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# Midkine Rescues Wilms' Tumor Cells from Cisplatin-Induced Apoptosis: Regulation of Bcl-2 Expression by Midkine[1]

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**Midkine (MK) is a heparin-binding growth factor involved in diverse biological phenomena, *e.g.* neuronal survival, carcinogenesis, and tissue repair. MK expression is detected mainly in the kidney in adult mice. In this study, we show that, at a dose that can induce recoverable renal damage and induce apoptosis, cisplatin (CDDP) transiently suppressed MK expression in mouse kidney. *In vitro*, CDDP suppressed MK expression and induced apoptosis in cultured G401 cells, a Wilms' tumor cell line. Exogenous MK protein partially rescued G401 cells from CDDP-induced apoptosis. MK enhanced the expression of Bcl-2, but not that of Bcl-$x_L$, in G401 cells in a dose-dependent manner, and it prevented the Bcl-2 reduction due to CDDP. Moreover, Bcl-2 expression in mouse kidney was also transiently suppressed by CDDP treatment, the expression profile being similar to that of MK. These results imply that MK exerts cytoprotective activity toward a damaging insult, presumably at least in part through enhancement of the expression of Bcl-2.**

**Key words: apoptosis, Bcl-2, cisplatin, kidney, midkine.**

The process of cell death in metazoans is highly conserved and is called apoptosis, which is actively regulated by the cell. The evolutional force of multicellularity and cell specialization brought a need for the regulation of apoptosis through intercellular signaling (*1*). Extracellular signaling molecules, including membrane proteins and growth factors, that regulate apoptosis can be categorized into two groups: pro-apoptotic and anti-apoptotic proteins. Typical pro-apoptotic extracellular molecules are Fas, a membrane protein, and tumor necrosis factor, a growth factor, both of which utilize the FADD-caspase 8 intracellular pathway to induce apoptosis (*2*). Growth factors also act as anti-apoptotic factors for the cell (*3*). However, in many cases, the precise mechanism of the anti-apoptotic action of growth factors remains to be elucidated. In addition, although apoptosis is involved in the development and homeostasis of organs, only a few growth factors have been found to be important for a specific organ, *e.g.* epidermal growth factor, insulin-like growth factor–1 and hepatocyte growth factor for the kidney (*4*). Of the diverse biological functions of a growth factor, its anti-apoptotic activity is sometimes indispensable for another of its functions such as that in the proliferation, differentiation or transformation of cells.

MK is a growth factor with a molecular weight of 13 kDa, and rich in basic amino acids and cysteine (*5*, *6*). MK and pleiotrophin (PTN, also called heparin-binding growth-associated molecule or briefly HB-GAM) comprise a family of heparin-binding growth factors, and are not related to other heparin-binding growth factors such as fibroblast growth factor or hepatocyte growth factor (*7–9*). MK has been reported to promote neuronal survival and induce neurite outgrowth (*10*, *11*), enhance plasminogen activator activity (*12–14*), exhibit chemotaxis to neutrophils (*15*), and be involved in neural tissue formation in *Xenopus* (*16*, *17*). MK also plays roles in carcinogenesis: *e.g.* it causes the oncogenic transformation of NIH3T3 cells (*18*), and promotes angiogenesis (*19*). Moreover, strong MK expression has been found in a wide range of primary human tumors, including Wilms' tumors (*20*). MK is extensively expressed not only in advanced tumors, but also in early stages of carcinogenesis in human colorectal carcinoma (*21*).

During development, MK is intensely expressed in mid-gestation mouse embryos and thereafter becomes restricted to certain tissues, such as kidney (*22*). In adult mouse, MK expression is mainly found in the kidney, and MK mRNA is localized in the proximal tubular epithelium of the renal cortex (*23*). The MK protein is detected in both the proximal and distal tubular epithelium (*24* and unpublished observation). However, its biological functions in this organ remain uninvestigated. In addition, the preferential expression of MK in carcinogenesis suggests that MK may function as a potent anti-apoptotic factor giving cells a growth advantage. To investigate the roles of MK in the kidney

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Abbreviations: BUN, blood urea nitrogen; CDDP, cisplatin [*cis*-diamminedichloroplatinum (II)]; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MK, midkine; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; TUNEL, TdT-mediated dUTP nick end labeling.

and in carcinogenesis, we used both *in vivo* and *in vitro* systems of cisplatin [*cis*-diamminedichloroplatinum (II), CDDP]-induced apoptosis in the present study. CDDP is a chemotherapeutic drug used to treat a wide range of malignant tumors. One hindrance to its clinical use is that it causes several side effects, among which the most important is severe nephrotoxicity (*25*, *26*). The main site that suffers CDDP damage in the kidney is the proximal tubular epithelial cells (*25*).

The Bcl-2 protein family constitutes one of the relevant classes of apoptotic regulatory gene products, acting at the effector stage of apoptosis (*27*, *28*). The family consists of two subgroups: the first one includes anti-apoptotic proteins such as Bcl-2, Bcl-$x_L$, Bcl-w, Bfl-1, Brag-1, Mcl-1, and Al; and the second one includes pro-apoptotic proteins such as Bax, Bcl-$x_S$, Bad, Bak, Bid, Bik, and Hrk. The first identified member of this family, Bcl-2, is a 26 kDa proto-oncogene product and acts as an anti-apoptotic protein (*27*, *29*). It has also been reported that CDDP treatment can reduce the Bcl-2 level and induce apoptosis in several cell lines (*30–32*). Overexpression of the Bcl-2 protein in cultured cells can block or suppress the apoptosis induced by various stimuli including CDDP (*33*, reviewed in Ref. *34*).

