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# Effects of metal ions on osteoblast-like cell metabolism and differentiation

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The objective of this study was to evaluate the effects of metal ions, which may be released from orthopedic or dental implants, on osteoblast metabolism and differentiation. ROS 17/2.8 cells were cultured in F-12 medium for 7 days. Then Al<sup>+3</sup>, Co<sup>+2</sup>, Cr<sup>+3</sup>, Ni<sup>+2</sup>, Ti<sup>+4</sup>, and V<sup>+3</sup> were added at concentrations less than their cytotoxic concentrations. After 3 days, DNA synthesis, succinate dehydrogenase activity, alkaline phosphatase (ALP) activity, and culture calcification were assessed. Northern blots were performed for ALP, osteocalcin (OCN), and osteopontin (OPN) mRNA transcription. The data indicated that Cr<sup>+3</sup> and Al<sup>+3</sup> had few inhibitory effects on ROS cell metabolism below their cytotoxic concentrations, Ni<sup>+2</sup>, Co<sup>+2</sup>, Ti<sup>+4</sup>, and V<sup>+3</sup> affected all these parameters of ROS

cell metabolism at concentrations below cytotoxic levels. For RNA analysis, Al<sup>+3</sup> significantly suppressed the expression of ALP, OCN, and OPN at both cytotoxic and noncytotoxic concentrations. Co<sup>+2</sup> specifically suppressed ALP expression at cytotoxic concentrations. Cr<sup>+3</sup> and Ni<sup>+2</sup> inhibited OCN, OPN, and ALP gene expression only at cytotoxic concentrations. For Ti<sup>+4</sup> and V<sup>+3</sup> ions, gene expression at cytotoxic levels was not significantly affected as compared with the effects at noncytotoxic level. These results show that metal ions may alter osteoblast behavior even at subtoxic concentrations, but do not always affect the expression of all genes similarly. © 1997 John Wiley & Sons, Inc.

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## INTRODUCTION

Metals and alloys have a wide range of applications as prosthetic materials for bone and tooth tissue reconstruction. The most common metals or alloys in orthopedics and dentistry are cobalt–chromium–nickel, titanium–aluminum–vanadium, and commercially pure (cp) titanium. The suitable mechanical and physical properties of these materials have made them attractive as implant materials. However, *in vivo* corrosion of these metals is a major potential disadvantage of these implants. Generally, corrosion can cause two kind of changes: 1) reduction of the mechanical properties and shortening of the implant life time, and 2) harmful reactions to the host body both locally and systemically brought on by products of corrosion.

A number of investigations have demonstrated that metal ions can be released from metallic implants as the result of corrosion.<sup>1-3</sup> Trace element analysis has shown that metal ions can be localized in blood or

serum,<sup>4</sup> urine,<sup>5</sup> and other organs,<sup>6-8</sup> as well as in bone tissue adjacent to implants.<sup>9,10</sup> The biologic consequences of these released ions on the tissues or cells have been extensively studied *in vitro* and *in vivo*. Recent research results have shown that at concentrations less than those which cause a 50% depression in general cell activity, Ti and Co ions still can inhibit the specific cellular functions such as alkaline phosphatase (ALP) activity, extracellular calcification, and bone-specific gene expression.<sup>11,12</sup>

To identify a material as biocompatible to bone, it is more informative to pay attention to the effect of the material on specific cellular functions than to evaluate general cytotoxicity. Previous studies have investigated the effects of metal ions and alloys on bone tissue function. Wataha et al. compared the cytotoxicity of several metal ions by using a fibroblast cell line (Balb/c 3T3) and osteoblast-like cell line (ROS 17/2.8). For Ti the osteoblast-like cell line showed more sensitivity than the fibroblast cell line.<sup>13</sup> Haynes et al. reported that Ti-6Al-4V particles elicited high levels of cytokines and prostaglandin E<sub>2</sub> from human monocytes. These biologic mediators have the ability to initiate an inflammatory response and bone resorption.<sup>14</sup> Furthermore, Massa et al. compared titanium and hydroxyap-

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atite (HA) on ALP activity as well as the response to parathyroid hormone (PTH) in rat calvarium cells. The results indicated that cells contacting HA have higher ALP activity and higher response to PTH than cells contacting titanium.<sup>15</sup> Puleo et al. found that neither Ti-6Al-4V alloy nor HA ceramic had an effect on expression of the bone matrix protein genes of osteonectin and osteopontin (OPN).<sup>16</sup>

Although these studies provide valuable information about cellular response to implant materials, the mechanisms of cell-implant interactions are still unclear. Many experimental problems need to be solved. First, the cytotoxicity of a material is often evaluated by a fibroblast culture *in vitro*. To some extent, the results can explain how cytotoxic the material is. But, as we know, cells of different origins have different cellular reactions to foreign bodies. Thus, the results from fibroblasts should not predicate the cytotoxicity for bone cells or other tissue cells. Second, alloys, rather than component ions, are frequently studied for biocompatibility. When using the alloy, it is difficult to determine which element of the alloy is responsible for the adverse action. In addition, the conventional cytotoxicity evaluation which focuses on the viability of cells does not explain the material's influence on specific cellular functions and differentiation processes.

