenic mice harboring temperature-sensitive SV40 large T antigen gene derived from tsA58 virus. With these mice, immortalized cell lines have already been established from liver, kidney, bone marrow (Yanai et al. 1991a,b,c), and stomach (Sugiyama et al. 1993). Cell lines used in this study were established from microdissected single nephron segments of kidney, therefore they have definite segmental origin like primary cultures.

Our cells derived from S3 segments have shown some typical characteristics of proximal tubule. They have alkaline phosphatase activity (data not shown, and to be published) and ammoniagenic activity (Sekine et al. 1994), but we failed to detect their cyclic AMP response to PTH and gamma-glutamyl transpeptidase activity (data not shown). Cyclic AMP response to PTH was found in pars recta of the proximal tubule of C57 BL/6 J mouse (Brunette et al. 1979). However, the magnitude of cAMP responses to PTH also decreased in another proximal tubule cell line (Romero et al. 1992) and LLC-PK1 cell also lost the response (Malmström and Mürer 1986). Although no exact information of S3 segment concerning PTH response is available, we speculate that the lack of response to PTH seen in cultured S3 cells may also be characteristics for the fresh mouse S3 segment. With respect to gamma-glutamyl transpeptidase, the activity was lost even in primary culture (Chung et al. 1982), and may be lost easily also in the established cell line.

The cell line derived from OMCD represents tubular epithelium and it showed a cAMP response to AVP. This is the first report about a cell line derived from OMCD segment. To stimulate cAMP production by AVP, we used permeabilized cells, since intact cells attach to the bottom of the dishes on their basolateral side, and we therefore expected difficulties for AVP access to their basolateral V2 receptor. On the other hand, cell suspensions of CCD cells lost their cAMP response to AVP after trypsin treatment (data not shown). Permeabilized cells showed a cAMP response to AVP, but the magnitude of their response was lower than that of intact nephron segments (Fig. 3A,B).

We applied these two cell lines to the study of cisplatin nephrotoxicity. Other cell lines derived from transgenic mice harboring the same gene showed temperature-sensitive growth, with growth stopped at non-permissive temperature, 39 °C (Yanai et al. 1991b). Our cell line derived from CCD also grew in a temperature-sensitive manner. We experimented in basal medium supplemented with 5% fetal bovine serum at 33 °C, as a dedifferentiation and growth condition. At permissive temperature, the expressed T-antigen might affect on the result, although the cells did not suffer a spontaneous decrease in their viability. Generally temperature-sensitive cells show differentiated characters at non-permissive temperature, but the cells established by Yanai et al. (1991b) responded to AVP at permissive temperature, 33 °C, but not at non-permissive temperature, 39 °C. Thus, in SV40-transformed renal cell lines, large T antigen expression may be essential for the expression of differentiated characteristics. This phenomenon may not be related to large T antigen, but may be related only to temperature inactivation of the vasopressin receptor system.

Concerning serum concentrations, other studies have used serum-free conditions (Tay et al. 1988; Courjault et al. 1993). Serum, however, seems to be essential for an expression of cisplatin nephrotoxicity, because cisplatin-serum metabolites were more nephrotoxic (Daley-Yates and McBrien 1984). In this experiment, we also wanted to keep

cell viability; thus we studied cisplatin toxicity with the cells cultured in the medium supplemented with 5% FBS. Further investigations are needed to clarify the relationship between serum concentration and cisplatin-induced nephrotoxicity.

In the time course of cisplatin toxicity (Fig. 4C), the ATP content in control S3 cells (1.476 pmol/μg protein) was near the value of ATP content in microdissected mouse S3 segment, 1.351 pmol/μg protein, reported by Uchida and Endou (1988). Thus, we expected that these cells were almost in the same condition as in vivo. During the experiment, ATP content in control S3 cells increased more than cell number or protein content. Intracellular ATP content seemed to be affected by a change of culture medium.

Studying the dose-response of cisplatin toxicity, we observed that mouse S3 cells were more vulnerable to cisplatin than OMCD cells. In particular, the protein contents of OMCD cells did not change with cisplatin at lower concentration than 2.5×10^{-5} M after 40 h exposure. This value, 2.5×10^{-5} M, seems to correspond to the no-observed adverse effect level or the virtually safety dose for OMCD cells.

In general, established cell lines are not highly sensitive to cisplatin: toxic effects to LLC-PK1 cells (Dedon and Borch 1987) and OK cells (Courjault et al. 1993) were never detected with concentrations lower than 100 μM after 24 h exposure. Only NRK-52E, an established cell line of normal rat kidney released enzymes into culture medium with 1 μM cisplatin after 72 h exposure (Fukuishi and Gemba 1989). On the other hand, primary proximal tubule cell cultures are more sensitive to cisplatin, e.g. cell viability was reduced to 40% of the control with 100 μM cisplatin after 24 h exposure (Tay et al. 1988). This concentration is close to peak blood levels in cancer patients (Campbell et al. 1983; Dumas et al. 1985). Our cell line derived from S3 segments showed a decreased protein content, to 10% of the control after 20 h exposure with 100 μM cisplatin, and seems to be as sensitive to cisplatin as primary proximal cell cultures. Possible reasons for the sensitivity of our S3 cells to cisplatin are: 1) S3 cells the same mechanism of toxicity as in vivo, 2) the expression of large T antigen may be sensitive to cisplatin, and therefore cells whose viability is more dependent on the large T antigen are more sensitive to cisplatin, 3) the difference of serum concentration used in the experiments, or 4) interference with toxicity of antibiotics used in our study. To use this cell line as an alternative of experimental animals, it is important to clarify the possibilities of 2, 3, and 4.

Several authors have suggested that DNA, RNA and protein synthesis are successively inhibited in mammalian cells following cisplatin exposure (Harder and Rosenberg 1970; Tay et al. 1988). Tay et al. reported that the inhibition of these syntheses was evident as early as 6 h post-treatment, and DNA synthesis was suppressed even by 5 μg/ml (20 μM). They concluded that inhibition of renal synthetic activity by cisplatin, rather than its effect on enzyme activity, precedes the onset of cell lethality and may, therefore, be an important event in the initiation of cisplatin-induced nephrotoxicity.

If the inhibition of renal synthetic activity occurs at the initiation step of cisplatin-induced nephrotoxicity, the inhibition of large T antigen expression in our cells may relate to cell lethality. Nevertheless, our cells did not change their protein contents after 1 day culture at a non-permissive temperature, 39.5 °C. Therefore the changes in protein contents after 24 h exposure with cisplatin must represent the cisplatin toxicity.

In summary, we established renal tubule cell lines derived from S3 and OMCD segments dissected from transgenic mice harboring temperature-sensitive SV40 large T antigen gene. They expressed some, not all differentiated characters of original nephron segments yet cells derived from S3 segment kept sensitivity to cisplatin like primary culture cells. They are valuable for studies about intrarenal site-specific actions and possible mechanisms of pharmacological and toxic substances.

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