We report here that MK has a cytoprotective function, and that Bcl-2 is possibly involved in this process.

## MATERIALS AND METHODS

*Materials*—CDDP was obtained from Bristol-Myers Squibb (Tokyo). MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) and the kit used to measure BUN (blood urea nitrogen) were purchased from Wako (Osaka). The kit for *in situ* apoptosis detection (TUNEL method) was purchased from Takara Biomedicals (Osaka). The Wilms' tumor cell line, G401, was obtained from the Japanese Cancer Research Resource Bank (Tokyo) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Recombinant mouse MK was expressed by baculovirus and purified as described previously (*11*). The procedure for producing recombinant human MK with yeast will be described elsewhere, and recombinant human MK has the same activity as recombinant mouse MK (*10*, *35*, *36*). Antibodies against bacteria-produced mouse MK were raised in a rabbit as described previously (*37*). The antibodies react with both human and mouse MK but no other proteins (*21*, *37*). Monoclonal anti–human Bcl-2 antibodies were purchased from Dako (Glostrup, Denmark). Monoclonal anti–actin antibodies were purchased from Chemicon International (CA, USA). The Hoechst dye (33258) staining kit was a product of ICN Biomedicals (Ohio, USA).

*Animal Treatment*—Male C57 BL/6J mice (Japan SLC) of 8-weeks-old weighing 20–23 g were used in this experiment. The animals were housed under controlled environmental conditions, and were provided with standard food and water *ad libitum*. The animals were randomly divided into two groups. The body weight was determined just before injection. The mice were injected intraperitoneally with 0.5 ml saline or CDDP at the dose of 14 mg/kg-bodyweight. Blood was taken from the suborbital sinus on the indicated days after injection. After centrifugation, 20 µl serum was used to measure BUN. Animals were sacrificed on the indicated days, and their kidneys were removed immediately. One kidney was frozen in liquid nitrogen and stored at –80°C for RNA and protein extraction. The other kidney was fixed in 4% paraformaldehyde and then embedded in paraffin.

*Northern Blot Analysis*—The cDNA probe for *bcl-2* detection was derived by RT-PCR with a cDNA library of G401 cells. The primers used for PCR were: 5′-TGTGGTCCAC-CTGGCCCTCC-3′ and 5′-GTGATGCAAGCTCCCACCAG-3′. The 420-bp PCR product corresponds to the 1731–2150 fragment of human *bcl-2* cDNA (GenBank M13994).

From kidney tissue or cultured cell samples, RNA was extracted by the acid guanidium thiocyanate–phenol–chloroform method (*38*). Twenty micrograms total RNA was denatured with formaldehyde and then separated on a 1.2% agarose gel. Northern blot analysis was performed as described previously (*18*). The membrane was finally exposed to a Fuji imaging plate and hybridization signals were analyzed with a BAS 2000 reader (Fuji Film, Tokyo).

*Protein Extraction and Western Blotting*–MK protein extraction and Western blot analysis were performed as described previously (*18*).

For Bcl-2 analysis, G401 cells were lysed in cold lysis buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 mM PMSF, 0.1 mg/ml aprotinin, and 40 nM leupeptin), collected with a rubber scraper, and then passed about 10 times through a 26-gauge needle. To detect Bcl-2 in a kidney, half of the kidney was homogenized in lysis buffer. The lysate was centrifuged at 12,000 ×*g* for 30 min and then the protein in the supernatant was quantified with a BCA kit (Pierce Chemical, Rockford, US). Fifty micrograms of each protein sample was separated by 12% SDS-PAGE and then blotted onto a nitrocellulose membrane (Schleicher & Schuell, Germany). After blocking with 5% skim milk in PBS–0.1% Tween-20, the membrane was probed sequentially with mouse monoclonal anti–human Bcl-2 antibodies (1:500) and horseradish peroxidase coupled anti–mouse antibodies (1:5,000), and finally the signal was visualized with an ECL kit (Amersham Life Science, UK). The membrane was then stripped and reprobed with anti-actin antibodies.

*MTT Assay*—G401 cells were seeded onto 96-well plates at $1 \times 10^4$ cells per well in DMEM supplemented with 10% FCS. After the cells had become attached to the substratum, they were washed 3 times with DMEM containing 0.1% FCS. Then the cells were incubated in 100 µl of this medium containing MK. Six hours later, the cells were treated with 100 µM CDDP for 2 h. CDDP in the medium was removed by washing three times with 0.1% FCS/DMEM, and then incubation of the cells was continued in 100 µl of the same medium containing MK. The MTT assay was carried out as described previously (*39*). At the indicated times, 15 µl MTT (5 mg/ml in PBS) was added to each well, followed by incubation at 37°C for 4 h. The cells were lysed by adding 100 µl of lysis buffer (20% SDS, 10 mM HCl) and then incubated for 24 h at room temperature. Absorbance was measured at the wavelength of 560/630 nm with a mini-plate reader (System Instruments, Tokyo). Linearity of the optical density *vs.* cell number was obtained in the range of 0.01–0.30 of O.D.