In this article, we have evaluated the effect of Cr, Al, Ni, Co, Ti, and V ions, which are ions which may be released from metallic implants *in vivo*,<sup>28</sup> on osteoblast-like cell metabolism. We have tried to explain how these ions, at cytotoxic and noncytotoxic concentrations, affect the metabolism and bone-specific gene expression of osteoblasts. Since the bone tissue is probably chronically exposed to low levels of ions released from the implant, the focus of this study is on the effects of sublethal levels of the metal ions. Our hypothesis is that metal ions can affect bone cell differentiation and cell-specific functions at sublethal concentrations. Information from this study should help clarify the effects of metal ions on bone tissue and improve our understanding of bone-implant interactions.

## MATERIALS AND METHODS

Six metal ions, Al<sup>+3</sup>, Co<sup>+2</sup>, Cr<sup>+3</sup>, Ni<sup>+2</sup>, Ti<sup>+4</sup>, and V<sup>+3</sup>, were selected for this study because they are basic components of current metallic implants in orthopedics and dentistry. All metal salts were purchased as the chlorides (Aldrich, Milwaukee, WI) and stock solutions were prepared in sterile water, then were diluted such that 10  $\mu$ L would provide the desired final concentration in 200  $\mu$ L of F-12 culture medium. Pilot experiments showed that addition of these concentra-

tions altered pH by  $<0.05$ , and induced no precipitation.

To measure cellular proliferation and metabolism, the DNA synthetic rate (<sup>3</sup>H-TdR incorporation) and mitochondrial dehydrogenase activity [succinate dehydrogenase (SDH)] were used. Previous studies have shown that both of them were reliable and sensitive parameters for studying cellular growth and viability, and have been extensively used in the determination of cytotoxicity of metal ions as well as other biomaterials.<sup>17,18</sup> To determine the expression of osteoblast-related phenotypes, ALP activity was measured, von Kossa histologic staining was used to measure culture mineralization. Finally, to evaluate cellular differentiation for the osteoblast-like cells, expression of ALP (cDNA for mouse liver/bone/kidney ALP was a gift from Dr. Rennie Franceschi (University of Michigan, School of Dentistry, Ann Arbor, MI), osteocalcin [(OCN) mouse cDNA from Genetics Institute, Cambridge, MA], and OPN (mouse cDNA from Dr. G. A. Rodan, Merck Sharp and Dohme, West Point, PA) genes were measured using Northern blot analysis. OCN and OPN have been identified as noncollagenous extracellular proteins in bone matrix and are synthesized by osteoblasts and osteoblast-like cells.<sup>19</sup> Their biologic function in bone tissue is not completely known, but they generally relate to the formation, growth, and regulation of bone tissue and mineralization. Since they are synthesized mainly by osteoblasts, they usually are selected as unique markers for studying bone cell differentiation and bone tissue mineralization. As result, in the study of osseous tissue integration with biomaterials, the expression of bone-specific proteins in the local region is an important signal to indicate when bone tissue formation is occurring, and may give insight into the responsiveness of bone cells to the new environment created by the artificial implant.

Concentrations of metal ions were selected as follows. Concentrations which caused a 50% reduction of SDH activity in ROS cells were used as the highest concentrations, except for Al and Cr.<sup>13</sup> Subsequent concentrations (four others) were decreased sequentially by half. The ions and their concentrations ranges are shown in Table I. For Al and Cr, the maximum concentrations which did not cause precipitation in the medium were used, since 50% reduction in SDH activity could not be reached. The DNA synthesis and ALP activity were performed on these five concentrations. Because of interactions between the Cr, Al, Ni, and V ion solutions and von Kossa staining solution, the mineralization assay (von Kossa stain) was performed only for Ti and Co ions. Furthermore, effects of the ions on gene expression of these cells were determined at two concentrations. One concentration was selected because it was not cytotoxic by the SDH activity assay and another concentration was selected because it was

TABLE I  
Metal Ion Concentrations and Sources

Metal Ion	Conc. Range Tested ( $\mu\text{mol/L}$ )*	TC <sub>50</sub> Conc. ( $\mu\text{mol/L}$ ) <sup>†</sup>	Source
Al <sup>+3</sup>	195, 390, 780, 1560, 3120	3120 <sup>‡</sup>	AlCl <sub>3</sub> <sup>3</sup>
Co <sup>+2</sup>	13, 26, 52, 104, 208	208	CoCl <sub>2</sub> ·6H <sub>2</sub> O
Cr <sup>+3</sup>	120, 240, 480, 960, 1920	1920 <sup>‡</sup>	CrCl <sub>3</sub> ·6H <sub>2</sub> O
Ni <sup>+2</sup>	38, 76, 152, 304, 608	608	NiCl <sub>2</sub> ·H <sub>2</sub> O
Ti <sup>+4</sup>	13, 26, 52, 104, 208	208	TiCl <sub>4</sub> <sup>§</sup>
V <sup>+3</sup>	1.25, 2.5, 5, 10, 20	20	V Cl <sub>3</sub> <sup>§</sup>

\*Lowest and highest concentrations tested.

