Construction of HeLa cell lines overexpressing metallothioneins-1 and -2 and their cytoprotective effects against oxidative damage induced by hydrogen peroxide

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Abstract

It has been suggested that metallothioneins (MTs) play a role to protect cells from toxic effects of oxidative stress and heavy metals. On the other hand, the ability of Cu-containing MT to protect cells from oxidative damage is controversial. In the present study, we have constructed HeLa cell lines overexpressing metallothioneins-1E and -2A for the first time, and have examined cytoprotective effects of MT against oxidative damage induced by hydrogen peroxide (H_2O_2) in these cell lines pretreated with or without Cu. As a result, the HeLa cells overexpressing MT have suppressed oxidative damage induced by H_2O_2 at low concentrations of Cu, suggesting that Cu-containing MT has cytoprotective effects against oxidative damage induced by H_2O_2 if the amount of MT is sufficient to contain Cu in the protein and not to liberate Cu bound to it.

Key words: cell viability, copper, cytoprotective effect, HeLa cell, metallothionein, oxidative stress, overexpression

Introduction

Oxidative stress induced by reactive oxygen species can cause cytotoxic effects. It has been suggested that sulfhydryl-containing agents help to protect cells against oxidative damage. Metallothioneins (MTs) are low-molecular-weight sulfhydryl-rich metal-binding proteins [1]. There is increasing evidence that the proteins can play a role to protect cells from the toxic effects of oxidative stress and heavy metals. The ability of copper-containing MT, however, to protect cells from oxidative damage is controversial. Our previous study suggested that MT induced by copper (Cu) in HeLa cells facilitated oxidative stress induced by hydrogen peroxide (H_2O_2) [2]. On the other hand, Cu-MT was also reported to protect yeast from oxidative stress [3]. Insufficient information is available to clarify cytoprotective effects of Cu-containing MT against oxidative damage. In the present study, we have constructed HeLa cell lines overexpressing metallo-thioneins-1 and -2 for the first time, and have examined cytoprotective effects of MT against oxidative damage induced by H_2O_2 in these cell lines pretreated with Cu.

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Materials and methods

Cell culture

HeLa cells were maintained as a monolayer in Minimum essential medium alpha medium supplemented with 7% heat-inactivated fetal bovine serum, 50 U/ml penicillin G and 50 μg/ml streptomycin at 37°C under a humidified atmosphere of 5% CO_2.

RNA extraction

HeLa cells were plated at a density of approximate 1 x 10^6 cells per petri dish (8 cm in diameter) and incubated for 24 h at 37°C. In order to induce MT mRNA, cells were treated with 100 μM $ZnSO_4$ for 24 h at 37°C. Total RNA was extracted using an RNeasy Mini kit (QIAGEN, Germany).

Gene construction

Coding regions of the human MTs-1A, -1B, -1E, -1F, -1G, -1H, -1L, -1X, -2A and -3 were amplified from total RNA of HeLa cells using an RNA PCR Kit (AMV Ver2.1, TaKaRa, Japan). The PCR products were inserted in pcDNA3.1 plasmid (Invitrogen, USA) and transformation of competent *E. coli* JM109 (Promega, USA) was performed. The specificity of the insert was verified with DNA sequencing.

Transfection of HeLa cells

Approximate 3 x 10^5 HeLa cells were cultured into 6-well microplate and incubated for 24 h at 37°C. Transfection was carried out using 1 μg plasmid DNA and LipofectAMINE PLUS Reagent (Invitrogen, USA). Forty-eight hours after transfection, the cells were incubated with complete medium

containing 400 μg/ml of neomycin for 1 week. After this incubation, the medium including neomycin was replaced with complete medium not containing neomycin.

