A possibility for new evaluating method of cytotoxicity by using heat shock protein assay

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To determine whether heat shock proteins can be utilized as a biomarker for cytotoxicity of dental materials the induction of synthesis of heat shock proteins by mercuric chloride was examined. To analyse the synthesis of heat shock proteins, HeLa cells were labelled with [^{35}S] methionine, and the labelled proteins were separated by SDS-PAGE and autoradiographed. Incubation of the cells in a medium containing 1.25 to 40 μM mercuric chloride markedly increased the synthesis of HSP70. At 20 or 40 μM mercuric chloride in medium, HeLa cells synthesized HSP70 at 2 h after exposure, maximally at 4–7 h, and gradually diminished thereafter. Examination of the cytotoxicity of mercuric chloride by the conventional neutral red uptake assay revealed a reduction of uptake of the dye in the presence of mercuric chloride at 40 μM and above. These findings suggest that the induction of synthesis of HSP70 is one of the most sensitive cellular responses caused by mercury ion, and the heat shock protein assay can be utilized for evaluation of the cytotoxicity of dental materials.

1. Introduction

Various stressors other than heat, including amino acid analogues, heavy metals, and oxidizing agents, can induce heat shock proteins (stress proteins) in various cells [1–5]. Heat shock proteins have been classified into several families: HSP105, HSP90, HSP70, HSP60, and the small HSP family [6, 7]. They have structural similarities in a wide variety of organisms and seem to be conserved through evolution. Most of them are expressed in considerable amounts in unstressed cells, and appear to be indispensable for normal cellular functions [6, 7]. In fact, HSP70 and HSP60 proteins appear to be required for assembly of cellular proteins to oligomeric structures or for transfer of proteins into various cellular compartments. Heat shock proteins also seem to be involved in cell protection and the repair of cell damage caused by various stressors [6, 7]. Among heat shock protein families, the HSP70 family seems to be essentially involved in cell protection and repair of cell damage caused by various stressors [8–12].

For evaluation of cytotoxicity of dental materials, *in vitro* studies have several advantages in terms of reproducibility, quantification, rapidity, simplicity and low cost, over *in vivo* experiments using animals, which have been challenged worldwide by animal rights groups and considerably restricted in recent years [13]. A variety of methods have been developed to evaluate the cytotoxicity of noxious substances. Abnormal membrane permeability has been assessed by a dye exclusion test [14], by the release of ^{51}Cr from labelled cells [15], or the release of vital stains [16] or lactate dehydrogenase [17]. On the other hand, the neutral red uptake assay is a commonly used simple assay, by which weakly cationic neutral red enters cells by non-ionic diffusion through the cell membrane, and accumulates in the lysosomes of living cells [18, 19]. The cytotoxicity of many dental materials including chemical agents has been tested with the neutral red uptake assay [20].

We have found that mammalian cells exposed to heavy metals, such as copper, zinc, and arsenate, synthesize not only metallothionein but also heat shock proteins [21, 22]. Since mercury ion is known to be cytotoxic and is one of the main components of dental restoratives, we examined the synthesis of heat shock proteins by mercuric chloride, in comparison with the cytotoxicity evaluated by the neutral red uptake assay using the HeLa cells.

2. Materials and methods

2.1. Cell culture and treatment of cells with mercuric chloride

HeLa 229 cells were maintained as a monolayer in Eagle's minimum essential medium (MEM, Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated calf serum (Gibco BRL, Life Technologies Inc., Grand Island, NY) at 37 °C in a CO_2 incubator (5% CO_2 in air). For experiments, exponentially growing HeLa 229 cells (6×10^5/35-mm dish) were incubated in the medium containing various concentrations of mercuric chloride for various periods.

