Stress protein assay for the evaluation of cytotoxicity of dental amalgam

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To evaluate the cytotoxicity of mercury in dental amalgams, a stress protein assay was performed and the results were compared with the cytotoxicity evaluated by a neutral red uptake assay. The induction of a major stress protein, hsp70, was analyzed at levels of mRNA, synthesis and accumulation in human HeLa cells treated with extracts from amalgam, metal mercury and mercuric chloride. Mercuric chloride induced an increase in the synthesis of hsp70 at concentrations of mercury half those used for the neutral red uptake assay. The extracts from dental amalgam and metal mercury induced an increase in hsp70 mRNA at concentrations of mercury half those causing the inhibition of neutral red uptake into cells. Furthermore, the extracts from dental amalgam or metal mercury increased the synthesis of hsp70 and inhibited the uptake of dye at concentrations of mercury 1/10–1/50 lower than those at which mercuric chloride acted. These results suggest that the stress protein assay is more sensitive than the conventional neutral red assay for the evaluation of the cytotoxicity of mercury in dental amalgams and that the methods used in the preparation of metal solutions seem to be critical to the evaluation of cytotoxicity of dental materials.
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1. Introduction
Dental amalgams are restorative materials that contain several metals such as mercury, copper, silver and tin. Of these metals, mercury consists of approximately 50% dental amalgams and can enter the body by various routes [1]. The amalgams inside the oral cavity continuously release mercury vapor, which is inspired, expired and swallowed. Mercury as well as corrosion products of dental amalgams are considered to have deleterious effects [2, 3]. A ring inhibits mitochondrial enzymic activity in cultured cells adjacent to cast base metal crown and bridge alloys, but not polished alloys, probably due to degradation products of the materials [2]. Hence, cellular responses caused by not only mercuric chloride but also mercury extracted from dental amalgams seems to be important for evaluation of the biocompatibility of amalgams.

For evaluation of the cytotoxicity of noxious substances, a variety of methods such as measurements of cell viability, cell proliferation, protein synthesis, membrane permeability and dye uptake have been developed. Among them, the cytotoxicity of many dental materials has been conveniently tested by neutral red assay, which evaluates the ability of cells to incorporate the weakly cationic neutral red into lysosomes [4]. Recently, the stress protein assay was established as a new tool for toxicological research, in both cell lines and whole organisms [5, 6]. The rationale is that many environmentally relevant insults can elicit an increase in the expression of stress proteins. In fact, heavy metals such as copper, zinc and arsenate induce the synthesis of not only metallothionein but also stress proteins in mammalian cells [7, 8]. Using the stress protein assay, we have shown that the induction of a major stress protein hsp70 is one of the most sensitive cellular responses induced by mercuric chloride, and have suggested that the stress protein assay can be used for evaluation of the cytotoxicity of dental materials [9].

In this study, to examine the cellular response caused by metals in dental amalgams and extracts containing ingredients released from amalgams, we analyzed the induction of hsp70 at levels of mRNA, synthesis and accumulation in human cells as a stress protein assay, and compared the results with the cytotoxicity evaluated by neutral red uptake assay.

2. Materials and methods
2.1. Chemicals
Metal compounds that consist of dental amalgam were used in this study; mercuric chloride (Ishizu Seiyaku); stannous chloride (Nacalai Tesque); silver sulfate (Nacalai Tesque); and cupric chloride (Wako Pure Chemicals).

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2.2. Preparation of extracts from amalgams and metal mercury

For the preparation of dental amalgam, amalgam alloys (Spherical-D, Shofu, 109467, Kyoto) containing 59.5% silver, 27.5% tin and 13% copper were mixed with mercury (99.9%, Shofu, 69817, Kyoto) at a ratio of 1 : 0.9 in a spherical amalgam mixer (Shofu, Kyoto), and poured into Teflon molds (diameter 12.5 mm; length 2 mm). Following sterilization by UV light for one day, three amalgam discs were extracted dynamically in 10 ml of Eagle's minimum essential medium (Nissui) at 37 °C for one week by gyration (240 rpm). Metal mercury (10 g, 99.9%, Ishizu, 32466, Osaka) was also extracted as above. Concentrations of Hg, Sn, Ag, Cu and Zn in the extracts were measured using an atomic absorption spectrophotometer (Hitachi 180-70).

