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Protective effect of metallothioneins against oxidative stress evaluated on wild type and MT-null cell lines by means of flow cytometry

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Abstract

It is generally accepted that metallothioneins (MTs) are devoted to the regulation of the metabolism of essential trace metals and to chelation of toxic metals. Nowadays, there is increasing evidence that MTs also act as free radical scavengers. We employed wild type mouse embryo fibroblast cell line, GKA1, and its MT-null variant, GKA2, in order to correlate the presence of MTs to the sensitivity of cells to reactive oxygen species (ROS), spontaneously generated by the aerobic cellular metabolism, or chemically induced by hydrogen peroxide. The absence of MTs in GKA2 cells was unambiguously correlated to higher sensitivity to ROS attack, as evaluated by detection and quantification of 8-oxo-2′-deoxyguanosine (8-oxo-G), the first product of oxidative attack to DNA, using Fluorescence-Activated Cell Sorter (FACS). When compared to MT-null cell line, the wild type cells (GKA1) were less sensitive to ROS attack. In GKA1 cells, MT biosynthesis is readily induced by Cd^{2+} treatment, and such an induction caused a further decrease in sensitivity to ROS injury. On the contrary, the MT-null cells (GKA2) expressed no detectable metallothioneins either constitutively, or after heavy metal pretreatment. Indeed, in GKA2 cell line, pretreatment with Cd^{2+} did not reduce but even enhanced the oxidative stress.

Introduction

Metallothioneins (MTs) are small (6–7 kDa), cysteine-rich (ca. 30%), cadmium-binding proteins, which participate in protective stress response (Shaw *et al.* 1991). Although a single essential function of MTs has not been demonstrated, MTs of eukaryotes have deputed to regulate essential metal levels, and their distribution within cells and organisms. These proteins also have protective role against toxic effects evoked by some metals.

In mice, among the four known MT genes, the MT-I and -II genes are most widely expressed. Transcription of these genes is rapidly and dramatically up-regulated in response to cadmium, organic chemicals, and various kinds of stress (Suzuki 1996; Abe 1997; Coyle *et al.* 2002).

The biological role of MTs has been extensively studied *in vivo* using transgenic MT-overexpressing and MT-knockout mice. Moreover, cells grown in culture provide an invaluable tool for examining *in vitro* the importance of MTs in the cellular responsiveness to stress (Andrews 2000; Klaassen & Liu 1998; Klaassen *et al.* 1999).

Oxygen derived species such as superoxide radical and H_2O_2 are produced in mammalian cells during normal aerobic metabolism (Fridovich 1986). These reactive oxygen species (ROS) are thought to promote chemical changes in DNA which are potentially mutagenic (Frenkel 1992; Breen & Murphy 1995). Modification of DNA bases is frequently observed in cells subjected to oxidative stress (Aruoma *et al.* 1989; Zastawny *et al.* 1995). Addition of H_2O_2 to cells in culture can lead to transition metal

ion-dependent $OH^{\bullet}$-mediated oxidative DNA damage (Halliwell *et al.* 2000).

Recent evidences support a role for MTs as radical scavengers *in vitro* (Lazo *et al.* 1995). Mechanisms underlying this function may include: i) direct interception of free radicals; ii) chelation of transition metal ions responsible for depletion of thiol-containing antioxidants and enzymes (Ercal *et al.* 2001) or for radical generation (through Fenton reaction, provided they are redox active such as iron and copper). MT overexpression after direct gene transfer in cultured cells decreases cytotoxicity caused by ROS; on the other hand, enhanced intracellular oxidation is observed in cells derived from mice lacking MT genes (Lazo *et al.* 1998).

In the current study, we have examined embryonic fibroblasts from wild type (GKA1) and MT-null (GKA2) mice, and characterized their sensitivity to ROS attack produced during normal cell metabolism or induced by H_2O_2 using classic cytotoxic tests and quantification of 8-oxo-G, the first product of oxidative attack to DNA (Steenken & Jovanovic 1997), by means of flow cytometry.

Materials and methods

Chemicals

All chemicals and solvents, unless noted otherwise, were purchased from Sigma Chemical Co.