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2.2. Cell labelling and SDS-PAGE analysis

To examine the induction of synthesis of heat shock proteins by mercuric chloride, HeLa cells were washed once with methionine-free Eagle's MEM supplemented with 10% calf serum and were incubated in 1 ml of methionine-free medium containing various concentrations of mercuric chloride and 10 μCi/ml [^{35}S] protein labelling mix (Amersham, Buckinghamshire, England) for 6 h. For analysis of the induction kinetics of HSP70 by mercuric chloride, HeLa cells were incubated in the medium supplemented with 10% calf serum and mercuric chloride. After appropriate times, the cells were washed once with methionine-free Eagle's MEM supplemented with 10% calf serum and then labelled with 1 ml of methionine-free medium containing 20 μCi/ml [^{35}S] protein labelling mix for 1 h with mercuric chloride.

After the cells were labelled, the radioactive medium was removed, and the cells were washed twice with cold phosphate-buffered saline, solubilized in 400 μl of 0.1% SDS, and boiled for 2 min [23]. The radioactivity incorporated into the protein was assayed as the counts precipitated with hot trichloroacetic acid. The protein concentration was assayed by the dye binding method [24]. The labelled proteins (10 μg) were electrophoresed on a SDS-10% polyacrylamide slab gel [25, 26], and autoradiographed at $-80\,^{\circ}$C. The density of the gel bands was measured with an image analyser (Toyobo image analyzer V1, Toyobo, Osaka, Japan).

2.3. Neutral red uptake assay

Cytotoxicity was measured by a neutral red uptake assay [20]. HeLa cells (1.2×10^5 cells/well, 96-well microplate) were incubated in medium without or with various concentrations of mercuric chloride for various periods. After appropriate times, 1 ml of NR-medium (50 μg/ml neutral red in culture medium) was added to each well, which was then incubated for 3 h. NR-medium was then removed and cells were rapidly washed three times with phosphate-buffered saline. To extract neutral red, 1 ml of 1% acetic acid–50% ethanol was added to each well, and kept for 20 min at room temperature. The absorbances are measured with a microplate reader equipped with a 540-nm filter. The readings, usually in quadruplicate, were averaged and the results expressed as percentage of control culture.

3. Results

Total protein synthesis by HeLa cells incubated in the presence of various concentrations of mercuric chloride for 6 h was only slightly inhibited by mercuric chloride at concentration up to 20 μM, but was significantly inhibited by mercuric chloride at 40 μM, and completely inhibited by that at 80 μM and above (Fig. 1). However, under these conditions, more than half of the proteins in the control cells were recovered from cells treated with mercuric chloride at 80 μM and above.

