Protective Effects of Thiol Compounds on Chromate-Induced Cytotoxicity in HeLa Cells

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ABSTRACT. The effects of several thiol compounds on the cytotoxicity induced by chromate (potassium dichromate) were examined. HeLa cells were incubated in Eagle’s minimum essential medium (MEM) with or without the chromate alone, or with both chromate and any one of L-cysteine ethyl ester (LCysEE), L-cysteine methyl ester (LCysME), N-acetyl-L-(+)-cysteine, 2,3-dimercaptosuccinic acid (DMSA), 2, 3-dimercapto-1-propanesulfonic acid (DMPS), or dithiothreitol. After a given period of incubation, the number of viable cells was counted using the trypan blue exclusion test and the chromium content of the cells was estimated by atomic absorption spectrophotometry. The results obtained were as follows. 1) Chromate-induced cytotoxicity evaluated by inhibition of cell growth at 3 days of incubation was diminished by all of the thiol compounds tested when the cells were incubated in MEM with 2.5 to 10.0 μM chromate and 25 to 100 μM thiol compounds. 2) All of the thiol compounds produced a concentration-dependent reduction of chromate when a solution of the thiol compound (12.5 to 100 μM) was mixed with a solution of chromate (10 μM) in distilled water. 3) When cells were incubated in MEM with both 10 μM chromate and 25 to 100 μM thiol compounds, the chromium content of the cells at 6 hr of incubation was decreased in a concentration-dependent manner. 4) When these thiol compounds were added to MEM 1 hr before or after chromate, no or little protective effects of these thiol compounds against chromate-induced cytotoxicity and chromium uptake by the cells were observed. The results of this study demonstrate that the thiol compounds, especially LCysEE, LCysME, DMSA, and DMPS are useful for treating chromate-induced cytotoxicity when they are given immediately after intake of the metal and suggest that a part of this effect may be due to decrease in chromium uptake by the cells accompanying the reduction of chromate. — key words: chromate-induced cytotoxicity, chromium, HeLa cell, thiol compound.


A number of thiol compounds are available for the treatment of heavy-metal intoxication. For example, cysteine, penicillamine, 2,3-dimercaptosuccinic acid (DMSA), 2, 3-dimercaptoopropane-1-sulfonate and dithiothreitol (DTT) are effective in treating poisoning by compounds of cadmium [1, 5, 15], mercury [7, 8, 18], lead [13, 14], arsenic [3, 12], and other heavy metals.

It is known that chromium dermatitis is prevented by the application of ascorbic acid. The mechanism for the inactivation of hexavalent chromium by ascorbic acid was thought to involve reduction to trivalent chromium and subsequent complex formation with the trivalent species [26]. Susa [28] reported that DL- penicillamine diminished chromate-induced cytotoxicity, which was closely related to the reduction of chromium uptake by the cultured HeLa cells. Furthermore, Susa et al. [30] reported that combined administration of hexavalent chromium and DL-penicillamine caused not only diminished chromium accumulation within the tissues, but also increased urinary excretion of chromium, and thus, DL-penicillamine prevented the lethal effects of chromium in mice.

Ascorbic acid and thiol-containing molecules such as cysteine, cysteamine, glutathione, unithiol, penicillamine, DTT, mercaptoethanol, lipoic acid, DMSA, and thiolactic acid, effectively reduce chromate under physiologic conditions [6]. This finding suggests the possibility that the thiol-containing compounds may be useful as an antichrome agent for the prevention and treatment of chromium poisoning.

The purpose of this paper is to investigate the effectiveness of the monothiols, L-cysteine ethyl ester (LCysEE), L-cysteine methyl ester (LCysME), and N-acetyl-L-(+)-cysteine (NAC), and that of the dithiols 2, 3-dimercapto-1-propanesulfonic acid (DMPS), DMSA, and DTT, on the cytotoxicity induced by potassium dichromate as hexavalent chromium. Inhibition of cell growth was used in the present study as a parameter of cytotoxicity induced by chromate.