Cell Culture

The simian virus 40 (SV-40) transformed mouse embryo fibroblasts from MT-I and MT-II double nullizygous (knockout) mice, named GKA2, and from normal mice of the same strain, named GKA1, were kindly provided by Prof. Dr. G.K. Andrews, University of Kansas Medical Center. Cells were maintained in Dulbecco's modified Eagle's medium (MEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and antibiotics (penicillin 100 IU/ml and streptomycin 100 IU/ml). Cells were grown at 37 °C in 5% CO_2 and passaged every 3 days.

Pretreatment with cadmium

In a recent study (Mahboobi *et al.* 2003) we reported a ca. 3-fold increase in MT content of GKA1 cells and no detectable variation in MT content of GKA2 cells following treatment with 0.4 μM $CdCl_2$ for 6 days. This treatment condition did not alter the viability of the cell lines. Therefore in the current study the cells were pretreated with $CdCl_2$ under the same conditions. Control cells were allowed to grow in complete medium containing no $CdCl_2$.

Cell viability

Cell sensitivity to H_2O_2 was determined using colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann 1983). Briefly, exponentially growing (control and $CdCl_2$-pretreated) cells were harvested by means of treatment with 0.1% trypsin/0.02% EDTA for 3 min and the resulting single cell suspension was plated in 6-well plates at 60 000–70 000 cells/well. Cell growth was continued at 37 °C and subconfluent cells were exposed to 40 μM H_2O_2 for 1 h in the dark. The medium containing H_2O_2 was then aspirated off and replaced with fresh medium. After 24 h recovery period at 37 °C, 100 μl of MTT stock solution (5 mg/ml) was added into each well and the cells were incubated at 37 °C for 1 h. The converted dye was solubilized with acidic propanol (0.1 N HCl in absolute propanol). The viability of cells was quantified by measuring the absorbance of converted dye at 570 nm with background subtraction at 630 nm. Data are expressed as percentage of the untreated controls, and values represent means $\pm$ SD of two independent experiments. T-test was performed to assess the statistical significance.

8-oxo-G for detecting DNA damage

Oxidative DNA damage was evaluated using the OxyDNA assay kit. Exponentially growing cells were exposed to 40 μM H_2O_2 for 1 h at 37 °C, in the dark. Following oxidant exposure, cells were washed with cold phosphate-buffered saline (PBS) and were detached from the culture dishes by means of trypsinization. The cells were then processed for the OxyDNA assay according to manufacturer's (Calbiochem, CA) instructions. Briefly, 2×10^6 cells were washed 2 times with PBS and were incubated with 2% paraformaldehyde for 15 min on ice. After 2 washing steps with PBS, the cell pellets were resuspended in ice-cold 70% ethanol. The fixed and ethanol-treated cells were washed twice with PBS, and once with the washing solution (Tris-buffered saline/TWEEN 20, containing Thimerosal). Non-specific binding sites of the resulting pellets were blocked by incubation with the blocking solution for 1 h at 37 °C. After 2 washing steps

