Journal of Biochemistry and Molecular Biology, Vol. 36, No. 5, September 2003, pp. 468-474

JOURNAL OF Biochemistry and Molecular Biology

Cobalt Chloride-induced Apoptosis and Extracellular Signal-regulated Protein Kinase Activation in Human Cervical Cancer HeLa Cells

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Received 21 February 2003, Accepted 14 April 2003

The molecular mechanism of hypoxia-induced apoptosis has not been clearly elucidated. In this study, we investigated the involvement of extracellular signal-regulated protein kinase (ERK 1/2) in hypoxia-induced apoptosis using cobalt chloride in HeLa human cervical cancer cells. The cobalt chloride was used for the induction of hypoxia, and its IC_{50} was 471.4 μM. We demonstrated the DNA fragmentation after incubation with concentrations more than 50 μM cobalt chloride for 24 h, and also evidenced the morphological changes of the cells undergoing apoptosis with electron microscopy. Next, we examined the signaling pathway of cobalt chloride-induced apoptosis in HeLa cells. ERK1/2 activation occurred 6 and 9 h after treatment with 600 μM cobalt chloride. Meanwhile, the pretreatment of the MEK 1 inhibitor (PD98059) completely blocked the cobalt chloride-induced ERK 1/2 activation. At the same time, the activated ERK 1/2 translocated into the nucleus and phosphorylated its transcriptional factor, c-Jun. In addition, the pretreatment of PD98059 inhibited the cobalt chloride-induced DNA fragmentation and apoptotic cell death. These results suggest that cobalt chloride is able to induce apoptotic activity in HeLa cells, and its apoptotic mechanism may be associated with signal transduction via ERK 1/2.

Keywords: Apoptosis, Cobalt chloride, c-Jun, Hypoxia, ERK 1/2

Introduction

Hypoxia is a common feature of human tumors. It has increasingly been recognized as having an important role in determining not only the response to therapy but also tumor progression (Moulder and Rockwell, 1984; Hockel *et al.*, 1996; Brown and Giaccia, 1998; Fyles *et al.*, 1998). Furthermore, hypoxia provides a physiological pressure on tumor cells and has been shown to induce apoptosis (Graeber *et al.*, 1996; Kim *et al.*, 1997). Both angiogenesis and apoptosis that are induced by hypoxia can accelerate the progression of tumor cells to malignancy, thereby creating a poorer prognosis, irrespective of which cancer treatment is used (Bae *et al.*, 1998; Brown, 1999).

The representative hypoxic responses of tumor cells have anaerobic metabolism, erythropoiesis, vasodilatation, angiogenesis, and increased breathing. These adaptive responses to hypoxia are achieved by the coordinated expression of a number of genes. These include the following; erythropoietin (EPO), vascular endothelial growth factor (VEGF), glycolytic enzymes, glucose transporter (Glut), inducible NO synthase (i-NOS), and many others (Guillemin and Krasnow, 1997; Kim *et al.*, 2002). Induction of these genes is mediated by the hypoxia inducible factor 1(HIF-1), a transcription factor known as a global regulator of the hypoxic gene expression (Guillemin and Krasnow, 1997; Chandel *et al.*, 1998). HIF-1 consists of 120-kDa HIF-1α and 91- to 94-kDa HIF-1β subunits (Wang and Semenza, 1995), and HIF-1α is the subunit that is regulated by hypoxia (Minet *et al.*, 2000).

In vitro, hypoxic conditions were achieved by using a hypoxia chamber or by treatment with cobalt chloride or desferrioxamine mesylate (DFX), which induced hypoxia (Chandel *et al.*, 1998; Kitazono *et al.*, 1998; Wang *et al.*, 2000). Recent work reported that the presence of cobalt chloride not only induced hypoxia but also induced DNA damage and activated the cellular DNA damage response (Wang *et al.*, 2000).

