Role of Metallothionein in the Cell Cycle: Protection against the Retardation of Cell Proliferation by Endogenous Reactive Oxygen Species

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The growth rate of metallothionein (MT)-null (MT–/–) cells was significantly lower than that of wild-type (MT+/+) cells, the cell cycle being arrested at the G_2/M phase in MT–/– cells and endogenous reactive oxygen species (ROS) accumulating with the progress of the cell cycle. Exogenous ROS generated on treatment with H_2O_2 arrested the cell cycle of MT–/– cells at the G_2/M phase more severely than that of MT+/+ cells. These observations suggest that MT maintains the normal cell cycle as an antioxidant against excessive endogenous ROS that are generated synchronously with the cell cycle and disturb the normal cell cycle.

Key words —— metallothionein, reactive oxygen species, cell cycle, cell viability, proliferation

INTRODUCTION

Reactive oxygen species (ROS) including superoxide anions ($O_2^{-\bullet}$), hydrogen peroxides (H_2O_2), and hydroxyl radicals (HO•) can be generated in several cellular events such as respiratory bursts of activated leukocytes and energy metabolism in the electronic transfer system.[1] Physiological amounts of ROS such as H_2O_2 can act as a possible second messenger in signal transduction by regulating cell proliferation,[2] differentiation,[3] transformation,[4] and apoptosis.[5] However, excessive amounts of ROS induce not only damage to cellular constituents such as DNA and proteins but also retardation and/or arrest of the cell cycle at various phases.[6] We recently observed that the amounts of intracellular ROS stained with dichlorodihydrofluorescein diacetate (H_2DCFDA) increased at specific phases (S and G_2/M), and that treatment with exogenous ROS such as H_2O_2 arrested the cell cycle at the G_2/M phase in a mouse fibroblast cell line, BALB 3T3 cells (unpublished observation).

Metallothionein (MT) is a low molecular weight, cysteine-rich protein.[7] Many biological roles based on its unique structure have been proposed, *i.e.*, detoxification of non-essential and excessive essential metals, homeostatic regulation of essential metals, scavenging of free radicals and ROS, and intracellular transport of metals.[8–11] In addition to these functions, a role of MT in cell proliferation was recently proposed.[12,13] Namely, MT is required for cell proliferation to supply essential metals, especially zinc (Zn) to Zn-requiring enzymes.[14] Actually, mice bearing null mutations in the MT-1 and MT-2 genes developed significantly less epidermal hyperplasia than wild-type mice on treatment with proliferative agents.[15] However, the other important role of MT, that is its protective role against ROS during cell proliferation, has not been evaluated despite that intracellular ROS are actively produced during cell proliferation.[16]

In the present study, we intended to find a role of MT and to explain our finding that the growth rate of MT-null (MT–/–) cells is lower than that of wild-type (MT+/+) cells. We examined the protective role of MT in the cell cycle against physiologically and exogenously generated ROS using immortalized fibroblasts established from MT-null and wild-type mice, and suggested the biological significance of MT in cell proliferation.

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MATERIALS AND METHODS

Chemicals —— H_2DCFDA, propidium iodide (PI), and RNase A were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., while H_2O_2 and cadmium chloride ($CdCl_2$) from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other reagents were from commercial sources, unless stated otherwise.

Cell Culture —— MT+/+ and MT–/– cells were established from embryonic fibroblasts of 129Sv MT wild type mice and MT-null mutant mice transformed with SV40 large T antigen, respectively, by Kondo *et al*.,[17] and were kindly donated by Professor S. Himeno (Tokushima Bunri University, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) of the high glucose type (4500 mg/l), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicilin and 100 μg/m.

Cytotoxicity of Cadmium —— MT+/+ and MT–/– cells were plated on 96-well plastic plates and then cultured for 48 hr. Then, the medium was changed to Cd-containing serum-free DMEM, at the concentrations being 0, 0.5, 1, 5, 10, 25, 50 and 100 μM, respectively. After exposure to Cd for 24 hr, cell viability was measured with a CellTiter 96® (Promega Co., Madison, WI, U.S.A.) according to the manufacturer's instructions.

Determination of Growth Rate —— MT+/+ and MT–/– cells were plated on 96-well plastic plates and then cultured in DMEM. Cell numbers were determined at 0, 19.5, 34.5, 49, 60, 74 and 90.5 hr after inoculation with the CellTiter 96®, and the growth rate was calculated from the absorbance at 490 nm because of the linear relation between the cell number and absorbance.