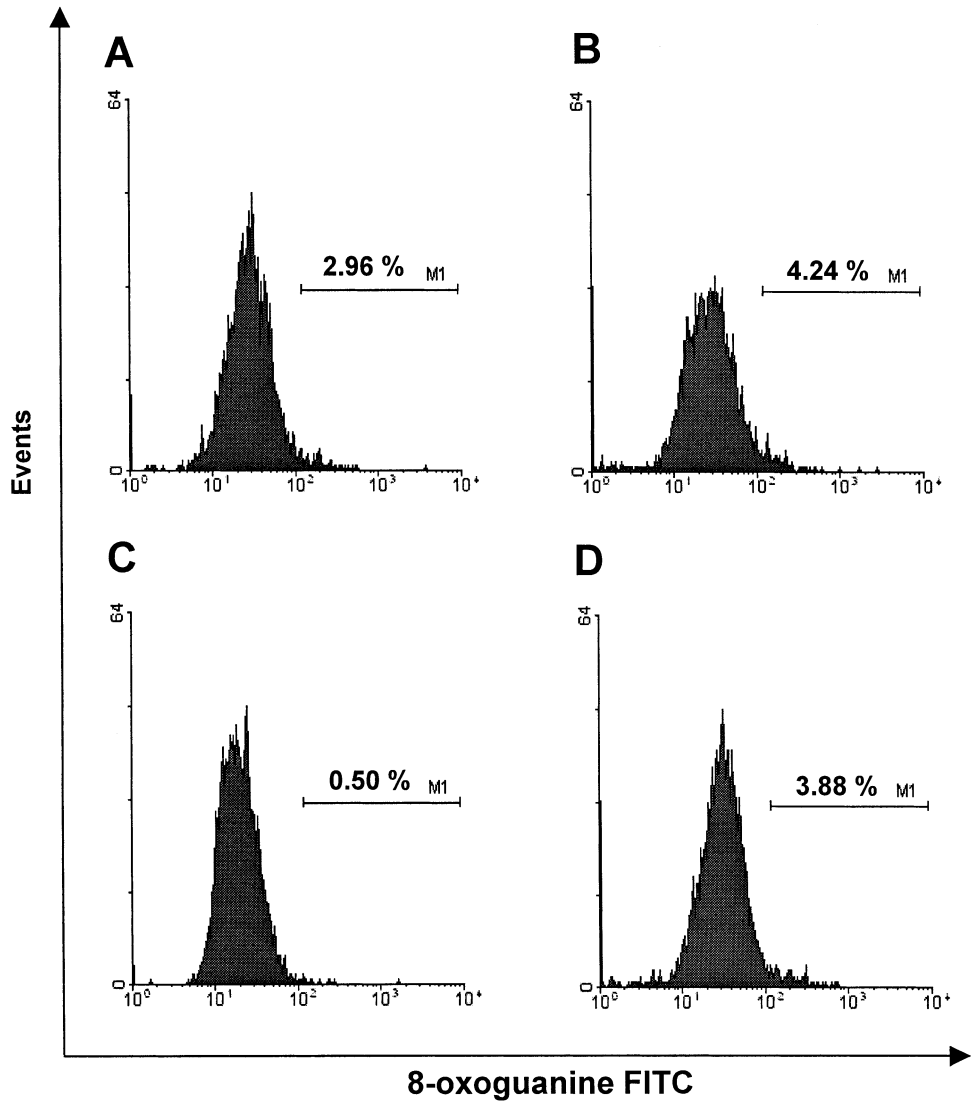

Fig 1. Evaluation of oxidative stress level in control and Cd-pretreated wild type mouse embryonic fibroblast cells (GKA1). The *x*- and *y*-axis of the histogram display the fluorescence (FITC) intensity and the number of collected cells (events) per sample, respectively. Panel A: untreated (control) cells. Panel B: cells treated with H_2O_2 (40 μM, 1 h). Panel C: $CdCl_2$-(0.4 μM, 6 days)-pretreated cells. Panel D: $CdCl_2$-(0.4 μM, 6 days)-pretreated cells challenged with H_2O_2 (40 μM, 1 h).

with the washing solution, the pellets were incubated with 100 μl of FITC-Conjugate (binding protein conjugated to Fluorescein, containing Thimerosal) for 1 h at room temperature in the dark. The samples were then washed twice with the washing solution and once with PBS. The pellets were finally resuspended in FACS fluid and read with Becton Dickinson FACScan (excitation 495 nm, emission 515 nm).

Results and discussion

Oxidative stress can be defined as the loss of balance between the production of reactive oxygen species (ROS) and the cellular antioxidant defense system. ROS are persistently produced in cells by the action of the mitochondrial electron transport system, but a complex and efficient antioxidant system blocks their damaging action. This system includes enzymes that inactivate ROS, i.e. superoxide dismutase for O_2^-, and catalase and glutathione peroxidase for H_2O_2. There