Apoptosis is a regulated cell death process that is characterized by cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation (Kerr *et al.*, 1972). Apoptosis begins with the condensation of nuclear chromatin at the nuclear periphery, followed by blebbing of the nuclear and

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cytoplasmic membranes, and culminating in the fragmentation of the residual nuclear structure into discrete membrane-bounded apoptotic bodies (Allen, 1987; Kerr *et al.*, 1987). The morphological alterations of apoptosis are accompanied by a variety of biochemical changes (Cho *et al.*, 2002). Elevations in the cytosolic free calcium (McConkey *et al.*, 1990) and cytoplasmic hydrogen ion (Barry *et al.*, 1992) are followed by internucleosomal DNA degradation (Lockshin *et al.*, 1990; Arends *et al.*, 1991) and sharp decreases in cellular NAD levels (Denisenko *et al.*, 1989). Reportedly, the exposure of cells to hypoxia-induced DNA fragmentation is characteristic of apoptosis and ultimately leads to apoptosis (Yao *et al.*, 1995; Bae *et al.*, 1998).

Recently, mitogen-activated protein (MAP) kinase cascades, which are well known for their cell proliferation and differentiation-inducing pathways, were reported to be associated with the apoptotic pathway. MAP kinase families include extracellular regulated protein kinase (ERK 1/ERK 2), p38, and stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK) (Davis, 1993; Jung *et al.*, 2002). Members of the MAP kinase family that have been extensively studied are ERK 1/2, which can be triggered by growth factors, and phorbol esters through the Ras-dependent activation of the Raf-MEK-ERK pathway (Davis, 1993; Stokoe *et al.*, 1994). The JNK cascade is operated by a parallel signaling module that consists of the MEKK-1 / SEK 1/ JNK cascade (Kyriakis *et al.*, 1994; Coso *et al.*, 1995). Unlike ERK, JNK is strongly activated by environmental stress, such as inflammatory cytokine (Martin *et al.*, 1997), ultraviolet C (Kyriakis *et al.*, 1994), gamma irradiation (Yu *et al.*, 1996), and DNA-damaging drugs (Jimenez *et al.*, 1997). In addition, the phosphorylated MAP kinase in cytosol is translocated into the nucleus (Chen *et al.*, 1992) and is involved in the regulation of transcription factors, including c-Jun, c-fos, and c-Myc, which influence apoptosis (Cavigelli *et al.*, 1995; van Dam *et al.*, 1995; Baek *et al.*, 1996). It was recently reported that hypoxia activated the MAP kinase, Raf (Muller *et al.*, 1997; Conrad *et al.*, 1999; Seimiya *et al.*, 1999), phosphatidylinositol 3-kinase (PI 3-kinase) (Mazure *et al.*, 1997), PKC, c-fos (Yao *et al.*, 1994; Muller *et al.*, 1997; Bae *et al.*, 1998; Seimiya *et al.*, 1999) and c-Jun (Yao *et al.*, 1994; Bae *et al.*, 1998). However, the molecular mechanism of the hypoxia-induced apoptosis has not been clearly elucidated.

In this study, we investigated the involvement of the MAP kinase to hypoxia-induced apoptosis by using cobalt chloride in HeLa human cervical cancer cells. The cobalt chloride was used for the induction of hypoxia. We examined the cytotoxicity and apoptotic effect of cobalt chloride in HeLa cells. In particular, the potent activation of ERK 1/2 and its nuclear translocation were observed after the cobalt chloride treatment. The significance of these observations has been discussed.

Materials and Methods

Materials The cobalt chloride was purchased from the Sigma Chemical Co. (St. Louis, USA). Anti-HIF-1, Anti-phospho-ERK 1/2, anti-phospho-c-Jun antibodies, and MEK 1 inhibitor (PD98059) were purchased from New England Biolabs (Beverly, USA). All of the other chemicals and reagents were the highest grade commercially available.

Cell culture and *in vitro* cytotoxicity assay HeLa cancer cells were maintained in RPMI 1640 that was supplemented with 10% fetal bovine serum without antibiotics and antifungal agents. All of the cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Cytotoxicity was measured by the microculture tetrazolium (MTT) method. Exponentially-growing cells were inoculated to 5×10^3 cells/well using a 96 well microplate that was supplemented with 100 μl RPMI 1640. For the experiments that studied the cells in the plateau growth phase, the cells were permitted to grow for a minimum of 72 h before they were exposed to drugs. The cells were exposed to various concentrations of cobalt chloride. Distilled water was used to dissolve the cobalt chloride. After the treated cells were incubated for 24 h, 50 μl MTT (1 mg/ml, Sigma) was added. The plates were incubated at 37°C for 4 h. To dissolve the formazan, 150 μl DMSO was added. The plates were measured at 540 nm by a spectrometer. The IC_{50} value was determined by plotting the drug concentration versus the survival ratio of the treated cells.