*Detection of Apoptosis by the TUNEL Method and Hoechst Dye Staining*—A paraffin-embedded mouse kidney was cut into 6 µm sections. The detection of *in situ* apoptosis using the TUNEL method was performed following the

manufacturer's instructions.

Cells ($2 \times 10^4$) were seeded in 1.0 ml 10% FCS/DMEM onto 22 × 22 mm coverslips in 3.5-cm culture dishes. After incubation at 37°C overnight for the cells to become attached to the substratum, 1.0 ml of the same medium was added and the cells were incubated further for 24 h. The cells were then washed with 0.1% FCS/DMEM for 3 times and further incubated in this medium for 12 h. After that, the cells were treated with MK and CDDP in the same way as for the MTT assay. Twelve or 24 h after CDDP exposure, Hoechst staining was performed according to the manufacturer's instructions. Briefly, the cells were fixed (methanol:acetic acid = 3:1) for 5 min, and then fixed again with the same fixative for 10 min. The coverslips were air-dried for 30 min, and then placed in 1.0 ml staining solution (0.05 μg/ml Hoechst 33258 in 1 × HBSS buffer) for 30 min, followed by washing three times (1 min each) with distilled water. After the coverslips had been air-dried, photos were taken under a fluorescence microscope (Olympus-BX60, Tokyo), and finally the total and apoptotic cell numbers were determined in the photos. For each condition, about 300 cells were counted.

*Thymidine Incorporation*—Cells ($5 \times 10^4$) were seeded onto a 24-well plate in 10% FCS/DMEM and then treated as described for the MTT assay. Twelve or 24 h after CDDP treatment, 25 μl $^3$H-thymidine (0.5 μCi) was added to each well, followed by incubation at 37°C for 12 h. After fixation with 1.0 ml methanol:acetic acid (3:1) for 1 h, the unincorporated thymidine was removed by washing two times with 1.5 ml 80% methanol. The cells were detached with 0.5 ml trypsin (0.2 mg/ml) for 1 h at room temperature, and then lysed by the addition of 0.5 ml 1% SDS and incubation for 5 min. One hundred microliters of the cell lysate in each well was counted with a Beckman Scintillation Counter.

## RESULTS

*CDDP Induces Apoptosis in the Kidney*—CDDP was injected intraperitoneally into C57 BL/6J mice, and then the condition of their kidneys was followed by measuring BUN. Our initial purpose was to monitor the MK expression profile during CDDP-induced kidney damage as well as in the course of recovery. We tested several doses of CDDP, and found that at the dose of 14 mg/kg-body-weight it caused renal injury and that most mice could recover later. Therefore, this dose was chosen for our experiments. As reported by Choie and colleagues (*25*), we observed a transient increase in the BUN level after CDDP administration. Three days following CDDP injection, the BUN level began to increase, reaching the maximal value on the fifth day. Afterwards it decreased and had returned to nearly the normal level on the tenth day (data not shown). We also performed histological studies. Hematoxylin and eosin staining revealed that CDDP induced cell death in kidney tissues, mainly in the proximal tubular epithelium, and the histological damage was most severe on the fifth day after injection (data not shown). To determine whether or not the CDDP-induced cell death in the kidney is apoptotic, we carried out *in situ* apoptosis detection using the TUNEL method. CDDP treatment induced TUNEL-positive cells (apoptotic cells) in the kidney (Fig. 1). Apoptotic cells appeared on the third day, increased in number until the fifth day, and then gradually decreased. However, on the tenth day we could still observe a few positively stained cells. The apoptotic cells were distributed in the cortex, and most of the positive cells were proximal tubular epithelial cells. The distal and collecting tubules were less affected. These data indicate that, at the dose of 14 mg/kg-body-weight, CDDP can induce apoptosis and transient, recoverable renal failure.

