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Mode of Cell Death and Molecular Change of Oral Squamous Carcinoma Cells Exposed to Metal Ions

Hyung Il Kim[1], Chang Jun Baek[1], In Ryoung Kim[2], Hyung Keun Kim[2], and Hae Ryoun Park[2]*

[1]*Department of Dental Materials, College of Dentistry, Pusan National University, Pusan, Korea*
[2]*Department of Oral Pathology, College of Dentistry, Pusan National University, Pusan, Korea*

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Concerns remain regarding the biocompatibility and adverse effects of dental casting alloys. The aim of this study was to understand the cytopathogenic effect of metal ions, which might be released from dental alloys, on oral squamous carcinoma (OSC) cells. The cellular morphology, viability, the type of cell death and molecular change in response to metal ion salt solutions including aluminum (Al), cobalt (Co), copper (Cu) and nickel (Ni) were examined. The TC_{50} values for the metal ions with the exception of Al were estimated to be between 400 and 600 μM. The cells treated with the metal ions showed apoptotic change with the exception of Al ions. Metal ion-induced apoptosis was further confirmed using flow cytometric analysis. This study showed that the cytotoxicity and the mode of cell death by metal ions clearly depend on the cell type, the type of metal ion and the duration of exposure. The protein level of Rb, a tumor suppressor that affects apoptosis para-doxically, was higher in the cells treated with Co, Cu and Ni. It is believed that apoptosis and cell damage in the OSC cells treated with Co, Cu or Ni can be evoked by the regulation of Rb.

Key words: dental alloys, metal ions, oral squamous carcinoma cells, apoptosis.

Introduction

Heavy metals, such as nickel (Ni), cobalt (Co) and copper (Cu), are quite abundant in the environment and are components of many ordinary objects. These metals may be in the form of alloys that are used in dentistry, which are in long-term intimate contact with the oral tissue. Consequently, the biocompatibility of dental casting alloys is a critical issue and many concerns have been raised regarding the adverse effects of these alloys (Craig and Hanks, 1990; Wataha *et al.*, 1993; Bumgardner and Lucas, 1995). It is well known that the metal ions are released from various dental casting alloys (Wataha *et al.*, 1991; Hsu and Yen, 1998; Wataha *et al.*, 1998). Many studies have evaluated the cellular changes caused by the metal ions, and have shown that certain metal ions have cytopathogenic effects *in vitro* and *in vivo* (Schedle *et al.*, 1995; Messer and Lucas, 1999; Ermolli *et al.*, 2001). The allergic and carcinogenic potential of metals have been also documented (Dunnick *et al.*, 1995; Kasprzak, 1995; Bouchard *et al.*, 1996; Oller *et al.*, 1997). These pathological phenomena are accompanied by changes in the intracellular molecules, which might evolve a secondary reaction. However, the sequential biological processes as well as the basic mechanism of the cellular response to metal ion exposure are not clearly understood.

The use of solid samples of alloys is most desirable when the cellular biological responses evoked by dental casting alloys and their clinical significance are evaluated. In order to create the right conditions for a cytotoxic response and to observe the accumulative effect of metal ions released from dental casting alloys in cultured cells, the cells should be maintained in the same culture medium without any discharge for a long period of time. It is difficult to accumulate metal ions released from alloys without contaminating the culture medium. It is also impossible to evaluate the effects of the individual ions using the culture medium. By using a metal-ion solution, the effect of each type of metal ions can be evaluated individually, and their concentration and

*Corresponding author: Hae Ryoun Park, Dept. of Oral Pathology, College of Dentistry, Pusan National University, Ami-dong 1-10, Seo-gu, Busan 602-739, Korea. Tel.: +82-51-240-7839; Fax.: +82-51-245-2777; E-mail: parkhr@pusan.ac.kr.

duration of exposure can be controlled. Therefore, solutions of metal ions were used instead of exposing the cells to the dental alloys for lengthy periods. This type of approach was more favorable in investigating the biological response by metal ions accumulated in cells. The metal ions selected were based on previous studies, which demonstrated their release from dental alloys (Wataha *et al.*, 1991; Hsu and Yen, 1998; Wataha *et al.*, 1998).