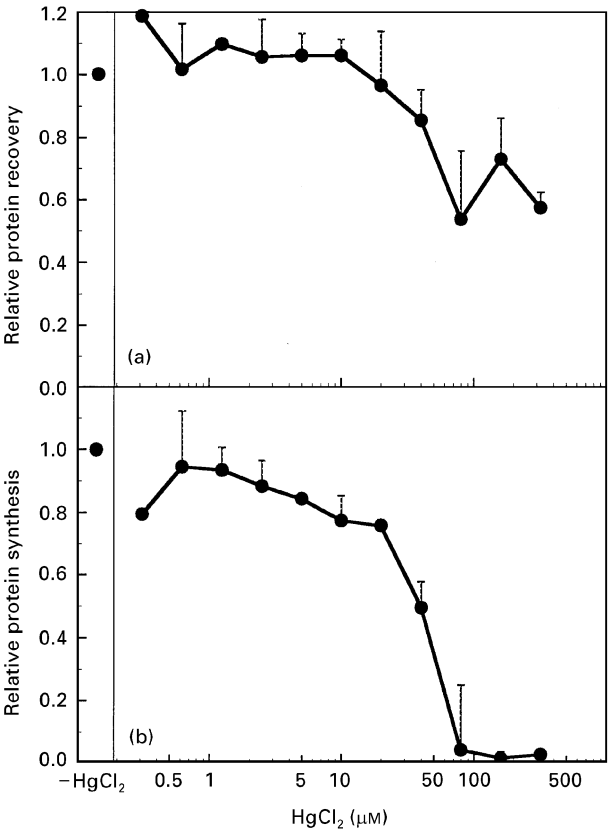

Figure 1 Protein synthesis of HeLa cells treated with various concentrations of $HgCl_2$. HeLa cells were incubated in medium without or with 0.315, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160, or 320 μM mercuric chloride in the presence of 10 μCi/ml [^{35}S] protein labelling mix for 6 h, then cellular proteins were recovered. Radioactivity incorporated to proteins was assayed as hot trichloro-acetic acid precipitable counts, and proteins were assayed by the dye-binding method. Experiments on cell culture and treatment of cells with mercuric chloride and SDS-PAGE analysis were repeated four times. (a) Relative protein recovery is shown as a ratio to the proteins recovered from the control cells. (b) Relative protein synthesis (radioactivity incorporated/protein recovered) is shown as a ratio to that of the control cells. Each value is the mean ± S.D. of at least four experiments.

To analyse the induction of synthesis of heat shock proteins, the labelled cellular proteins were separated by SDS-PAGE and autoradiographed (Fig. 2a). The syntheses of HSP70, HSC70, HSP90, and actin were measured by densitometry of the gel bands (Fig. 2b). The synthesis of HSP70 was significantly increased by treatment of cells with 1.25 to 40 μM mercuric chloride, whereas the synthesis of HSC70, HSP90 and actin did not increase under these conditions (Fig. 2). At 80 μM mercuric chloride, synthesis of all these proteins was completely inhibited (Fig. 1).

We further examined the induction kinetics of HSP70 by mercuric chloride by 1 h pulse labelling. Since the cells proliferated during the experiment, the proteins recovered from control cells increased, and proteins recovered from cells treated with even 5–20 μM mercuric chloride also increased gradually. However, the amount of protein in the cells treated with 40 μM mercuric chloride decreased (Fig. 3). On the other hand, total protein synthesis of HeLa cells was inhibited to less than half of the control levels immediately after treatment with even 5 μM mercuric chloride. The inhibition of protein synthesis recovered

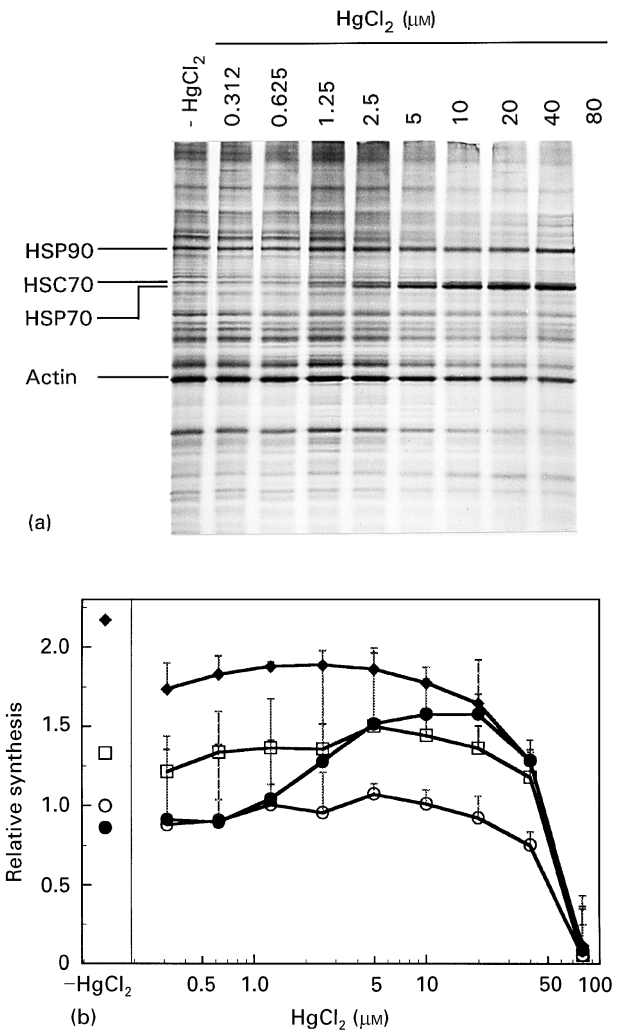

Figure 2 Effects of $HgCl_2$ on induction of heat shock proteins in HeLa cells. (a) 10 μg of the labelled proteins, obtained as mentioned in Fig. 1, were separated by SDS-PAGE, and autoradiographed. (b) The density of the gel bands was measured by densitometry, and syntheses of HSP70 (●), HSC70 (○), HSP90 (□), and actin (◆) are shown as a fraction of synthesis of HSC70 of the control cells. Experiments on cell culture and treatment of cells with mercuric chloride and SDS-PAGE analysis were repeated four times. Each value is the mean ± S.D. of at least four experiments.