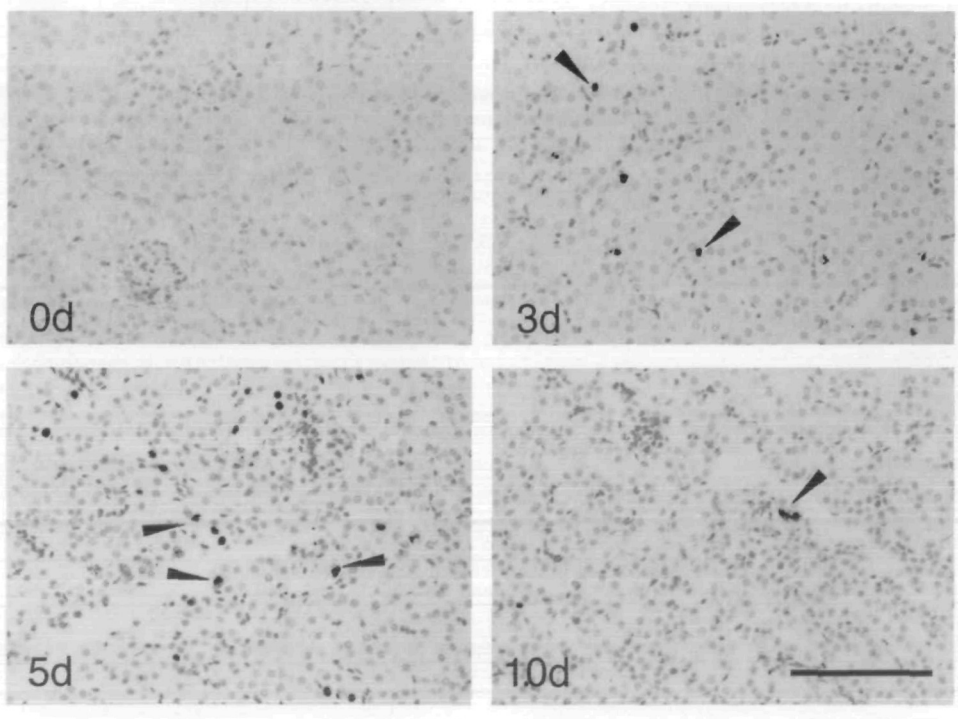


Fig. 1. ***In situ* apoptosis detection in CDDP-treated mouse kidney.** Paraffin-embedded kidney tissues were cut into 6 μm sections. The sections were then subjected to *in situ* apoptosis detection with the TUNEL method. Samples at 0, 3, 5 and 10 days are shown here. Arrowheads indicate some of the positively stained cells. Bar, 100 μm.

*CDDP Transiently Suppresses MK Expression in the Mouse Kidney*—To determine whether or not there is any relationship between renal damage and MK expression, we analyzed the MK expression level in the injured kidney. In a group of mice treated with CDDP, we detected a dramatic, transient decrease of MK expression in the kidney at both the RNA (Fig. 2A) and protein (Fig. 2B) levels. On the third day, when some histological and functional injury had appeared, MK expression had already decreased (Fig. 2A, lane 2; Fig. 2B, lane 2). On the fifth day, when the injury including apoptosis showed the most prominent manifestations, MK expression had reached its minimum level (Fig. 2A, lane 3; Fig. 2B, lane 3). Compared with the control, CDDP suppressed MK expression by more than 3-fold. While this reduction was maintained for a short duration, along with the decrease of apoptosis, MK expression gradually returned to its normal level. Thus, in the CDDP-treated kidney, the MK expression profile was closely related to alterations of the renal condition and apoptosis (Figs. 1 and 2).

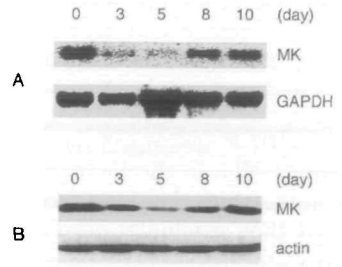


Fig. 2. **MK expression in the kidneys of mice treated with CDDP.** Mice were injected with CDDP (14 mg/kg-body-weight) as described. At the indicated times, animals were sacrificed, and their kidneys were used for RNA and protein extraction. The MK level was examined by Northern blotting and Western blotting. Experiments were performed twice and gave similar results. Representative results are shown here. A, MK mRNA level in CDDP-treated mouse kidney. Upper panel, MK; lower panel, GAPDH. B, MK protein level in CDDP-treated mouse kidney. MK protein extracted from 10 mg tissue was loaded on each lane. Fifty μg lysate of each sample before MK protein extraction was used for actin detection by separate SDS-PAGE. Upper panel, MK; lower panel, actin.

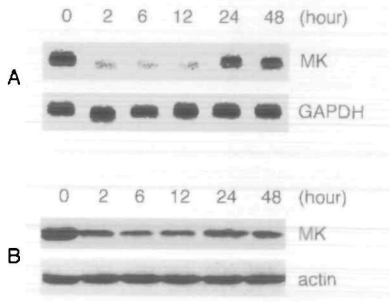


Fig. 3. **MK expression in CDDP-treated G401 cells.** G401 cells were exposed to 100 μM CDDP for 2 h, and then CDDP in the medium was removed by washing 3 times. MK expression was checked at several time points. Experiments were performed twice and gave similar results. Representative results are shown here. A, MK mRNA level in CDDP-treated G401 cells. Upper panel, MK mRNA; lower panel, GAPDH. B, MK protein from CDDP-treated G401 cells detected on immunoblotting. MK extracted from 120 μg protein of cell lysate was loaded on each lane. Fifty micrograms cell lysate of each sample before MK protein extraction was used for actin detection by separate SDS-PAGE. Upper panel, MK; lower panel, actin.