Although epithelial cells first come in contact with metals within the oral environment, which can act as a local reservoir of the metals, only a few histopathologcial and cytotoxicological studies have described the oral squamous cell damage caused by these metals (Picardo *et al.*, 1990; Little *et al.*, 1996; Brosin *et al.*, 1997). Most studies were carried out to determine the cytotoxic effects of metal ions on mesenchymal cells (Wataha *et al.*, 1993; Bumgardner and Lucas, 1995; Schedle *et al.*, 1995; Messer and Lucas, 1999). Oral epithelial cells in human tissues may react differently to metal ions than gingival fibroblasts. Therefore, experiments using oral squamous cells may be meaningful in determining the biological behavior of metal ions within the oral cavity. Accordingly, this study examined the response and molecular changes in epithelial cells to metal ion exposure using human oral squamous carcinoma (OSC) cells. The aim of this study was to clarify the sequential events along with the elements involved in the cellular responses.

Materials and Methods

Cell culture

The human oral squamous carcinoma (OSC) cell line established from an oral squamous carcinoma patient was used in these experiments. The OSC cells were a generous gift from Professor J. I. Yook (College of Dentistry, Yonsei University, Seoul, Korea) (Chun *et al.*, 2002). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY) at 37°C in a humidified air containing 5% CO_2.

Chemicals

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT), dimethyl sulfoxide (DMSO), aluminum (Al), cobalt (Co), copper (Cu), nickel (Ni) were purchased from Aldrich (SIGMA-ALDRICH, Milan, Italy). Aqueous solutions of the metal ions were prepared from the chloride salts and only the filtered portions were used in the experiments.

Cell viability tests

The viability of the cultured cells was estimated using the MTT method. The cells were plated in a 96-well plate and incubated for 24 h. Metal ions were added to the cells and incubated for further 24 h. Subsequently, the cells were treated with 1 mg/ml MTT in DMEM and incubated for 4 h at 37°C in a 5% CO_2 incubator. The medium was aspirated and the formazan crystals, which were formed from a reaction with MTT and NADH-generating dehydrogenases in metabolically active cells, were dissolved in 200 μl DMSO. The cell viability was evaluated by measuring the intensity of the blue color (OD at 570 nm) by a multiwell reader (μQuant, Pharm-Tek, Little Chalfont, UK) and compared with that of the control culture (taken as 100%). The assay was performed in triplicate.

DNA fragmentation assay

1×10^6 cells were plated in 100 mm culture dishes. The cells were grown for 24 h and incubated for 24 h after being treated with metal ions (Sigma). Both the attached and unattached cells were harvested. The cells were washed in phosphate buffered saline (PBS), and lysed with a 0.5 ml lysis buffer (0.5% Triton X-100; 5 mM Tris, pH 7.5; 20 mM EDTA) for 20 min on ice. RNase was then added to the lysate. After 2 h, proteinase K was added to a final concentration of 100 μg/ml, and the mixture was incubated overnight at 55°C. After centrifugation at 15,000×g for 10 min, the DNA was extracted three times with an equal volume of phenol and chloroform : isoamyl alcohol (24 : 1, v/v). The samples were adjusted to 0.3 M sodium acetate (using 3 M stock sodium acetate, pH 7.0), and the DNA was precipitated with two volumes of cold ethanol. After incubation overnight at -20°C, the DNA was collected by centrifugation (15,000× g, 4°C) for 10 min, washed in 500 μl of 70% ethanol with centrifugation, and air dried. The pellets were dissolved in 10 μl of a Tris/EDTA buffer (pH 7.4). The DNA was run on a 2.25% agarose gel containing 1 μg/ml ethidium bromide in a TAE buffer at 50 V for 1.5 h, and visualized using an UV illuminator and photographed with a Polaroid camera. The size of the DNA was compared with a standard ladder (Bio-Rad, Hercules, CA).

Flow cytometry analysis

The harvested cells were washed with PBS containing 1% bovine serum albumin and centrifuged at 2,000 rpm for 10 min. The cells were resuspended in 3 ml of ice-cold 75% ethanol for 24-72 h at 4°C, and incubated with DNase-free RNase (0.1 mg/ml; Sigma) and propidium iodide (50 μg/ml; Sigma) at 4°C for at least 1 h prior to flow cytometric analysis. The propidium iodide-stained cells were assayed at 488 nm using an EPICS-XL cytometer (Beckman Coulter, Miami, FL) that is equipped with an air-cooled 20 mW argon laser. The cell cycle was analyzed using Multicycle software (Advanced version; Phoenix Flow Systems, San Diego, CA).