Measurement of ROS —— MT+/+ and MT–/– cells were cultured for 48 hr on plastic 6-cm diameter dishes, and then the cells were treated with 5 μM H_2DCFDA for 20 min. The amounts of intracellular ROS in 20000 cells were determined as the green fluorescence of dichlorofluorescein (DCF) with a flow cytometer (EPICS ELITE ESP; Beckman Coulter, Fullerton, CA, U.S.A.) using an argon laser with a 525-nm band pass filter.

Cell Cycle Analysis —— MT+/+ and MT–/– cells were cultured for 48 hr on plastic 6-cm diameter dishes, and then both types of cells were further cultured in the presence and absence of 0.05 mM H_2O_2 for 24 hr. Both types of cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested with 0.1% trypsin plus 125 μM EDTA in PBS (PET), and then the cells were resuspended in 1000 μl of ice-cold PBS. 2 μl of RNase (20 μg/ml) was added to the cell suspension, and then the suspension was incubated at room temperature for 30 min. Then, 25 μl of PI (25 μg/ml) was added to the cell suspension, and the suspension was stored in ice until use. The cell cycle in 20000 cells was analyzed as the intensity of the PI fluorescence with a flow cytometer using an argon laser with a 570 nm band pass filter.

Cell Viability Analysis —— The necrotic, apoptotic and viable cells among MT+/+ and MT–/– cells were detected using a Vybrant® Apoptosis Assay Kit (Promega) according to the manufacturer's instructions. MT+/+ and MT–/– cells were plated on 6-cm diameter plastic dishes and then cultured for 48 hr. Both types of cells were washed with PBS and harvested with PET, and then the cells were resuspended in 1000 μl of ice-cold PBS. PI (an indicator of necrotic cells) and YO-PRO®-1 (an indicator of apoptotic cells) fluorescence was detected with a flow cytometer using an argon laser with 525 nm (YO-PRO®-1) and 570 nm (PI) band pass filters, respectively.

RESULTS AND DISCUSSION

We observed for the first time that the growth rate of MT–/– cells was significantly lower than that of MT+/+ cells (Fig. 1), and assumed that the retardation was caused by ROS produced synchronously with the progress of the cell cycle. Indeed, the amount of intracellular ROS determined from the DCF fluorescence in MT–/– cells in the logarithmic growth phase was significantly higher than that in MT+/+ cells (Fig. 2). As the source of these endogenous ROS produced with cell proliferation, several biological events can be considered, such as the electron transfer in energy metabolism. However, further examination was not performed on the source of ROS in the present study.

As for the growth rate, Kondo *et al*. reported that there were no differences in the growth rate and doubling times between MT+/+ and MT–/– cells in their original report when they established these two cell lines, the doubling time being 18 hr for both cell lines.[17] However, our present results indicated that the doubling times of MT+/+ and MT–/– cells were 27.4 and 37.8 hr, respectively (Fig. 1), despite that both cell lines responded correctly as to sensitivity

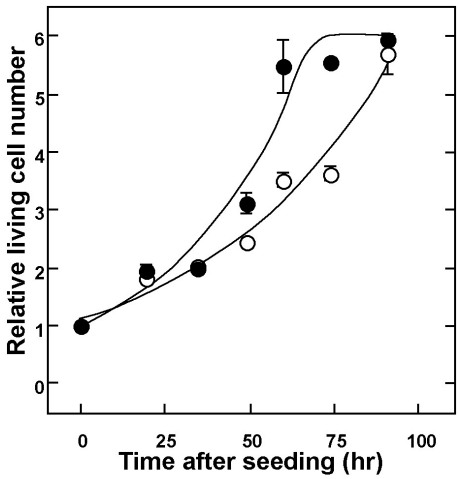

Fig. 1. Growth Curves of MT+/+ and MT–/– Cells

Both MT+/+ (closed circles) and MT–/– (open circles) cells were seeded on plastic 96-well plates at 6000 cells. The relative cell numbers after the inoculation were determined with a CellTiter 96®. Values are means ± S.D. for four different experiments.

