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Cellular Toxicity of Cadmium Ions and Their Detoxification by Heavy Metal–Specific Plant Peptides, Phytochelatins, Expressed in Mammalian Cells[1]

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The apoptotic cell death of Jurkat cells due to Cd^{2+} toxicity was studied by fluorescence microscopic observation and DNA fragmentation assaying. It was suggested that the apoptotic response to Cd^{2+} was less clear than that to a typical apoptosis inducer, ultraviolet light (254 nm). Examination of MAP kinase phosphorylation (p38, JNKs, and c-Jun) due to Cd^{2+} toxicity indicated that the phosphorylation was very slowly activated (4 h after stimulation), while UV light could activate the phosphorylation immediately. Therefore, it was suggested that Cd^{2+} may not be a typical apoptosis inducer. Antioxidants [glutathione (GSH) and *N*-acetylcysteine (NAC)] could detoxify Cd^{2+}, indicating that the toxicity is a kind of oxidative stress. The detoxification effect of antioxidants showed cooperation with Bcl-2, suggesting that Cd^{2+}-treatment causes diversified toxic signals including oxidative stress. On the addition of a plant-specific peptide, phytochelatin [PC_7, $(\gamma Glu\text{-}Cys)_7$-Gly], to the medium, the detoxification of Cd^{2+} and cooperation with Bcl-2 were more intense than in the cases of GSH and NAC. Using an appropriate vector, a PC synthase gene was transferred from *Arabidopsis thaliana* to the Jurkat cell. The transfectant exhibited resistance to Cd^{2+} and production of plant-specific PC ($PC_{2\text{-}6}$).

Key words: cadmium ion, MAP kinase, oxidative stress, phytochelatin, phytochelatin synthase.

Cd^{2+} is an environmental pollutant with well-known mutagenic, carcinogenic, and teratogenic effects (*1*). Cd^{2+} is known to accumulate in the human kidney for a relatively long time and at high doses, and is also known to have harmful effects on the respiratory system and has been associated with bone diseases (*1, 2*). At the molecular and cellular levels, a lot of studies on apoptosis and stress kinase activation by Cd^{2+} have been performed (*3–7*). However, possibly because diverse cellular responses to the toxicity of Cd^{2+} were included, the mechanism of the toxicity is not fully understood.

Plants respond to heavy metal toxicity *via* a number of mechanisms. One such mechanism involves the chelation of heavy metal ions by a family of peptide ligands, the phytochelatins [PCs, $(\gamma Glu\text{-}Cys)_n$-Gly, $n \geqq 2$] (*8–10*). Recently, we reported a rapid method for the detection and detoxification of heavy metal ions in water environments involving PCs (*11*). Moreover, molecular genetic approaches have brought important advances in our understanding of phytochelatin (PC) biosynthesis. Genes encoding the PC synthases from *Arabidopsis thaliana, Schizosaccharomyces pombe,* and wheat have been reported. These genes, designated as *AtPCS1* (*CAD1*), *SpPCS,* and *TaPCS1*, respectively, encode 40–50% sequence-similar 50–55 kDa polypeptides active in the synthesis of PC from GSH (*12–14*). Mammalian cells can not synthesize PC because of their lack of the key enzyme for phytochelatin biosynthesis, PC synthase.

In this study, we examined the apoptotic cell death caused by Cd^{2+} and activation of stress kinases in comparison with a typical apoptosis inducer, UV light (254 nm). The effects of antioxidants and PC on the detoxification of Cd^{2+} were also studied. Furthermore, we attempted to express the plant-specific peptides (PCs) in mammalian cells by transfecting Jurkat cells with a plant PC synthase gene (*AtPCS1*).

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Abbreviations: GSH, glutathione reduced form; JNKs, c-Jun N-terminal kinases; MAP kinase, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; PC, phytochelatin; UV, ultraviolet.

MATERIALS AND METHODS

Antibodies—Antibodies against p38, JNKs and c-Jun, and their phosphorylated forms were purchased from New England Biolabs (Beverly, MA, USA). Alkaline phosphatase-conjugated secondary antibodies were from ICN Biochemicals Inc. (Costa Mesa, CA, USA).

Phytochelatin—Homogeneous phytochelatin (PC_7) was chemically synthesized by a slightly modified Fmoc-method, as explained in our previous paper (*11*).