Western blot analysis

The cells were lysed with a RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml phenylmethylsulfonyl fluoride) on ice for 1 h. The cell lysates

containing 60 μg of the proteins were separated on Tris-glycine SDS-polyacrylamide gel and electrotransferred to a polyvinylidene fluoride membrane (Bio-Rad) using a wet transfer kit. The membranes were incubated for 1 h in Tris-buffered saline (TBS, pH 7.6) containing 0.1% Tween and 5% non-fat milk, and then incubated with the primary antibody. The blots were washed in TBS containing 0.1% Tween and incubated with the secondary antibody. The blots were then washed with the same solution four times, and visualized on Kodak X-OMAT AR films (Rochester, NY) using an Enhanced Chemiluminescence detection kit (Amersham Life Sciences, Little Chalfont, UK). All the procedures were carried out at room temperature. The antibodies against bcl-2, bax and Rb were purchased from Transductions laboratories (San Diego, CA).

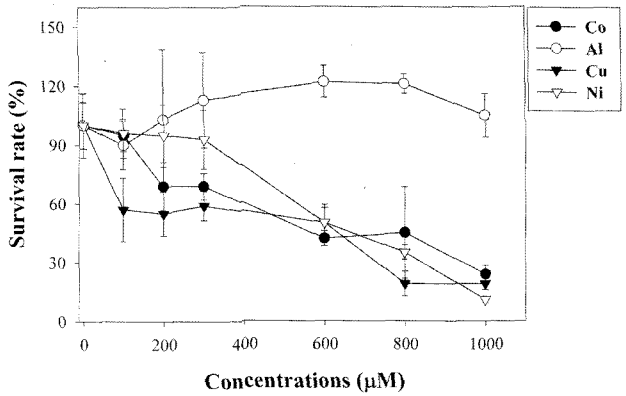

Fig. 1. MTT test for the viability of subconfluent OSC cells grown in DMEM for 24 h in the presence of increasing concentrations of Al, Co, Cu, or Ni. The bars represent ± S. D.

Results

Cytotoxic effect of Co, Cu and Ni ions on OSC cells

The effects of an array of metals, including Al, Co, Cu and Ni, on OSC cells were analyzed in order to determine the cytotoxicities of various ions. The effective doses of the metal compounds reducing the viability of the cells were first tested using a MTT assay. The logarithmically growing cells were treated with 0, 100, 200, 400, 600, 800 and 1,000 μM of the metal ions for 24 h. Al was not found to be cytotoxic in the concentration range tested. The TC_{50} values for the metal ions with the exception of Al were estimated to be between 400 and 600 μM (Fig. 1). Therefore, a 1 mM Al solution and 500 μM solutions of the other metal were used throughout the study.

When the cellular morphology was evaluated using inverted microscopy, the majority of Co, Cu and Ni treated cells, which originally had an epithelial cell-like appearance, became shrunken and had a round shape, and the number of the cells was significantly reduced. The remaining viable cells appeared to have neurite-like processes (Fig. 2). There was almost no difference between the Al treated and control cells.