DNA extraction and electrophoresis Five million cells, which were treated with cobalt chloride for 24 h, were washed twice in a solution of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl, then lysed with a 500 μl lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 20 mM EDTA). The lysates were harvested by $1,000 \times g$ for 10 min. The supernatants were incubated for 4 h at 37°C with 50 μg/ml RNase A, 120 μg/ml proteinase K. Then phenol/chloroform/isoamylalcohol (25 : 24 : 1, Sigma) extracted the DNA. After precipitation, the pellets were resuspended in a 30 μl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Each DNA sample was electrophoresed through 1.8% agarose gel that contained ethidium bromide. The gel was visualized by UV fluorescence.

Electron microscopy The cells that were treated with 600 μM cobalt chloride for 60 h and 72 h were centrifuged at $400 \times g$, fixed with 2% glutaraldehyde in PBS for 24 h, washed in 0.1 M caocodylate, pH 7.4, and fixed with 0.1% OsO_4 in 0.1 M caocodylate for 1.5 h. After fixation, the cells were washed with in 0.1 M caocodylate, pH 7.4, then dehydrated in graded ethanol. Next, the cells were impregnated with prophylene oxide and embedded in Polybed 812 (Polyscience, Inc., Warrington, USA). After a 60°C-incubation, the cells were cut and stained with uranyl acetate and lead citrate.

Preparation of cytosolic and nuclear fractions Exponentially-growing cells were starved for 24 h, then exposed to 600 μM cobalt

chloride for 6, 9, 12, and 15 h. To analyze the PD98059 effects on the cobalt chloride-treated cells, the starved cells were exposed to 10 μM PD98059 for 1 h, then treated with 600 μM cobalt chloride for 6, 9, 12, and 15 h. The treated cells were washed twice and collected into a 1 ml hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 40 μg of PMSF per ml, and 10 μg of both pepstain and leupeptin per ml, pH 7.5). The cellular suspensions were homogenized and harvested by 600 × *g* for 5 min. To prepare the cytosolic fraction, the supernatant was obtained and centrifuged at 12,000 × *g* for 20 min. The pellets were suspended by 0.25 M sucrose in 1.5% citric acid, then passed three times through a 26 gauge needle and loaded onto 1 ml of a 0.88 M sucrose cushion in 1.5% citric acid. To obtain the nuclear fraction following centrifugation at 900 × *g* for 10 min, the pellets were dissolved with a hypotonic lysis buffer that contained 0.5% Igepal CA-630, 0.1% deoxycholate, 0.1% Brij-35, and then centrifuged at 10,000 × *g* for 10 min. All of the fractional procedures were completed on ice.

The purity of the nuclear fraction was determined by measuring the lactate dehydrogenase activity as the cytosol marker. The nuclear fractions were then confirmed by a Western blot analysis.

Immunoblot analysis We first examined the protein concentrations of the prepared cytosolic and nuclear extracts with a Bradford assay solution (100 mg/l Coomassie brilliant blue G-250, 50 ml/l 95% ethanol, 100 ml/l 85% phosphoric acid). The same protein sample concentrations were SDS-PAGE on 4% stacking gel and 10% running gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes, blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.15% Tween-20) that contained 10% skim milk. The membranes were then incubated with anti-HIF-1, anti-phospho-ERK 1/2, anti-phospho-c-Jun antibodies at adequate dilutions in TBS for 1 h at room temperature. After washing, the blots were incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies at a 1 : 1,000 dilution for 1 h at room temperature, washed 3 times in TBST, and then detected with the enhanced chemiluminescence detection method by immersing the blots for 1 min in an 1 : 1 mixture of chemiluminescence reagents A and B (Amersham, Piscataway, USA). They were then exposed to Kodak film for a few minutes.

