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Evidence that HSP70 Gene Expression May be Useful for Assessing the Cytocompatibility of Dental Biomaterials

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In the current studies, we examined the possibility of using HSP70 gene regulation as a cytocompatibility test for dental biomaterials. For this reason, we assessed the effects of three metal salts, $HgCl_2$, $CuSO_4$ and $NiCl_2$ on HSP70 gene expression in HeLa S3 cells using real-time Taqman quantitative PCR. Incubation of the cells for 4 h in medium containing $HgCl_2$ (20 or 40 μM), $CuSO_4$ (157, 313, 625 or 1250 μM) or $NiCl_2$ (5000 and 10000 μM) significantly induced HSP70 mRNA. The real-time Taqman quantitative PCR was able to detect HSP70 mRNA induction at 4-fold lower concentrations of $HgCl_2$ and 8-fold lower concentrations of $CuSO_4$ than the Neutral Red cell viability assay. These results indicate that real-time Taqman quantitative PCR, in combination with the monitoring of cell viability, may be a valuable tool for distinguishing between specific HSP70 mRNA induction and cytocompatibility of metals in dental biomaterials.

Key words: HSP70 mRNA, Real-time PCR, Cytocompatibility

INTRODUCTION

Stress proteins (HSPs) are considered markers of cellular stress, and their use has been suggested for environmental monitoring of toxic compounds[1−3]. In fact, a previous study[3] reported that the induction of HSP70 synthesis is one of the most sensitive cellular responses to mercury ion. HSP70 gene is also induced by other metals[4,5]. Therefore, HSP70 gene expression could be used to monitor the cytocompatibility of metal-containing dental materials.

Several methods exist for quantitation of mRNA expression. For example, Northern blotting and ribonuclease protection assays have been widely used. However, these two methods can require large amounts of RNA as well as radioactivity[6]. Conventional RT-PCR may also be used, but it is limited by the need for parallel reactions for a single sample, post-amplification steps and by its relatively narrow dynamic range[7].

Recently, real-time Taqman PCR has been developed to overcome some of these limitations. This method measures PCR product accumulation with a double-labeled fluorogenic probe and this method provides accurate and reproducible quantitation of gene copies[7]. We therefore investigated the regulation of HSP70 genes by three metal salts in HeLa S3 cells using real-time Taqman quantitative PCR.

MATERIALS METHODS

Cell culture and treatment of cells with metal salts

HeLa S3 cells were maintained as a monolayer in Eagle's minimum essential medium (MEM, Nissui, Tokyo, Japan) supplemented with 10% heat-incubated calf serum. Mercury chloride ($HgCl_2$, ISHIZU SEIYAKU LTD., Osaka, Japan), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, Wako Pure Chemical Industries LTD., Osaka, Japan) and nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$, Wako Pure Chemical Industries LTD., Osaka, Japan) were dissolved in H_2O and then diluted with cell culture medium containing 10% fetal bovine serum (GIBCO BRL, Life Technologies Inc., Grand Island, NY). Final concentrations were 10-160 μM for $HgCl_2$, 157-2500 μM for $CuSO_4$, and 625-10000 μM for $NiCl_2$. HeLa S3 cells were seeded into 96-well at 5×10^3 cells/100 μL and 12-well multi-plates at 5×10^4 cells per well /ml containing 10% fetal bovine serum, and then cultured for 48 h at 37℃ in a humidified atmosphere containing 5% CO_2.

Assay of quantitative gene expression

Cells were grown in 12-well microtiter plates as described above. The medium in each well was replaced with the various concentrations of metal salts and grown for the indicated amount of time. Total RNA was isolated from the cell cultures using a Total RNA extraction kit (Trizol, invitrogen, CA, USA). Single strand cDNA was synthesized from 1 μg of total RNA using an oligo(dT)20 primer (Toyobo, Osaka, Japan) and a ReverTra Ace-α first strand cDNA syntheses kit (Toyobo, Osaka, Japan). For quantitative real-time PCR, sequences for the HSP70 mRNA probe (5'-ATCCTGAGCGTGACAGCCACTGACAG-3') and primers (forward: 5'-AGGTGACCTTTGACATTGATGCT-3', reverse: 5'- CTTGTCATTGGTGATGGTGATCTT-3') were designed by application of the computer software Primer Express (PE Biosystems, Foster City,