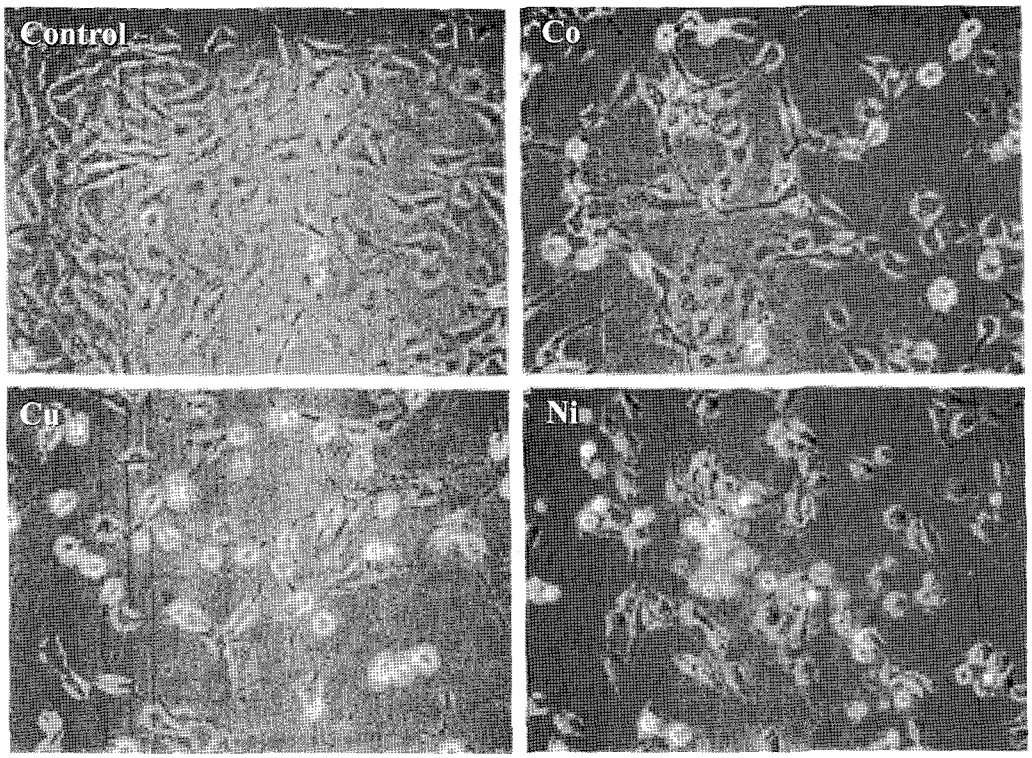

Fig. 2. The morphology of the OSC cells treated with no chemicals (control), 500 μM Co, 500 μM Cu, or 500 μM Ni for 24 h.

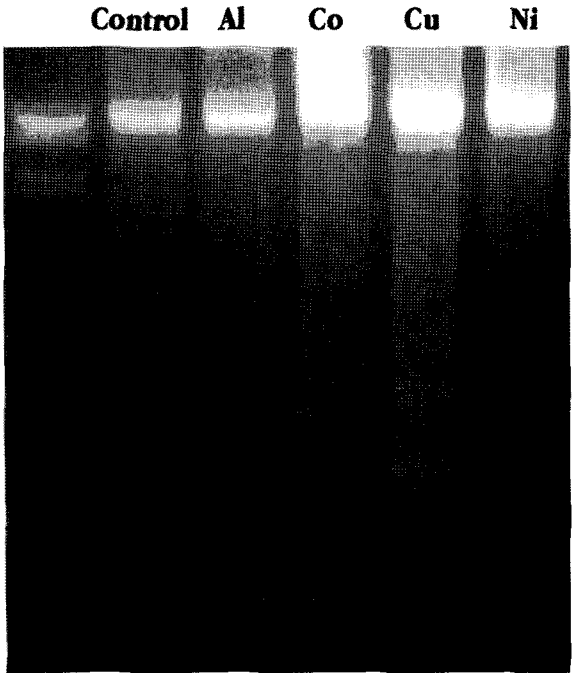

Fig. 3. DNA fragmentation assay. The cells were treated with no chemicals (control), 1 mM Al, 500 μM Co, 500 μM Cu, or 500 μM Ni for 24 h. The DNA was extracted and run on 2.25% agarose gels containing 1 μg/ml ethidium bromide in TAE buffer.