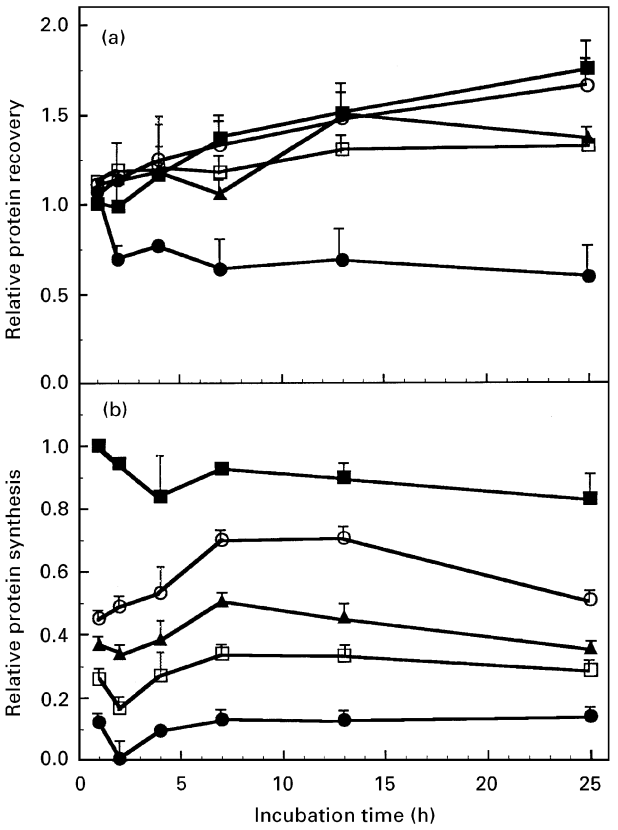

Figure 3 Time course analysis of protein synthesis of HeLa cells treated with $HgCl_2$. HeLa cells were incubated in medium containing 0 (■), 5 (○), 10 (▲), 20 (□) or 40 (●) μM $HgCl_2$ for 0, 1, 3, 6, 12 and 24 h, then incubated with 20 μCi/ml [^{35}S] protein labelling mix and corresponding $HgCl_2$ for 1 h. Radioactivity incorporated to proteins was assayed as hot trichloro-acetic acid precipitable counts, and proteins were assayed by the dye-binding method. Experiments on cell culture and treatment of cells with mercuric chloride and cell labelling were repeated four times. (a) Relative protein recovery is shown as a ratio to the proteins recovered from the control cells. (b) Relative protein synthesis is shown as a ratio to that of the control cells. Each value is the mean ± S.D. of at least four experiments.

by 7 h, but protein synthesis still remained at low levels thereafter. Fig. 4 shows autoradiographs of the labelled cell proteins separated by SDS-PAGE, and syntheses of HSP70, HSC70, HSP90 and actin were quantified by densitometry of gel bands. Synthesis of HSP70 began to increase at 2 h after treatment with 20 μM mercuric chloride, and reached a maximum at 4 to 7 h, and gradually decreased by 13 h. However, syntheses of HSC70, HSP90 and actin, did not significantly increase under the same conditions.

We next examined the cytotoxicity of mercuric chloride by the conventional neutral red uptake method. The uptake of neutral red by HeLa cells treated with various concentrations of mercuric chloride for 6 h was not affected by mercuric chloride at a concentration up to 20 μM, and was significantly decreased at a concentration of 40 μM and above (Fig. 5a). The time course experiment revealed no decrease of uptake of the dye in the cells treated with 5 μM mercuric chloride (Fig. 5b). The uptake decreased 13 h after treatment with 10 or 20 μM mercuric chloride, but decreased only 2 h after treatment with 40 μM mercuric chloride.

