임프란트에 관련된 금속이온의 조골세포에 대한 세포독성에 미치는 Hsp27의 영향에 대한 실험적 연구

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Abstract

HSP27 MODULATION OF IMPLANT- ASSOCIATED METAL ION CYTOTOXICITY OF OSTEOBLASTIC CELLS

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Objectives: The extent of bone formation that occurs at the interface of metallic implants and bone is determined by the number and activity of osteoblastic cells. Stress proteins may be contributing determinants of cell viability in altered environments. Hsp27 is a small Mr hsp which is known as a molecular chaperone.

Methods: To better understand how heat shock protein 27 contributes to endosseous implant - associated metal ions affects on osteoblastic cell viability, the effect of chromium and titanium ions were compared to effects of cadmium ions in the ROS17/2.8 osteoblastic cell line.

Results: ROS17/2.8 osteoblastic cell line demonstrated ion - specific reductions in growth; reductions were significantly greater for cadmium than for chromium or titanium. Chromium impaired growth of cultures without altering cell viability measured using the MTT assay. A stable transformed cell line expressing additional hsp27(clone "A7") was resistant to the toxic effects of titanium and partially protected from cadmium toxicity.

Conclusions: A role for hsp27 in protection of osteoblastic cells from metal ion toxicity is supported by the chromium - induced elevations in hsp27 abundance and the behavior of the A7 cell line in response to metal ions in culture. Similar biochemical responses to altered cellular environments may contribute to the fate of tissues adjacent to select metallic implants.

Key words : Metallic implants, Heat shock protein 27, Chromium, Titanium, Cadmium

Ⅰ. Introduction

The extent of bone formation that occurs at different endosseous implant surfaces is dependent on many factors affecting cell viability. Limiting the implant - related perturbation of the local environment is essential to the successful osseointegration of metallic endosseous devices. One of many factors affecting cell function at endosseous implants is the effect of implant - derived ions on cellular activity at the implant - tissue interface[1]. While different endosseous implants are made of biocompatible materials such as stainless steel, nickel chrome or titanium; the chromium, cobalt,

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and nickel ions that are potentially released at alloplastic - tissue interfaces are cytotoxic[2]. Various metal salts have been shown to be cytotoxic to osteoblastic cells[3]. Because titanium is rapidly oxidized, the absence of free titanium ions has been considered to be of clinical advantage[4] and any effect of free titanium has not been examined.

Eukaryotic cells possess innate defenses against environmental toxicants. The heat shock proteins(hsp) are protective proteins that prevent cell death following exposure to a variety of different environmental irritants[5]. While the role of hsp in protecting cells against toxic metal ions such as cadmium has been defined[6], the contribution of hsp in protecting cells from toxicity associated with ions from metallic orthopedic implants has not been considered.

Hsp27 has been reported to be a constitutive product of osteoblastic cells[7]. It is a small Mr hsp(low molecular weight hsp) with demonstrable function as a molecular chaperone[8]. In addition, several independent studies have indicated that the presence of hsp27 in cells is associated with increased resistance to a variety of different environmental stressors and toxicants[9,10]. In this report, the effect of different ions associated with metallic implants on osteoblast viability was investigated as a function of hsp27 abundance. Using rat osteosarcoma(ROS17/2.8) cells[11] and a stable transformed derived clone with 12 - fold increased abundance of hsp27(clone A7), the role of this stress protein in protection of osteoblastic cells from metal ion toxicity was investigated using cell culture techniques.