<sup>†</sup>Concentration required to reduce SDH activity by 50% compared to controls (without metal ions).

<sup>‡</sup>3120  $\mu\text{mol/L}$  of Al only suppressed SDH activity by 20%. Higher concentrations could not be used because of precipitation.

<sup>§</sup>Obtained from atomic absorption standard solutions.

<sup>‡</sup>1920  $\mu\text{mol/L}$  of Cr did not suppress SDH activity significantly, but was the highest concentration which could be used because of precipitation.

significantly cytotoxic by the SDH activity assay. Since Al and Cr were not cytotoxic at any concentration tested, the highest and lowest concentrations were chosen for gene expression tests.

ROS 17/2.8 osteoblast-like cells were kindly given to us by Dr. Robert Majeska (Mt. Sinai Hospital, New York, NY) and were maintained in F-12 medium (Gibco, Grand Island, NY) containing: 28 mM HEPES (Calbiochem, La Jolla, CA), 1.1 mM CaCl<sub>2</sub> (Allied Chemical, Monistown, NJ), 5% NuSerum (Collaborative Res, Bedford, MA), and 25 mM L-glutamine and 125 U/mL penicillin-streptomycin (GIBCO). Cells were plated at 30,000 cells/cm<sup>2</sup> in 200  $\mu\text{L}$  of medium in 96-well plates (Costar, Cambridge, MA) and were kept at 37°C in a humidified 95% air-5% CO<sub>2</sub> atmosphere. At 3-4 days, the medium was changed. At 7 days, the cultured cells became confluent. Control wells were fed with the following medium: F-12 medium and 50  $\mu\text{g/mL}$  ascorbic acid (AA) (J. T. Baker, Phillipsburg, NJ), 10 mM  $\beta$ -glycerophosphate (GP; Sigma, St. Louis, MO), and 100 nM dexamethasone (Dex; Sigma, St. Louis, MO). Experimental wells were treated with various concentrations of metal ions in addition to medium supplements at 7 days. At 10 days, determinations of cell proliferation, mineralization and specific mRNA expression were carried out. For every test group, there were a total of six replicates.

The procedure for [<sup>3</sup>H]thymidine (TdR) incorporation is described in detail elsewhere.<sup>20</sup> <sup>3</sup>H-TdR was purchased at 1  $\mu\text{Ci/mL}$  and 65 Ci/mmol (ICN, Irvine, CA). <sup>3</sup>H-TdR at a final concentration of 0.05  $\mu\text{Ci/mL}$  was added to the cells in the culture for 2 h. At the end of incubation, the cultures were washed by 5% trichloroacetic acid (TCA) solution at 4°C three times, and the solution was removed. 100  $\mu\text{L/well}$  of 6.7N perchloric acid (PCA) solution was added and stirred for 5 min. Then 75  $\mu\text{L}$  of the PCA extract was added to

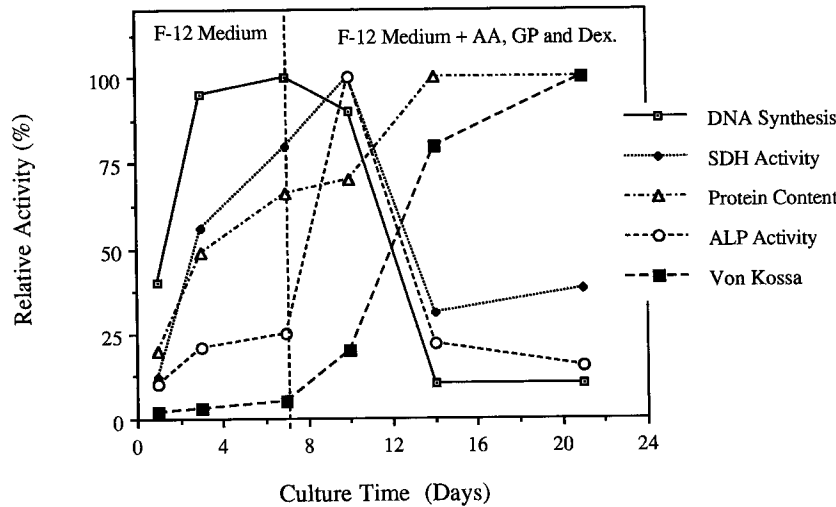
3 mL Bio-Safe II liquid scintillation solution (Research Products, Mount Prospect, IL) and allowed to rest for 12 h. Scintillation counting [counts per minute (cpm)] was performed in a 1209 RackBeta liquid scintillation counter (LKB Wallac, Turku, Finland) after 12 h.

Succinate dehydrogenase (SDH) activity was determined by the colorimetric MTT method [a tetrazolium salt: 3-(4,5-dimethyl-thiazoly(-2)-2,5-diphenyl tetrazolium bromide] (Sigma).<sup>21</sup> Then 100  $\mu\text{L/well}$  1% MTT solution was added and incubated with the cells at 37°C for 1 h. The MTT solution was removed and cells were washed with phosphate-buffered saline (PBS). The blue formazan product was dissolved in 100  $\mu\text{L/well}$  dimethyl sulfoxide (DMSO), and the absorption (Abs) of MTT-formazan was read at 560 nm.