2.3. Cell culture and chemical treatments

Human HeLa 229 cells, obtained from the Japan Cancer Research Resource Bank, were maintained in Eagle's minimum essential medium (Nissui) supplemented with 5% heat-inactivated calf serum (Gibco BRL) in a CO_2 incubator (5% CO_2 in air) at 37 °C. Exponentially growing HeLa cells (5×10^5 cells/35-mm dish) were incubated in the medium containing various concentrations of chemicals or extracts from amalgam or metal mercury for 6 h.

2.4. Northern blotting analysis

Total cellular RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform method [10]. Aliquots of RNA (5 µg) were electrophoresed on 1% agarose gels, and transferred onto nitrocellulose membranes. Filters were washed with 2X SSC, and hybridized in a solution containing 50% formamide, 5X SSC, 0.6X Denhardt's solution, 1% SDS, and 100 µg/mL salmon sperm DNA for 24 h at 42 °C. Denatured ^{32}P-labeled probe was added to the solution for an additional 16 h at 42 °C. Human hsp70 DNA [11] was used as a probe. Filters were washed in 2X SSC, and then 0.5% SDS/2X SSC at 65 °C, and subjected to autoradiography at − 80 °C. To estimate amounts of RNA loaded onto the gels, 28S rRNA was used as a reference.

2.5. Metabolic labeling of cells

HeLa cells were washed once with methionine-free Eagle's minimal essential medium and were incubated in 1 mL of the methionine-free medium supplemented with 5% calf serum containing various concentrations of chemicals or extracts from amalgam or metal mercury and 10 µCi/mL ^{35}S-protein labeling mix (Amersham) for 6 h. After labeling of cells, the radioactive medium was removed, and the cells were washed twice with cold phosphate-buffered saline (PBS), solubilized in 0.1% SDS, and boiled for 2 min. Protein concentration was assayed by the dye binding method [12]. The labeled proteins (10 µg) were electrophoresed on a SDS-10% polyacrylamide slab gel [13], and autoradiographed at − 80 °C.

2.6. Western blotting and immunological detection

Ten micrograms of the protein sample from HeLa cells was electrophoresed on SDS-10% polyacrylamide gels, and the proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes by electrotransfer [14]. The membranes were incubated with a 1 : 5000 dilution of mouse anti-hsp70 monoclonal antibody (Sigma), and then visualized by the immunoperoxidase method (Vectastain ABC kit, Vector Laboratories).

2.7. Neutral red uptake assay [4]

HeLa cells (1.2×10^5 cells/well, 96-well micro plate) were incubated in medium with or without various concentrations of chemicals or extracts from amalgam or metal mercury for 6 h. Then, 0.1 mL of NR-medium (50 µg/mL neutral red in culture medium) was added to each well, and cells were further incubated for 3 h. The NR-medium was then removed, and cells were rapidly washed three times with PBS. To extract neutral red from cells, 0.1 mL of 1% acetic acid-50% ethanol was added to each well, and the cells kept for 20 min at room temperature. Absorbance at 540 nm was measured using a microplate reader. Values represent rates relative to the control and are expressed as means and standard deviations from at least three separate experiments. The differences between control and chemically exposed cells were evaluated by the Student *t* test.

3. Results

3.1. Stress protein assay for evaluation of cytotoxicity of mercuric chloride

To evaluate the cytotoxicity of mercury in dental amalgam, mercuric chloride was used as a model compound for the stress protein assay. When HeLa cells were incubated with 0.6–10 µM mercuric chloride, the level of hsp70 mRNA was not significantly changed (Fig. 1(A)). However, the synthesis of hsp70 was clearly up-regulated in cells treated with mercuric chloride at 2.5 µM and above (Fig. 1(B)). The accumulation of hsp70 was not increased by the treatment with mercuric chloride at the concentrations examined (Fig. 1(C)). In the conventional assay, the uptake of neutral red by HeLa cells was significantly decreased at 5 µM mercuric chloride and above (Fig. 1(D)). Thus, the stress protein assay at the level of hsp70 synthesis was twice as sensitive as the conventional neutral red uptake assay for evaluation of the cytotoxicity of mercuric chloride.