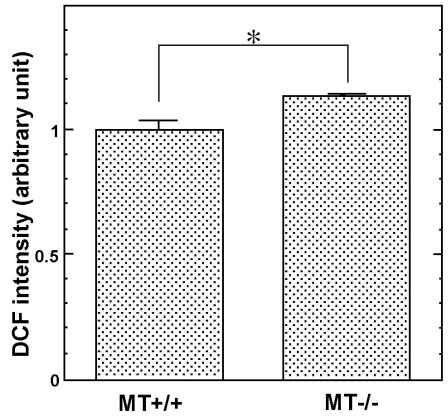

Fig. 2. Intracellular ROS in MT+/+ and MT–/– Cells

MT+/+ and MT–/– cells were cultured for 48 hr on plastic 6-cm diameter dishes, and then the cells were treated with 5 μM H_2DCFDA for 20 min. The amounts of intracellular ROS in 20000 cells were determined as the green fluorescence of DCF with a flow cytometer. Values are means ± S.D. for three different experiments. *significantly different between the wild-type and MT-null mutant at $p < 0.05$.

to Cd, a reliable and typical marker for distinguishing MT–/– and MT+/+ cells. The concentrations of Cd required for 50% lethality (IC_{50} values) for MT+/+ and MT–/– cells were 4.56 and 10.4 μM, respectively (Fig. 3). This discrepancy may be explained by that oxidative stress caused by endogenous ROS accumulate in cells during several passages.

The percentile distribution of cell numbers at the G_2/M phase in the cell cycle was significantly higher in MT–/– cells (20.5%) than in MT+/+ cells (15.5%)

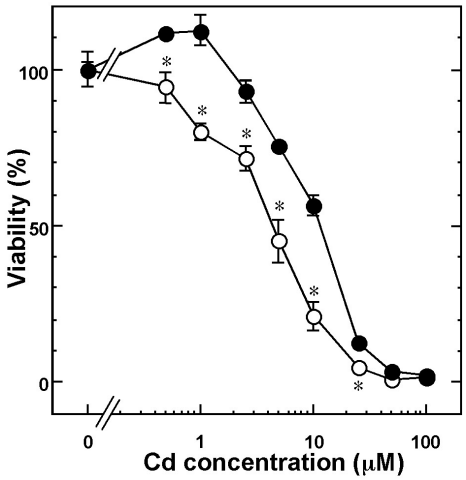

Fig. 3. Dose–Response Relation between the Cadmium Concentration in the Medium, and Cell Viability of MT+/+ and MT–/– Cells

MT+/+ (closed circles) and MT–/– (open circles) cells were plated on 96-well plastic plates and then cultured for 48 hr. Then, the medium was changed to serum-free DMEM containing Cd at the concentrations of 0, 0.5, 1, 5, 10, 25, 50 and 100 μM, respectively. After exposure to Cd for 24 hr, the cell number was determined with a CellTiter 96®. Values are means ± S.D. for three different experiments. *significantly different from the wild-type at $p < 0.05$.

(Fig. 4), indicating that the absence of MT arrests the cell cycle at the G_2/M phase. The percentile distribution at the G_2/M phase also increased significantly on treatment with H_2O_2 in both types of cells, suggesting that the cell cycle is arrested by excessive exogenous H_2O_2 at the G_2/M phase (Fig. 4). In accordance with the increase in cell number at the G_2/M phase, the percentile distribution of cells at the G_1 phase decreased for both MT+/+ and MT–/– cells on treatment with H_2O_2, while the distribution at the S phase was not changed in either type of cells (Fig. 4).

To determine the difference in the cell viability between MT+/+ and MT–/– cells at the logarithmic growth phase of the normal cell cycle, the numbers of apoptotic, necrotic and viable cells were determined by flow cytometry. There were no differences in cell viability (distributions among apoptotic, necrotic and viable cells) between MT+/+ and MT–/– cells (Fig. 5), suggesting that MT–/– cells did not exhibit induction of apoptosis and necrosis but merely a delayed normal cell cycle.

There are no physiological and biological differences in the phenotypes, such as litter size, growth rate and life span, between MT-null and MT-wild type mice at the whole body level.[18] However, MT-null mice are more sensitive to various stresses such

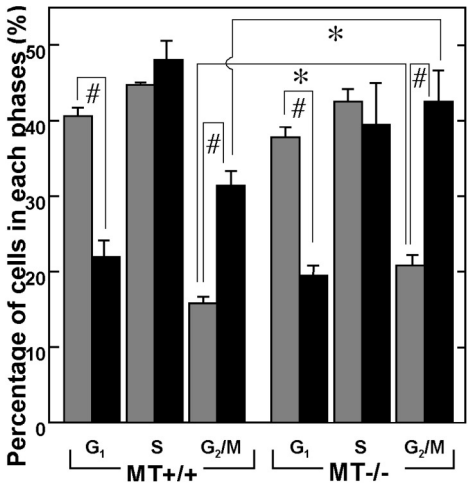

Fig. 4. Cell Cycle analysis of MT+/+ and MT–/– Cells Treated with or without H_2O_2