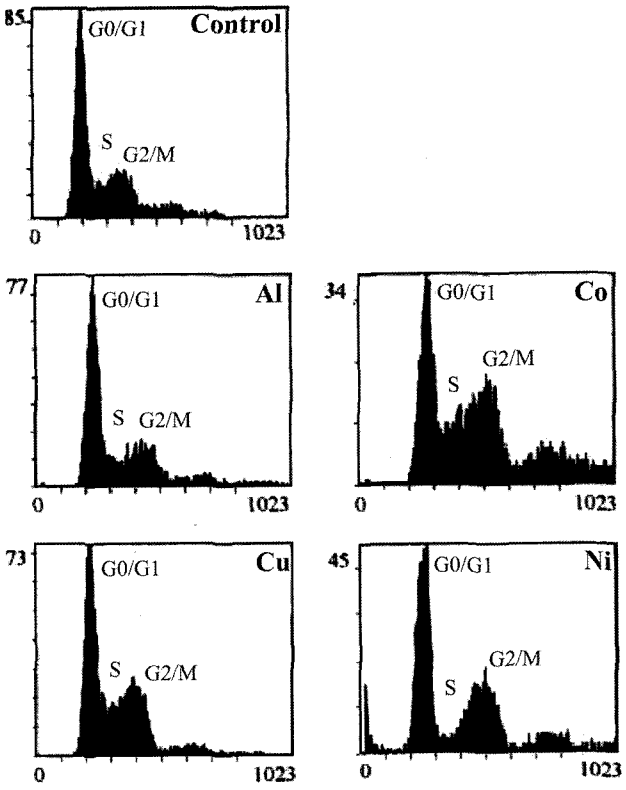

Fig. 4. Flow cytometric analysis. The cells were treated with the metal ions for 24 h and fixed with 70% ethanol. After adding RNase, the cells were stained with propidium iodide (50 μg/ml in PBS) at 4°C for at least 1 h and analyzed using a flow cytometer.

Co, Cu and Ni ions induced apoptosis in OSC cells

The type of cell death induced by metal ions was examined by determining the extent of DNA fragmentation, which is the biochemical hallmark of apoptosis, although not always demonstrable. As shown in Fig. 3, the DNA ladder was clearly detected in the cells treated with metal ions with the exception of Al ions. Its intensity increased markedly after 24 h exposure.

Metal ion-induced apoptosis was further confirmed using flow cytometric analysis (Fig. 4). In flow cytometric analysis, the occurrence of a sub-G0/G1 peak, which is indicative of apoptosis, was detected in the cells treated with Ni for 24 h. Apoptotic peaks in the Al, Co and Cu treated cells were not prominent in the graph.

Molecules involved in the cytotoxic response depend on the type of metal ions

The main aim of this study was to investigate the cytotoxicity of Co, Ni, Cu and Al with relation to their subsequent molecular mechanism. In order to identify the molecules responsible for the cellular response, expression levels of bcl-2, bax and Rb, on the apoptotic process or cell cycle regulation were examined by western blot analysis. The levels of the bax and bcl-2 proteins were lower in the cells treated with Cu and Ni. The Rb protein level was higher in the cells treated with the metal ions. In particular, the Co, Cu, or Ni treated cells showed the significant up-regulation of the Rb protein. These results showed that the Rb protein might be involved in the metal-ion induced apoptosis of OSC cells (Fig. 5).

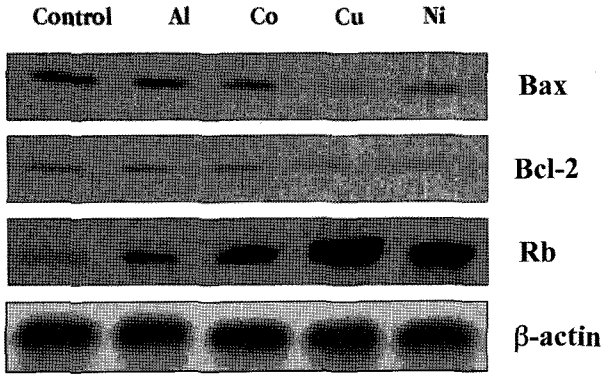

Fig. 5. Western blot analysis of bax, bcl-2 and Rb in the OSC cells treated with metal ions. The cells were harvested after 24 h incubation with Al, Co, Cu, or Ni. The amount of protein was normalized to the 60 μg/lane.

Discussion

Many transition metals are toxic to cells when present in excess quantities. Several experiments have tested the cell