3.2. Stress protein assay for evaluation of cytotoxicity of extracts from amalgam

To assess the cytotoxicity of dental amalgam from which the ingredients may be released and dissolved into saliva, amalgam discs were extracted in culture medium by one week-dynamic gyration. Concentrations of mercury, tin, silver and copper released into the amalgam extracts were 27.9, 26.5, 7.5 and 393.5 µM, respectively (Table I). As a reference, when metal mercury was extracted in culture medium by one week-dynamic gyration in the same way, the concentration of mercury in the mercury

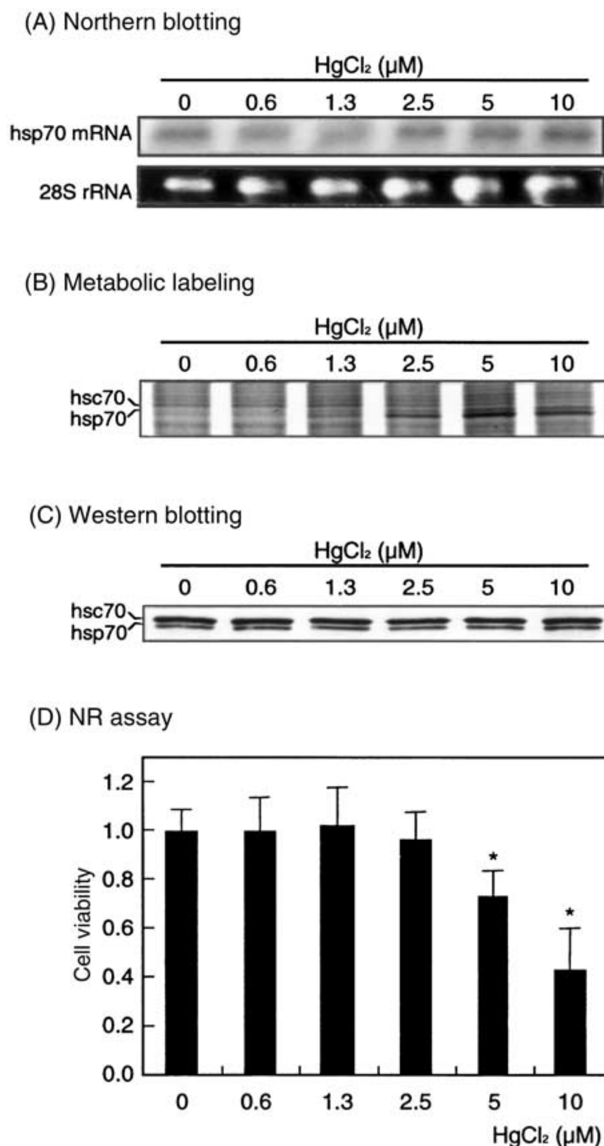

Figure 1 Induction of stress response by mercuric chloride in HeLa cells. HeLa cells were incubated with or without 0.6–10 µM mercuric chloride for 2 h (A) or 6 h (B–D). (A) The amount of hsp70 mRNA was analyzed by Northern blotting using human hsp70 DNA. Ethidium bromide staining of 28S rRNA is shown as a reference. (B) The synthesis of hsp70 was analyzed by the metabolic labeling of cells with ^{35}S-protein labeling mix. (C) Accumulation of hsp70 was measured by Western blotting using anti-hsp70 antibody. (D) Cytotoxicity was measured by neutral red uptake assay, and values are the mean ± S.D. of at least three experiments. *Significant difference ($p < 0.05$) from control.

TABLE I Metals in the extracts from dental amalgam and mercury obtained by dynamic extraction (µM)

Extracts	Hg	Ag	Sn	Cu
Dental amalgam	27.9	7.5	26.5	393.5
Metal mercury	124.6	—	—	—

extracts was approximately 125 µM, four times higher than in the extracts from dental amalgams.