Ⅱ. Materials and methods

1. Cell culture

ROS17/2.8 cells[11] were maintained in DMEM/F12 media containing 5% Bovine Calf Serum and 25 μg/ml penicillin/streptomycin at 37℃ in a 5% CO_2 / air humid atmosphere. A ROS17/2.8 derived cell line that displays 12 - fold increase in constitutive hsp27 expression(clone A7) was maintained under identical conditions and in the additional presence of 500 μg/ml G418 antibiotic(Life Technologies, Gaithersburg, MD). Cells were passaged every third day. ROS17/2.8 cells were transfected with an hsp27.a - encoding expression vector. The hsp27.a open reading frame was cloned into the Pst1 and BamH1 sites of the pcDNA3.1 expression vector(Invitrogen, Carlsbad, CA) and supercoiled plasmid DNA was prepared by an affinity matrix method(Qiagen, Valencia, CA). Stable transformation of ROS 17/2.8 cells was performed using liposomes (Lipofectamine™, LifeTechnologies). A mixture of 4 mg DNA, 18 ml of Lipofectamine in 4 mL of Optimem™(LifeTechnologies) was placed onto a 100 mm tissue culture plate of subconfluent cells. After 5 hours of incubation, additional media and serum was added to 10 mL at 10% BCS. 72 hours following transfection, cells were split 1 : 20 into DMEM / F12 containing 5 % BCS, penicillin / streptomycin, and 500 mg/mL G418 antibiotic (LifeTechnologies). After three weeks, 50 colonies were selected for clonal growth and these were screened for increased constitutive hsp27 expression by Western blotting. From a single 100 mm dish of transfected cells, over 100 colonies formed and of 50 selected colonies, 11 colonies demonstrated elevated hsp27 expression.

2. Heat shock

Some cultures were pre-conditioned by transfer of culture dishes to a 42.5℃ incubator for 2 hours(a sub-lethal heat shock). Then the cultures were recoveried by returning the culture dishes to the 37℃ tissue culture incubator for 3 hours.

3. Measurement of proliferation

The effect of metal ions on cell proliferation was measured directly by counting of cell number. Cells were plated in 12 well dishes(5×10^4 cells / well) and after attachment and overnight incubation, cells were rinsed three times with phosphate buffered saline(PBS) and media was replaced with 1 ml of fresh media containing 0.0 - 0.3 mM

chromium($CrCl_3 \cdot 6H_2O$, Sigma, St. Louis, MO), 0.0 - 0.2 mM titanium(Titanium atomic absorption standard solution, Sigma) or 0.0 - 0.05 mM cadmium($CdCl_2$, Sigma). After 24, 48 and 72 hours of growth in these media, wells were rinsed three times with PBS, cell layers were stained with trypan blue and vital, adherent cells were manually counted. Three fields were counted per well and the average number of cells per treatment group were compared.

4. Measurement of Cell Viability

Cell viability following treatment with 0.0 - 0.3 mM chromium, 0.0 - 0.2 mM titanium or 0.0 - 0.05 mM cadmium was evaluated by the MTT assay[12]. Cells were plated in a 96 well format (5×10^4 cells/well). Following overnight incubation and 24, 48 or 72 hour growth in Ti, Cr, or Cd - ion containing media, The MTT reagent(Thiazolyl blue 5 mg/ml, Sigma) was added to cultures for 4 hours and following incubation at 37℃, 5% CO_2, absorbance at 570 nm and 630 nm was measured and the difference calculated. Four wells per time point and per ion concentration were evaluated in duplicate experiments. Individual pairs of data were compared by two - tailed Student's T - test.

5. Western Blot Determination of hsp expression

To evaluate hsp expression in ROS17/2.8 cells following heat shock preconditioning, cells plated in 6 well dishes were heat shocked at 42.5℃ for 2 hours and incubated for 3 hours at 37℃. Cell layers were then rinsed three times with cold PBS and lysed directly into 150 ㎕ RIPA buffer[13]. Total protein concentrations were determined using the BCA assay and 30 ㎍ total protein aliquots of control and heat shock treated ROS 17/2.8 cell lysates were subjected 12% Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)[14]. Separated proteins were transferred to a nitrocellulose filter by a semi -dry apparatus in a methanol - based buffer[15]. The filter was blocked with non-fat dry milk[16] and immunoblotting was performed using a 1:1000 dilution of anti hsp27 antibody in Blotto and 1:10,000 dilution horseradish peroxidase - conjugated goat anti - rabbit secondary antibody. Immunocomplexes were subsequently identified using chemiluminescence(ECL, Amersham, Beverly, MA).

