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European Journal of Pharmacology 578 (2008) 11–18
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Cadmium induces apoptotic cell death through p38 MAPK in brain microvessel endothelial cells

Yi-Sook Jung [a,b,1], Euy-Myoung Jeong [a,1], Eun Kyung Park [a], You-Mie Kim [a,c], Seonghyang Sohn [d], Soo Hwan Lee [a,c], Eun Joo Baik [a,c], Chang-Hyun Moon [a,c,*]

[a] *Department of Physiology, School of Medicine, Ajou University, Suwon 443-749, Republic of Korea*
[b] *BK21 for Molecular Science and Technology, Ajou University, Suwon 443-749, Republic of Korea*
[c] *BK21 Graduate Program for Medical Science, Ajou University, Suwon 443-749, Republic of Korea*
[d] *Laboratory of Cell Biology, Ajou University Institute for Medical Science, Suwon 443-749, Republic of Korea*

Received 3 July 2007; received in revised form 23 August 2007; accepted 30 August 2007
Available online 20 September 2007

Abstract

Cadmium (Cd), an ubiquitous heavy metal, is known to be accumulated outside of the blood–brain barrier. In this study, we investigated whether Cd has cytotoxicity in mouse brain microvascular endothelial cells (bEnd.3). Results from the cell viability assay showed that Cd caused a remarkable decrease in cell viability in a dose-dependent manner. The cell death induced by Cd appeared to involve apoptosis, based on our results from annexin V staining, electron microscopy and TUNEL staining. And the cell death induced by Cd was inhibited by caspase inhibitor ZVAD-fmk. To further investigate the mechanism of the Cd-induced cell death, we examined the effects of selective inhibitors for mitogen activated protein kinase (MAPK) pathways on the cell death. The Cd-induced cell death was significantly inhibited by p38 MAPK inhibitor SB202190, but not by either, c-Jun N-terminal kinase (JNK) inhibitor SP600125 or extracellular signal-regulated kinase (ERK) inhibitor U0126. Phosphorylations of p38 MAPK, JNK and ERK were stimulated by treatment with $CdCl_2$. In summary, our results suggest that Cd can induce apoptotic cell death, at least in part, through the p38 MAPK pathway in brain microvascular endothelial cells.

Keywords: Cadmium; Brain microvascular endothelial cells; Apoptosis; p38 MAPK

1. Introduction

The cerebrovascular endothelial cells, the cellular component of the blood–brain barrier, play an essential role in forming a barrier which limits access of potentially harmful blood-borne substances to the brain under physiological conditions (Rubin and Staddon, 1999; Demeuse et al., 2002). Nevertheless, in pathological situations such as stroke and multiple sclerosis, a disruption in blood–brain barrier integrity occurs, resulting in permeability increase, edema, and thereby exacerbation of brain damage (Petty and Lo, 2002). Mechanisms by which the disruption of blood–brain barrier occurs have been demonstrated to involve abnormal changes of the junctional complexes of the cerebrovascular endothelium (Krizbai et al., 2005; Fischer et al., 2005), and transmigrations of leukocytes across the cerebrovascular endothelium mediated through adhesion molecules such as integrin and intercellular adhesion molecule-1 (ICAM-1) (Bolton et al., 1998; Bolton and Perry, 1998). Furthermore, a recent report suggested that apoptotic death of the cerebrovascular endothelial cells may be also an important cause of the disruption of the blood–brain barrier integrity (Zhang et al., 2000).

Heavy metals, including lead and manganese, are well known to cause neurotoxicity (Bressler et al., 1999; Mergler et al., 1999). Cadmium (Cd), a highly ubiquitous heavy metal, has also been demonstrated to induce brain edema and hemorrhage, and blood–brain barrier disruption (Webster and Valois, 1981; Shukla et al., 1996). In support of these Cd-induced brain

* Corresponding author. Department of Physiology, School of Medicine, Ajou University, Suwon 443-749, Republic of Korea. Tel.: +82 31 219 5041; fax: +82 31 219 5049.
E-mail address: yisjung@hanmail.net (C.-H. Moon).
[1] These authors contributed equally.

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doi:10.1016/j.ejphar.2007.08.049

damages, we previously has demonstrated that Cd induces ICAM-1 expression through NF-κB activation in cerebrovascular endothelial cells, suggesting that Cd may cause an increase in the blood–brain barrier permeability through increased leukocyte migration mediated by ICAM-1 (Jeong et al., 2004). However, it has not been evaluated whether Cd-induced blood–brain barrier alteration involves apoptotic death of the cerebrovascular endothelial cells.