Cell Treatments and Cell Viability Assay—Jurkat cells, the human leukemic T cell line, were used, and cells transfected with the pUC-CAGGS vector (*15*) with the human *bcl-2* gene inserted (*16*) (Jurkat Bcl-2 cells), and with the pUC-CAGGS vector (Jurkat CAGGS cells) were prepared. All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. Exponentially growing cells at 60–80% confluency were diluted to a concentration of 5×10^4 cells/ml, and then a stock solution of 10 mM $CdCl_2$ was added to the culture medium to give final concentrations of 10–100 μM. The cells were incubated for 48 h. For UV irradiation, the cells were placed in a tissue culture dish and then exposed to UV light at an intensity of 1 W/m^2 for 1 min (about 40 J/m^2) using a UV-C (254 nm) light source (Spectroline ENF-260 C/J; Spectronics Co., NY, USA). Cell viability was measured with a Cell Titer 96 AQ_{ueous} Cell Proliferation assay kit (Promega, Madison, WI, USA).

Observation of Apoptosis and DNA Fragmentation—Fluorescence microscopic assaying was performed as described previously (*17*) to determine the apoptotic cell death. Hoechst 33342 and propidium iodide were added to the medium to final concentrations of 30 and 10 μM, respectively. After incubation at 37°C for 10 min, the cells were examined under a fluorescence microscope (Olympus BX50, Tokyo) with UV excitation at 360 nm. The nuclei of viable, necrotic, early apoptotic, and late apoptotic cells were blue round, pink round, fragmented blue and fragmented pink, respectively. DNA fragmentation was detected with an ApopLadder EX kit (Takara Biomedicals, Kyoto).

Western Blotting—Whole cell proteins were fractionated by 12% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk in TBS-T buffer [30 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5], the membrane was incubated with primary antibodies at 4°C for 16 h, followed by extensive washing with TBS-T buffer. Then the membrane was incubated with secondary antibodies at room temperature for 1 h. After the washing step, the immune complexes were detected with an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Uppsala, Sweden).

Plasmid Construction—The cDNA for the PC synthase gene (*AtPCS1*) was amplified by PCR using the cDNA phage library of *A. thaliana* (Arabidopsis Biological Resource Center, Columbus, OH, USA) as a template. For the PCR reaction, primers [PSF1, 5′-ACCATGGCTATGGCGAGTTTATATCGGCG-3′ (containing an *Nco*I site, underlined); PSR1, 5′-CGGGATCCCTAATAGGCAGGAGCAGCGAGATCATCC-3′ (carrying a *Bam*HI site, underlined)] were used for amplification of 1.5 kbp cDNA containing the whole coding region of the PC synthase. The amplified cDNA was digested with *Bam*HI, and then subcloned into the pUC18 plasmid digested with *Hinc*II and *Bam*HI (Takara Biomedicals). The nucleotide sequence of the fragment was compared with the reported nucleotide sequence of the PC synthase gene (*13*). The cDNA was digested with *Hin*dIII and *Bam*HI, and then the fragment was inserted into the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) so that the gene would be expressed under the cytomegalovirus (CMV) promoter. The resultant plasmid was designated as pcDNA3-PCS.

Transfection—Electroporation was performed according to the protocol recommended by the manufacturer. Briefly, 1×10^7 cells subcultured 3 days earlier (60–80% confluent) were mixed with 60 μg of plasmid DNA, which was digested with *Bam*HI into its linear form, in a total volume of 0.5 ml of phosphate-buffered saline (PBS). Electroporation was performed at $V = 470$ V, $C = 975$ microfarads using a Gene Pulser II (Bio-Rad, Richmond, CA). Following electroporation, the cells were cultured for 1 day in RPMI 1640 medium supplemented with 10% FBS, and for 14 days with 10% FBS and 800 μg/ml G418.

Fluorescence In Situ Hybridization (FISH)—FISH was carried out to detect a plant gene (*AtPCS1*) in mammalian cells by the procedures reported by Ohmido and Fukui (*18*). In order to prepare a DNA probe of *AtPCS1*, a DNA fragment corresponding to the sequence of nucleotide numbers 470–1121 of *AtPCS1* was amplified, and labeled with biotin-16-dUTP (Roche Molecular Biochemicals, Böehringer Mannheim, GmbH, Germany) simultaneously, from the pcDNA3-PCS by PCR using primers (PSF2, 5′-GAAAAGTGACCATTCCCAGTCTGC-3′; PSR2, 5′-TGAAGACAGTCTGACTTATGCTGC-3′). The labeled probe was hybridized to the metaphase chromosomes derived from transfected Jurkat cells with the pcDNA3-PCS gene in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in a mixture of Fluorescein Avidin DCS and Biotinylated anti-Avidin D (Vector Laboratories, Burlingame, CA, USA), followed by counterstaining with 4′,6-diamidino-2-phenylindole (Sigma, St. Louis, MO, USA).