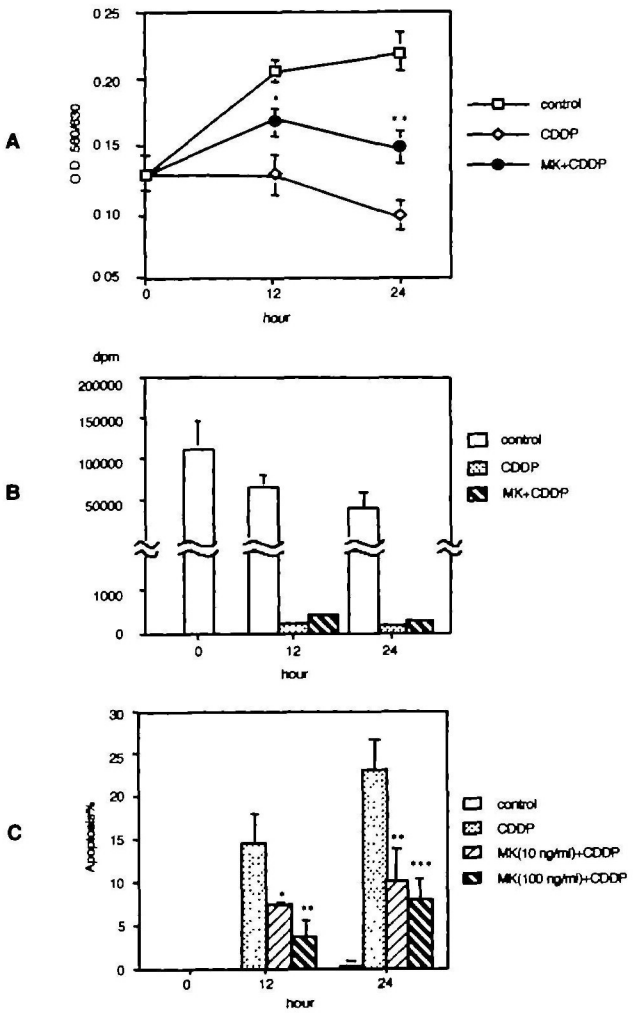


Fig. 4. **Apoptosis of cultured G401 cells treated with CDDP and the MK protein.** G401 cells were treated with human MK and/or CDDP, and then the cellular response was assessed by means of the MTT assay (A), thymidine incorporation (B), and Hoechst dye staining (C). A, cell viability. G401 cells were seeded onto 96-well plates and then treated as described under "MATERIALS AND METHODS." At 12 or 24 h, cell viability was monitored by means of the MTT assay. The results of a representative experiment of three independent ones are shown here. Each condition was examined in triplicate. Control, 0.1% FCS/DMEM; CDDP, 100 μM CDDP; MK+CDDP, 100 ng/ml human MK and 100 μM CDDP. $^{*}p < 0.05$; $^{**}p < 0.01$ (paired *t* test; *versus* CDDP). B, thymidine incorporation. G401 cells were seeded onto 24-well plates, and then treated with human MK and CDDP. Thymidine incorporation was monitored as described under "MATERIALS AND METHODS." The values are the averages for triplicate samples. Representative results of three independent experiments are shown. Control, CDDP and MK+CDDP, same as in A. C, apoptotic cell percentage. The numbers of cells with a fragmented or integrate chromosome were determined in photos after Hoechst dye staining, and the percentages of apoptotic cells are shown here. Control, 0.1% FCS/DMEM; CDDP, 100 μM CDDP; MK (10 ng/ml) + CDDP, 10 ng/ml human MK and 100 μM CDDP; MK (100 ng/ml) + CDDP, 100 ng/ml human MK and 100 μM CDDP. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (paired *t* test; *versus* CDDP).

*CDDP Suppresses MK Expression in Cultured G401 Cells*—We examined whether or not CDDP affects MK expression *in vitro*. G401 cells, a Wilms' tumor cell line originating from kidney epithelium, were used in this experiment. As reported previously (*40*), we detected abundant MK expression in G401 cells, and 2-h-exposure to 100 μM CDDP notably reduced MK expression in this cell line (Fig. 3). On Northern blotting and Western blotting, from 2 to 12 h after CDDP treatment, a decrease of MK expression was detected (Fig. 3A, lanes 2–4; Fig. 3B, lanes 2–4). Densitometrical analysis showed that at 6 and 12 h MK expression was repressed by at least 2-fold. At 24 h, it began to recover (Fig. 3A, lane 5; Fig. 3B, lane 5), but at 48 h it was still lower than the normal level (Fig. 3A, lane 6; Fig. 3B, lane 6). We could not observe it for a longer time, because most cells died.

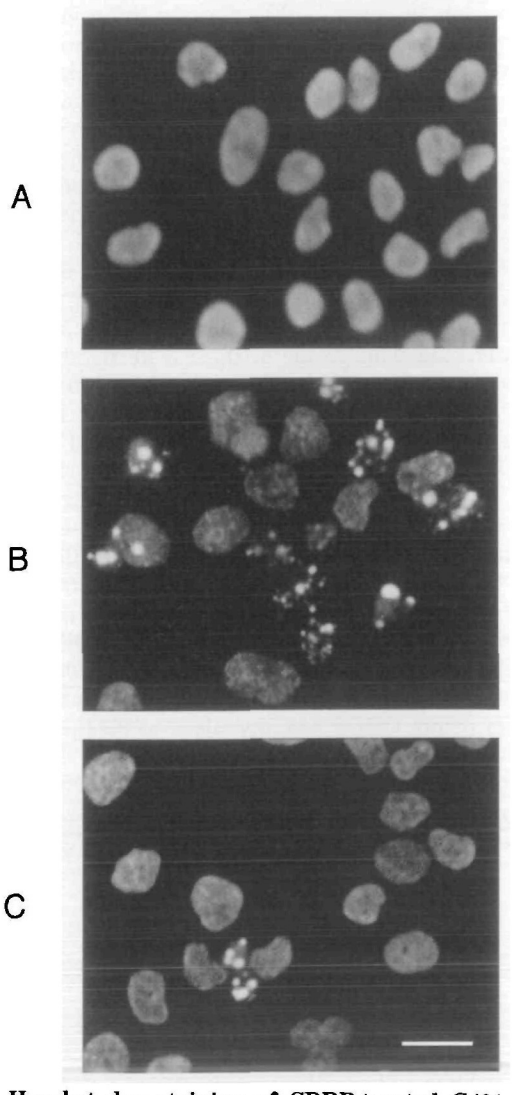


Fig. 5. **Hoechst dye staining of CDDP-treated G401 cells.** G401 cells were seeded onto cover slips and then treated as described under "MATERIALS AND METHODS." At 12 h, the cells were stained with Hoechst dye. The DNA staining pattern was examined under a fluorescence microscope, and photos of 6 fields in each condition were taken. A, control; B, CDDP, 100 μM; C, MK+CDDP, 100 ng/ml human MK and 100 μM CDDP. Bar, 40 μm.