6. Immunocytochemistry

ROS17/2.8 cells and A7 cells were plated on glass cover slips and after 24 hour incubation, cells were fixed in cold methanol. Fixed cells were briefly permeabilized in 0.1% Triton X -100 and then incubated in 1:100 dilution of anti hsp27 antibody(Stressgen, Victoria, BC, Canada), followed by FITC conjugated goat anti-rabbit antibody(Vector Lab. Burlingame, CA). Cells were then examined by immunofluorescence using an Olympus BH12 microscope and photographed using T - Max 100 film (Kodak, Rochester NY).

Ⅲ. Results

1. Hsp27 expression in parental and transfected cells

The expression of hsp27 in ROS17/2.8 cells and cells transfected with an hsp27 expression vector(clone A7) was demonstrated by immunofluorescent microscopy(Fig. 1). Staining with affinity purified hsp27 antibody revealed the presence of hsp27 in the cytoplasm of ROS17/2.8 cells and this was increased in intensity in the clonally derived A7 cells.

2. Metal ion effects on osteoblast proliferation

The effect of titanium, chromium and cadmium ions on ROS17/2.8 cell and clone A7 cell fate was first examined by determining the growth rates of these cells. Both the parental cell line and clone A7 displayed similar growth rate (doubling time, 21 hrs). In the presence of increasing concentra-

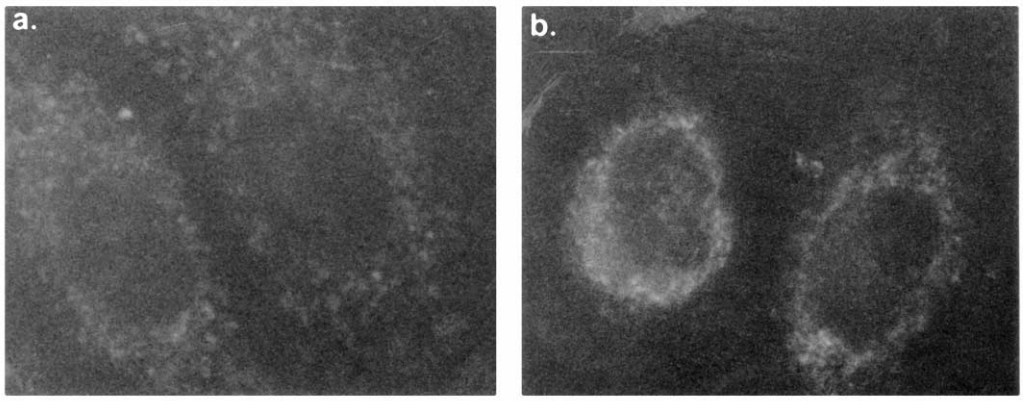

Fig. 1. Hsp27 expression in cultured ROS17/2.8 cells (a) and A7 cells (b). Immunofluoresence microscopy using purified anti - hsp27 antibody and FITC conjugated - goat - anti rabbit secondary antibody reveals hsp27 in the cytoplasm of cells(X400).