Results and Discussions

***In vitro* cytotoxic effects** Exponentially-growing cells were exposed to various concentrations of cobalt chloride. After a 24 h exposure to cobalt chloride, the cytotoxic activity was evaluated by the MTT method against HeLa cells. The minimal concentration of cobalt chloride to inhibit the growth of HeLa cells by 50% (IC_{50}) was then determined. The cobalt chloride showed that the concentration-dependent cytotoxicity and IC_{50} value of cobalt chloride was 471.4 μM (Fig. 1).

HIF-1 expression To demonstrate the induction of hypoxia by the cobalt chloride treatment, we measured the HIF-1 expression through immunoblotting. The HeLa cells were exposed with 600 μM cobalt chloride for 6, 9, 12, and 15 h.

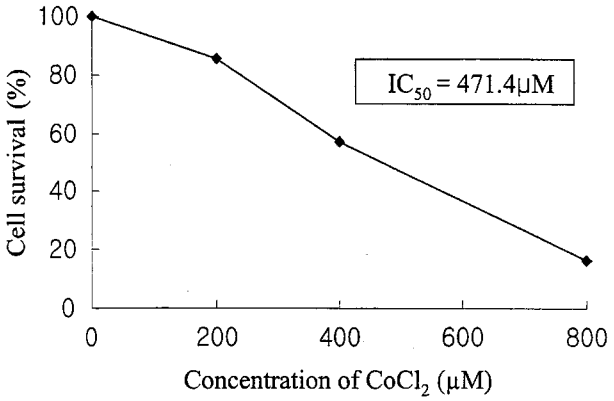

Fig. 1. Cytotoxic effect of cobalt chloride in HeLa cells.

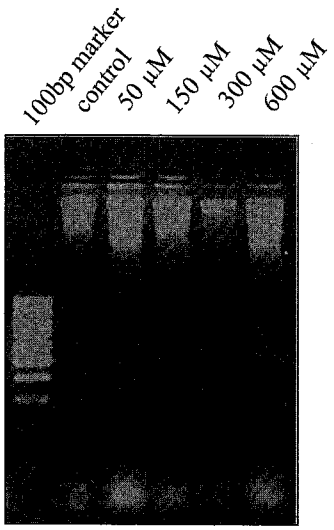

Fig. 2. DNA fragmentation induced by cobalt chloride. HeLa cells were treated with various concentrations of cobalt chloride for 24 h. Extracted DNA was fractionated by electrophoresis and stained by ethidium bromide.

The result was that cobalt chloride increased the HIF-1 expression 9 h after the treatment. The activation mechanism of HIF-1 is currently poorly understood. Phosphorylations seem to be involved in the stabilization of the HIF-1α subunit in hypoxia. During hypoxia, HIF-1 is expressed, binds to DNA, and induces the transcriptional activation of VEGF, known as a major mediator of vasculogenesis and angiogenesis (Forsythe *et al.*, 1996; Risau, 1997; Shweiki *et al.*, 1997).

DNA fragmentation In order to determine the apoptotic effect of cobalt chloride, we examined the apoptotic response, as judged by the appearance of a DNA ladder by 1.8% gel electrophoresis at various concentrations of cobalt chloride. The DNA ladder patterns with cobalt chloride appeared at concentrations of more than 50 μM (Fig. 2). Thus, cobalt chloride exhibited DNA fragmentations in broader