Immunocytochemical staining of MT

HeLa cells were fixed with 4% paraformaldehyde for 20 min at room temperature. The fixed cells were washed in PBS (phosphate buffered saline) and then permeabilized in PBS plus 0.2% Triton X-100 for 20 min at room temperature. After washing in PBS, the nonspecific binding was blocked with 2% BSA in PBS for 20 min at room temperature, followed by incubation with a commercial monoclonal antibody against MT (Clone E9, Dako Cytomation, USA) diluted with PBS containing 2% BSA for 1 h at room temperature. The cells on coverslip were incubated with Fluorescein-5-isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (ICN Biomedicals Inc., USA) for 1 h at room temperature. After washing with PBS for 5 min twice and washing with PBS for 20 min once, each coverslip was mounted on a slide glass with DAPI-mounting Slow Fade Antifade kit (Molecular Probes, USA) containing glycerol in PBS. Fluorescence microscopy was performed with a microscope (BX60, OLYMPUS OPTICAL Co., Ltd., Japan) equipped with filters for FITC and DAPI.

Evaluation of oxidative stress induced by H_2O_2

Cell viability was employed to evaluate cell damage caused by oxidative stress induced by H_2O_2. After treatment with 0, 150, 300 or 450 μM of $CuCl_2$ for 24 h, the medium including or not including the metal ions was recovered. Then the cells were washed with PBS, supplied with 1 ml of medium containing 0, 20 or 50 mM of H_2O_2, and incubated for 1 h. Viable cells after the treatments were counted by trypan blue (0.5% in PBS) exclusion. Cell viability was expressed as percentages against the total number of counted cells. Approximate 200-500 cells were subjected to observation. The chi-square test was used to determine treatment effects on cell viability.

Results and Discussion

Construction of HeLa cell lines overexpressing metallothioneins-1 and -2

We tried to amplify eight isoform genes of MT-1, MT-2A and MT-3. It was confirmed by DNA sequencing that two HeLa cell lines expressing the human MT-1E and MT-2A coding regions were obtained. In order to examine the expression levels of MT, an indirect immunofluorescence technique was employed. The fluorescence intensity of the cells overexpressing MT-2A was found to be markedly higher than that of wild-type cells, which showed very low intensity [2].

Cytoprotective effects of MT against oxidative damage induced by H_2O_2

Sensitivity of cells to H_2O_2-mediated cytotoxicity was quantified by measuring cell viability based on trypan blue exclusion. All the cell lines exposed to H_2O_2 showed dose-dependent reduction in cell viability (Fig. 1). Compared to wild-type cells, MT-overexpressing cells exhibited significantly higher cytoprotective effect against H_2O_2 toxicity at concentration of 20 mM and/or 50 mM H_2O_2. This result shows that MT overexpressed in HeLa cells has cytoprotective effects against oxidative damage induced by H_2O_2. Our previous study demonstrated that MT whose biosynthesis was induced by zinc (Zn) had cytoprotective

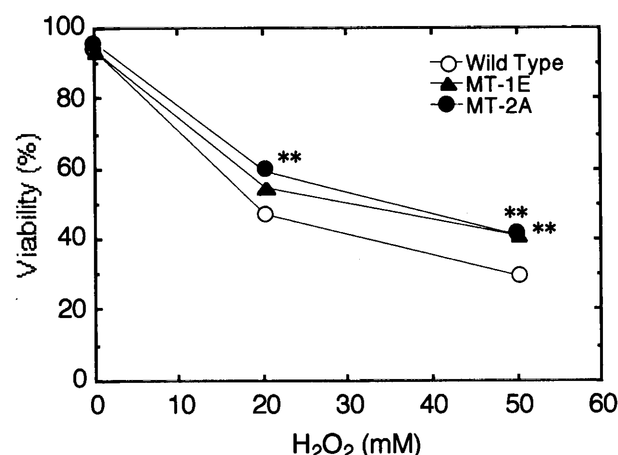

Fig. 1. Viability of HeLa cells exposed to H_2O_2 and level of significance for comparison of MT-overexpressing cells vs. wild-type cells using the chi-square test. Wild Type, wild-type cells; MT-1E, cells overexpressing MT-1E; MT-2A, cells overexpressing MT-2A. **, $p < 0.01$.