CA). The internal fluorogenic probes were labeled at the 5' end with the reporter dye FAM, and at the 3' end with the quencher dye TAMRA (Applied Biosystems, Ca, USA). β-Actin, a housekeeping gene probe labeled with the VIC reporter dye at its 5' end and the TAMRA quencher dye at its 3' end, was purchased from Applied Biosystems (Applied Biosystems, Ca, USA). This allowed simultaneous determination within a single reaction of the concentration of the target message and of β-actin. Thermal cycling and fluorescence detection were performed in a real-time Light Cycler (ABI Prism 7700 sequence detection system, Applied Biosystems, Ca, USA). The thermal cycling conditions were as follows: an initial denaturation for 10 min at 95℃, followed by 40 cycles of 15 sec at 95℃ and 1 min at 60℃. Quantities of the target were established by comparison to a standard curve. Values were normalized to expression of a housekeeping gene and fold-increase was expressed over the values from untreated cells.

Cell viability

Cell survival was assessed in 96-well microtiter plates using the Neutral Red (NR) assay (Nakarai, Kyoto, Japan). The medium in each well was replaced with

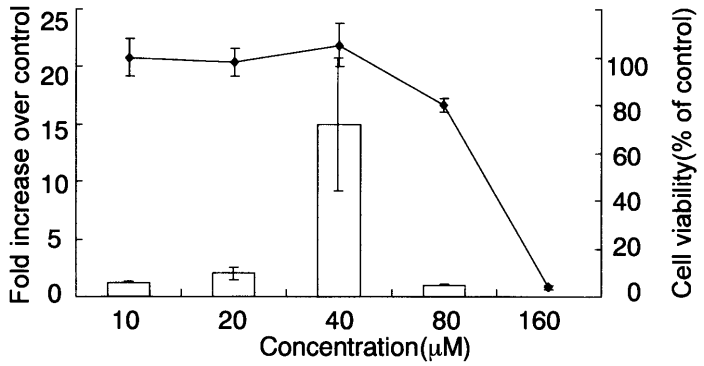

Fig. 1 Dose-response relationships of HSP70 mRNA induction and cytotoxicity.
Cells were exposed for 4 h to $HgCl_2$. Results represent three independent experiments (mean±standard deviation, n=3 wells per concentration). Bars represent fold-increase over untreated cells; Curves represent percentage of untreated control. Expression of both HSP70 and β-actin mRNA was not detected in medium containing 160 μM $HgCl_2$.

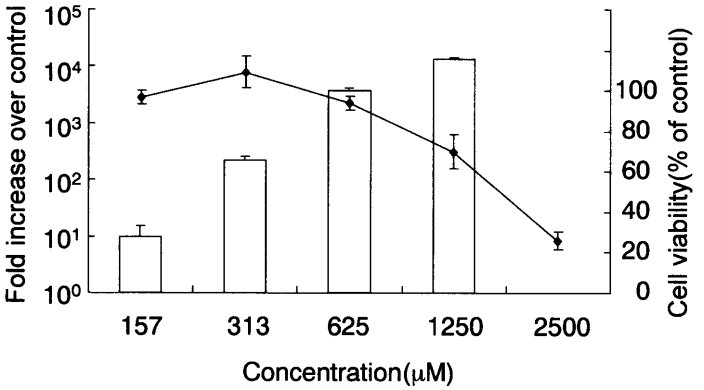

Fig. 2 Dose-response relationships of HSP70 mRNA induction and cytotoxicity.
Cells were exposed for 4 h to $CuSO_4$. Results represent three independent experiments (mean±standard deviation, n=3 wells per concentration). Bars represent fold-increase over untreated control; Curves represent percentage of untreated cells. Expression of both HSP70 and β-actin mRNA was not detected in medium containing 2500 μM $CuSO_4$.

medium containing various concentrations of the metal salts and cells were grown for the indicated periods of time. At appropriate times, 100 μl of NR medium (50 μg/ml neutral red in culture medium) was added to each well and cells were incubated for 3h. NR medium was then removed and cells were rapidly washed with phosphate-buffer saline. NR was extracted from each well with 100 μl of 1% acetic acid in 50% ethanol. The absorbance of each at 540 nm was determined. Absorbance readings were averaged and cell viability was expressed as a percentage of the values from untreated cells.