Detection of PC Synthesis in Jurkat Cells—The postcolumn derivatization method involving DTNB (5,5′-dithiobis-2-nitrobenzoic acid) was used for sensitive detection of PCs in extracts of Jurkat cells. Cells were exposed to 20 μM $CdCl_2$ for 40 h, and then harvested by centrifugation (100 ×*g*, 5 min). After the addition of 700 μl of 0.5 N NaOH containing 0.5 mg/ml $NaBH_4$, the cells were sonicated for 3 min and then kept on ice for 10 min to reduce the disulfides. The extracted sample was neutralized by the addition of 1 ml of 3.6 N HCl to stop the reducing reaction with $NaBH_4$ and then centrifuged (5,000 ×*g*, 10 min, 4°C) to remove insoluble debris. The clear supernatant was subjected to reversed-phase high-performance liquid chromatography (HPLC) [column: Hibar HPLC-cartridge 250-4 LiChrospher 100RP-18 (5 μM); solvent system: A: 0.02% TFA, 5.0 mM octanesulfonic acid, B: 100% acetonitrile, 0.02% TFA, 5.0 mM octanesulfonic acid; gradient 13–30% B in 30 min; flow rate, 1.5 ml/min], and the eluted sample was continuously mixed with the thiol-reactive solution (10% acetonitrile, 75 μM DTNB, 50 mM potassium phosphate, pH 8.0) to detect the PC and GSH at 412 nm.

RESULTS

Observation of Apoptotic Bodies and DNA Fragmentation—Jurkat cells were exposed to $CdCl_2$, and then apoptotic responses such as morphological changes in the cell nucleus and fragmentation of the chromosomal structure into nucleosomal units (*ca.* 180 bp) were examined. The results were compared with in the case of UV irradiation (254 nm), a typical apoptotic stress signal. The ratio of apoptotic bodies to the total dead cells with Cd^{2+} toxicity (*ca.* 4%) was much lower than that with UV irradiation (*ca.* 70%) (data not shown). Moreover, smear DNA fragmentation could only be observed with around 30 μM $CdCl_2$ (Fig. 1, lane 3), and no DNA fragmentation could be detected with higher concentrations (50 μM) (Fig. 1, lane 4), although very clear DNA fragmentation could be observed in the case of UV light (Fig. 1, lane 1). These results indicate that Cd^{2+} could not induce typical apoptotic cell death.

Activation of p38 and JNK Signal Pathways by Cd^{2+}—The activation of MAP kinase pathways by Cd^{2+} toxicity was studied by detecting phosphorylated p38, JNKs, and c-Jun in cells exposed to $CdCl_2$. With 30 μM $CdCl_2$, phosphorylation of p38 and JNKs in Jurkat CAGGS was induced at 4 and 8 h after the exposure, respectively (Fig. 2B). It was also shown that the stimulation of the p38 pathway by Cd^{2+} was stronger than that of the JNK pathway (Fig. 2B). In the case of UV light, the levels of phosphorylated p38 and JNKs markedly increased soon after the irradiation (Fig. 2A). These results indicated that the p38 and JNK pathways were very slowly activated by Cd^{2+}, while the pathways were immediately activated by UV light. This delayed and weak activation of MAP kinases is consistent with the above-mentioned findings on microscopic observation and DNA fragmentation assaying of apoptotic cell death caused by Cd^{2+}.

Effects of Bcl-2 on Cd^{2+} Toxicity—Bcl-2 is a well-known suppressor of apoptosis (*19, 20*). Indeed, when Jurkat Bcl-2 cells expressing a higher level of Bcl-2 were exposed to Cd^{2+}, the intensity of expression and phosphorylation of p38 and JNKs were weaker than in the case of Jurkat CAGGS cells (Fig. 2, B and C). Therefore, to determine whether or not Bcl-2 can suppress Cd^{2+} toxicity in Jurkat cells, Jurkat Bcl-2 cells were exposed to 10–100 μM $CdCl_2$ and then cell viability was examined. Suppression of Cd^{2+} toxicity by Bcl-2 could be observed at 20–60 μM $CdCl_2$ (Fig. 3A), but the most intensive effect was observed at 30 μM. Jurkat Bcl-2 cells were completely resistant up to 30 μM $CdCl_2$, while 40% of the control cells could not survive at 30 μM $CdCl_2$. This is in good agreement with the finding that apoptotic cell death and DNA fragmentation were observed

