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 G2/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 H2O2 arrested the cell cycle of MT−/− cells at the G2/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 (O2−•), hydrogen peroxides (H2O2), 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 H2O2 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 (H2DCFDA) increased at specific phases (S and G2/M), and that treatment with exogenous ROS such as H2O2 arrested the cell cycle at the G2/M phase in a mouse fibroblast cell line, BALB 3T3 cells (unpublished observation).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.
MATERIALS AND METHODS

Chemicals —— H$_2$DCFDA, propidium iodide (PI), and RNase A were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., while H$_2$O$_2$ 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/ml of streptomycin.

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® (Promega Co., Madison, WI, U.S.A.) according to the manufacturer’s instructions.

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$_2$DCFDA 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$_2$O$_2$ 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.

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...
to Cd, a reliable and typical marker for distinguishing MT−/− and MT+/+ cells. The concentrations of Cd required for 50% lethality (IC50 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 G2/M phase in the cell cycle was significantly higher in MT−/− cells (20.5%) than in MT+/+ cells (15.5%) (Fig. 4), indicating that the absence of MT arrests the cell cycle at the G2/M phase. The percentile distribution at the G2/M phase also increased significantly on treatment with H2O2 in both types of cells, suggesting that the cell cycle is arrested by excessive exogenous H2O2 at the G2/M phase (Fig. 4). In accordance with the increase in cell number at the G2/M phase, the percentile distribution of cells at the G1 phase decreased for both MT+/+ and MT−/− cells on treatment with H2O2, 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. However, MT-null mice are more sensitive to various stresses such
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 G2/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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Fig. 4. Cell Cycle analysis of MT+/+ and MT–/– Cells Treated with or without H2O2

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 H2O2 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 H2O2 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.

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