MATERIALS AND METHODS

Cell cultures: HeLa cells originating from human cervix carcinoma (Flow Laboratories LTD., U.S.A.) were maintained as monolayers and
routinely passaged in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with L-glutamine (0.292 g/l), kanamycin (60 μg/ml), NaHCO₃ (2.2 g/l) and 10% heat-inactivated calf serum (Irvine Scientific, Santa Ana, Calif.). The cells were grown at 37°C in an atmosphere of 5% CO₂-95% air and 95 to 100% humidity. Four replicate cultures were used for each treatment per experiment.

**Evaluation of cytotoxicity:** To determine the effects of chemicals on cell growth, the cells were seeded at 5×10⁵ cells per 60 mm glass petri dish with 5 ml of the medium. One day after incubation, the medium was exchanged with a fresh medium containing chromium alone, or both chromium and one of the thiol compounds, in which the cells were then incubated for 3 days. For the control experiment, the cells were incubated in the medium with neither chromate nor thiol compounds in the same manner as described above. No medium change was made during exposure to the chemicals. After 3 days of additional incubation, the number of viable cells was counted by the trypan blue exclusion test according to the method described in a previous paper [29] and the growth-inhibitory ratio, Y, for each dose of test chemical was calculated using the equation, Y (\%) = (C-T)/(C-Co)×100 where T is the cell count for each dose after 3 days incubation; C is the mean cell count for the control after 3 days; Co is the mean cell count at the start of chemical treatment.

**Evaluation of chromate reduction:** For evaluating chromate reduction, a solution of potassium dichromate dissolved in distilled water was mixed with one of the solutions of thiol compounds dissolved in distilled water. After the mixture was incubated at 37°C for 5 min, amount of the chromate in the mixture was determined spectrophotometrically (Hitachi Spectrophotometer, model 220) at 540 nm by the colored reaction complex with 1,5-diphenylenecarbazide in H₂SO₄-acidified solutions (diphenylenecarbazide method) [25].

**Chromium uptake by the cells:** To determine the effects of thiol compounds on cellular chromium uptake, the cells were seeded at 1×10⁶ cells per 100 mm plastic petri dish (Corning) with 10 ml of medium. Three days after incubation, the medium was exchanged with serum free medium containing chromium alone, or both chromium and any one of the thiol compounds. After 6 additional hours of incubation, the medium was discarded and the cell layer was rinsed twice with phosphate-buffered saline (PBS). The cells were then scraped from the dishes with a rubber policeman and suspended into an aliquot amount of PBS to analyze the cellular chromium and protein contents. Estimation of chromium was carried out with an atomic absorption spectrophotometer (Simazu AA-650) fitted with a graphite furnace atomizer (Simazu GFA-2) following wet ashing of the sample by concentrated nitric acid in a test tube on a hot plate. Cellular protein content was determined by the method of Lowry et al. [20]. The chromium content of the cells was expressed as μgCr per mg cell protein.

**Chemicals:** The chemicals used were: potassium dichromate (Kanto Chemical Co., Inc., Tokyo), L-cysteine ethyl ester hydrochloride (LCysEE, Nakarai Chemicals Ltd., Kyoto), L-cysteine methyl ester hydrochloride (LCysME, Nakarai Chemicals Ltd., Kyoto), N-acetyl-L-(+)-cysteine (NAC, Kanto Chemical Co., Inc., Tokyo), 2, 3-dimercapto-p-tartronic acid (DMSA, Nakarai Chemicals Ltd., Kyoto), 2, 3-dimercapto-1-propanesulfonic acid sodium salt (DMPS, Sigma Chemical Company, St. Louis, Mo. U.S.A.), and dithiothreitol (DTT, Nakarai Chemicals Ltd., Kyoto). All chemicals employed were of commercial reagent-grade quality. Each chemical was dissolved in distilled water just prior to use at 100 times the final concentration and then sterilized by Millipore filtration (0.45 μm). These solutions were further diluted as final concentration with the culture medium.

**Statistical analysis:** All the results were expressed as the mean and standard error of 4 experiments. The differences between the mean values for the data were evaluated by the Student's t-test for equal variance or Welch's t-test for unequal variance, and a P value less than 0.05 was considered to be statistically significant.