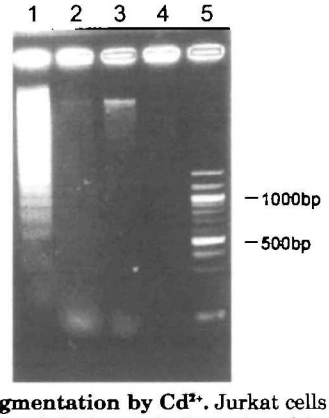

Fig. 1. **DNA fragmentation by Cd^{2+}.** Jurkat cells (5×10^4 cells/ml) were exposed to UV light for 1 min (lane 1), nothing, control (lane 2), or 30 μM $CdCl_2$ (lane 3), or 50 μM $CdCl_2$ (lane 4) for 6 h, respectively. Lane 5, molecular size markers.

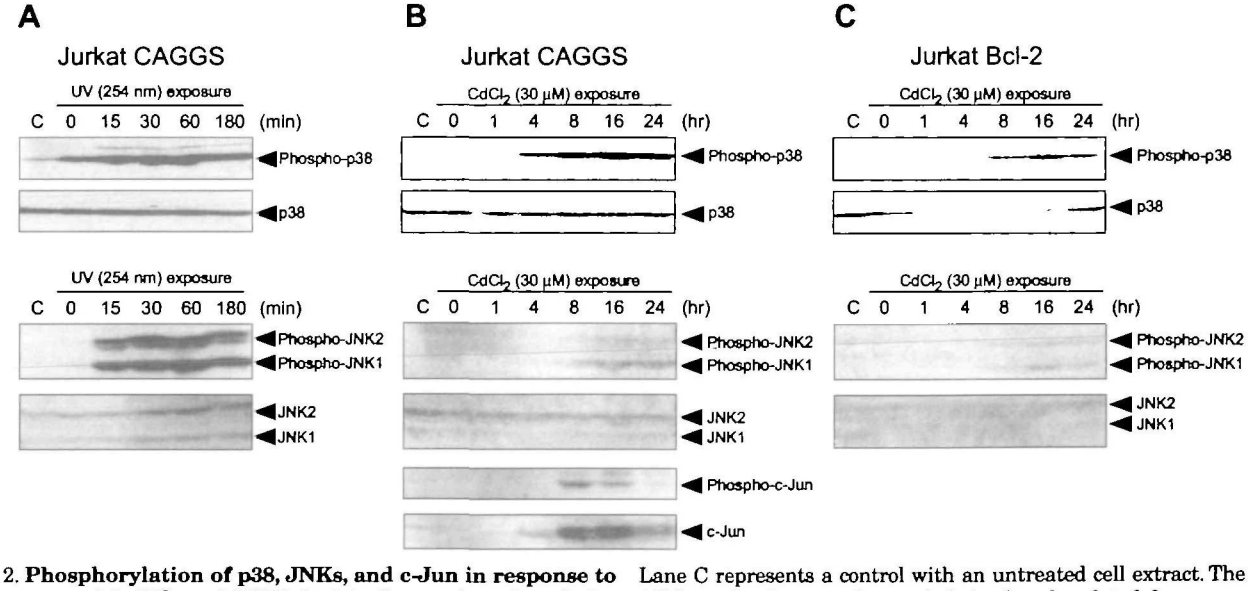

Fig. 2. **Phosphorylation of p38, JNKs, and c-Jun in response to treatment with Cd^{2+} and UV light.** The figures show the relative levels of phosphorylated p38 and JNKs in Jurkat CAGGS cells treated with UV light (A), and 30 μM $CdCl_2$ (B), and in Jurkat Bcl-2 cells treated with 30 μM $CdCl_2$ (C). The phosphorylation of c-Jun in Jurkat CAGGS cells treated with 30 μM $CdCl_2$ is also shown (B). Lane C represents a control with an untreated cell extract. The p38, JNKs, and c-Jun proteins, and their phosphorylated forms were detected with corresponding antibodies. The two bands with molecular weights of 46 and 55 kDa detected with anti-JNK antibodies correspond to JNK1 (*39*) and JNK2 (*40*), respectively.

optimally at 30 μM $CdCl_2$.

Effects of Antioxidants on Cd^{2+} Toxicity—Jurkat Bcl-2 and Jurkat CAGGS cells were incubated respectively in complete medium containing an antioxidant, GSH or NAC (equivalent to 500 μM thiol group), with 10–100 μM $CdCl_2$. With the addition of GSH or NAC to the medium, suppression of Cd^{2+} toxicity could be observed within the range of 30–70 μM $CdCl_2$, and the effect of NAC was slightly stronger than that of GSH (Fig. 3B). When Jurkat Bcl-2 cells were used, cell growth was completely inhibited at 70 μM $CdCl_2$. However, GSH and NAC cooperatively suppressed Cd^{2+} toxicity with Bcl-2 with a wider range of $CdCl_2$ concentrations (Fig. 3C).