Based on these considerations, we here investigated whether Cd can induce apoptotic death in brain microvessel endothelial cells (bEnd.3) and if so, what the signaling pathways for its actions are, especially focusing on the mitogen activated protein kinase (MAPK) signaling pathways, which have been extensively studied for their roles in apoptotic death pathways in various cell types and tissues (Matsumoto et al., 2002; Zhang et al., 2001).

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and other culture reagents were purchased from Life Technologies Inc. (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT, USA). SB202190, SP600125, U0126, z-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk) were purchased from Calbiochem (San Diego, CA, USA). Fluorescein-conjugated annexin V antibody was purchased from BD Biosciences (San Jose, CA, USA). Cadmium chloride ($CdCl_2$) and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise specified.

2.2. Cell culture

The immortalized mouse brain microvessel endothelial cell line, bEnd.3 (Montesano et al., 1990), was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in DMEM supplemented with 450 mg/dl glucose, 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were cultured at 37 °C in a 5% CO_2–95% air humidified incubator. $CdCl_2$ was dissolved in distilled-deionized water and treated at the indicated concentrations. Inhibitors were prepared fresh in dimethyl sulfoxide (DMSO) at appropriate concentrations; the final concentration of the solvent in cell suspension never exceeded 0.1%. Cells were treated with $CdCl_2$ for indicated periods in the presence or absence of caspase inhibitor or MAPK signaling inhibitors.

2.3. Mitochondrial activity as an index of cell viability

Cell viability was determined by 3-[4,5-dimethyl-triazolyl-2] 2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded in 24-well plates at a density designed to reach 90% confluency at the time of assay. Cells were treated with various concentrations of $CdCl_2$ in triplicate. After 12 and 24 h of $CdCl_2$ treatment, MTT was added at 0.5 mg/ml finally, and the plate was incubated for 2 h at 37 °C. Cells having functional mitochondrial succinate dehydrogenase can convert MTT to formazan that generates a blue color when dissolved in dimethyl sulfoxide (DMSO). The medium containing MTT was removed, and 200 μl of solubilizing solution (2.5% acetic acid, 2.5% 1 N HCl, 50% *N*,*N*-dimethylformamide, and 20% (w/v) sodium dodecyl sulfate (SDS) in water, pH 4.7) was added and the absorbance was read at 540 nm using a Spectra Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Annexin V staining

The annexin V-fluorescein isothiocyanate (FITC) binding assay was adopted for determination of apoptosis. Briefly, after treatment with $CdCl_2$, the cells were washed twice with PBS and suspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). The cells were stained with annexin V-FITC (7.5 ng/μl) for 20 min and propidium iodide (5 ng/μl) for 20 s in the dark. The stained cells were analyzed using confocal laser scan microscope (Olympus, Hamburg, Germany) and flow cytometry (BD Biosciences).