Measurement of ALP activity was performed as described by Bessey et al.<sup>22</sup> Briefly, cultured cells were rinsed with PBS and 100  $\mu\text{L/well}$  of 50mM Tris-HCl buffer (pH 7.45) was added and the cultures were stored at -20°C until ready for assay. The cultures were incubated at 37°C for 10 min with 20  $\mu\text{L/well}$  enzyme substrate mixture solution containing: 2mM Na<sub>2</sub>-nitrophenylphosphate (Na<sub>2</sub>-PNPP) (Sigma) and 2mM MgCl<sub>2</sub> in 0.1M 2-amino-2methyl-1-propanol buffer (pH 10.5) in individual wells of a 96-well tissue plate. The reaction was stopped with 100  $\mu\text{L/well}$  of 1N NaOH. Enzyme activity was read at 405 nm. Protein concentration was determined using a micro-BCA protein assay kit (Pierce, Rockford, IL). The ALP activity was expressed in terms of micromoles per minute per milligram protein.

Mineralization of the cultures was measured by von Kossa stain. The matrix was washed with PBS, fixed by sequential treatment of the cultures with 70, 90, and 100% ethanol for 5 min, respectively, and then hydrated with H<sub>2</sub>O. The fixed cultures were treated with 5% silver nitrate solution 100  $\mu\text{L/well}$  in the dark at 37°C for 1 h. The excess silver nitrate solution was then completely washed away by double-distilled H<sub>2</sub>O (dd-H<sub>2</sub>O) and the culture plate was exposed to strong light for 30 min. The metallic silver produced brown-black precipitates, and the optical density of the stained matrix was measured at 405 nm.

Total RNA isolation and Northern blot analyses were carried out by the method as described by Towle.<sup>23</sup> Total RNA was extracted and purified by using phenol-chloroform and isoamyl alcohol. RNA was precipitated in sodium acetate and collected by centrifugation at 10,000 $\times g$  for 20 min at 4°C. A total of 20  $\mu\text{g}$  of total RNA was electrophoresed in 0.8% agarose/formaldehyde gel and blotted onto a nylon membrane with 20 $\times$  SSC (NaCl, sodium citrate, and NaOH) solution. After crosslinking, prehybridization was performed in 50% formamide, 5 $\times$  SSC, 5 $\times$  Denhardt's solution, 50 mM phosphate buffer (pH 6.5), 1% sodium dodecyl sulfate (SDS), 250  $\mu\text{g/mL}$  salmon sperm DNA at 42°C for 4 h. Hybridization was performed at 45°C



**Figure 1.** Metabolism characteristics of osteoblast-like ROS 17/2.8 cells at different culture times. Cells were plated at 30,000 cells/cm<sup>2</sup> and cultured in F-12 medium from 7 days. After 7 days, cells were cultured in F-12 medium with AA, GP, and Dex. The DNA synthesis, SDH activity, ALP activity, total protein content, and von Kossa stain were performed on 1, 3, 7, 10, 14, and 21 days, respectively. All terms are described as the relative activity, i.e. the percentage of the maximum level of measurements.

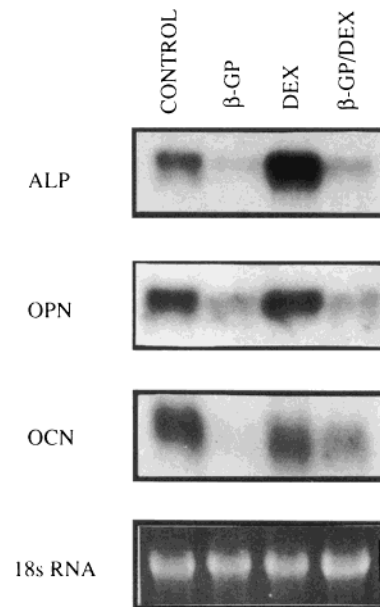
for 18 h in the same buffer containing  $1 \times 10^6$  dpm [<sup>32</sup>P]dCTP-labeled denatured cDNA probe, using a random primer kit (Boehringer Mannheim, Indianapolis, IN). After hybridization, blots were washed twice at room temperature and once at 65°C with 2× SSC and 0.1% SDS and then twice at 65°C with 1× SSC and 0.1% SDS. Blots were visualized by autoradiography.

Normal cell metabolic characteristics were tested from 1 to 21 days to select the best time to add the metal ions and measure cell response. Figure 1 shows the DNA synthesis rate, SDH activity, ALP activity, total protein content, and matrix calcification (von Kossa) over 21 days in culture with no metal ions. All parameters are described as percent activity relative to controls. From data shown in Figure 1, the 10-day cultures were determined to be the most suitable cultures for testing the effects of the metals. This time was chosen for several reasons. Although the mineralization was still moderate compared to the 14- and 21-day cultures, the ALP activity, SDH activity, and DNA synthesis rate were highest at 10 days. In addition, the expression of ALP, OCN, and OPN mRNA were also detectable at this time. Figure 2 presents the Northern blot results of gene expression at 10 days without metals present. Throughout these experiments, the exposure time of the metals to the ROS cells was 3 days (from days 7 to 10). We chose this interval because the early addition of the metal ions would have the greatest chance of inhibiting cell proliferation and changing cell phenotypes.