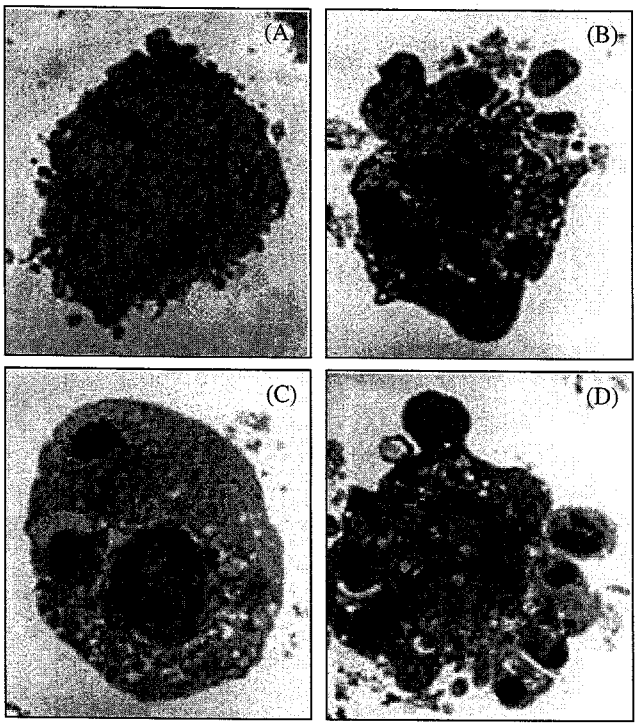

Fig. 3. Electron micrographs in HeLa cells treated with cobalt chloride. HeLa cells were treated with 600 μM cobalt chloride for 60 h (B), 72 h (C, D), and the control (A).

concentration ranges in HeLa cells. Many studies reported that hypoxia triggered DNA fragmentation in various cell lines (Yao *et al.*, 1995; Bae *et al.*, 1998), but there has been no research about how cobalt chloride-induced hypoxia provokes apoptosis.

Electron microphotography When the condensed nuclei were fixed with glutaraldehyde and examined by thin section EM, the treatment of HeLa cells with cobalt chloride resulted in morphological changes that are consistent with the apoptosis process. Initially, in the nucleus, a rim of heterochromatin appeared at the nuclear periphery and the nucleolus simultaneously disappeared (Fig. 3C). In this case, mitochondria and the plasma membrane remained intact throughout the course of these morphological changes. Consequently, ~50% of the cells continued to exclude trypan blue. The nuclei then subsequently fragmented (Fig. 3B). Finally, the packaging of the nuclear fragments into multiple membrane-enclosed apoptotic bodies were discovered (Fig. 3D).

Activation and translocation into nucleus of ERK 1/2 HIF-1α stabilization was observed in the presence of EGF (Feldser *et al.*, 1999). EGF is known to activate the ERKs MAPK pathway (Lee *et al.*, 1992; Feldser *et al.*, 1999). In order to search for kinases that are activated in hypoxic conditions, we investigated the putative role of ERKs in HIF-1 activation. Furthermore, the MAP kinase pathway is involved

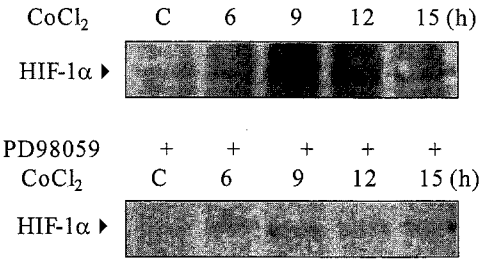

Fig. 4. The inhibition of HIF-1 activation by PD98059. HeLa cells were treated with 600 μM $CoCl_2$ and HeLa cells were also treated with 10 μM PD98059 for 1 h, then treated with 600 μM $CoCl_2$ for 6, 9, 12, and 15 h (lanes 2-5), and the control (lane 1).

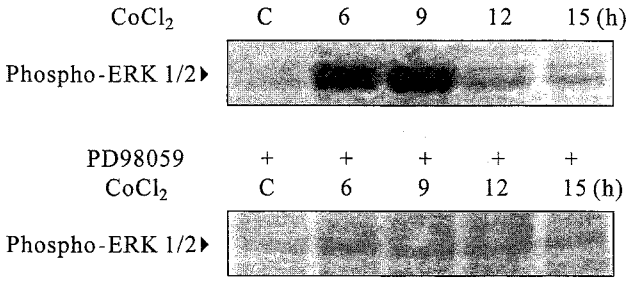

Fig. 5. Immunoblotting analysis of ERK 1/2 activation by cobalt chloride and the inhibition of phospho-ERK 1/2 by PD98059. (A) HeLa cells were treated with 600 μM $CoCl_2$ for 6, 9, 12, and 15 h (lane 2-5), and the serum-free control (lane 1). (B) HeLa cells were treated with 10 μM PD98059 for 1 h and then treated with 600 μM $CoCl_2$ for 6, 9, 12, and 15 h.