Using the amalgam extracts, stress protein and neutral red uptake assays were performed to evaluate the cytotoxicity. When HeLa cells were incubated with the diluted amalgam extracts, the level of hsp70 mRNA was increased in cells treated with 512-fold diluted extracts

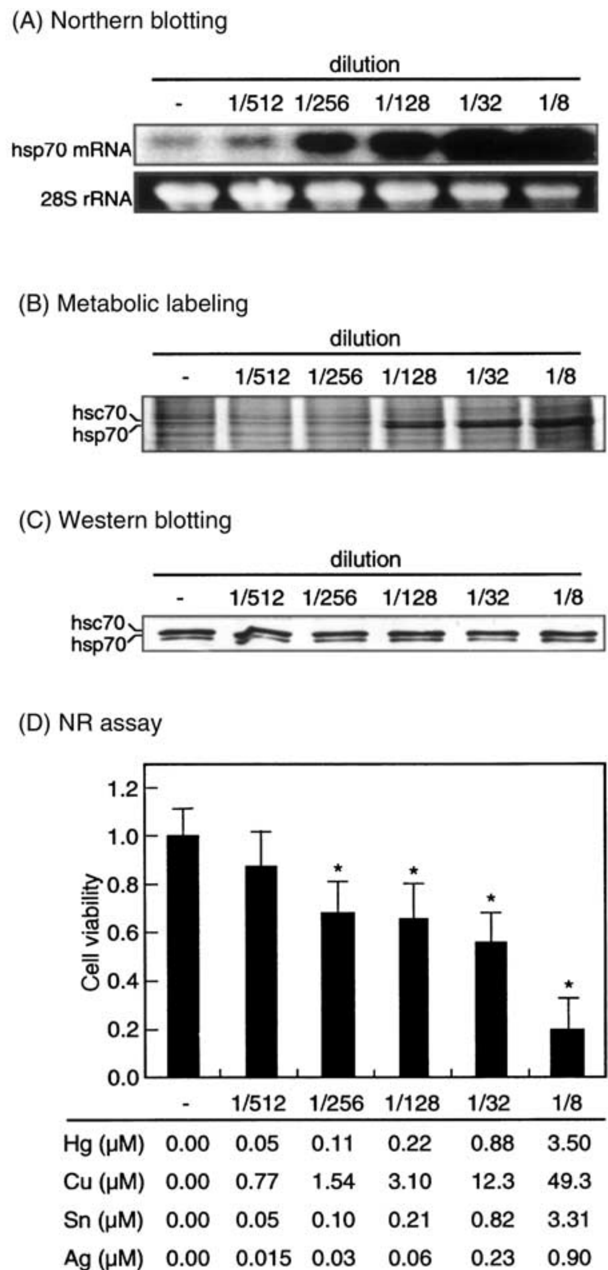

Figure 2 Stress protein assay for the amalgam extracts. HeLa cells were incubated with or without the diluted amalgam extracts for 2 h (A) or 6 h (B–D). (A) The amount of hsp70 mRNA was analyzed by Northern blotting, and ethidium bromide staining of 28S rRNA as a reference. (B) The synthesis of hsp70 was analyzed by the metabolic labeling of cells. (C) Accumulation of hsp70 was measured by Western blotting. (D) Cytotoxicity was measured by a neutral red uptake assay, and values are the mean ± S.D. of at least three experiments. *Significant difference ($p < 0.05$) from control.

containing 0.05 µM mercury, 0.77 µM copper, 0.015 µM silver and 0.05 µM tin (Fig. 2(A)). The synthesis of hsp70 was markedly enhanced in cells treated with 128-fold diluted extracts containing 0.22 µM mercury, 3.1 µM copper, 0.06 µM silver and 0.21 µM tin (Fig. 2(B)). However, the accumulation of hsp70 was not markedly increased in cells treated with the extracts at any dilution examined (Fig. 2(C)). The uptake of neutral red by HeLa cells was significantly decreased in cells treated with 256-fold diluted extracts containing 0.11 µM mercury, 1.54 µM copper, 0.03 µM silver and 0.1 µM tin (Fig. 2(D)). Thus, the stress protein assay at the level of hsp70 mRNA induction was twice as sensitive as the neutral red uptake assay for the evaluation of the cytotoxicity of the

amalgam extracts. However, these results were quite different from those for mercuric chloride. The increase in hsp70 mRNA was induced by the amalgam extracts, not by mercuric chloride. The synthesis of hsp70 was induced by amalgam extracts containing 0.22 μM mercury, 3.1 μM copper, 0.06 μM silver and 0.21 μM tin, and by mercuric chloride at a concentration of 1.3 μM mercury.