toxicity of metal ions but the findings have been inconsistent (Wataha *et al.*, 1993; Bumgardner and Lucas, 1995; Dominguez *et al.*, 1995; Schedle *et al.*, 1995; Bellows *et al.*, 1999; Messer and Lucas, 1999). Wataha *et al* (1993) incubated fibroblasts with Al for 24 h and reported that Al was not toxic. Under different experimental conditions Al was found to be toxic to cultured human fibroblasts and reduced the level of cell survival after an exposure period of 4 days (Dominguez *et al.*, 1995). However, in the current study, Al was not cytotoxic to squamous cells after a 24 h culture even at concentrations up to 1.0 mM and induced no specific changes. This suggests that the cytotoxicity of Al might be due to the exposure time to Al rather than by the cell types or the concentration of Al ions used. There are a few reports showing that Co and Ni ions play a role in the toxicity to macrophages, lymphocytes, alveolar cells and fibroblasts (Granchi *et al.*, 1996; Catelas *et al.*, 2001; Cross *et al.*, 2001). This study confirmed that Co and Ni are cytotoxic to the OSC cells. However, there were no differences in the potency of those metal ions. Based on these findings and previous reports, Co and Ni ions are toxic to all types of cells but the sensitivity of the cells to Co and Ni ions may depend on the cell type. There are two arguments against the cytotoxicity of Cu. Most reports have shown that Cu is toxic to many kinds of cells (Schedle *et al.*, 1995). However, some investigators could not observe any cytotoxicity of Cu toward HepG2 cells, human dermal fibroblasts or human arterial smooth muscle cells at concentrations up to 500 μM (Tsang *et al.*, 1996; Hu, 1998). The present study showed that Cu is toxic to the OSC cells, indicating that the cytotoxicity of Cu is dependent on the cell types.

The OSC cells exposed to Co, Cu and Ni had a shrunken and round shape in this study, which is common finding for damaged cells. Interestingly, the undamaged and viable cells developed neurite-like extensions. This change in the cell morphology to an elongated phenotype was observed in Ni-treated Chinese Hamster Ovary cells, and Shiao *et al* (1998) suspected that this change is associated with the up-regulation of cAMP. It is possible that the change in the OSC cell shape by Co, Cu and Ni is also related to cAMP.

The extent of cell damage caused by the metal ions along with the resultant death observed in this study might be due to either apoptosis or necrosis. Among the rigorous sets of biochemical criteria for apoptosis, the most reliable biochemical marker for apoptosis is the double-strand cleavage of the nuclear DNA at the linker regions between the nucleosomes, which is represented as a ladder form (Cohen *et al.*, 1992). The observation of DNA laddering with the presence of a sub G0/G1 peak after the Co, Cu and Ni treatment suggest that the OSC cells die through an apoptotic process. Co and Ni induce apoptosis in many kinds of cells (Shiao *et al.*, 1998; Zhai *et al.*, 2000; Lee *et al.*, 2001). Zou *et al* (2001) reported that cobalt chloride triggered the apoptosis of neuronal PC12 cells in a dose- and time-dependent manner. In contrast, L-929 murine fibroblasts and gingival fibroblasts after exposure to Co, Cu and Ni showed signs of necrosis but no signs of apoptosis (Schedle *et al.*, 1995). Overall, the mode of cell death and the magnitude of apoptosis clearly depend on the cell type, metal ions type and the duration of exposure.

Proteins from bcl-2 family are important regulators of cell life and death, with some functioning to prevent or to promote apoptosis, such as bcl-2 and bax, respectively. It was reported that when bcl-2 is overexpressed, it heterodimerized with bax and repressed cell death. Bax overexpression accelerates the rate of apoptotic death of cells triggered by certain apoptotic stimuli. It was recently shown that the bcl-2 to bax ratio is major determinant on cell survival or death after an apoptotic stimulus (Boise *et al.*, 1993; Oltvai *et al.*, 1993). This study observed a decrease in the bcl-2 protein level by Cu and Ni, which was accompanied by a decrease in the bax level. Therefore, there was no significant change in the bcl-2 to bax ratio by the metal ions, and the ratio of these proteins does not appear to regulate the apoptotic death in the OSC cells. Many reports have suggested that the proteins of the bcl-2 family are regulated by tumor suppressor gene, p53 (Haldar *et al.*, 1994; Miyashita *et al.*, 1994). The OSC cells, which do not possess the endogenous tumor suppressor gene p53, were used in this study (Chun *et al.*, 2002). This means that the apoptosis caused by the above-mentioned metal ions does not involve the p53-pathway, and other molecules are probably involved in the regulation of metal-ion induced cell death. Rb is known to induce G1 arrest when it is hypophosphorylated and can provide apoptotic signal under some conditions (Weiberg, 1995; Krek *et al.*, 1995). It is possible that apoptosis and cell damage in cells treated with Co, Cu or Ni can be evoked by the regulation of Rb rather than bcl-2 family. A further study will be needed to determine the precise role of Rb in metal-ion induced apoptosis.

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