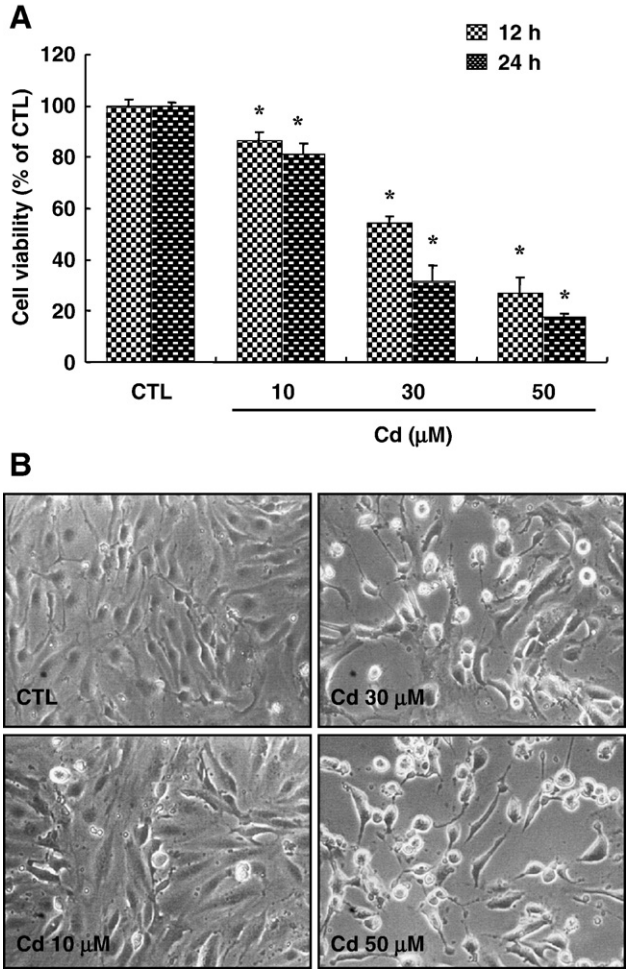

Fig. 1. Effect of Cd on cell viability in brain microvessel endothelial cell line, bEnd.3. After the treatment with various concentrations of $CdCl_2$ for 12 h and 24 h, the cytotoxicity was determined by MTT assay A and microscopy at 24 h B. Data shown are the mean ± S.E.M. ($n = 5$). *$P < 0.05$ *vs.* untreated control.