**RESULTS**

**Effects of thiol compounds on chromate-induced cytotoxicity:** Preliminary experiments were performed to determine the growth-inhibitory effects of thiol compounds. The cells were incubated with medium containing each thiol compound alone for 3 days. As shown in Fig. 1, LCysEE and LCysME at 125 to 1,000 μM markedly inhibited the cell growth in a concentration-dependnet manner, while no remarkable inhibition of cell growth was observed when exposed to NAC, DMSA, DMPS or DTT at the range of concentrations tested (125–1,000 μM).
Fig. 1. Inhibitory effects of several thiol compounds on the growth of HeLa cells. After 24 hr incubation, the medium was exchanged with the thiol compound-containing medium, in which the cells were then incubated for 3 days. The number of viable cells was then counted using the trypan blue exclusion test after 3 days exposure to each thiol compound and the growth-inhibitory ratio for each dose of test chemical was calculated as described in MATERIALS AND METHODS. Control cells were incubated without thiol compound. Each value represents the mean of four replicate cultures for each exposure concentration.

Based on these results, nontoxic concentrations of the thiol compounds were used in the following experiments.

In the experiments summarized in Fig. 2, the cells were incubated in medium with or without 2.5 to 10 μM chromate alone, or both chromate and one of the thiol compounds for 3 days. The growth-inhibitory ratio of the cells in medium with chromate alone was increased in a concentration-dependent manner, however, inhibition of cell growth induced by chromate was diminished as rise in a concentration of the thiol compounds, except DTT which not showed a similar tendency.

When the cells were incubated in medium containing a thiol compound alone at the range of concentration tested in Fig. 2 (25 to 100 μM), significant difference in rate of cell growth was not observed between control without thiol compound (data is not shown).

In the second experiment, the cell incubation medium was exchanged for fresh medium one day after incubation and the thiol compounds (100 μM) were added to the medium 1 hr before or after addition of chromate (5 μM), in which the cells were then incubated for 3 days. The growth-inhibitory

Fig. 2. Effects of several thiol compounds on the growth-inhibitory effect of chromate in HeLa cells. After 24 hr incubation, the medium was exchanged with a medium containing 2.5 to 10 μM chromate alone (□) or chromate and a thiol compound (25 μM; □, 50 μM; □, 100 μM; □), in which the cells were then incubated for 3 days. The number of viable cells was then counted using the trypan blue exclusion test after 3 days exposure to the compounds. Control cells were incubated in medium with neither chromate nor thiol compounds. Results from the cells incubated in medium with a thiol compound alone (25 to 100 μM) were not significantly different from control result. Each value represents the mean±S.E. of four replicate cultures for each exposure concentration.

*and **: Significantly different from chromate alone; each asterisk indicates P<0.05 and P<0.01, respectively.
Fig. 3. Effects of pre- or post-treatment of several thiol compounds on the growth-inhibitory effect of chromate in HeLa cells. After 24 hr incubation the medium was exchanged for a fresh medium and a thiol compound (100 μM) was added to the medium 1 hr before (□) or after (○) addition of 5 μM chromate (□; chromate alone), in which the cells were then incubated for 3 days. The number of viable cells was then counted using the trypan blue exclusion test after 3 days exposure to the compounds. Control cells were incubated in medium with neither chromate nor thiol compounds. Each value represents the mean±S.E. of four replicate cultures for each exposure.

*: Significantly different from chromate alone, P<0.05.

ratios (%) of the cells obtained at 3 additional days of incubation are shown in Fig. 3. A significant difference in the growth-inhibitory ratio induced by chromate in the cells was not observed between the cultures with both chromate and thiol compound, except with DTT which slightly prevented the growth-inhibitory effect of chromate when added to the medium prior to chromate.