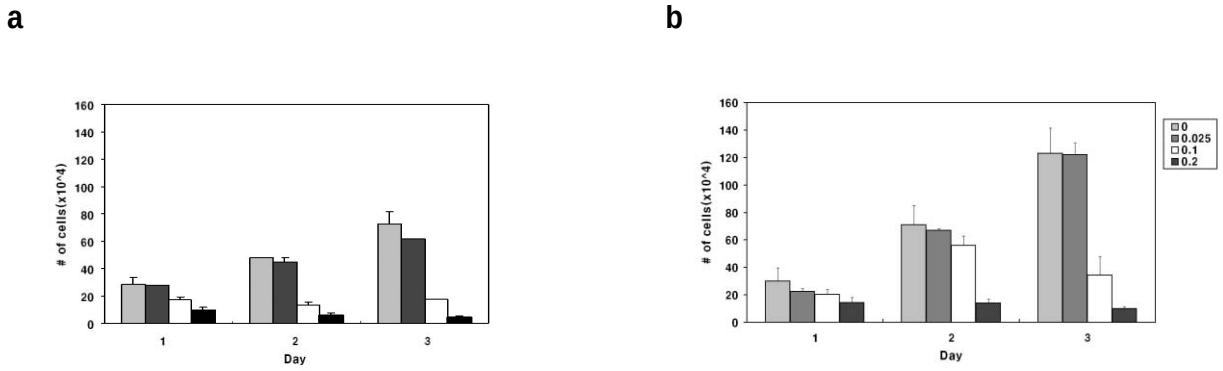

Fig. 2. Cell number following continuous exposure to titanium. (a) Number of ROS17/2.8 cells present after growth in 0 - 0.2 mM titanium(as indicated) for 1, 2 or 3 days. (b) Number of A7 cells present after growth in 0 - 0.2 mM titanium (as indicated) for 1, 2 or 3 days.(std. error indicated above bar)

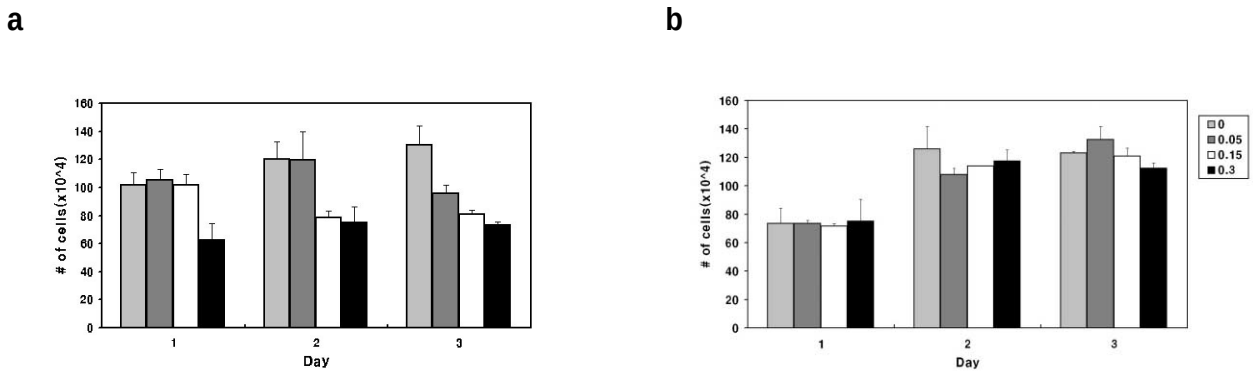

Fig. 3. Cell number following continuous exposure to chromium. (a) Number of ROS17/2.8 cells present after growth in 0 - 0.3 mM chromium(as indicated) for 1, 2 or 3 days. (b) Number of A7 cells present after growth in 0 - 0.2 mM chromium(as indicated) for 1, 2 or 3 days.(std. error indicated above bar)