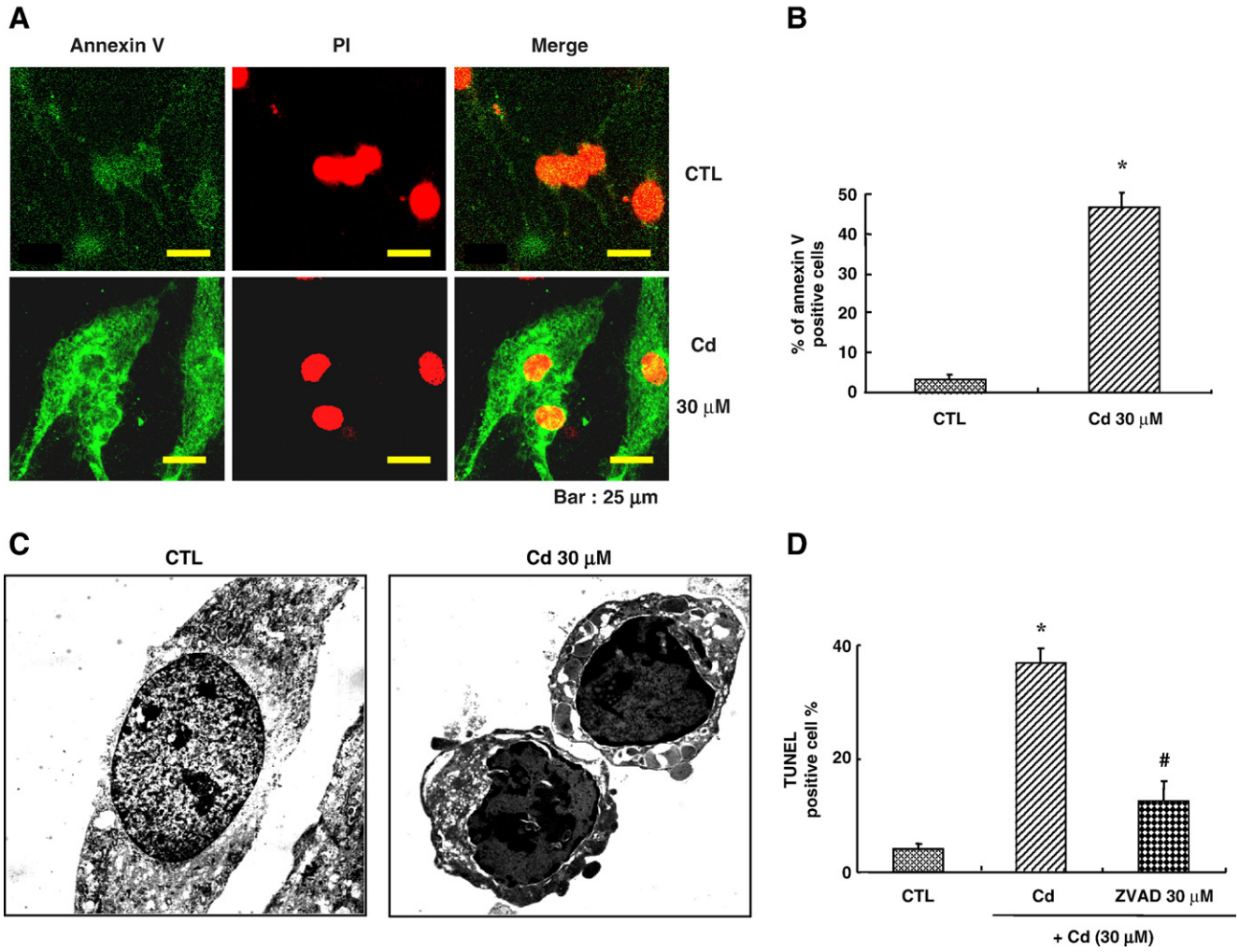

Fig. 2. Cd induces apoptosis in bEnd.3 cells. Cells treated with $CdCl_2$ (30 μM) for 8 h were stained by annexin V-FITC and propidium iodide (PI), and the fluorescence intensity was analyzed by confocal laser scan microscope A and FACscan B. C Electron microscopy on the cells treated with $CdCl_2$ (30 μM) for 24 h. D Caspase-dependent apoptosis measured by TUNEL-positive cells. Cells were treated with $CdCl_2$ (30 μM) for 8 h. Caspase inhibitor (ZVAD-fmk) 30 μM was co-treated with $CdCl_2$. Data shown are the means±S.E.M. ($n=3$). *$P<0.05$ *vs.* untreated control. #$P<0.05$ *vs.* $CdCl_2$-treated cells.

2.5. Transmission electron microscopy

The $CdCl_2$-treated cells were fixed with Karnovsky's fixative solution (1% paraformaldehyde, 2% glutaraldehyde, 2 mM calcium chloride, 100 mM cacodylate buffer, pH 7.4) for 2 h and washed with cacodylate buffer. After post-fixing with the fixative solution containing 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h, cells were stained with *en bloc* in 0.5% uranyl acetate, embedded in Poly/Bed 812 resin (Pelco, Redding, CA, USA). The cells were sectioned by Reichert Jung Ultracut S (Leica, Wetzetlar, Germany) and stained with uranyl acetate and lead citrate. The cells were observed and photographed under transmission electron microscope (Zeiss, Leo, Oberkochen, Germany).

2.6. Terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) staining

TUNEL staining was carried out using ApopTag Plus kit (Oncor, Gaithersburg, MD, USA) according to the manufacturer's protocol. Briefly, cells were fixed with fresh 4% paraformaldehyde in phosphate-buffered saline (PBS), and post-fixed with ethanol:acetic acid (2:1) at −20 °C for 5 min. Cells were washed and incubated with blocking solution (0.3% H_2O_2 in PBS) at 4 °C for 15 min and then treated with equilibrium buffer at room temperature for 10–15 s, followed by TUNEL reaction mixture (terminal deoxynucleotidyl transferase and modified nucleotides) for 60 min at 37 °C. Cells were incubated with anti-digoxigenin peroxidase for 60 min at 37 °C and the staining color of the immunolabeling was developed with metal enhanced diaminobenzidine (DAB)-substrate solution. Cells were counted (positive *vs.* total) from five fields per well at 400× using optical microscopy (Leica).

2.7. Western blot analysis

Cells were collected after the indicated treatment. Cells were washed twice with PBS and lysed with lysis buffer (62.5 mM Tris–HCl, pH 7.4, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin,

200 μM sodium orthovanadate, 1 mM sodium fluoride, and 10 mM EDTA) for 5 min on ice. Lysed cell extracts were sonicated, heated for 5 min, and centrifuged at 13,000 *g* for 10 min. Equal amounts of protein (20 μg) were loaded onto each lane and run on SDS-PAGE under reducing conditions. Samples were then electroblotted onto nitrocellulose filters (Bio-Rad Laboratories, CA, USA), blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Triton X-100