## RESULTS

Table I shows the concentration required to cause a 50% decrease in SDH activity (TC<sub>50</sub>) for each ion. For

aluminum, only the TC<sub>25</sub> concentration could be determined, which was the concentration required to cause a 25% decrease of SDH activity. For Cr, no suppression of SDH activity was evident at the highest concentration.



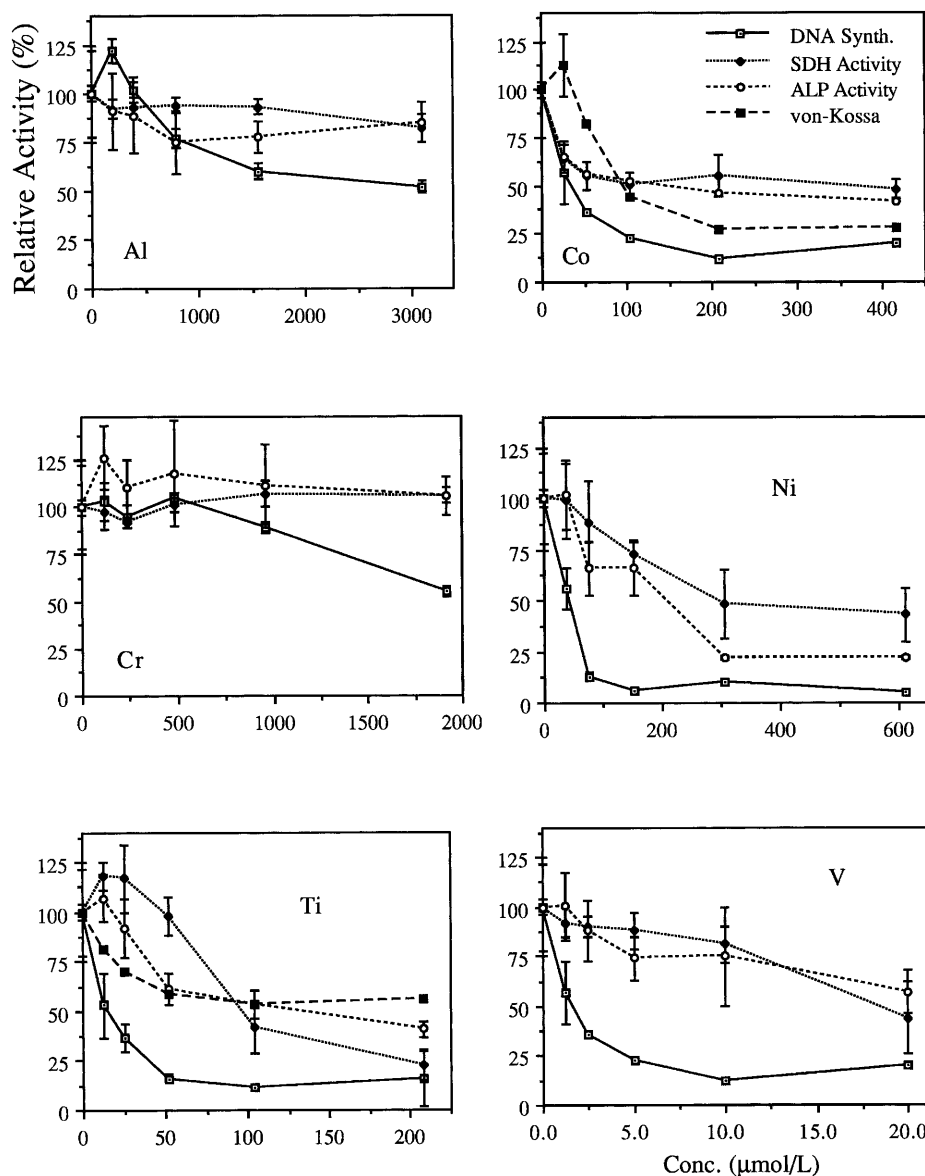
**Figure 2.** ALP, OCN, and OPN gene expression by ROS 17/2.8 cells. RNA was isolated from 10-day cultured cells. Lane 1: cells were cultured only in F-12 medium with AA. Lane 2: cells were cultured in F-12 medium with AA and GP. Lane 3: cells were cultured in F-12 medium with AA and Dex. Lane 4: cells were cultured in F-12 medium with AA, GP, and Dex. Results show that Dex can stimulate the expression of ALP gene, but not of OCN and OPN genes in ROS 17/2.8 cells.

The only significant effect of the Cr ion on the ROS cells was to suppress the DNA synthesis about 50% at the TC<sub>50</sub> value (Fig. 3). SDH and ALP activities were maintained at high and stable levels (Fig. 3). RNA analysis indicated that Cr ion did not inhibit ALP, OCN, and OPN gene expression at 120  $\mu\text{mol/L}$ , but significantly inhibited ALP, OCN, and OPN gene expression at 1920  $\mu\text{mol/L}$  (Fig. 4 and Table II).