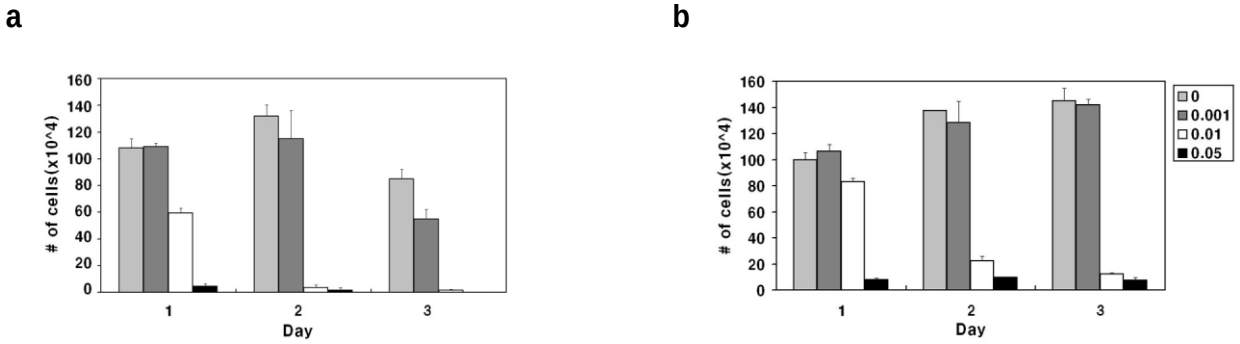

Fig. 4. Cell number following continuous exposure to cadmium. (a) Number of ROS17/2.8 cells present after growth in 0 - 0.2 mM cadmium(as indicated) for 1, 2 or 3 days. (b) Number of A7 cells present after growth in 0 - 0.05 mM cadmium(as indicated) for 1, 2 or 3 days.(std. error indicated above bar)

tion of titanium(0.0 - 0.2 mM), a marked reduction in growth was observed for the ROS17/2.8 cells. Clone A7 cells were affected to a lesser degree(Fig. 2). Chromium effects(0.0 -0.3 mM) on the growth rate were less pronounced(Fig. 3). Cadmium(0.0 - 0.05 mM) dramatically limited growth of both the ROS17/2.8 cells and the A7 cells(Fig. 4).

3. Metal ion effects on hsp27 expression

When the effect of chronic exposure of titanium, chromium or cadmium was examined at the level of protective hsp27 expression, Western blot analysis indicated that chromium exposure, but not titanium exposure resulted in modest elevation of hsp27(Fig. 5). Exposure to cadmium, known to induce hsp gene expression, also resulted in the increased expression of hsp27 in the osteoblastic cell lines.

4. Metal ion effects on osteoblast viability

The MTT assay was used to measure viability of ROS17/2.8 and A7 cells grown in the presence of increasing amounts of titanium, chromium or cadmium. The viability of ROS17/2.8 cells was affected differently by each metal(Fig. 6a). The ROS17/2.8 cell viability was reduced by exposure to titanium and cadmium, but not chromium. ROS17/2.8 cell resistance to chromium toxicity was again revealed(Fig. 6b). Compared to the parental cell line, the A7 cell displayed resistance to titanium($p<0.003$, Fig. 6a) and partial resistance to cadmium($p<0.05$, Fig. 6c), but not chromium(p , 0.26).

5. Heat shock preconditioning effects on metal ion toxicity in osteoblasts

To assess the role of endogenous heat shock proteins on resistance to these ions, ROS17/2.8 cells were preconditioned by 2 hour 42.5℃ heat shock and three hour recovery to allow for the expression of hsp. Under these circumstances, increased ROS17/2.8 survival following exposures to metals was not generally observed(Fig. 7). In contrast, preconditioning increased the viability of A7 cells measured after exposure to metal ions. This was most notable for the cadmium treatment ($p<0.004$). While heat shock preconditioning resulted in a classical heat shock response with increased levels of hsp27, hsp68 and hsp90 proteins as indicated by Western blot analysis in both the ROS17/2.8 cell and the A7 cell(not shown), heat shock alone failed to protect the ROS17/2.8 from titanium or chromium toxicity. Heat shock protected ROS17/2.8 cells from cadmium toxicity at lower doses(Fig. 6c, 7c).