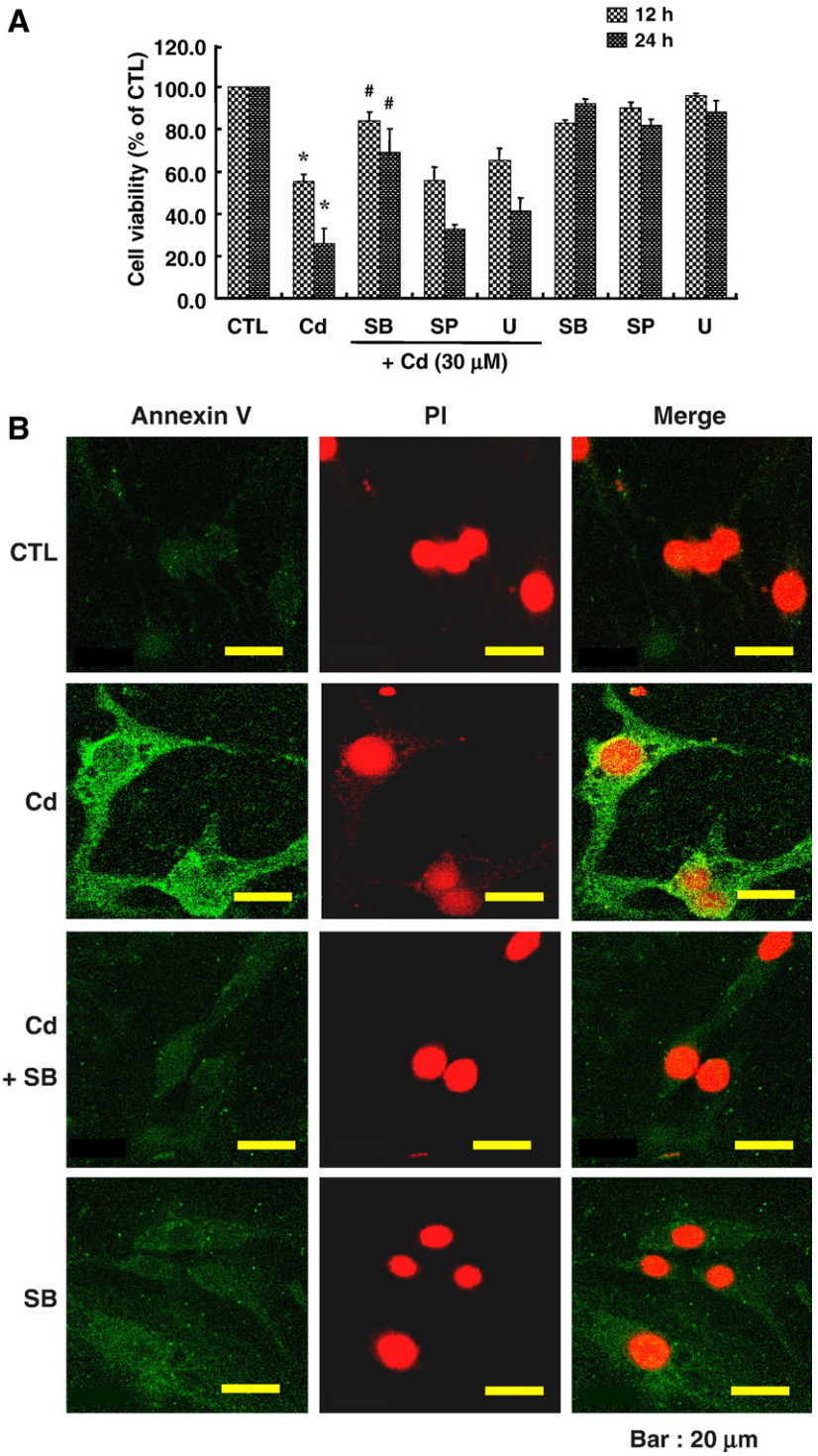

Fig. 3. Inhibitory effects of MAPK inhibitors on Cd-induced apoptosis. A The cell viability was determined by MTT assay after the treatment with $CdCl_2$ in the presence or absence of inhibitors for MAPK family, 20 μM SB202190 (for p38 MAPK), 5 μM SP600125 (for JNK) and 20 μM U0126 (for ERK). Data shown are the means±S.E.M. ($n=5$). *$P<0.05$ *vs.* untreated control. $^{\#}P<0.05$ *vs.* $CdCl_2$-treated cells. B Effect of 20 μM SB202190 on Cd-induced annexin V staining. Cells were treated with $CdCl_2$ (30 μM) in the presence or absence of SB202190 (20 μM) for 8 h, and stained for annexin V. Fluorescence intensity was analyzed by confocal laser scan microscope.

(TBST), and incubated overnight at 4 °C with different primary antibodies (anti-phospho-extracellular signal-regulated kinase (ERK), anti-ERK, anti-phospho-c-Jun N-terminal kinase (JNK), anti-JNK, anti-phospho-p38 MAPK, anti-p38 MAPK; Calbiochem). Incubation was followed by the reaction with horseradish peroxidase-conjugated specific secondary antibodies for 1 h at room temperature. The immunoreactive bands were revealed by ECL with a luminometer (Amersham, Piscataway, NJ, USA).

2.8. Statistical analysis

Student's *t*-test was used to analyze differences of quantitative variables between control and experimental groups. Results were expressed as mean±S.E.M. A P value <0.05 was considered significant.