Statistic

One-way ANOVA was used to determine the statistical significance of the differences between different metal salt treatment or time course. When the ANOVA detected statistically significant difference, the Scheffe-test was used to describe the significance of the differences between the individual groups.

RESULTS

Quantitative gene expression assay

We examined the ability of various concentrations of the three metal salts to induce HSP70 mRNA in

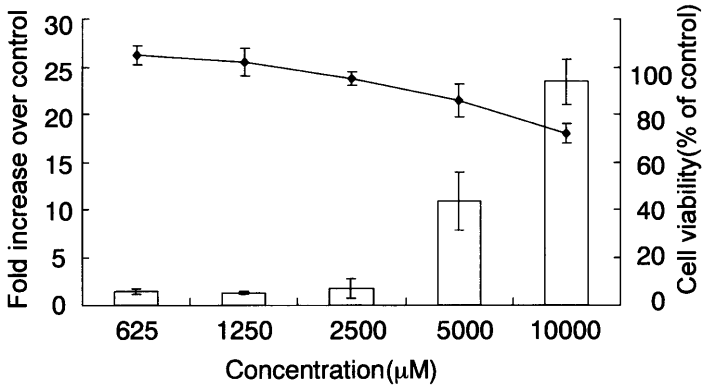

Fig. 3 Dose-response relationships of HSP70 mRNA induction and cytotoxicity.
Cells were exposed for 4 h to $NiCl_2$. Results represent three independent experiments (mean±standard deviation, n=3 wells per concentration). Bars represent fold-increase over untreated control; Curves represent percentage of untreated cells.

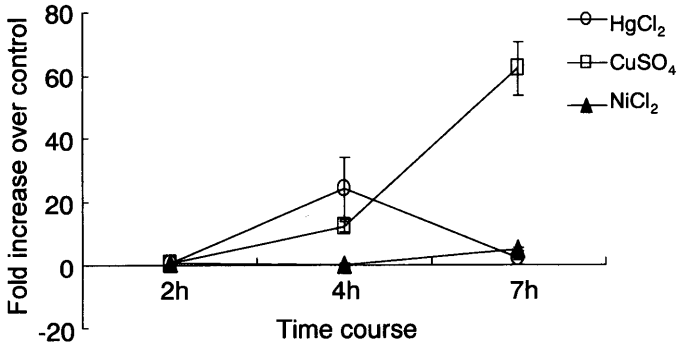

Fig. 4 Time-dependence of HSP70 mRNA induction. Cells were incubated in the presence of 40 μM $HgCl_2$, 625 μM $CuSO_4$ or 5000 μM $NiCl_2$ for the indicated periods. Results represent three independent experiments (mean±standard deviation, n=3 wells per concentration). Expressed as fold increase over the 2 h treatment.

HeLa cells. The amount of HSP70 mRNA present in these cells was compared to untreated controls. Cells were incubated for 4 h in medium containing the following concentrations of the metal salts: 20 and 40 μM $HgCl_2$; 157, 313, 625 and 1250 μM $CuSO_4$; or 5000 and 10000 μM $NiCl_2$. These treatments significantly enhanced the expression of HSP70 mRNA ($p<0.05$). The highest fold increase of the expression was observed in medium containing 40 μM $HgCl_2$, 1250 μM $CuSO_4$ and 10000 μM $NiCl_2$, respectively ($p<0.05$). Expression of both HSP70 and β-actin mRNA was not observed in medium containing 160 μM $HgCl_2$ and 2500 μM $CuSO_4$ due to strong cytotoxicity (Fig. 1-3).

To investigate the time dependence of HSP70 mRNA induction, we exposed the cells for 2, 4 or 7 h at a single concentration of each metal salt. The concentrations were selected to be sufficient to induce HSP70 mRNA. The greatest expression level was observed in the presence of 40 μM $HgCl_2$ in the following order: 4h>7h>2h ($p<0.05$). Both 625 μM $CuSO_4$ and 5000 μM $NiCl_2$ showed the same order of induction with regard to time (Fig. 4).