Detoxification of Cd^{2+} by PC—Phytochelatin (PC) is a heavy metal ion binding peptide produced by plants, algae, and some fungi (*8–10*). With the addition of the chemically synthesized PC_7 (equivalent to 500 μM thiol group) to the culture medium containing 10–100 μM $CdCl_2$, the cell survival ratio of Jurkat CAGGS cells clearly recovered (Fig. 4A). Furthermore, in the case of Jurkat Bcl-2 cells, the cooperative suppression by PC_7 and Bcl-2 of Cd^{2+} toxicity became more significant (Fig. 4B) than in the cases of GSH and NAC (Fig. 3C). Interestingly the cell survival ratio of Jurkat Bcl-2 cells in the medium containing PC_7 exceeded 100% in the range of 40–60 μM $CdCl_2$.

Transfection of Jurkat Cells with the PC Synthase Gene—To determine whether or not PC can be synthesized in mammalian cells with the introduction of the PC synthase gene (*AtPCS1*) from *A. thaliana*, the *AtPCS1* gene was amplified by PCR and then inserted into the pcDNA3 vector to construct pcDNA3-PCS. The plasmid DNA was transfected into Jurkat cells and stable transfectants were selected with an antibiotic (G418). The transfection of Jurkat cells with pcDNA3-PCS was confirmed by PCR using primers specific to *AtPCS1*, a positive signal corresponding to the predicted size of the gene being observed (1.5 kbp) (Fig. 5B). Furthermore, with FISH, the plant gene, *AtPCS1*, integrated into the medium-sized chromosome of the Jurkat cells could be clearly observed (Fig. 5C). From the condensation pattern and morphological features of the fluorescein-probe bound chromosome, we expected the integration of *AtPCS1* had occurred on chromosome 12. Further analysis is required to determine the correct chromosomal number. The constructed transfectant, *i.e.* Jurkat cells carrying *AtPCS1*, was designated as Jurkat PCS.

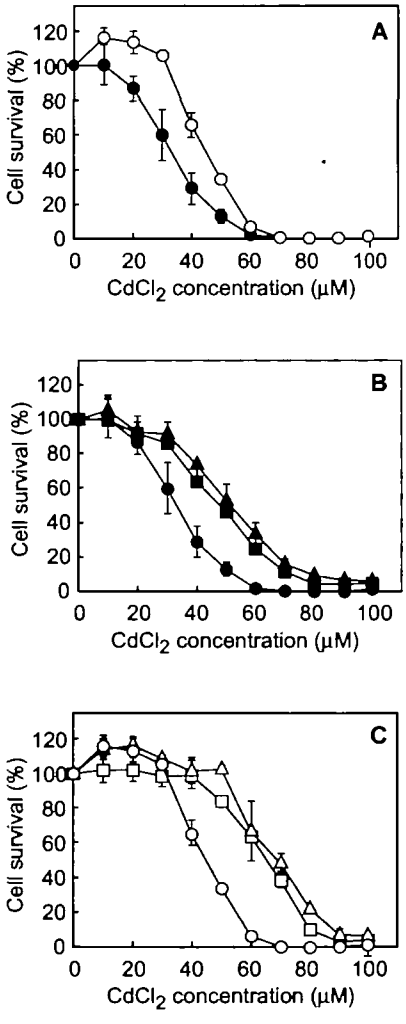

Fig. 3. **Effect of Bcl-2 on Cd^{2+} toxicity and cooperative detoxification with an antioxidant (GSH or NAC).** Jurkat Bcl-2 (open circles) and Jurkat CAGGS cells (closed circles) were treated with 10–100 μM $CdCl_2$ (A). The cell survival ratio in medium containing $CdCl_2$ (10–100 μM) with GSH (squares) or NAC (triangles), or without an antioxidant (circles) was determined for both Jurkat CAGGS (B) and Jurkat Bcl-2 cells (C). Antioxidants, GSH and NAC, equivalent to 500 μM thiol group were added to the medium, respectively. Error bars represent ± standard deviation for three separate experiments.