MT+/+ and MT–/– cells were cultured for 48 hr on plastic 6-cm diameter dishes, and then further cultured in the presence (closed columns) and absence (dotted columns) of H_2O_2 at 0.05 mM for 24 hr. The distributions of cells at each phase were determined for 20000 cells as the intensity of the PI fluorescence with a flow cytometer. Values are means ± S.D. for three different experiments. *significantly different between the wild-type and MT-null mutant at $p < 0.05$. #significantly different between the cells with and without H_2O_2 treatment at $p < 0.05$.cadmium concentration in the medium, and cell viability of MT+/+ and MT–/–cells.

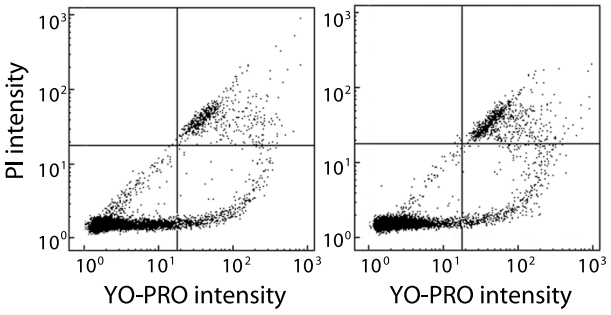

Fig. 5. Cell Viability analysis of MT+/+ and MT–/– Cells

MT+/+ and MT–/– cells were cultured for 48 hr on 6-cm diameter dishes. The necrotic, apoptotic and viable cells among MT+/+ and MT–/– cells were determined as the intensity of the PI fluorescence (an indicator of necrotic cells) and YO-PRO®-1 fluorescence (an indicator of apoptotic cells) with a flow cytometer.

as heavy metals, ROS and some kinds of anti-tumor drugs than MT-wild type mice.[19–21)]

It is not clear how the present observations for MT as to cell proliferation reflect the phenotypes at the whole animal level. However, we speculate as follows. First, the cell lines used in the present study exhibited high growth rates, due to the transformation with SV 40 large T antigen, to somatic cells at the whole animal level. Endogenous ROS can be produced through energy metabolism. Therefore, cultured cell lines respond more sensitively to endogenous ROS than somatic cells. According to this scenario, it may be expected that the effect of MT on cell proliferation is more obvious in the rapidly growing somatic cells in a whole animal as observed in the present cultured cells.

Second, the rapidly growing cells in a whole animal may contain other nuclear antioxidants in addition to MT. Indeed, it has been reported that male germ cells contain cysteine-rich and MT-homologous protein, tesmin.[22)] Tesmin is also localized in the nuclei on meiosis during spermatogenesis or with various stresses,[23)] suggesting that it acts as a nuclear antioxidant against endogenous ROS and compensates for the role of MT even in MT–/– cells.

To summarize our observations, endogenous ROS accumulate more in MT–/– cells with the progress of the cell cycle than in MT+/+ cells owing to the absence of an antioxidant, MT, and the MT–/– cells are arrested at the G_2/M phase, resulting in slower proliferation than in the case of MT+/+ cells. Exogenous ROS also retard proliferation more obviously in MT–/– cells than in MT+/+ cells. Based on these observations, it is suggested that MT plays a role as a specific antioxidant against endogenous ROS generated in the cell cycle, especially in the nuclei.

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REFERENCES

1) Kamata, H. and Hirata, H. (1999) Redox regulation of cellular signalling. *Cell. Signal.*, **11**, 1–14.
2) Pani, G., Colavitti, R., Bedogni, B., Anzevino, R., Borrello, S. and Galeotti, T. (2000) A redox signaling mechanism for density-dependent inhibition of cell growth. *J. Biol. Chem.*, **275**, 38891–38899.
3) Suzukawa, K., Miura, K., Mitsushita, J., Resau, J., Hirose, K., Crystal, R. and Kamata, T. (2000) Nerve growth factor-induced neuronal differentiation requires generation of Rac1-regulated reactive oxygen species. *J. Biol. Chem.*, **275**, 13175–13178.
4) Arnold, R. S., Shi, J., Murad, E., Whalen, A. M., Sun, C. Q., Polavarapu, R., Parthasarathy, S., Petros, J. A. and Lambeth, J. D. (2001) Hydrogen peroxide mediates the cell growth and transformation caused