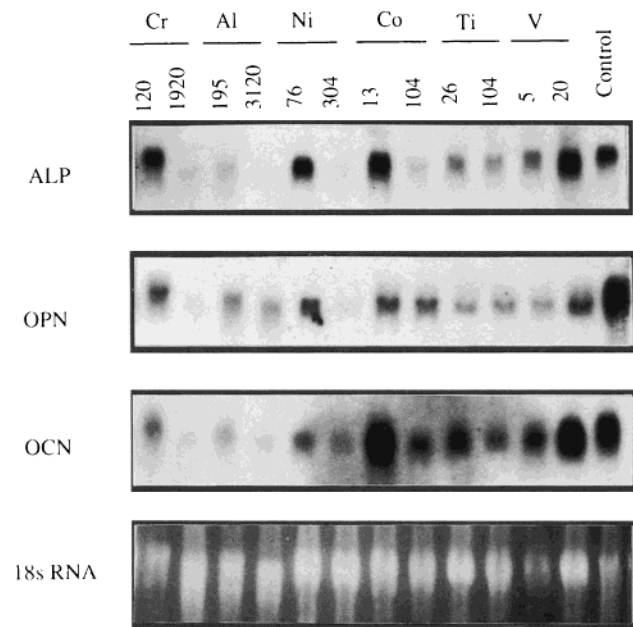
A similar pattern for DNA synthesis, SDH activity, and ALP activity were observed for Al ion. However, the DNA synthesis rate was inhibited about 40% at 1560  $\mu\text{mol/L}$  (Fig. 3). Compared to controls, SDH activity and ALP activity were maintained at relatively stable levels (Fig. 3). RNA analysis demonstrated

that Al ions significantly inhibited the ALP, OCN, and OPN gene expression at both TC<sub>25</sub> concentration (3120  $\mu\text{mol/L}$ ) and noncytotoxic concentration (195  $\mu\text{mol/L}$ ). The ALP gene expression was almost completely suppressed (Fig. 4 and Table II).

ROS cells showed similar responses to Co, Ni, Ti, and V ions. For these ions, cytotoxicity was a function of the concentrations below the TC<sub>50</sub> values. As the concentration increased, the cytotoxicity of these ions was also increased. The DNA synthesis was the most sensitive of the metabolic assays (Fig. 3). In addition, it was found that the presence of Co and Ti ions could inhibit the mineralization process during 3 days exposure to ROS, 17/2.8 cells (Fig. 3). However, the effects



**Figure 3.** Effects of six metal ions on ROS 17/2.8 cell DNA synthesis, SDH activity, ALP activity, and mineralization (von Kossa stain). Al, Co, Cr, Ni, Ti, and V ions at five different concentrations were added to ROS cells for 3 days. Because of interactions between the Cr, Al, Ni, and V ion solutions and the von Kossa stain solution, von Kossa staining was performed only for Ti and Co ions. All test items are expressed as a percentage of control samples.



**Figure 4.** Effects of six metal ions on ROS 17/2.8 cell expression of ALP, OCN, and OPN genes. For each ion, there were two concentrations ( $\mu\text{mol/L}$ ) selected for the RNA analysis.

of these ions on gene expression did not have the same pattern (Fig. 4 and Table II). Ni ion suppressed OCN gene, but did not suppress the ALP and OPN mRNA expression at the noncytotoxic concentration ( $76 \mu\text{mol/L}$ ), and significantly inhibited all of them at cytotoxic concentration ( $304 \mu\text{mol/L}$ ). Co ion did not affect levels of mRNA of ALP, OCN, and OPN at noncytotoxic concentration ( $13 \mu\text{mol/L}$ ), but did suppress ALP and OCN mRNA significantly at the cytotoxic concentration ( $104 \mu\text{mol/L}$ ). Ti and V did not significantly inhibit the expression of these three mRNA levels at either cytotoxic or noncytotoxic concentrations.

## DISCUSSION

ROS 17/2.8 osteoblast-like cells originate from rat bone sacroma.<sup>24</sup> These cells are reported to contain a large proportion of proliferative, phenotypically immature cells, closely resembling progenitor cells, and ongoing maturation of these cells may be responsible for the increase in ALP activity and hormones, and responsiveness to growth factors.<sup>25</sup> Glucocorticoids are reportedly responsible for regulation of ALP activity in bone-cell culture. They may increase ALP levels up to sevenfold in ROS 17/2.8 culture.<sup>26</sup> This effect has been associated with reduced cell growth.<sup>27</sup> Recent evidence has emerged that dexamethasone (Dex) has a specific activity on ALP gene expression.<sup>28</sup>  $\beta$ -Glycerophosphate (GP) is required for mineralization in several bone cell or tissue culture systems *in vitro*. Although its mechanism is still unclear, it is generally thought that GP is a source of inorganic phosphate ions (Pi).<sup>29</sup> When GP is added to the bone-cell culture, it may be hydrolyzed by ALP giving a high level of Pi in a local environment. This elevated Pi may thereby provide the chemical potential for mineral deposition.<sup>30</sup> In our cell culture system,  $100\text{nM}$  Dex and  $10\text{mM}$  GP in F-12 medium increased ALP activity and its gene expression, and promoted the matrix calcification (Figs. 1 and 2). These observations make ROS 17/2.8 cells a suitable model for cytotoxicity as well as cell differentiation studies.