4. Discussion

The synthesis of heat shock proteins and cytotoxicity have been examined using tissue culture of HeLa cells. Our findings revealed that established cells such as HeLa cells were more sensitive in expressing the cytotoxicity levels of various dental materials [27–29]. Synthesis of HSP70 was induced in cells treated for 6 h with mercuric chloride at concentrations as low as 1.25 μM. Synthesis of HSP70 increased at 2 h after treatment with 20 μM mercuric chloride, and was maximum at 4 to 7 h. Protein synthesis was significantly inhibited in cells treated with mercuric chloride, at 40 μM and above, and the inhibition was observed at only 1 h after treatment. This suggested that synthesis of HSP70 was induced at concentrations of mercuric chloride lower than those at which protein synthesis was inhibited.

Heat shock proteins are involved in cell protection and the repair of cell damage caused by various

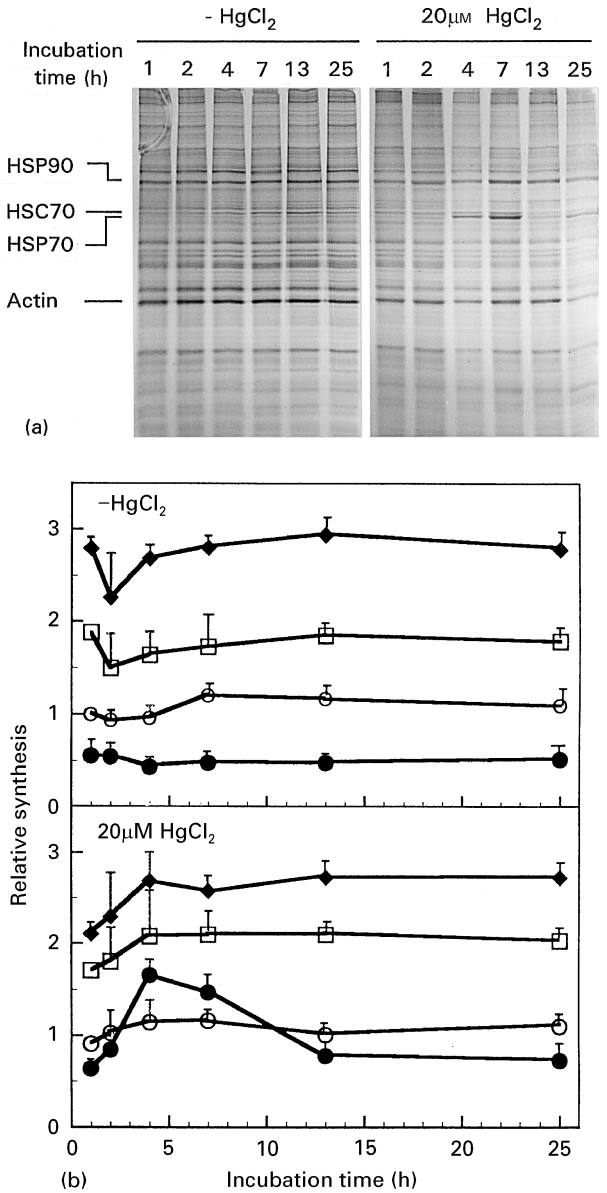

Figure 4 Induction kinetics of syntheses of heat shock proteins of HeLa cells by $HgCl_2$. (a) 10 μg of the labelled proteins, obtained as mentioned in Fig. 3, were separated by SDS-PAGE, and autoradiographed. (b) The density of the gel bands, shown in (a) was measured by densitometry, and syntheses of HSP70 (●), HSC70 (○), HSP90 (□), and actin (◆) are shown as a fraction of synthesis of HSC70 of the control cells. Experiments on cell culture and treatment of cells with mercuric chloride and SDS-PAGE analysis were repeated four times. Each value is the mean ± S.D. of at least four experiments.