3.3. Stress protein assay for evaluation of cytotoxicity of extracts from metal mercury

Since the amalgam extracts which contained low concentrations of Hg, Cu, Sn and Ag were more toxic than mercuric chloride, we next examined the cytotoxicity of mercury extracts using stress protein and neutral red uptake assays. As shown in Fig. 3(A), the level of hsp70 mRNA was markedly increased in cells treated with 2048-fold diluted mercury extracts containing 0.06 μM mercury. The synthesis of hsp70 was also markedly up-regulated in cells treated with 512-fold diluted extracts containing 0.24 μM mercury (Fig. 3(B)). However, the accumulation of hsp70 was not increased in cells treated with the mercury extracts, as in the case of amalgam extracts (Fig. 3(C)). The uptake of neutral red by HeLa cells was significantly decreased in cells treated with 1024-fold diluted extracts containing 0.12 μM mercury (Fig. 3(D)). Thus, the stress protein assay at the level of hsp70 mRNA induction was also twice as sensitive as the neutral red uptake assay for the evaluation of the cytotoxicity of the mercury extracts, as in the case for the amalgam extracts.

The amalgam extracts contained not only mercury, but also tin, silver and copper, but the mercury extracts contained only mercury. However, the induction of hsp70 mRNA expression and hsp70 synthesis and the inhibition of neutral red uptake by the amalgam and mercury extracts were observed at similar concentrations of mercury in both extracts. Thus, among the metals in the amalgam extracts only mercury seemed to be responsible for the production of stress protein and for the cytotoxicity without synergism with other metals in the extracts. In fact, when HeLa cells were incubated with cupric chloride, an increase in hsp70 mRNA and the synthesis and accumulation of hsp70 were only observed at 50 μM cupric chloride and above (data not shown). Furthermore, 50–800 μM stannous chloride and 3–50 μM silver sulfate did not induce an increase of hsp70 mRNA or the synthesis and accumulation of hsp70 at all (data not shown).