The cytotoxicity rank as determined by the SDH assay of the six metal ions in this study was in the following order:  $\text{V}^{+3} > \text{Ti}^{+4}, \text{Co}^{+2} > \text{Ni}^{+2} > \text{Cr}^{+3}, \text{Al}^{+3}$ . In general, this rank is similar to other results determined in other types of cell cultures. Wataha demonstrated the cytotoxicity rank order of several metal ions by Balb/c 3T3 cell line as:  $\text{V}^{+3} > \text{Co}^{+2} > \text{Ni}^{+2} > \text{Ti}^{+4}$

**TABLE II**  
Effects of Six Metal Ions on ROS 17/2.8 Cell Metabolism and Gene Expression

Ion Conc. ( $\mu\text{mol/L}$ )*	Cell Activity (%) <sup>†</sup>			Osteogenic Gene Expression <sup>‡</sup>			
	DNA Synth.	SDH Activity	ALP Activity	ALP	OCN	OPN	
$\text{Al}^{+3}$	195	122 <sup>§</sup>	92 <sup>§</sup>	91 <sup>§</sup>	+	+	++
	3120	52	82	85	-	±	++
$\text{Co}^{+2}$	13	57	64	65	++++	++++	+++ / ++
	104	12	55	46	+	++	+++ / ++
$\text{Cr}^{+3}$	120	103	97	126	++++	++	+++ / ++
	1920	55	106	106	+	-	-
$\text{Ni}^{+2}$	76	13	88	66	++++	+	+++
	304	10	48	22	-	-	-
$\text{Ti}^{+4}$	26	37	117	92	++	+++	++
	104	11	42	53	++	++	++
$\text{V}^{+3}$	5	36	90	88	++	+++	++
	20	20	42	57	++++	++++	+++
Control		100	100	100	++++	++++	++++

\*The low concentration had low cytotoxicity and the high concentration had high cytotoxicity as determined by SDH activity.

<sup>†</sup>Percentage of control (without addition of metal ions).

<sup>‡</sup>Determined by comparing the visual band density of sample with control sample (without addition of metal ions).

<sup>§</sup>The error for the mean is about 15%.

> Cr<sup>+3</sup>, Al<sup>+3</sup>.<sup>13</sup> Takeda reported the cytotoxic order to L-929 cell line by extracted solutions of pure metals as: Cr > Co > V > Ni > Ti, Al.<sup>31</sup> Comparing these results, there is a common agreement about the toxic order of V, Co, Ni, and Al, but there are also several differences. The TC<sub>50</sub> value of Ti is 1050  $\mu$ M/L for the Balb/c 3T3 cell line, but only 200  $\mu$ M/L for the ROS 17/2.8 osteoblast-like cell line. This is an order of magnitude difference and suggests that the osteoblast is more sensitive to titanium than the fibroblast. The second difference is that the Cr was listed as the most toxic element in the group of metals by Takeda. This difference may be explained by the existence of a hexavalent form of Cr in Takeda's cell-culture test, since Cr<sup>+6</sup> is more toxic than Cr<sup>+3</sup>.<sup>32,33</sup> Finally, aluminum is considered a toxic element to several tissues including bone,<sup>34</sup> kidney, liver,<sup>35</sup> and brain.<sup>36</sup> However, the results of present study indicated little cytotoxicity of Al in ROS 17/2.8 cells. This low cytotoxicity of Al *in vitro* has been observed in other studies.<sup>13</sup> The reason for variation in cytotoxicity is not known. There has been a report of long-term cytotoxicity of Al, indicating that exposure time may play a key role in Al cytotoxicity.<sup>37</sup>

Comparing the data in Figure 3 with TC<sub>50</sub> values from pilot studies (Table I), it was found that the Cr ion at the established TC<sub>50</sub> concentration did not cause a 50% reduction of SDH activity, and that other ions did cause a 50% reduction of SDH at lower concentrations than expected. The reason might be that the TC<sub>50</sub> value in the pilot study was for 24 h, compared to 72 h for this study. To promote cell differentiation and mineralization, AA, GP, and Dex were introduced into the culture medium from days 7 to 10 after plating. These agents could also alter the cell metabolism and sensitivity to metal ions.