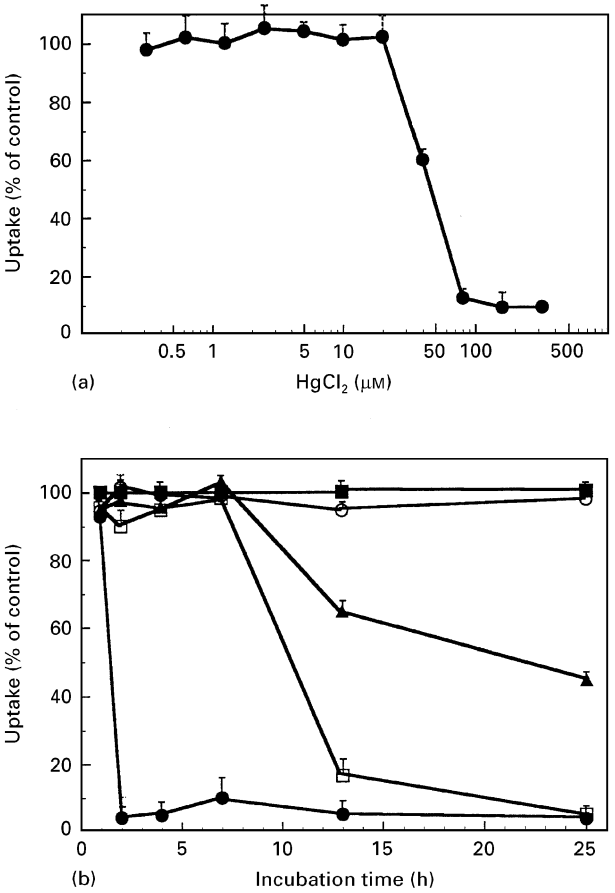

Figure 5 Cytotoxicity assayed by neutral red uptake method. (a) HeLa cells were incubated in medium without or with 0.312, 0.625, 1.25. 2.5, 5, 10, 20, 40, 80, 160, or 320 μM mercuric chloride for 6 h, and then uptake of dye was assayed by the neutral red uptake method. (b) HeLa cells were incubated in medium without (■) or with 5 (○), 10 (▲), 20 (□) or 40 (●) μM mercuric chloride for 0, 1, 2, 4, 7, 13, 25 h and then uptake of dye was assayed. Experiments on neutral red uptake assay were repeated three times. Each value is the mean ± S.D. of at least 30 experiments.

stressors [6, 7]. Among the heat shock proteins, the HSP70 family seems to be essentially involved in cell protection and repair of cell damage caused by various stressors [8–12]. Metallothioneins are also induced by heavy metals [30–32], and can detoxify heavy metals, such as cadmium, mercury and copper, in mammalian cells [33–35]. Although we did not analyse the induction of metallothioneins by mercuric chloride, we have previously shown that HSP70 does not participate in the detoxification of heavy metals as does metallothionein, but it may function in repairing damage caused by the metal [21, 22]. Here, we showed that an inducible HSP70, but not a constitutive HSC70 and HSP90, was induced by the treatment of cells with mercury ion. Thus, inducible HSP70 may have a principle role in the protection of cells from the toxic metal.

The neutral red uptake assay is a commonly used method for evaluation of cytotoxicity of toxic agents. The dye may enter cells by non-ionic diffusion through the cell membrane, and accumulate in the lysosomes of living cells [18–20]. Neutral red uptake method was not affected by treatment of cells with up to 20 μM mercuric chloride for 6 h. The uptake was decreased only after treatment with 10 or 20 μM mercuric chloride for 13 h. Thus, induction of synthesis of HSP70 could be detected more rapidly at one tenth to one twelfth lower concentrations of mercuric chloride than those at which uptake of the dye was decreased.

These findings suggest that induction of the synthesis of HSP70 is a sensitive response caused by mercury ion. However, we examined only mercuric chloride. Further studies on various substances including the present findings are necessary to determine whether the heat shock protein assay, which uses the induction of synthesis of HSP70, is an efficient evaluation method for cytotoxicity of dental materials or not. These findings will also provide a useful clue to the mechanism of the cellular response to stimulus and toxic substances, and will help

develop an efficient method of evaluating cytotoxicity of dental materials.

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