Reduction of chromate by thiol compounds: The thiol compounds are effective in reducing chromate [6], so the chrome concentration of distilled water containing 10 μM chromate with or without thiol compounds (12.5–100 μM) was measured by the diphenylcarbazide method. As shown in Fig. 4, all of the thiol compounds tested produced a concentration-related reduction of chromate. With a solution containing both 10 μM chromate and 100 μM thiol compounds, chromate concentration of a solution decreased to 3% for LCysEE, 2% for LCysME, 72% for NAC, 43% for DMSA, 13% for DMPS, and 5% for DTT of that of a solution containing chromate alone, respectively.

Effects of thiol compounds on chromium uptake by cells: Experiments were performed on chromium uptake by the cells to explain the mechanism of the restorative effects of thiol compounds against chromate-induced cytotoxicity. The chromium content of the cells in serum-free medium with 10 μM chromate was about 0.36 μg Cr/mg cell protein at 6 hr of incubation. As shown in Fig. 5, the chromium content of the cells decreased by all of the thiol compounds, and the significant differences were observed for more than 25 μM of LCysEE, LCysME and DMPS, for more than 50 μM of NAC, DMSA and DTT.

In the other experiment, thiol compounds were added to the medium 1 hr before or after chromate (5 μM), and the chromium content of the cells was measured at 6 hr after addition of the chromate. As shown in Fig. 6, the chromium content of the cells decreased slightly by addition of LCysME, DMPS and DTT before and after chromate. However, no significant changes were induced by LCysEE, NAC or DMSA.

DISCUSSION

The effects of several thiol compounds on the cytotoxicity induced by chromate (potassium dichro-
Fig. 5. Effects of several thiol compounds on chromium uptake by HeLa cells from chromate-containing medium during a 6 hr incubation period. After 3 days of incubation, the medium was exchanged with a medium containing 10 μM chromate alone (□) or chromate and a thiol compound (25 μM; □, 50 μM; □, 100 μM; □), in which the cells were then incubated for 6 hr. The chromium content of the cells was estimated by atomic absorption spectroscopy as described in MATERIALS AND METHODS. The rate of chromium content was expressed as percentage of each control incubated with chromate alone. Each value represents the mean±S.E. of four replicate cultures for each exposure concentration. The chromium content of control in medium with 10 μM chromate alone was approximately 0.36 μgCr/mg cell protein at 6 hr incubation. * and **: Significantly different from chromate alone; each asterisk indicate P<0.05 and P<0.01, respectively.

Fig. 6. Effects of post- or post-treatment of several thiol compounds on chromium uptake by HeLa cells from chromate-containing medium during a 6 hour incubation period. After 3 days incubation, the medium was exchanged for a fresh medium and 100 μM thiol compound was added to the medium 1 hr before (□) or after (□) addition of 5 μM chromate (□; chromate alone), in which the cells were then incubated for 6 hr after addition of the chromate. The rate of chromium content was expressed as percentage of control incubated with chromate alone. Each value represents the mean±S.E. of four replicate cultures for each exposure. * and **: Significantly different from chromate alone; each asterisk indicates P<0.05 and P<0.01, respectively.
in chromium content of the cells (Fig. 5), while this effect of NAC was lower than that of LCysEE and LCysME. These results suggest that chromate-induced cytotoxicity is diminished as a result of the reduction of chromium uptake by the cells accompanying the reduction of chromate because of cell membrane impermeability to the trivalent chromium [11]. However, these L-cysteine derivatives tested did not restore the growth rate of the cells (Fig. 3), nor did they reduce their chromium content (Fig. 6) when the cells were treated with these compounds 1 hr before or after chromate. Toohey et al. [33] reported that added sulphydryl compounds, such as cysteine, thioethanolamine, DTT, oxidize rapidly in the MEM tissue culture system containing serum and cells. Thor et al. [32] reported that added cysteine, NAC, and methionine oxidize rapidly in the medium. In this study, L-cysteine derivatives tested as well as DMSA, DMPS, and DTT produced reduction of chromate in distilled water even 24 hr after adjustment, and the same rate of chromate reduction as result shown in Fig. 4 was observed (data is not shown). From these of view, it is considered that these L-cysteine derivatives oxidize rapidly in the medium, and chromate-induced cytotoxicity and chromium content of the cells is not diminished by these compounds added to the medium 1 hr before chromate. Furthermore, it might be suspected that chromate is taken up rapidly by the cells, and thus, L-cysteine derivatives added 1 hr after chromate could not inactivate intracellular chromium.