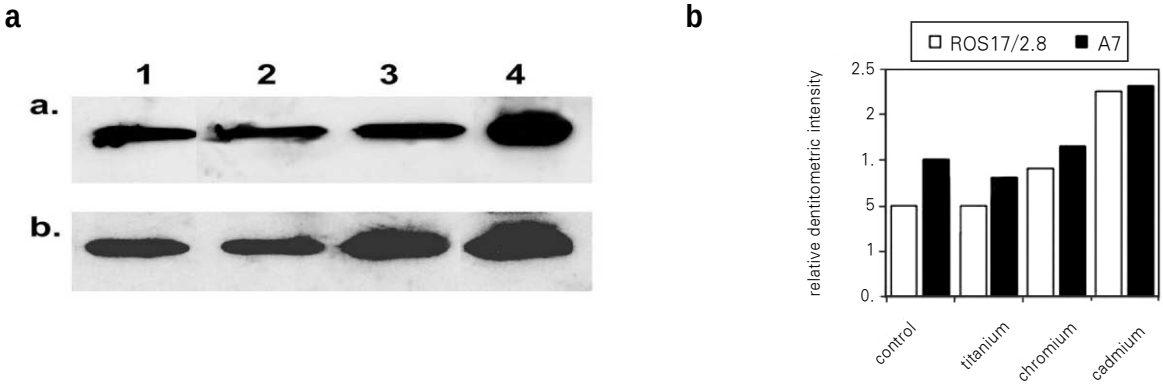

Fig. 5. (a) Western blot analysis of hsp expression following exposure to titanium, chromium, or cadmium reveals relative abundance of hsp27 present in lysates from ROS17/2.8 cells panel a) or A7 cells(panel b) after no treatment(lane 1) or cells treated with titanium(lane 2), chromium(lane 3), or cadmium(lane 4).
(b) Result of densitometric tracing of images in (a). The relative densitometric intensity of each band is compared to the untreated ROS17/2.8 cell result(panel a, lane 1).

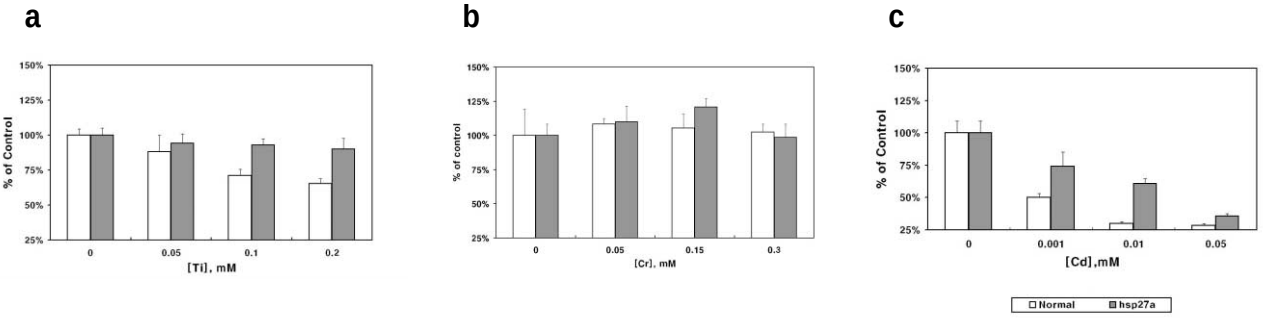

Fig. 6. The cytotoxicity of titanium (a), chromium (b) and cadmium (c) in ROS17/2.8 cells(open bars) and A7 cells(filled bars). Cell viability was measured by the MTT assay and plotted as percent of untreated cell viability(treated - control /100) versus mM concentration of treatment ion.(std. error indicated above bar)