3. Results

3.1. Effect of Cd on cell viability in bEnd.3 cells

To investigate the effect of Cd on the cell viability, MTT assay was performed after the treatment of cells with various concentrations of $CdCl_2$ for 12 and 24 h in the mouse brain microvessel endothelial cells, bEnd.3. As shown in Fig. 1A, the treatment with 10–50 μM $CdCl_2$ decreased significantly the cell viability in a dose-dependent manner (control:100±3.55%, 10 μM:86.2±3.95%, 30 μM:52.7±3.36% and 50 μM:28.1±4.83% for 12 h, control:100±2.33%, 10 μM:81.2±5.01%, 30 μM:32.6±5.80% and 50 μM:18.0±1.58% for 24 h, respectively). A considerable inhibitory effect on cell viability was observed when cells were exposed to $CdCl_2$ at concentrations not less than 10 μM. Fig. 1B shows the increase of Cd-induced cytotoxicity observed by phase contrast microscope. Control cultures have normal cell bodies and intact features. However, $CdCl_2$-treated bEnd.3 cells became rounded and condensed and lost adhesion from the monolayer and showed reduced cell density.

3.2. Cd induces apoptosis in bEnd.3 cells

To examine whether Cd-induced cell death is apoptotic, the cells were stained with annexin V. Annexin V stains the flipped-out phosphatidylserine on the outer side of the plasma membrane, Flipping out of the phosphatidylserine from the inner side to the outer side of the plasma membrane is known to be one of the characteristics of apoptotic cells. Confocal laser microscopy using immunofluorescence demonstrated that annexin V binding increased dramatically in the cells treated with 30 μM $CdCl_2$ for 8 h. (Fig. 2A). In addition, annexin V-positive cells measured by flow cytometry were significantly increased by 15 fold in $CdCl_2$-treated cells compared with control (Fig. 2B).

To confirm Cd-induced apoptosis, transmission electron microscopy was performed for the cells treated with 30 μM $CdCl_2$ for 24 h. As shown in Fig. 2C, $CdCl_2$-treated cells were shrunken with increased electron density and had characteristic apoptotic nuclei showing heterochromatin condensation, while the control cells were observed to have the well-defined nuclei and intact nuclear membrane. And plasma membrane blebbing was observed in $CdCl_2$-treated cells. The morphology was identical to that of the typical apoptotic cells.

Cd-induced apoptosis was also confirmed by TUNEL staining and the number of TUNEL-positive cells was remarkably increased in $CdCl_2$-treated cells. To further examine whether the Cd-induced apoptosis is caspase pathway dependent, we examined the effects of general caspase inhibitor, ZVAD-fmk (30 μM) (Slee et al., 1996), on the increase of TUNEL-positive cells. As shown in Fig. 2D, the increase was significantly inhibited by ZVAD-fmk.

3.3. Involvement of MAP kinase pathway in Cd-induced cell death

To investigate the mechanism of Cd-induced apoptosis, we examined the effects of inhibitors for MAPK family, p38 MAPK, JNK and ERK, on the cell death induced by Cd in bEnd.3 cells. The cells were treated with $CdCl_2$ (30 μM) in the presence or absence of specific inhibitors for p38 MAPK (SB202190), JNK (SP600125) and ERK (U0126). We used those inhibitors at concentrations which have effective inhibitory activity (Fig. 4) but not toxic effect (Fig. 3B). As shown in Fig. 3A, the treatment with SB202190 (20 μM) reversed significantly Cd-induced cell death observed by MTT assay, while neither SP600125 (5 μM)