in apoptotic signal transduction (Wang *et al.*, 1998). To determine if ERK 1/2, one member of the MAP kinase family, was activated by the cobalt chloride stimulation of inducing an apoptotic response, we examined the phosphorylation of ERK 1/2 by an immunoblot analysis. HeLa cells were exposed to 600 μM cobalt chloride for 6, 9, 12, and 15 h. As a result, cobalt chloride activated ERK 1/2 at 6 and 9 h after treatment. When the cells were exposed to 600 μM cobalt chloride in the presence of 10 μM PD98059, a specific inhibitor of the MAP kinase kinase 1 (MEK 1), there was no activation of ERK 1/2 (Fig. 5). It has been proposed that the prolonged activation of the MAP kinase is accompanied by the translocation of the enzyme to the nucleus (Chen *et al.*, 1992) with subsequent alterations in the gene expression (Marshall *et al.*, 1995). To confirm the nuclear translocation of ERK 1/2, we prepared the separation of nuclei from the cells that were exposed to 600 μM cobalt chloride for 6, 9, 12, and 15 h and examined it with an immunoblot analysis. As shown in Fig. 6, ERK 1/2 (44/42kD) was phosphorylated and translocated into nucleus by cobalt chloride using the same conditions of inducing apoptosis. Accordingly, ERK 1/2 may be involved in the signal transduction of cobalt chloride-induced apoptosis.

c-Jun phosphorylation To study the possible regulation of the nuclear target by cobalt chloride, the nuclear fractions

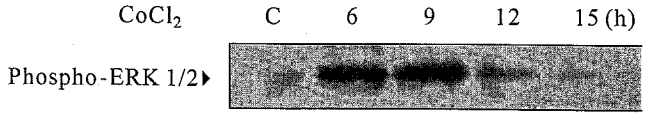

Fig. 6. Immunoblotting analysis of phospho-ERK 1/2 translocation from cytosol to nucleus. HeLa cells were treated with 600 μM $CoCl_2$ for 6, 9, 12, and 15 h (lane 2-5), and the serum free control (lane 1).

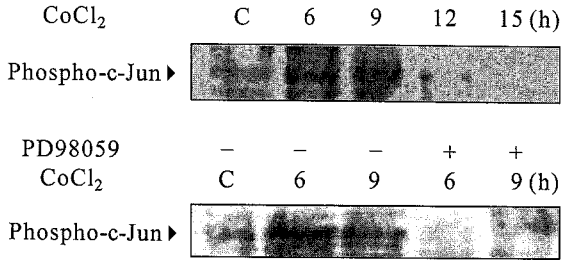

Fig. 7. Immunoblotting analysis of c-Jun activation by cobalt chloride and the inhibition of phospho-c-Jun by PD98059. (A) HeLa cells were treated with 600 μM $CoCl_2$ for 6, 9, 12, and 15 h (lane 2-5), and the serum free control (lane 1). (B) HeLa cells were treated with 600 μM $CoCl_2$ for 6 and 9 h with or without 10 μM PD98059 for 1 h (lane 2-5), and the serum free control (lane 1).

from the 600 μM cobalt chloride-treated HeLa cells were examined for the activity of the transcriptional factor, c-Jun, by an immunoblot with phospho-c-Jun Ab. We reported recently that c-jun is downstream of the ERK1/2 activation in the apoptotic response of the HeLa cells (Lee *et al.*, 2001). This work also shows that cobalt chloride comparatively activated c-Jun at 6 and 9 h after treatment. The activation of c-Jun did not occur when the cells were exposed to 600 μM cobalt chloride, the presence of 10 μM PD98059 (Fig. 7). Therefore, these results suggest that c-Jun is a downstream effector of ERK 1/2 activation that is responsive to treatment with cobalt chloride.