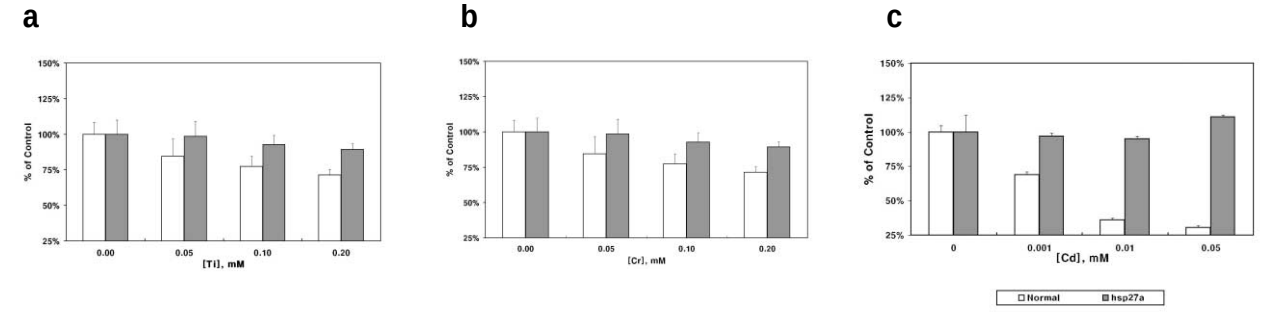

Fig. 7. The cytotoxicity of titanium (a), chromium (b) and cadmium (c) in heat shock preconditioned ROS17/2.8 cells (open bars) and A7 cells (filled bars). Cell viability was measured by the MTT assay and plotted as percent of untreated cell viability (treated - control /100) versus mM concentration of treatment ion.(std. error indicated above bar)

Ⅳ. Discussion

An hsp27 - transformed osteoblastic cell line was shown to possess increased resistance to thermal stress. The parental cell line, ROS 17/2.8 has been used to demonstrate that osteoblastic cells are negatively affected by the presence of metallic implant - associated ions including chromium, cobalt and the cadmium[3,17]. Hsp27 expression correlates with embryonic stem cell resistance to metal toxicity[18]. Therefore, to begin to define the biological responses that implant metallic ions may engender in osteoblastic cells, the role of hsp27 in protection of osteoblastic cells from orthopedic metallic ion toxicity was studied using the hsp27 - transformed cell line A7.

Different orthopedic device - related metal ions have distinct effects on osteoblast proliferation and viability.

When the effects of two orthopedic metallic ions were compared with cadmium toxicity, both the parental ROS17/2.8 cell line and the derived A7 cell line demonstrated ion - specific toxic responses. In general, cadmium was most toxic. Titanium effectively reduced the growth of cultures(70-85% reductions) and chromium had a more modest inhibitory effect on growth(30-40% reductions).

This comparative examination using ROS17/2.8 cells suggests that osteoblastic cells have different resistance to these three ions. To gain further understanding of the cell's responses to these metals, cell viability was directly measured by the MTT assay. Interestingly, chromium did not cause acute cytotoxicity(24 hour death) in the range of doses tested. Ionic titanium was toxic, leading to approximately 40% cell death at 24 hours. As expected, cadmium was toxic, causing greater than 70 % cell death during the 24 hour exposure.

Chromium effects were distinct when comparisons of proliferation and toxicity data were made. Chromium was not acutely toxic. Chromium may be released from metallic implants and sequestered by red blood cells as well as fibroblastic cells[19]. Previously, chromium did not affect the proliferation of rat bone marrow stromal cells[20]. In studies of rat osteoblastic cell viability using the MTT assay, Cr^{+6} was more toxic than Cr^{+3}. Recently, studies using the ROS17/2.8 cell indicated that subtoxic levels of Cr^{+3} had little affect on this cell's expression of bone specific mRNAs and viability[17]. It has been suggested that Cr^{+6} is more toxic because it is membrane permeable[21]. These different results may reflect cell line - and cell culture technique - related effects which complicate any effort to extrapolate in vitro data to the clinical environment.

Aside from acute and general effects on cell physiology, chronic metallic ion effects on the more differentiated osteoblast physiology have also been considered. McKay et al[21] suggest that the differentiation marker, alkaline phosphatase, is a more sensitive test for osteoblast cytotoxicity. Seven day administration of chromium inhibited osteoblast - specific protein gene expression at cytotoxic concentrations[20] and differentiation of bone marrow stromal cells to osteoblastic cells (measured by osteocalcin secretion and matrix mineralization) was inhibited by exposure to Ti^{+4}, Al^{+3}, and $TiAl_6V_4$[22]. Titanium ion effects in cell culture may not be of direct relevance to bulk titanium activity in vivo; the oxidized titanium surface is not particularly reactive and has shown to be extremely biocompatible in the endosseous environment.