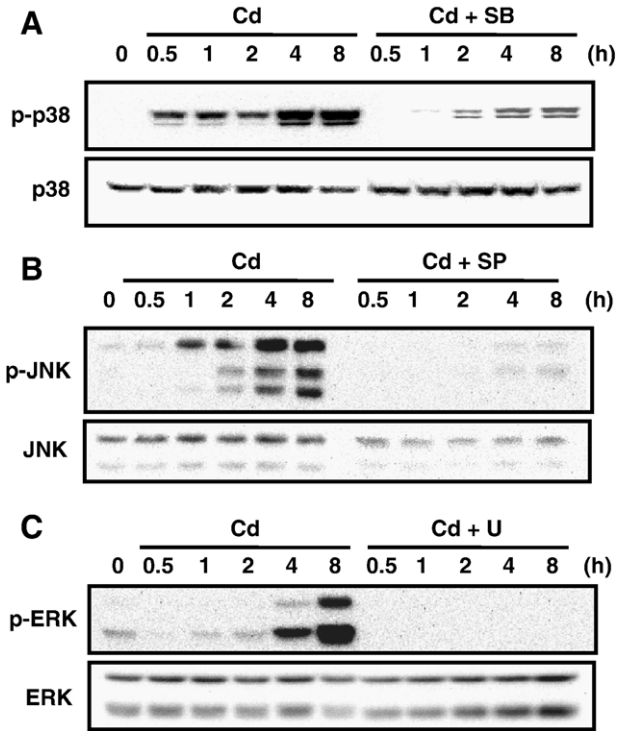

Fig. 4. Cd induces activations of MAPKs in bEnd.3 cells. Western blotting analyses of cell lysates treated with $CdCl_2$ (30 μM) for the indicated times in the absence or presence of MAPKs inhibitors were performed as described under Materials and methods. A Phosphorylation of p38 MAPK. B Phosphorylation of JNK. C Phosphorylation of ERK.

nor U0126 (20 μM) affected significantly (control:100±0.6%, Cd:55.0±3.12%, Cd+SB:83.1±5.68%, Cd+SP:55.5±6.52% and Cd+U:65.1±4.93% for 12 h, Cd:25.8±6.46%, Cd+SB:68.8±11.2%, Cd+SP:32.2±2.25% and Cd+U:41.7±5.82% for 24 h, respectively).

Since p38 MAPK among MAPK family appeared to be involved in Cd-induced cell death, the effect of SB202190 on apoptotic cell death induced by Cd was further investigated with annexin V staining. As shown in Fig. 3B, Cd-induced annexin V-positive staining was inhibited completely by SB202190.

To examine whether Cd can directly affect the activities of MAPKs, immunoblot analysis was performed with antibodies that recognize the activated phosphorylated forms of the kinases in $CdCl_2$-treated cells (Berra et al., 1998). As shown in Fig. 4, the phosphorylation of p38 MAPK increased as early as 30 min after $CdCl_2$ treatment and this increase was inhibited by the treatment with SB202190 (20 μM). Total p38 MAPK shows equal lane load. Similarly, the levels of phosphorylated JNK and ERK also increased after $CdCl_2$ treatment and these activations were inhibited by the treatment with their specific inhibitors. And the total forms of the kinases show equal lane load.

4. Discussion

In this study, we have found that Cd can induce apoptotic death, at least partially, through the p38 MAPK pathway in brain microvascular endothelial cells.

It was reported that a Cd intoxicated 3 year old boy died suddenly of brain intracellular accumulation with resultant cellular dysfunction, blood–brain barrier disruption, and lethal cerebral edema (Provias et al., 1994). Cd has also been demonstrated to induce brain edema and blood–brain barrier disruption in animals (Webster and Valois, 1981; Shukla et al., 1996). Since disruption of the blood–brain barrier integrity may be caused by apoptotic death of the cerebrovascular endothelial cells (Zhang et al., 2000), we hypothesized that Cd-induced blood–brain barrier alteration may involve apoptotic death of the cerebrovascular endothelial cells.

To investigate this hypothesis, we evaluated at first the cytotoxicity of Cd in bEnd.3 cells in terms of cell death. Our results showed that Cd (10 to 50 μM) did have significant and concentration-dependent toxic effect. Consistently, Shih et al. (2004) report that during the last decade, Cd has been shown to induce cell death *in vivo* and *in vitro* at varied concentrations from 1 to 300 μM. Although some reports indicate that Cd is pro-necrotic (Kim et al., 2003; Lopez et al., 2003), Cd-induced apoptosis has been demonstrated in many cell types by a majority of investigators (Szuster-Ciesielska et al., 2000; Li et al., 2003; Shih et al., 2004). And we investigated whether the cell death induced by Cd may occur *via* apoptosis by performing annexin V staining, electron microscopy and TUNEL staining. When the cells were treated with 30 μM $CdCl_2$, the percentage of annexin V-stained cells increased significantly (Fig. 2A and B), implying that Cd can cause apoptosis of the cells. Cd-induced apoptosis was also observed in electron microscopy, which showed plasma membrane blebbing, plasma condensation and shrinkage of nuclei (Fig. 2C). Cd-induced apoptosis was further confirmed by TUNEL staining and the number of TUNEL-positive cells remarkably increased in $CdCl_2$-treated cells (Fig. 2D).