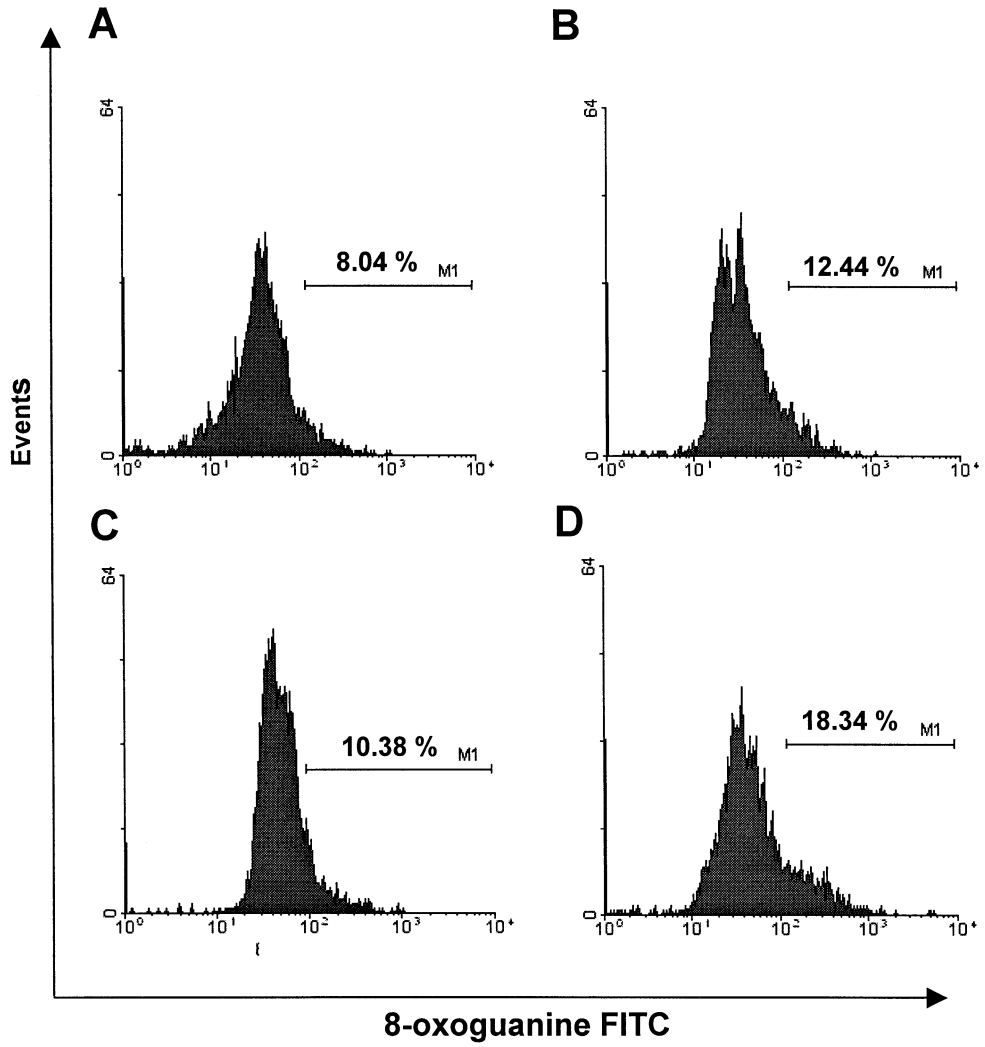

Fig 2. Evaluation of oxidative stress level in control and Cd-pretreated MT-null mouse embryonic fibroblast cells (GKA2). The *x*- and *y*-axis of the histogram display the fluorescence (FITC) intensity and the number of collected cells (events) per sample, respectively. Panel A: untreated (control) cells. Panel B: cells treated with H_2O_2 (40 μM, 1 h). Panel C: $CdCl_2$-(0.4 μM, 6 days)-pretreated cells. Panel D: $CdCl_2$-(0.4 μM, 6 days)-pretreated cells challenged with H_2O_2 (40 μM, 1 h).

is, however, no specific defense mechanism against $OH^{\bullet}$, which is the most dangerous ROS. Recently, the role of MTs as radical scavengers (especially towards $OH^{\bullet}$) has been highlighted (Sato & Bremner 1993, Viarengo *et al.* 2000).

The scope of this work is to further confirm the role of MTs as part of the antioxidant defense system. Since different cell types may have various physiological levels of MTs, the possibility of having the MT-null (knockout) cultured cell system, GKA2, and the corresponding wild type cultured cell system, GKA1, gives a better chance to evaluate the radical scavenging role of MTs. This comparison is more unambiguous than the previous ones (Schwarz *et al.* 1994) where MT-overexpressing cells, relative to wild type cells, were employed.

In a recent investigation, aimed to understand the resistance induced by MTs on antitumor drug action (Mahboobi *et al.* 2003), GKA1 and GKA2 cell lines showed significant difference in sensibility to radical injury caused by ferrocenium cations that are able to produce hydroxyl radicals in solution (Osella *et al.* 2000; Cassino *et al.* 2001; Tabbi *et al.* 2002). In GKA1 cells, this difference was further enhanced by $CdCl_2$ pretreatment that caused induction of MTs in this cell line (Mahboobi *et al.* 2003).