Hsp27 confers protection from orthopedic-related metal ion toxicity to osteoblasts

The overexpression of hsp27 in the A7 cell line was associated with protection of this osteoblastic cell line from chromium and titanium - associated reductions in growth. In addition, the A7 cell line showed greater resistance to cadmium - related reductions in growth and viability. This confirms previous reports that hsp27 confers resistance to cadmium toxicity[18].

The A7 cell was completely resistant to chromium effects at 0.3 mM. However, this cell was only partially resistant to titanium ion effects at 0.2 mM. At low doses of Cd, overexpression of hsp27

also protected cultures to a limited degree. This reflects the levels of hsp27 expression and is consistent with other observations that hsp27 overexpression is generally protective[9,23].

When the effect of hsp27 on osteoblast sensitivity to these ions was considered in the context of the overexpression of hsp27, the A7 cell line showed increased resistance to death at the range of titanium concentrations tested. The A7 cell line was also partially resistant to cadmium cytotoxicity. This comparison of metal ion effects on the parental ROS17/2.8 cell and the A7 cell indicate that hsp27 is able to confer some protection from the acute toxicity of metal ions. The additive effect of preconditioning heat shock and overexpression of hsp27 in protecting the A7 cell line from cadmium toxicity suggests that hsp27 works in concert with other inducible determinants of cell viability.

Specific stress responses to different metals were revealed at several levels. First, Western blot analysis indicated that exposure to titanium did not alter hsp27 levels, but exposure to chromium and to a greater degree, to cadmium, did increase the steadystate level of hsp27 in the ROS17/2.8 cell line. The absence of the titanium effect on hsp27 expression were associated with the ROS17/2.8 cell's sensitivity to this metal ion.

Second, a role for hsp27 in this biochemical protection of osteoblastic cells was shown by the present comparison of ion effects on the parental ROS17/2.8 cell line and the hsp27 transformed A7 cell line. The level of Hsp27 present in the A7 cells provided complete protection from chromium, but not titanium ion or cadmium reductions in cell growth.

Third, thermal preconditioning induced a classical heat shock response that was effective in limiting the effect of cadmium on cell viability only when additional hsp27 was present(A7 cells, compare figures 6c, 7c). However, only modest protection from titanium effects was noted. The increased tolerance invoked by this preconditioning indicates a role for the endogenous hsp in resistance to metallic ion toxicity.

Hsp27 may reflect a general molecular determinant of osteoblast responses to alloplastic environments

Hsp27 performs a number of important protective roles in eukaryotic cells. While it is presently unclear how hsp27 protects cells from metal ion cytotoxicity, several protective roles for hsp27 have been established. Hsp27 is a defined molecular chaperone[8]. It prevents intracellular protein aggregation and promotes denatured protein refolding. In a number of different cell lines, overexpression of hsp27 conveys thermoresistance and protection from different modes of cell death including apoptosis[23]. Hsp27 is also an inhibitor of actin polymerization and may play a role in the regulation of the cells actin cytoskeleton[24,25]. How hsp27 contributes to the demonstrated resistance to metal ion cytotoxicity requires further consideration. The hsps may be of increased importance to this cell line that has previously been reported to display an absence of metallothionin protein and sensitivity to cadmium[26].

Ⅴ. Conclusion

In summary, this study of metal ion - mediated cytotoxicity indicates that the overexpression of hsp27 in osteoblastic cells increased the resistance to the cytotoxic effects of chromium, titanium and cadmium. However, different orthopedic implant - related metal ions have distinct effects on cell viability and cell growth. This phenotypic modulation of the ROS17/2.8 cell provided protection that was similar in extent to that provided by the preconditioning induction of endogenous hsp in the ROS17/2.8 cell. Osteoblastic cells express a few proteins in response to stress that contribute to maintaining cell viability in the presence of environmental toxicants such as metal ions associated with endosseous metallic implants. Understanding these ion effects in terms of the biochemical responses they may allow for clinical preconditioning (e.g. pharmacological) of osteoblastic cells to support osteogenesis in altered environments.

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