DMSA and DMPS are water-soluble chemical analogs of BAL. In contrast to BAL, they have less toxicity, greater water solubility and limited lipid solubility, and are effective antidotes for metal poisoning when given orally [2]. In this experiment, DMSA and DMPS were less toxic to HeLa cells than LCysEE or LCysME (Fig. 1), and BAL as reported previously [28].

DMSA has been shown to be fairly effective in therapy for cadmium [5], mercury [7, 21, 22], lead [13, 14], and arsenic intoxication [12, 19]. Tablock and Aposian [31] reported that oral administration of DMSA and DMPS are effective in the protection of mice against the lethal effects of sodium arsenite. In this experiment, DMSA and DMPS were effective agents, as well as LCysEE and LCysME, for diminishing the chromate-induced cytotoxicity (Fig. 2) and decreasing the chromium content of the cells (Fig. 5). They also exhibited chrome-reducing ability (Fig. 4). These results suggest that the hexavalent chromium in the medium was reduced to the trivalent form by the addition of DMSA or DMPS, as in the case of L-cysteine derivatives, and that chromium-induced cytotoxicity was diminished as a result of reduced chromium uptake by the cells. DMPS is not likely to penetrate into the cells, and consequently, distributes primarily in the extracellular space [9]. From this point of view, it is also assumed that DMSA and DMPS form trivalent chromium complexes by reductive chelation with potassium dichromate, and that these complexes can not penetrate into the cells. It has been demonstrated that DMPS enhances the urinary excretion of mercury, when given before or after intake of mercury [8, 16]. Aposian et al. [3] reported that administration of DMSA or DMPS immediately after and 90 min after NaAsO2 increased the LD50 of NaAsO2 in mice. In this experiment, addition of DMSA or DMPS to medium before or after chromate did not restore chromate-induced cytotoxicity (Fig. 3) or remarkably diminished the cellular chromium content (Fig. 6). Form these results, it is suspected that DMSA and DMPS as well as L-cysteine derivatives oxidize in the medium, and chromate-induced cytotoxicity and chromium content of the cells is not diminished by these compounds added to the medium 1 hr before chromate. It is also considered that DMSA and DMPS could not affect to the intracellular chromium because of DMPS does not cross the cell membrane [9].

The metal-chelating and sulfhydryl-reducing agent, DTT, reverses the renal toxicity of mercury without concomitant reduction of the renal mercury concentration [17, 18]. In the present study, DTT belonged to the less toxic group of the thiol compounds tested (Fig. 1), and showed a restorative effect on chromate-induced cytotoxicity at the highest concentration (100 μM) tested (Fig. 2). However, DTT as well as the other thiol compounds tested except NAC reduced the chromate (Fig. 4), and diminished the chromium content of the cell (Fig. 5). DTT also slightly restored chromate-induced cytotoxicity when cells were treated with it before addition of the chromate (Fig. 4). Since DTT can penetrate living cells [10], it might be suspected that DTT restores the sulfhydryl status of cellular enzymes and thereby prevents severe dysfunction in cells treated with chromate since treatment with chromate reduces the concentration of intracellular sulfhydryl compounds (gluthathione) [34]. DTT also
diminished the chromium content of cells when given before or after the addition of chromate (Fig. 6), while the rate of decrease in chromium content was lower than that observed in the cells simultaneously treated with chromate and DTT (Fig. 5). From the above mentioned point of view, this effect of DTT may occur either as a result of chromium chelation, and concomitant prevention of chromium access to cell membrane, or sulfhydryl group regeneration. It needs more works to explain the mechanism of preventive effect of DTT to chromate-induced cytotoxicity.

In conclusion, the thiol compounds tested, especially LCysEE, LCysME, DMSA, and DMPS are useful for treating chromate-induced cytotoxicity when given immediately after intake of metal, and a portion of this effect may be due to the reduction of chromium uptake by the cells accompanying chromate reduction.

REFERENCES