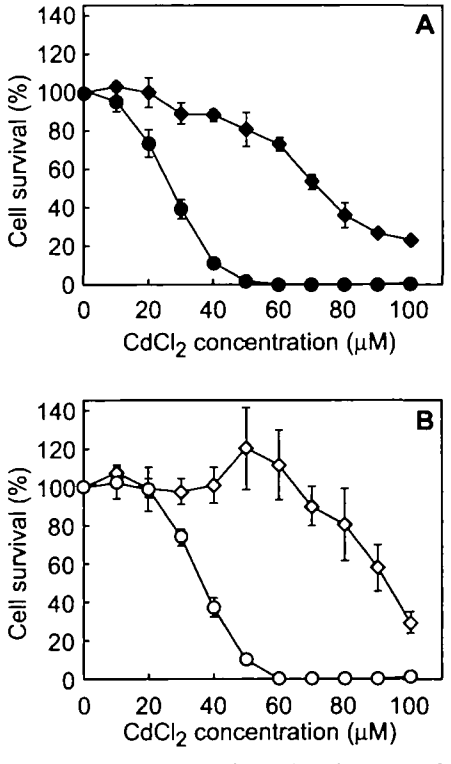

Fig. 4. **Cooperative effect of PC_7 and Bcl-2 on Cd^{2+} toxicity.** The cell survival ratios of Jurkat CAGGS cells (A) and Jurkat Bcl-2 (B) treated with 10–100 μM $CdCl_2$ with (diamonds) or without PC_7 (circles) were determined. PC_7 equivalent to 500 μM thiol group was added to the medium. Error bars represent ± standard deviation for three separate experiments.

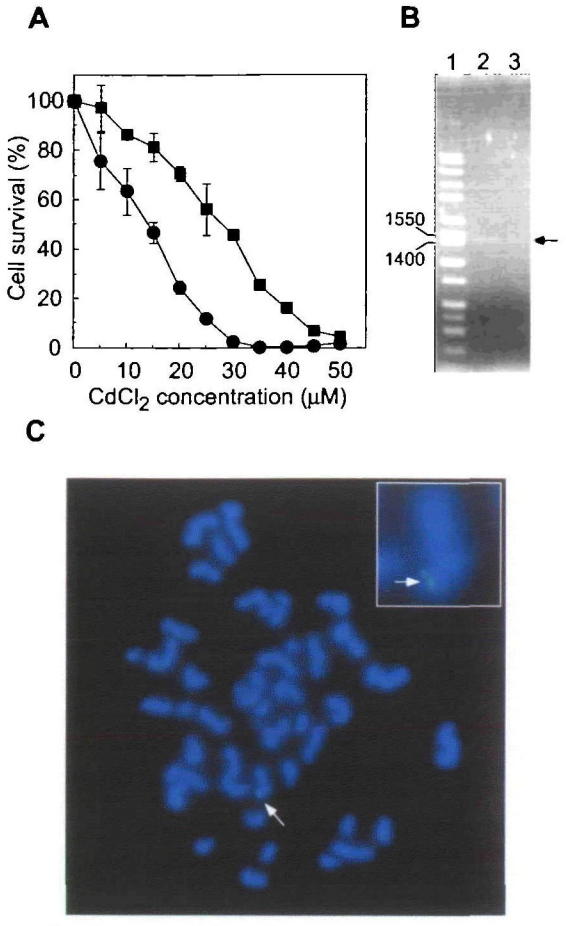

Fig. 5. **Cd^{2+} tolerance of *AtPCS1* gene-expressing Jurkat cells.** (A) Jurkat PCS (carrying the pcDNA3-PCS plasmid) (squares) and Jurkat DNA3 cells (carrying the empty pcDNA3 plasmid) (circles) were treated with 10–50 μM $CdCl_2$ for 48 h. Error bars represent ± standard deviation for three separate experiments. (B) The PCR-amplified products in Jurkat PCS (lane 2) and Jurkat CAGGS (lane 3) cells with the same primers as used for amplification of the *AtPCS1* gene were separated on a 1% agarose gel. Lane 1, molecular weight standards (Wide-Range Ladder; Takara Biomedicals, Kyoto, Japan). (C) FISH analysis of the metaphase chromosome using the biotin-labeled *AtPCS1* sequence (stained green). DNA was counter-stained with 4′,6-diamidino-2-phenylindole. The chromosome with green spots is enlarged.