As judged by SDH activity and DNA synthesis, the Cr and Al ions had low cytotoxicity within the concentrations tested. However, Ni, Co, Ti, and V were highly cytotoxic and their effects on cell viability were a function of their concentrations. Compared with SDH activity results, the DNA synthesis rate was the more sensitive assay. However, these cytotoxicity evaluation assays cannot totally explain cell-specific functions and cell differentiation processes for ROS 17/2.8 cells. For example, metabolic tests cannot show how the ions cause preosteoblasts and young osteoblasts to differentiate into mature osteoblasts. To answer these questions, it is necessary to determine the effects of these ions on the cellular differentiation processes. In the current study, ALP, OCN, and OPN were selected as markers for observing the process of cell differentiation and mineralization. ALP is highly expressed in osteoblasts and other mineral tissues. Its functions in these tissues are not identified, but it is commonly recognized that it is related to the mineralization process in mineral tissues.<sup>38</sup> OCN (bone gla protein) has high affinity to Ca or HA,<sup>39</sup> and may function in mainte-

nance and resorption of bone tissue by control of recruitment and differentiation of osteoclasts.<sup>40</sup> Further study has demonstrated that OCN expression occurs only when osteoblasts differentiate from stem cells or preosteoblasts to mature osteoblasts.<sup>41</sup> OPN also has high affinity to HA as well as collagen,<sup>42,43</sup> and functions in the attachment of osteoblasts to surrounding calcified matrix.<sup>44</sup> The synthesis of OPN can be detected at all developmental stages of osteoblast differentiation.<sup>45</sup>

For Cr and Ni, the reduction of expression of these genes was proportional to the metal ion's concentrations. The threshold level of inhibition for gene expression was not determined, but was between 120 and 1920  $\mu$ mol/L for Cr, and 76 and 304  $\mu$ mol/L for Ni. However, it seems that the effects of metal ions on ROS cell gene expression did not simply depend on metal ion concentration. Al had very strong effects on bone-specific gene expression, but was not especially cytotoxic at any concentration tested. In contrast, Ti and V at cytotoxic concentrations did not significantly suppress gene expression. Furthermore, Co ion specifically affected ALP expression at a noncytotoxic concentration without affecting the OPN expression. The current study therefore supports the hypothesis that each metal ion acts uniquely in its effects on gene expression in ROS cells.

Multiple mechanisms exist which control and regulate the transcription and translation in eukaryotic cells.<sup>46</sup> In addition, the post-transcriptional events influence the gene expression. Most important, gene transcription does not necessarily mean that the encoded protein will be found in the cell or extracellular matrix. Any disruption of transcription could lead to an abnormal or dysfunctional protein molecule. These experiments go a step beyond cytotoxicity testing to the level of gene expression. The results of this study allowed us to observe that the metal ions affected some of these processes. Generally, these effects could not be predicted from the cytotoxicity assays. However, the results raise new questions as to the nature of the interaction between the metal ions and various mechanisms of transcription control. For example, Cr and Al significantly suppressed ALP gene expression, but did not change ALP activity in ROS cells. The explanation for this may be that ALP activity of ROS cells was measured by a histochemistry method which measured all ALP isoenzyme activities. Each isoenzyme has its own genetic expression mechanism. The Northern blot analysis in this study was based upon only one specific ALP gene. This fact may explain the different results by the two different testing methods. However, the data may also indicate that the mRNA transcription process of this ALP is very sensitive to the presence of the Cr and Al ions.

Cr<sup>+3</sup> used in this study is recognized as having low cytotoxicity because of its membrane impermeability and biologic inactivity.<sup>33</sup> The cell metabolism data

agree with this statement. Interestingly, the bone-specific gene expression in the presence of  $\text{Cr}^{+3}$  was substantially suppressed even though the metabolism data showed that the highest concentration caused a 50% reduction of the DNA synthetic rate as the only metabolic effect. This result implies that we should reconsider the biologic behaviors of  $\text{Cr}^{+3}$  especially on cellular differentiation processes. Another finding was that Ti ions suppressed ALP gene expression at toxic and nontoxic concentrations to the same degree, but the ALP activity at the toxic concentration was 52% of control, while the ALP activity at nontoxic concentration was 92% level of control. There is about a 40% difference of ALP activity in culture cells between these two tested concentrations. These findings suggest that for Ti ions inhibition might start after transcription or in translation. For example, a recent study has shown that aluminum ions can interact with elements of signal transduction pathways [i.e., bind to the guanine nucleotide-binding proteins (G protein) and a phosphatidylinositol-4-5 diphosphate ( $\text{PIP}_2$ )-specific phospholipase C].

In this study, we have investigated the effects of metal ions on osteoblast-like cell metabolism and differentiation, and the findings show that metal ions may affect cellular metabolism and cellular differentiation by different mechanisms at sublethal concentrations. The results have shown that the inhibition of the gene expression is ion specific, gene specific, and concentration related. Furthermore, this inhibition is not predictable based on the cellular metabolism activity. However, the mechanisms for the gene suppression are complex and the results of this study only begin to clarify mechanisms. In addition, it is important to remember that *in vivo*, alloys containing Ti, Co, and Al frequently have passivated surfaces from which very few ions are released in the environment. For the concentrations of ions released, we would expect the results described in this study. For a comprehensive understanding of the biologic actions of the metal ions, it is important to know the concentration of ions released as well as transportation pathways of metal ions through the plasma membrane and between cellular organelles. Information about reactions between biologic moleculars and metal ions is also needed.

The authors thank Dr. R. T. Franceschi and Y. Q. Cui for their generous donation of DNA probes and technique support for the Northern blot analysis. This study was supported in part by NIDR Grant P50DE09296.

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Received October 4, 1995

Accepted February 19, 1996