Inhibition of HIF-1 expression by PD98059 Minet *et al.* (2000) recently investigated the role of the mitogen-activated protein kinases (MAPK) ERK1 and ERK2 in the activation of HIF-1 by hypoxia in endothelial cells. Inhibition of these kinases with PD98059 did not inhibit the hypoxic stabilization of the HIF-1α subunit, nor did it inhibit the DNA binding activity of HIF-1. However, in the cells that were treated with PD98059, they observed the inhibition of the HIF 1 transcriptional activity that is induced by hypoxia. (Minet *et al.*, 2000). We investigated the association of ERK 1/2 activation with the HIF-1 expression. When the cells were exposed to 600 μM cobalt chloride in the presence of 10 μM PD98059, a specific inhibitor of the MAP kinase kinase 1 (MEK1), there was no increase in the HIF-1 expression (Fig. 4).

Inhibition of DNA fragmentation by PD98059 In order to

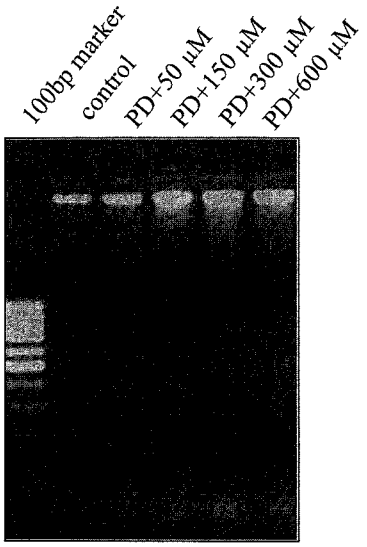

Fig. 8. The inhibition of cobalt chloride-induced DNA fragmentation by PD98059. HeLa cells were treated with 10 μM PD98059(PD) for 1 h, then individually treated with indicated concentrations of $CoCl_2$ for 24 h.

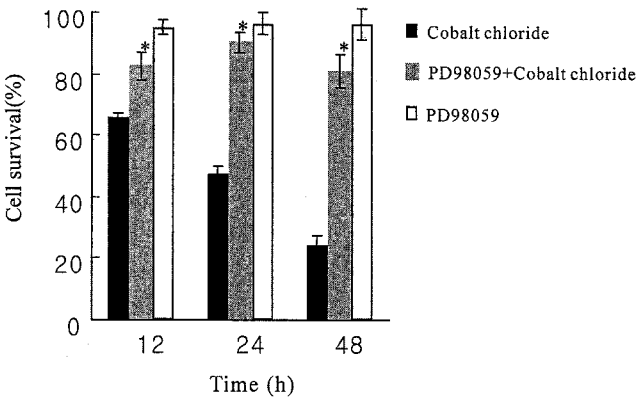

Fig. 9. The inhibition of cobalt chloride-induced apoptotic cell death by PD98059. HeLa cells were treated with 600 μM $CoCl_2$ and HeLa cells were also treated with 10 μM PD98059 for 1 h, then treated with 600 μM $CoCl_2$ for 12, 24, and 48 h.

confirm that ERK 1/2 relates to the cobalt chloride-induced DNA fragmentation, we added 10 μM PD98059 before treatment of cobalt chloride in the HeLa cells. The DNA ladder did not appear (Fig. 8). Accordingly, we showed that ERK 1/2 is a signaling pathway of cobalt chloride-induced apoptosis.

Inhibition of apoptotic cell death by PD98059 We also examined whether ERK 1/2 participates in cobalt chloride-induced apoptotic cell death. The cell survival rate (estimated by the MTT method after treatment of 600 μM cobalt chloride) gradually decreased. On the other hand, the pretreatment of 10 μM PD98059 maintained a cell survival rate of 80-90% (Fig. 9). Therefore, once more we affirmed the

association of ERK 1/2 with cobalt chloride-induced apoptosis. In summary, cobalt chloride has a cytotoxic effect against human cervical cancer HeLa cells and induces the apoptotic response. Also, our results imply that the activation and translocation into the nucleus of ERK 1/2 may be related to the apoptotic signal transduction of cobalt chloride and suggests that c-Jun is a downstream effector that is responsive to treatment with cobalt chloride.

Acknowledgments This work was supported by the Yonsei University Research Fund of 2001.

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