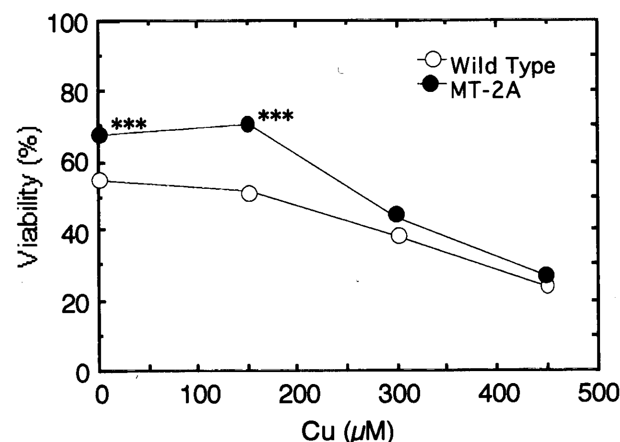

Fig. 2. Viability of HeLa cells pretreated with Cu and exposed to 20 mM H_2O_2, and level of significance for comparison of MT-2A isoform-overexpressing cells vs. wild-type cells using the chi-square test. Wild Type, wild-type cells; MT-2A, cells overexpressing MT-2A. ***, $p < 0.001$.

effects against oxidative damage by H_2O_2 in HeLa cells [2]. Girotti and co-workes [4] demonstrated that MT inhibited lipid peroxidation and speculated that the released Zn^{2+} participated in the antioxidant process. A recent study by Apostolova et al. [5] showed that subcellular localization of Zn was consistent with localization of MT. Several studies have shown that oxidation of MT by H_2O_2 and other reactive oxygen species can mediate Zn release from MT [6]. These studies imply that Zn-containing MT plays a crucial role to protect cells against oxidative damage.

Cytoprotective effects of MT against oxidative damage induced by H_2O_2 in cells pretreated with Cu

As shown in Fig. 2, both cell lines of wild type and overexpressing MT-2A showed dose-dependent reduction in cell viability at concentrations of 300 μM and 450 μM Cu pretreatment followed by exposure to 20 mM H_2O_2. The viability of cells overexpressing MT-2A was significantly higher than that of wild-type cells at concentrations of 0 and 150 μM Cu ($p < 0.001$ in both cases). This result shows that the cells overexpressing MT have significantly higher cytoprotective effects than those of wild-type cells at concentrations of 0 and 150 μM Cu in the presence of H_2O_2. Cu is an absolute prerequisite for aerobic metabolism, yet is highly cytotoxic and genotoxic. Cu can cause the formation and release of oxygen free radicals via Fenton-type chemistries. It is essential, therefore, to maintain Cu in a state that allows its physiological functions to be separated from its toxic activity. The present study has confirmed that Cu-potentiated oxidative stress can facilitate H_2O_2-mediated cell death. Moreover, the metal-binding protein, MT, has conferred significant protection against Cu toxicity in the cells overexpressing MT at lower concentrations of Cu, 150 μM or less. Thus it is suggested that the abundant existence of MT in the cells overexpressing MT would protect cells against Cu toxicity.

On the other hand, at concentrations of 300 μM and 450 μM Cu, there is no significant difference in cell viability between wild-type cells and MT-2A isoform-overexpressing cells (Fig. 2). Oxidant-induced release of Cu from MT could potentiate cell damage during oxidative stress [7-9]. Therefore it is considered that the facilitated cytotoxicity observed in the present study, namely decreases in cell viability and no significant difference in it between the two cell lines at concentrations of 300 μM and 450 μM Cu, would be due to excess of free Cu ions rather than Cu-containing MT itself under oxidative stress induced by H_2O_2 at the relatively high concentrations of Cu in the cells.

In conclusion, we have constructed HeLa cell lines overexpressing metallothioneins-1 and -2, and have examined cytoprotective effects of MT against oxidative damage induced by H_2O_2 in these cell lines pretreated with or without Cu. The results obtained in the present study strongly support the contention that Cu-containing MT has cytoprotective effects against oxidative damage induced by H_2O_2 if the amount of MT is sufficient to contain Cu in the protein and not to liberate Cu bound to it.

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