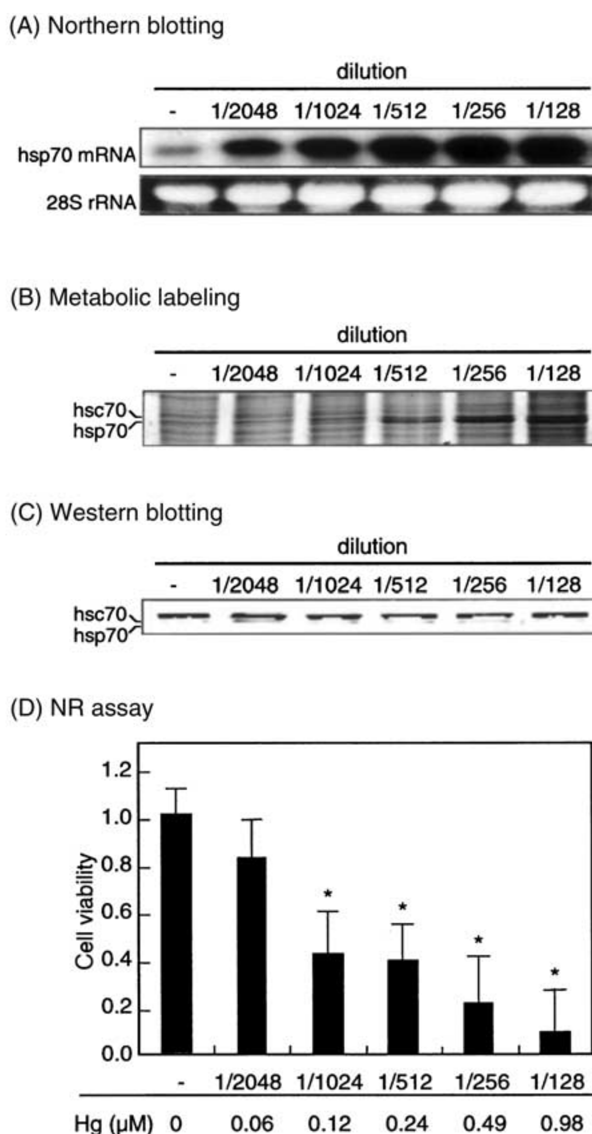

Figure 3 Stress protein assay for the mercury extracts. HeLa cells were incubated with or without the diluted mercury extracts for 2 h (A) or 6 h (B–D). (A) The amount of hsp70 mRNA was analyzed by Northern blotting, and ethidium bromide staining of 28S rRNA as a reference. (B) The synthesis of hsp70 was analyzed by the metabolic labeling of cells. (C) Accumulation of hsp70 was measured by Western blotting. (D) Cytotoxicity was measured by a neutral red uptake assay and values are the mean $\pm$ S.D. of at least three experiments. *Significant difference ($p < 0.05$) from control.

4. Discussion

Stress proteins are generally produced in response to heat shock, heavy metals, oxidative agents and pathophysiological stresses in a wide range of living organisms, and serve to protect cells from the cytotoxic effects of aggregated proteins produced by various types of stress [15]. These proteins are also necessary for essential cellular events such as folding, assembly, transport and degradation of proteins, as molecular chaperones [16]. In mammalian cells, heat shock and other forms of stress induce a rapid trans-activation of heat shock genes [17]. Consequently, the induction of mRNA of stress proteins is followed by the synthesis and accumulation of stress proteins in cells. Monitoing the induction of stress proteins can be exploited for the development of new and rapid toxicological assays.

Here, we showed that mercuric chloride induced an increase in the synthesis of hsp70 at concentrations of mercury half those needed for the inhibition of neutral red uptake into human cells. Furthermore, the amalgam extracts induced hsp70 mRNA expression at concentrations of mercury half those causing the inhibition of neutral red uptake into cells, and only mercury seemed to be responsible for the induction of stress responses and cytotoxic effects without any synergism with other metals in the amalgams. Thus, the stress protein assay at the level of mRNA or synthesis of hsp70 was a more

sensitive method than the conventional neutral red assay for evaluation of the cytotoxicity of mercury.

Although a stress response was induced both by mercuric chloride and by mercury in the amalgam or mercury extracts, there were some differences between them. The increase in mRNA and the subsequent synthesis of hsp70 were observed in cells treated with the amalgam or mercury extracts. However, the synthesis was promoted in cells treated with mercuric chloride, although the level of hsp70 mRNA was not increased at any concentration of mercuric chloride, suggesting that mercuric chloride enhanced the synthesis of hsp70 post-transcriptionally. These different responses induced by mercuric chloride and the amalgam and mercury extracts may be due to different chemical forms of mercury or different mercuric complexes with amino acids, proteins and other compounds in the medium, which may affect its incorporation into and actions in cells [18]. Proteins have binding sites, such as a cysteine moiety, that can easily form complexes with metal cations. As mercury tends to react with proteins in cells and medium, it may have significant effects on the conformation and activity of cellular proteins, and subsequently cellular metabolism [19–21]. Furthermore, as the accumulation of hsp70 was not increased in cells treated with either mercuric chloride or the extracts from amalgam and mercury, hsp70 protein synthesized in the presence of mercury may be unstable and degraded rapidly in the cells.

Interestingly, the synthesis of hsp70 was increased by the amalgam or mercury extracts containing 0.22–0.24 μM mercury, but was only increased by 2.5 μM mercuric chloride. The uptake of neutral red into cells was also inhibited by the amalgam or mercury extracts containing 0.11–0.12 μM mercury, but was only inhibited by 5 μM mercuric chloride. As the mercury extracted from the amalgam seemed to be more toxic than mercuric chloride, the methods used for the preparation of metal solutions seemed to be a critical factor in the evaluation of the cytotoxicity of metals. These findings provide not only useful clues for the evaluation of the cytotoxicity of mercury, but will also help us to develop more efficient, sensitive and convenient methods for evaluating the cytotoxicity of dental materials.

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