by the mitogenic oxidase Nox1. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 5550–5555.
5) Gotoh, Y. and Cooper, J. A. (1998) Reactive oxygen species- and dimerization-induced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor-alpha signal transduction. *J. Biol. Chem.*, **273**, 17477–17482.
6) Zhang, Z., Leonard, S. S., Huang, C., Vallyathan, V., Castranova, V. and Shi, X. (2003) Role of reactive oxygen species and MAPKs in vanadate-induced G2/M phase arrest. *Free Radic. Biol. Med.*, **34**, 1333–1342.
7) Kägi, J. H. R. (1991) Overview of metallothionein. *Methods Enzymol.*, **205**, 613–626.
8) Yoshida, M., Satoh, M., Shimada, A., Yasutake, A., Sumi, Y. and Tohyama, C. (1999) Pulmonary toxicity caused by acute exposure to mercury vapor is enhanced in metallothionein-null mice. *Life Sci.*, **64**, 1861–1867.
9) Suhy, D. A., Simon, K. D., Linzer, D. I. and O'Halloran, T. V. (1999) Metallothionein is part of a zinc-scavenging mechanism for cell survival under conditions of extreme zinc deprivation. *J. Biol. Chem.*, **274**, 9183–9192.
10) Cai, L., Satoh, M., Tohyama, C. and Cherian, M. G. (1999) Metallothionein in radiation exposure: its induction and protective role. *Toxicology*, **132**, 85–98.
11) Suzuki, K. T., Someya, A., Komada, Y. and Ogra, Y. (2002) Roles of metallothionein in copper homeostasis: responses to Cu-deficient diets in mice. *J. Inorg. Biochem.*, **88**, 173–182.
12) Takeda, A., Hisada, H., Okada, S., Mata, J. E., Ebadi, M. and Iversen, P. L. (1997) Tumor cell growth is inhibited by suppressing metallothionein-I synthesis. *Cancer Lett.*, **116**, 145–149.
13) Arora, V., Iversen, P. L. and Ebadi, M. (1998) Manipulation of metallothionein expression in the regenerating rat liver using antisense oligonucleotides. *Biochem. Biophys. Res. Commun.*, **246**, 711–718.
14) Jacob, C., Maret, W. and Vallee, B. L. (1998) Control of zinc transfer between thionein, metallothionein, and zinc proteins. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 3489–3494.
15) Hanada, K., Sawamura, D., Hashimoto, I., Kida, K. and Naganuma, A. (1998) Epidermal proliferation of the skin in metallothionein-null mice. *J. Invest. Dermatol.*, **110**, 259–262.
16) Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K. and Finkel, T. (1995) Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science*, **270**, 296–299.
17) Kondo, Y., Yanagiya, T., Himeno, S., Yamabe, Y., Schwartz, D., Akimoto, M., Lazo, J. S. and Imura, N. (1999) Simian virus 40-transformed metallothionein null cells showed increased sensitivity to cadmium but not to zinc, copper, mercury or nickel. *Life Sci.*, **64**, 145–150.
18) Michalska, A. E. and Choo, K. H. (1993) Targeting and germ-line transmission of a null mutation at the metallothionein I and II loci in mouse. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 8088–8092.
19) Liu, J., Liu, Y., Habeebu, S. S. and Klaassen, C. D. (1999) Metallothionein-null mice are highly susceptible to the hematotoxic and immunotoxic effects of chronic $CdCl_2$ exposure. *Toxicol. Appl. Pharmacol.*, **159**, 98–108.
20) Zheng, H., Liu, J., Liu, Y. and Klaassen, C. D. (1996) Hepatocytes from metallothionein-I and II knockout mice are sensitive to cadmium- and tert-butylhydroperoxide-induced cytotoxicity. *Toxicol. Lett.*, **87**, 139–145.
21) Kondo, Y., Woo, E. S., Michalska, A. E., Choo, K. H. and Lazo, J. S. (1995) Metallothionein null cells have increased sensitivity to anticancer drugs. *Cancer Res.*, **55**, 2021–2023.
22) Sugihara, T., Wadhwa, R., Kaul, S. C. and Mitsui, Y. (1999) A novel testis-specific metallothionein-like protein, tesmin, is an early marker of male germ cell differentiation. *Genomics*, **57**, 130–136.
23) Matsuura, T., Kawasaki, Y., Miwa, K., Sutou, S., Ohinata, Y., Yoshida, F. and Mitsui, Y. (2002) Germ cell-specific nucleocytoplasmic shuttling protein, tesmin, responsive to heavy metal stress in mouse testes. *J. Inorg. Biochem.*, **88**, 183–191.