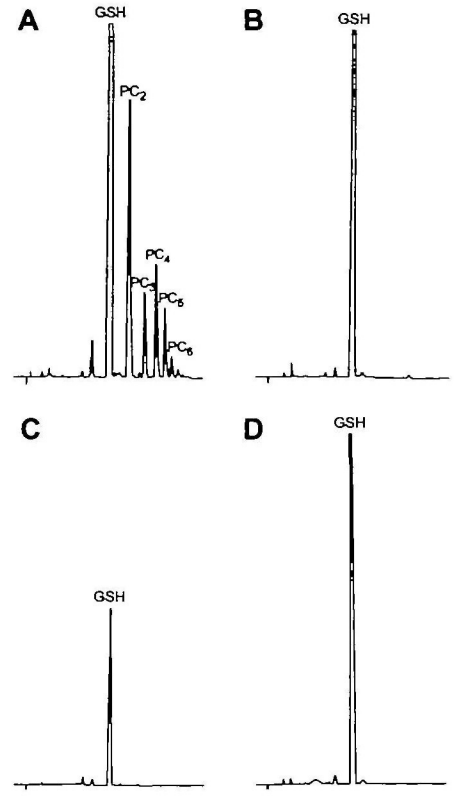

Fig. 6. **PC synthesis in Jurkat cells mediated by the *AtPCS1* gene.** Cell extracts of Jurkat PCS and Jurkat DNA3 cells were analyzed by HPLC using the postcolumn derivatization method. Jurkat PCS cells were treated with 20 μM $CdCl_2$ (A) or left untreated (B). As an experimental control, Jurkat DNA3 cells were also treated with (C) or without (D) 30 μM $CdCl_2$. The peaks designated as "GSH," "PC_2," "PC_3," "PC_4," "PC_5," and "PC_6" were identified as such on the basis of their retention times, respectively, compared with native PCs extracted from *Silene cucubalus* (*33*).

The tolerance to $CdCl_2$ (10–50 μM) and PC synthesis were examined, and compared with in the case of control cells transfected with pcDNA3 (Jurkat DNA3 cells). The survival ratio of Jurkat PCS cells was significantly higher than that of Jurkat DNA3 cells (Fig. 5A), indicating that PC synthase was expressed in mammalian cells and functioned properly. A cell lysate of the Jurkat PCS cells was analyzed by HPLC using a postcolumn derivatization method, as explained above. In the case of Jurkat PCS cells treated with 20 μM $CdCl_2$, PC synthesis corresponding to PC_2-PC_6 could be clearly observed (Fig. 6A), indicating that the PC synthase gene from *A. thaliana* could catalyze PC synthesis even in Jurkat cells. The intracellular concentrations of GSH and PC were estimated based on the cell size (12.5 μm diameter) and the HPLC profile (Fig. 6A). In the case of Cd^{2+}-untreated Jurkat-PCS cells, only GSH could be detected, its intracellular concentration being estimated to be 2.23 mM. In the case of 30 μM Cd^{2+}-exposed cells, the intracellular concentrations of GSH and PC (at the level of the γGlu-Cys unit) were 2.58 and 0.8 mM, respectively. These results indicated that the intracellular concentration of GSH was maintained even after induction of PC production. Considering that GSH is a non-allosteric effector for feedback inhibition of the rate limiting step of GSH synthesis (γGlu-Cys synthetase), PC did not inhibit γGlu-Cys synthetase and significantly increased the number of intracellular γGlu-Cys units. In cooperation with GSH, PC made the intracellular conditions less oxidative and tolerant to Cd^{2+} toxicity.

Furthermore, it has been reported that the activity of the PC synthase from *A. thaliana* was posttranslationally enhanced by Cd^{2+} (*21*). Indeed, the activity of the PC synthase in Jurkat cells was also stimulated by Cd^{2+}. Therefore, not only the catalytic activity but also the activation mechanism of the plant PC synthase was functional in the mammalian cells (Fig. 6).

DISCUSSION

Cell Death Due to Cd^{2+}-Induced Oxidative Stress—It has been reported that Cd^{2+} could induce apoptosis in various

types of cells (*22–27*). In this study, however, we showed that Cd^{2+}-induced apoptotic cell death was not dose-dependent, and that both apoptotic bodies and DNA fragmentation were not clear in comparison with in the case of the typical apoptosis induced by UV irradiation (Fig. 1). Western blotting analyses clearly showed that stress kinases such as p38 and JNKs were very slowly activated (>4 h) (Fig. 2), although they were activated immediately after the stimulus of UV irradiation. These observations indicated that Cd^{2+} was not necessarily a strong inducer of apoptosis, although the frequency of apoptotic cell death is dependent on the type of cell. The death signal caused by Cd^{2+} toxicity must be diversified for different signal cascade(s) causing cell death.