Cell viability assay

Using the NR assay, we next examined the cytotoxicity of the metal salts after 4 h of treatment of the HeLa cells. $HgCl_2$ showed cytotoxicity at 80 μM and hit a critical level at 160 μM ($p<0.05$). $CuSO_4$ produced no cytotoxicity at 157, 313 or 625 μM ($p>0.05$) but reached a critical level at 2500 μM ($p<0.05$). $NiCl_2$ produced no cytotoxicity at 625, 1250 or 2500 μM ($p>0.05$), but was cytotoxic at 5000 and 10000 μM ($p<0.05$) (Fig. 1-3).

DISCUSSION

The three metal salts that we have tested showed differences in their capacities to induce HSP70 mRNA. The increased synthesis of the HSP70 in response to physical or chemical stressors is preceded by a transcriptional activation of the HSP70 gene, which leads to elevated levels of HSP70 mRNA, and this can be readily detected by Real-time PCR. Our results show that these metal salts induce a variety of effects on HSP70 mRNA expression.

A similar gene induction study has been carried out using various metal salts[4,5]. Murata *et al.*[4] utilized Northern blotting to investigate the ability of metal salts to induce HSP70 mRNA in using HeLa S3 cells. The sensitivity to $HgCl_2$ and $CuSO_4$ in their study corresponded well with our findings, but they did not detect HSP70 mRNA induction by $NiCl_2$. On the other hand, Steiner *et al.*[5] used conventional RT-PCR to show that the three metal salts examined in this study are inducers of HSP70 mRNA. However, the magnitude and dose sensitivity of their results were less than we observed. These discrepancies may be due to the different cells used and the cell culture conditions. In addition, in contrast to Northern blotting, PCR amplifies the mRNA, and, therefore, may be a more sensitive method for assessing HSP70 mRNA induction. Finally, differences in the induction of HSP70 mRNA by the various metal salts are related to their ability to activate HSP70 gene transcriptional activation factors. For example, Zafarullah *et al.*[8] showed that $CdCl_2$ provoked a stronger stress response in chondrocytes than $CuCl_2$ and $ZnCl_2$ and that these differences were related to their relative cytotoxicities. In this study, the much higher fold increase over control obtained from the expression of HSP70 mRNA induced by the $CuSO_4$ compared to the other two metal salts tested might be associated with reactive oxygen species (ROS) formation induced by Cu^{2+}. Ma Y *et al.*[9] reported that HSP70 is important in increasing resistance to ROS induced in human promyelocytic leukemia HL-60 cells treated with copper.

We used the NR assay to measure cytotoxicity. The dye may enter cells by non-ionic diffusion through the cell membrane and accumulate in the lysosomes of living cells[10]. Of the metal salts tested, $HgCl_2$ was the most cytotoxic, followed by $CuSO_4$ and $NiCl_2$. Few studies have examined the cytotoxicity of metal ions over such a short period as we used in these studies. Akiyama *et al.*[11] investigated the cytotoxicity of pure metals extracts in Syrian hamster embryo cells by MTT assay. The concentration of Cu extract that caused cytotoxicity in their study corresponded well with our results, while the cytotoxicity of Hg and Ni extracts in their studies was lower than what we observed. These results may indicate that the cytotoxicity of a metal ion may be associated with its charge. The difference between Cu^{3+} and Cu^{6+} has been reported in studies of cytotoxicity and genotoxicity[12,13]. In addition, the difference in cells, methods and conditions of the cytotoxicity assays could explain discrepancies between our results and others'.

Metal restorations in the oral cavity are exposed to mechanical, physical and chemical attack. This may cause a release of metal ions into the oral environment. Metallic Hg is found in approximately half of dental amalgam, and it is regarded as hazardous[11]. Other metals, such as Cu and Ni, have been used as compositional elements in metal restorations. Allergic reactions to these three metals, especially Ni, have been documented[14,15]. The findings obtained in this study may provide useful information about the biological interactions between metallic dental restorations and oral or systemic tissues.

The present study was carried out using HeLa S3 cells, a cell line that has been frequently used to study HSP induction. Oshima *et al.*[3] demonstrated that HSP70 expression could be used along with a subspecies of these cells, HeLa 229, to evaluate the cytocompatibility of dental materials. In addition, a

number of studies[11,12] have revealed that established cell lines such as the HeLa 229 cells were more sensitive to the cytotoxicity of various dental materials.