*Exogenous MK Protein Rescues G401 Cells from CDDP-Induced Apoptosis*—Since CDDP suppressed MK expression (Figs. 2 and 3) as well as induced apoptosis, both *in vivo* and *in vitro* (Figs. 1 and 4), it was interesting to investigate the cause/consequence relationship between apoptosis and the reduction of the MK level. To do this, *in vitro* experiments were performed using G401 cells cultured in DMEM supplemented with 0.1% FCS. We chose this low concentration of FCS in order to minimize the effects of other growth factors in the serum, and under these conditions G401 cells could proliferate for several days and

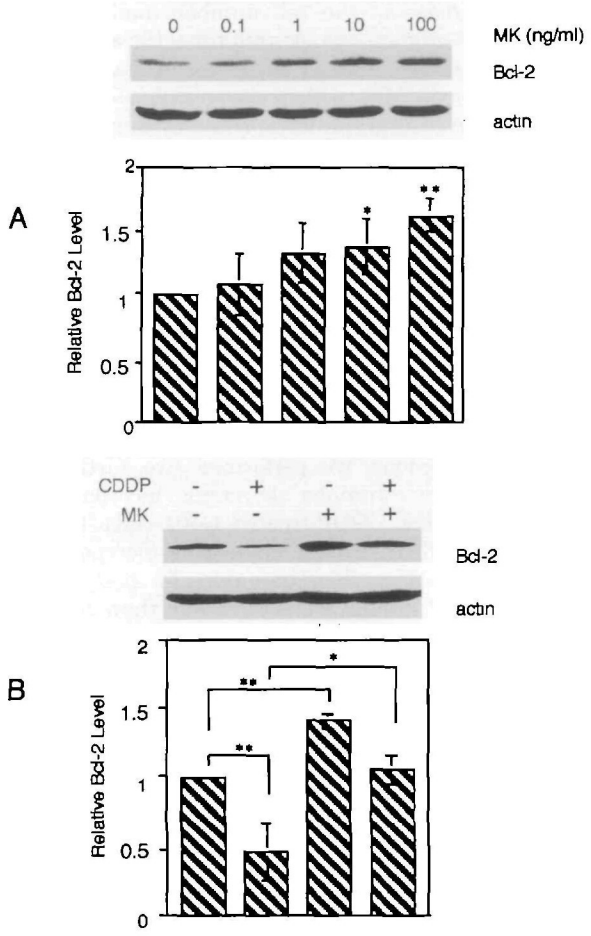


Fig. 6. **Bcl-2 protein expression in G401 cells upon treatment with MK and CDDP.** A, MK up-regulates Bcl-2 expression in a dose-dependent manner. G401 cells were pretreated with 0.1% FCS/DMEM for 12 h and then cultured with different concentrations (as indicated in the figure) of human MK for 12 h. Fifty micrograms protein of a whole-cell lysate was separated by 12% SDS-PAGE and then the protein was transferred to a nitrocellulose membrane. The membrane was blotted with anti–Bcl-2 antibodies (top panel), and then the same membrane was stripped and blotted with anti-actin antibodies (middle panel). The densimetric values of Bcl-2 were normalized as to actin, and the results of four independent experiments were summarized (bottom panel, mean ± SE). Representative results of Western blot are shown here. $^{*}p < 0.05$; $^{**}p < 0.01$ (paired *t* test; *versus* control). B, MK prevents the decrease in the Bcl-2 protein level induced by CDDP. G401 cells were treated as described for the MTT assay. Twelve hours after CDDP exposure, cells were lysed for Bcl-2 detection by immunoblotting. Upper panel, Bcl-2; middle panel, actin; lower panel, densimetric value (mean ± SE, $n = 3$). Representative results of Western blotting are shown here. $^{*}p < 0.05$; $^{**}p < 0.01$.

showed a negligible level of apoptosis, *i.e.* as low as that in 10% FCS. We added exogenous human MK to the culture system and then observed the CDDP-induced apoptosis. G401 cells were pretreated with MK at 100 ng/ml for 6 h and then exposed to 100 μM CDDP for 2 h. After removal of CDDP by washing, the cells were further incubated with medium supplemented with MK. First, we determined the total cell number by means of the MTT assay. Twelve hours after CDDP treatment, the cell number had decreased to 92.8% of the original one, while if exogenous MK at the concentration of 100 ng/ml was added, the cell number was 123.0%. At 24 h, the CDDP-treated group exhibited a cell number of 70.5% compared to the original one. MK inhibited this decrease in the cell number, maintaining it at 106.5% of the original one. At this time, the cell number in the MK+CDDP group was 1.5-fold that in the CDDP group (Fig. 4A). Mouse MK, which was purified with different methods, gave results similar to those by human MK (data not shown), confirming that MK but not some impurity exerted the effect. If MK was added simultaneously or after CDDP treatment, we did not observe any effect of MK on the cell number (data not shown). Thus, MK pretreatment appeared to be necessary for its cytoprotective activity toward CDDP-treated G401 cells.