At present, based on the observation of the suppressive effect of Bcl-2 or an antioxidant (NAC or GSH) on Cd^{2+} toxicity (Fig. 3, A and B), we hypothesize that Cd^{2+}-induced stress is a kind of oxidative stress. Indeed, the toxicity of Cd^{2+} has been reported to interfere with antioxidant enzymes and radical scavengers such as superoxide dismutases, peroxidases, and catalases (*28, 29*). The higher level of cytosolic reactive oxygen species (ROS), known as cell death signals (*30*), produced by Cd^{2+} might be removed by these enzymes and scavengers. Bcl-2 has broad anti-apoptotic effects by inhibiting the production of ROS and enhancing the steady state of mitochondrial transmembrane functions (*19*). Indeed, regarding our experimental results, MAP kinase activation in the case of Jurkat Bcl-2 was weaker than in that of control cells, although the interval before activation was the same (>4 h after stimulation) (Fig. 2C). Cd^{2+}-induced cell death could be very effectively suppressed by the cooperative effect of antioxidants and Bcl-2 (Fig. 3C). These results further supported that Cd^{2+} toxicity is diversified, including a kind of oxidative stress, and related to ROS production.

Cooperative Effect of PC and Bcl-2 on Cd^{2+} Detoxification—The most significant difference between PC and antioxidants (NAC or GSH) is that PC could physically chelate Cd^{2+} stronger than GSH (*11, 31*). Indeed, the effect of detoxification by PC added to the medium was much stronger than that of other antioxidants (Fig. 4A). However, in terms of cooperation, that of PC and Bcl-2 was more significant than in the cases of GSH and NAC (Fig. 4B), indicating that PC might be functional in not only the physical chelation of Cd^{2+} but also the suppression of the diversified toxicity of Cd^{2+} including oxidative stress and ROS production.

PC Synthesis in Jurkat Cells—The Cd^{2+} tolerance in mammalian cells is dependent on metallothionein, but in plants and algae it is mainly dependent on PCs. PC-deficient plants have been reported to be very sensitive to Cd^{2+} (*32*). We assumed that these PCs are important antioxidants that decrease the harmful oxidative stress due to UV light and heat as well as heavy metal ions. It would be interesting to determine how plant-specific PCs can function in mammalian cells whose vacuoles are not well developed like those in plant cells. Cell extracts of the transfectant, Jurkat PCS cells, apparently contained PCs of different sizes (PC_{2-6}) (Fig. 6A). Interestingly, the lengths of the PCs found in transfectants of mammalian cells are longer than those reported for plant cells (PC_{2-4}) (*33*). Without any physical stress, the intracellular concentration of GSH in mammalian cells is generally maintained at 1–8 mM (*34*). On the contrary, in *A. thaliana* cells, except in chloroplasts, the concentration of GSH is maintained at lower concentrations (50–250 μM) (*35*). Although further experiments are needed, this difference in the intracellular GSH concentration might be the reason for the synthesis of longer PCs in transfected mammalian cells. Nevertheless, it is interesting to note that the plant *AtPCS1* gene can be utilized to enhance the resistance to Cd^{2+} toxicity of mammalian cells (Fig. 5A). Using PC-expressing mammalian cells, the examination of tolerance against other types of external stress including UV light, heat shock and magnetism is now in progress. Additionally, analysis of the diversified cell death signals of Cd^{2+} is also in progress, involving DNA profiling with DNA chips.

Cd^{2+} Toxicity to Mammalian Cells—One possible mechanism for the diverse death signals might start with the membrane lipid peroxidation by ROS induced by Cd^{2+}. As many recent studies suggested, the process of cell death caused by Cd^{2+} toxicity might be closely related to the inflammatory process *via* the arachidonic cascade (*36, 37*), rather than the apoptotic signal cascade represented by stress kinase activation. Recently, it was reported that p38 is more important for oxidative stress and the inflammatory process (*38*) than JNK pathways. Our observation of stress kinase activation also showed that activation of the p38 signal was slightly stronger than that of JNK signals, although both types of signals were equally, strongly, and immediately activated by UV light (Fig. 2A). Therefore, we considered that this stronger activation of p38 might be characteristic of a death signal starting from ROS, and the result further supported the important linkage between oxidative stress and the inflammatory process. Indeed, the major pathological symptoms caused by Cd^{2+} are renal failure and bone damage. These symptoms seem to be caused by inflammatory responses rather than apoptotic ones.

Further studies on ROS production, membrane lipid peroxidation, intracellular calcium ion concentrations, and NO synthesis caused by the toxicity of Cd^{2+} are now in progress.

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