In the present study, HSP70 mRNA expression by the three metal salts was observed 2 h after exposure. HSP70 mRNA expression reached maximum after 4 h of treatment with $HgCl_2$ and after 7 h with $CuSO_4$ and $NiCl_2$. A difference in the time course of biochemical events induced by the various metal salts may be responsible for the variations in the rate of HSP70 mRNA induction. In contrast to our results, Steiner *et al.*[5] showed that maximal HSP70 mRNA induction occurred 4 h after treatment with $CuCl_2$, $NiCl_2$ and Hg $(NO_3)_2$. This difference may be due to the specific concentrations and time points tested. Oshima *et al.*[3] showed induction of HSP70 after 2 h in the presence of 20 μM Hg $(NO_3)_2$. This induction of HSP70 reached a maximum after 4-7 h. Their 2 and 4 h time points agreed with our results, although we found that HSP70 mRNA expression was decreased at the 7 h time point. Transcription of HSP70 in animal cells occurs within a few seconds after stimulation whereas translation occurs for several hours[3]. At the 7 h time point, transcription is complete and translation occurs, which suggests that our assessment of HSP70 gene transcription may more sensitive than the measurement of HSP70 expression. Finally, consistent with our findings, previous studies have shown that heavy metals induce HSP70 mRNA in HeLa cells 2 h after exposure with maximal induction after 4-7 h and gradual diminishment thereafter.

Our observations of HSP70 mRNA induction correlated with the cytotoxicity of $NiCl_2$, but did not correlate with the cytotoxicities of $HgCl_2$ and $CuSO_4$. Cell viability was decreased after a 4 h treatment with 80, 1250 and 5000 μM $HgCl_2$, $CuSO_4$ or $NiCl_2$. Thus, the mRNA induction assay was as sensitive as the NR assay for determining the toxicity of $NiCl_2$, although HSP70 mRNA induction could be detected more rapidly and at a four-fold lower concentration of $HgCl_2$ and an eight-fold lower concentration of $CuSO_4$ than in the NR assay. One possible explanation for the higher sensitivity of the mRNA induction is that HSP70 mRNA induction is more closely connected than overall cell viability to the biochemical changes, reaction with cysteine sulfhydryls, for example that occur upon exposure to heavy metals. Although this induction does not always correlate well with overall toxicity of the metal salts, the assay of quantitative gene expression using real-time Taqman PCR carried out in this study has been proven to be a suitably sensitive and reliable tool for rapid analysis of mRNA level.

Endpoints other than those used to measure toxicity might be more suitable for *in vitro* testing of dental materials to simulate *in vivo* conditions. Schmaltz *et al.*[16] showed that cytokines, which are important in the initiation of the inflammatory process, are released from human oral tissue culture cells after exposure to compounds found in dental materials. Other experiments have indicated that dental materials may cause chronic toxicity and contact allergy, and that HSP70 mRNA expression may be a marker for these responses[14,15].

The use of real-time Taqman quantitative PCR described in this study has several advantages over the conventional RT-PCR system. First, the use of the Taqman probe provides specificity to the system. The Taqman probe contains a reporter dye at the 5'-end and quencher dye at the 3'-end. An increase in the fluorescence is detected only if the target sequence is complementary to the probe and is amplified during PCR. Therefore, only specific amplification is detected. Second, sample throughput is increased dramatically with this method because of the use of a sequence detector for all 96 wells of the thermal cycler. Our method allowed the detection of an eventual decrease in HSP70 mRNA levels after the initial induction. This could be an early indication of the cytotoxicity of the metal salts. Therefore, quantification of HSP70 mRNA levels by real-time Taqman quantitative PCR could be, in combination with the monitoring of cell viability, a valuable tool to distinguish between specific HSP70 mRNA and cytocompatibility *in vitro*.

In the current studies, we examined only a single human metal-inducible gene along with three kinds of heavy metal salts. The induction kinetics on the other HSPs, a metallothionein protein and *c-fos* for example, might also be useful for the detection of heavy metal toxicity. Indeed, examination of cytocompatibility of not only metals but also dental polymers could be explored using HSP gene induction as an endpoint with the method of Taqman quantitative PCR.

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