The MTT assay can only determine the total cell number, which is the result of cell proliferation and cell death. There are two possible mechanisms for the maintenance of the cell number by MK: promotion of cell proliferation or/and inhibition of cell death. To determine through which mechanism MK functions, we performed two further experiments. First, we examined thymidine incorporation into human MK and/or CDDP-treated G401 cells. Twelve and 24 h after CDDP treatment, thymidine incorporation was greatly reduced (Fig. 4B). The values for both the CDDP and MK+CDDP groups were far lower than that for the control group. Therefore, MK did not affect the CDDP-induced decrease in thymidine incorporation in G401 cells. Second, we examined the morphological changes of CDDP-treated cells by means of Hoechst dye staining. Following CDDP treatment, G401 cells exhibited a typical apoptotic morphology, including nuclear condensation and chromatin fragmentation (Fig. 5). At 12 and 24 h, the percentages of apoptotic cells were 14.5 and 23.2%, respectively (Fig. 4C). While if human MK was added to the medium, the percentage of apoptotic cells was significantly decreased: with 10 ng/ml of MK, 7.4 and 10.2% at 12 and 24 h, respectively; and at 100 ng/ml of MK, 3.7 and 7.7% (Fig. 4C). The effects of mouse MK were comparable to those of human MK (data not shown). These results indicate that MK maintains the cell number by reducing apoptotic death, not by enhancing cell proliferation.

*MK Upregulates the Expression of Bcl-2 in G401 Cells and Maintains It Upon CDDP Exposure*—Members of the Bcl-2 protein family play important roles in regulating apoptosis. In a search for the mechanism(s) responsible for the cytoprotective effect of MK, we examined the expression of Bcl-2 and Bcl-$x_L$, both of which are known to be anti-apoptotic proteins. G401 cells expressed detectable endogenous Bcl-2, and human MK could enhance the Bcl-2 level in a dose-dependent manner (Fig. 6A). At the concentration of 100 ng/ml, MK increased the Bcl-2 level to around 1.6-fold. On the other hand, 2-h exposure to CDDP suppressed Bcl-2 expression in G401 cells (Fig. 6B). Twelve hours following CDDP administration, Bcl-2 expression was reduced to less than 50% of the control level (Fig. 6B, lane 2), whereas MK maintained the Bcl-2 level in CDDP-treated G401 cells (Fig. 6B, lane 4). The Bcl-2 level was approximately 2-fold in the MK+CDDP group as compared with that in the CDDP group (compare lanes 2 and 4 in Fig. 6B). Bcl-2 expression was also checked by Northern blotting (Fig. 7). In G401 cells three *bcl-2* transcript bands (8.5, 3.5, and 1.5 kb) were detected. The profile of the 8.5 kb band mRNA was comparable to that of the Bcl-2 protein: it was suppressed by CDDP (Fig. 7, lane 2), and enhanced by MK alone (Fig. 7, lane 3). MK prevented the CDDP-induced reduction of the 8.5 kb mRNA band (Fig. 7, lane 4). Mouse MK produced with different methods gave comparable results (data not shown). We also checked the Bcl-$x_L$ level in these cells, but it was barely detectable, and was affected by neither MK nor CDDP exposure (data not shown).

*CDDP Down-Regulates Bcl-2 Expression in the Mouse Kidney*—The above data showed that CDDP suppressed MK and Bcl-2 expression in cultured G401 cells, and that it suppressed the MK level in the mouse kidney. To determine whether or not CDDP has any effect on Bcl-2 expression *in vivo*, we analyzed the Bcl-2 protein level in the kidney of CDDP-treated mouse. After CDDP treatment, the Bcl-2 level began to decrease from the third day, reached the lowest level on the fifth day, and then gradually recovered from

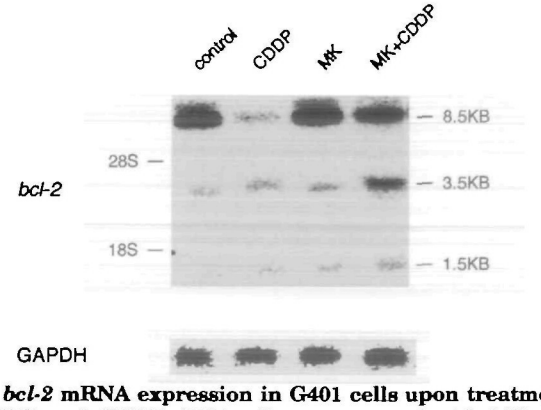


Fig. 7. ***bcl-2* mRNA expression in G401 cells upon treatment with MK and CDDP.** G401 cells were exposed with MK and CDDP as described in the legend to Fig. 6. Twelve hours after CDDP treatment, the cells were collected for RNA extraction. Twenty micrograms of total RNA was loaded on each lane. Upper panel, *bcl-2* expression; lower panel, GAPDH. Experiments were performed three times and gave similar results. Representative results are shown here.