The protective effect of MTs against oxidative DNA damage generated during normal cellular metabolic activity was confirmed by the comparison of the results obtained from experiments in which 8-oxo-G was measured in control and $CdCl_2$-pretreated cells of both lines (Figures 1 and 2). These results indicate lower oxidative DNA damage in $CdCl_2$- pretreated GKA1 cells with respect to control cells of the same cell line. The comparison evidences that the major antioxidant action of MTs consists of direct interaction with ROS instead of sequestering cadmium ions that may cause oxidative stress. In fact, $CdCl_2$-pretreatment of MT-null cells (GKA2), in which no induction of MTs is possible, led to a moderated increase in oxidative DNA damage.

To augment the level of oxidative stress, Cd-pretreated and control cells of both wild and MT-null type were challenged with H_2O_2, the precursor of the most active ROS, i.e. the hydroxyl radical. As expected, the level of 8-oxo-G was higher in H_2O_2-treated cells with respect to untreated ones (Figures 1 and 2). Comparison of the results in control and $CdCl_2$-pretreated GKA1 cells indicates that induction of MTs with Cd^{2+} provided protection against H_2O_2-induced oxidative DNA damage. However, this protection was less pronounced than that in normal cellular metabolic conditions. In GKA2 cell line, pretreatment with Cd^{2+} did not afford any protection against oxidative DNA damage caused by H_2O_2, on the contrary it increases the overall level of 8-oxo-G.

To assess the cytotoxic potential of H_2O_2, GKA1 and GKA2 cells were exposed to H_2O_2 for 1 h, and cell viability was determined after a recovery period of 24 hours by MTT assay, which evaluates the reduction of the tetrazolium salt by mitochondrial dehydrogenase in metabolically active cells. GKA1 cells challenged with H_2O_2 showed 62% survival (against 100% survival in untreated control cells of the same cell line, $p < 0.001$) (Figure 3). The percentage of survival increased to 73% as a result of pretreatment of the cells with $CdCl_2$ ($p < 0.05$). On the other hand, H_2O_2-treated GKA2 cells showed a lower viability (40% survival). Cd-pretreatment of GKA2 cells slightly increased the cytotoxic effect of H_2O_2 in this cell line (35% survival). This test confirms the role of MTs (eventually highlighted by Cd^{2+} induction) in protecting the cells against the hydroxyl radical attack, as previously reported by Suzuki *et al.* (2000) and You *et al.* (2002).

In conclusion, since both cell lines were treated in the same way with cadmium ions, the most potent inducers of MT synthesis in this kind of cells (Kondo *et al.* 1999), any possible resistance mechanism(s) other than induction of MTs should have occurred to the same extent for both cell lines. Thus, the decrease in sensitivity observed for Cd-pretreated GKA1 cells with respect to GKA2 is, in principle, due solely to the increase in MT level.

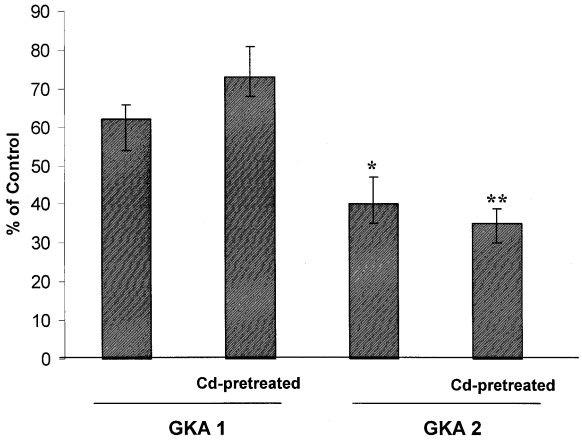

Fig 3. Viability of wild type (GKA1) and MT-null (GKA2) mouse embryonic fibroblasts challenged with H_2O_2. Cultures were treated with 40 μM H_2O_2 for 1 h. After 24 h of recovery in fresh growth medium, cell viability was measured by MTT assay. Data are expressed as percentage of the H_2O_2-untreated controls of each cell line and represent the mean value $\pm$ SD of two independent experiments. *vs. non pre-treated GKA1 $p < 0.05$, **vs. Cd-pretreated GKA1 $p < 0.001$.

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