The apoptotic signaling pathways induced by Cd remain unclear, although Cd-induced apoptosis was studied in many cell types (Shih et al., 2004). In the present study, we showed that caspase activity was involved in Cd-induced apoptosis using ZVAD-fmk (Fig. 2D). These results are consistent with other studies that report Cd-induced apoptosis occurring *via* a caspase-dependent pathway (Kim et al., 2000; Li et al., 2000). However, others demonstrate that Cd-induced apoptosis occurs through a caspase-independent pathway (Ishido et al., 1999; Shih et al., 2004). The reason for such inconsistent results is likely that the apoptotic signaling cascades are cell specific.

Recent reports identified a role of MAPK signaling pathway in Cd-induced apoptosis (Iryo et al., 2000; Kim and Sharma, 2004). The MAPK signaling pathways are critical regulators of a variety of cellular responses, including apoptosis (Miguel et al., 2005). Three subfamilies of MAPKs have been identified: ERKs, JNKs, and p38 MAPKs. It has been originally shown that ERKs are important for cell survival, whereas JNKs and p38 MAPKs were deemed stress responsive and thus involved in apoptosis. However, the regulation of apoptosis by MAPKs is more complex and often controversial. MAPK signaling may either protect or enhance sensitivity to apoptosis depending on the cell type, stimuli, and the latency of the activation of MAPKs (Wada and Penninger, 2004). The regulation of Cd-induced apoptosis by MAPKs is also complex and remains unclear. No uniform picture emerges with respect to the apoptotic mechanisms induced by Cd. ERK-mediated apoptosis was reported by Iryo et al. (2000) in human T lymphoblastoid cells treated with $CdCl_2$ while p38 MAPK was not involved in the apoptosis. On the other hand, Kim and Sharma (2004) have suggested that activation of JNK is pro-apoptotic in murine macrophages treated with $CdCl_2$. And a majority of investigators have demonstrated that p38 MAPK induce apoptosis provoked by Cd in various cells such as promonocytic cells, alveolar type 2 cells and Clara cells from rat lung (Galan et al., 2000; Miguel et al., 2005; Lag et al., 2005) as well as in endothelial cells derived from various tissues and species (Harfouche et al., 2003; Matsumoto et al., 2002; Hyman et al., 2002). We here examined the effects of inhibitors of signaling pathways (Singh et al., 1999; Bennett et al., 2001), which abrogate phosphorylations of p38 MAPK (SB202190), ERK (SP600125) and JNK (U0126), on Cd-induced cell death. Consistent with many reports, the present study shows the pro-apoptotic role of the p38 MAPK in Cd-induced bEnd.3 cell death, *i.e.*, SB202190 significantly blocked the Cd-induced cell death and annexin V staining for apoptotic cells while SP600125 or U0126 did not.

We determined Cd (30 μM) induced activations of all three MAPKs and the phosphorylations of three MAPKs were almost completely inhibited by their specific inhibitors (Fig. 4). In our previous report, however, only p38 MAPK, but not JNK or ERK, was activated by Cd in bEnd.3 cells (Seok et al., 2006). The reason for such discrepant results in the same cell line is due to treating cells with different concentrations of Cd; much lower

Cd concentration (2 μM) was used and time points later than 4 h were not studied in our previous study. Time points that MAPKs activations are observed are also important because the latent periods of MAPKs activations are different. In fact, p38 MAPK was activated earlier than JNK and ERK by various concentrations of Cd from 1 μM to 30 μM (data not shown). Considering altogether the results, it appears that p38 MAPK may play an upstream role in apoptotic cascade; however, once deleterious events are initiated by p38 MAPK, ERK and JNK contribute to, but are not necessary for further cell death.

In summary, this study has demonstrated that Cd can cause apoptotic cell death and the apoptosis can be regulated by caspase-dependent pathway in the brain endothelium-derived bEnd.3 cells. And this study suggests that Cd-induced apoptosis may occur, at least in part, through the activation of the p38 MAPK pathway in the brain microvascular endothelial cells. Pro-apoptotic activity of p38 MAPK in Cd-treated bEnd.3 cells remains to be confirmed with p38 MAPK gene transfectants in the future study. The findings of the current study will certainly aid in understanding an important cause of the disruption of the blood–brain barrier integrity by Cd intoxication.

Acknowledgement

This work was supported by the Ministry of Environment, Republic of Korea under the contract with the Eco-technopia 21 project.

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