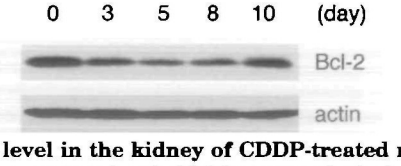


Fig. 8. **Bcl-2 level in the kidney of CDDP-treated mouse.** C57 BL/6J mice were injected with CDDP at 14 mg/kg-body-weight and then BUN was monitored as described in the legend to Fig. 1. At the indicated times, mice were sacrificed and their kidneys were removed for protein extraction. For each sample, 50 μg of protein was separated by 12% SDS-PAGE, and then Bcl-2 and actin were detected successively as in Fig. 6.

the eighth day (Fig. 8). Therefore, the profile of Bcl-2 expression was similar to that of MK (Figs. 2 and 8).

## DISCUSSION

In this paper, we report the cytoprotective activity of MK. CDDP induced apoptotic cell death in both murine kidney and cultured G401 cells, as shown by TUNEL and Hoechst dye staining, respectively (Figs. 1 and 5). In these two systems, a transient reduction of MK expression at both the mRNA and protein levels was observed (Figs. 2 and 3). The results were reminiscent of the enhanced programmed cell death of *C. elegans* with the loss-of-function mutation of *ced-9*, which encodes the Bcl-2 homologue of *C. elegans* (*41*). In fact, exogenous MK partially protected G401 cells from CDDP-mediated apoptosis (Fig. 4), and during this process, MK restored Bcl-2 expression (Figs. 6 and 7). These data imply that MK acts as an anti-apoptotic growth factor, and suggest that MK may exert the activity at least in part through the regulation of Bcl-2 expression. Pretreatment of cells with MK is necessary for the cytoprotective effect of MK, suggesting that MK activity is mediated through enhancement of the Bcl-2 level in advance.

MK has been implicated in the process of carcinogenesis. The relevant biological functions reported are promotion of cell growth, enhancement of angiogenesis and fibrinolysis, and induction of oncogenic transformation of NIH 3T3 cells (*10–14*, *18*, *19*, *42*). In addition to these functions, the present study provides evidence that MK can give cancerous cells a growth advantage through an anti-apoptotic activity. It is noteworthy that soluble (*43*) as well as plate-coated MK enhances neuronal survival (*10*, *35*). Moreover, MK expression is induced around the lesions in cerebral or heart infarction (*44*, *45*). MK also prevents the degeneration of photoreceptor cells induced by constant light exposure (*36*). Taking these data into account, the present data suggest that the cytoprotective function of MK is commonly involved in various physiological and pathological phenomena including carcinogenesis, neurogenesis and tissue repair.

CDDP has been reported to reduce Bcl-2 protein expression in other cell lines (*30–32*). In addition to Bcl-2 protein changes, on Northern blotting, we also detected three bands of Bcl-2 mRNA, 8.5, 3.5, and 1.5 kb (Fig. 7). These transcripts are derived through different splicing, and only the 8.5 kb mRNA contains all exons and therefore encodes the full length Bcl-2 protein (*46*). The level of 8.5 kb band mRNA was suppressed by CDDP and enhanced by MK (Fig. 7), suggesting that at the transcription level *bcl-2* gene expression is regulated by these two stimuli. Since the ratio of *bcl-2* transcripts appears to be influenced by various stimuli, *e.g.* MK, CDDP, and MK+CDDP (Fig. 7), these stimuli may also regulate the machinery of splicing. In addition, the changes at the transcription level appear to be more prominent than those at the protein level (Figs. 6 and 7), suggesting that the post-transcriptional regulation is partly involved in the regulation of *bcl-2* expression by MK.

The mechanisms by which Bcl-2 rescues cells from chemotherapy-induced apoptosis have not been completely defined. Zhang *et al.* recently reported that Bcl-2 blocks the whole apoptotic process in leukemia cells induced by chemotherapeutic reagents, such as etoposide and vincristine (*47*). DEVD, an inhibitor of caspase, only inhibits the final step of apoptosis, the execution phase (release of apoptotic bodies and vesicles), i.e. not the initial stages, namely apoptotic body formation, suggesting that Bcl-2, compared with DEVD, may act at a proximal step in the apoptotic pathway (*47*).

Bcl-2 expression has been reported in renal neoplasms, including Wilms' tumors, and is supposed to contribute to the development and progression of these tumors (*48*, *49*). Considering the high expression of MK in Wilms' tumors (*40*), and its ability to upregulate Bcl-2 expression, it is likely that MK and Bcl-2 collaborate during the oncogenesis process in renal tumors. Furthermore, MK expression is suppressed by WT1, the product of a tumor suppressor gene (*50*). Thus, the functional interactions of these carcinogenesis-related proteins, namely WT1, MK and Bcl-2, remain to be investigated.

Bcl-2 expression is detected in fetal kidney during embryogenesis and also in adult kidney (*48*, *51*, *52*). It is speculated that Bcl-2 is involved in kidney organogenesis (*52*, *53*). In fact, Bcl-2–deficient mice exhibit disorganized development of the kidneys and die of renal failure due to polycystic kidneys when a few months old (*54*, *55*). In addition, because the kidney metabolizes most electrolytes and some body waste, it is an organ in which cell renewal is prominent. Bcl-2–regulated apoptosis may play pivotal roles in maintaining the structure and function of the kidney. As the sites and time course of MK expression are correlated with those of Bcl-2 expression in the fetal and adult kidney (*22*, *23*, *52*), and the expression profiles of MK and Bcl-2 are consistent in the kidney of CDDP-treated mouse (Figs. 2 and 8), we speculate that there might be some interactions between the MK and Bcl-2 functions during kidney development and in maintenance of the homeostasis of adult kidney.

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