Regulatory role of metallothionein in NF-κB activation

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Abstract Metallothionein (MT), a low molecular weight, cysteine-rich metal binding protein, has been associated with cytoprotection from heavy metals and cellular oxidants. As MT has the ability to scavenge hydroxyl radicals, MT may control intracellular redox status. In the present study, we examined whether MT regulates the activity of nuclear factor-κB (NF-κB), which is one of the redox-regulated transcription factors, using the MT null embryonic cell lines established from MT null mice. We first found that tumor necrosis factor (TNF)-induced activation of the binding of NF-κB protein to DNA in wild type MT+/+ cells was lower than that in MT−/− cells. The NF-κB activation in MT-expressing cells established from MT−/− cells by the transfection of mouse MT-I gene was also significantly lower than that in MT−/− cells. In addition, transfection of the MT gene inhibited TNF-induced IκB degradation and suppressed NF-κB-dependent gene expression induced by TNF. These results demonstrate that MT may function as a negative regulator of NF-κB activity.

Key words: Metallothionein; Nuclear factor-κB; Redox status; Antioxidant

1. Introduction

Nuclear factor-κB (NF-κB) is a transcription factor that regulates a number of cellular genes, such as those encoding inflammatory cytokines, adhesion molecules, cyclooxygenase and nitric oxide synthase [1,2]. In most cell types, NF-κB is present as a heterodimer comprising p50 and p65 subunits that is anchored in the cytoplasm by tightly bound inhibitory protein IκB. Many of the signals known to activate NF-κB such as tumor necrosis factor (TNF), interleukin-1 and lipopolysaccharide result in phosphorylation and subsequent degradation of IκB, allowing NF-κB to translocate into the nucleus and activate these target genes. Recent studies indicated that NF-κB activity was regulated by intracellular redox status and reactive oxygen species (ROS) might be involved in this NF-κB activation cascade [1–3]. This is based on the observations that treatment of some cells with H_2O_2 can activate NF-κB and that certain antioxidants such as *N*-acetylcysteine or pyrrolidine dithiocarbamate can repress activation of NF-κB by blocking the signal-induced phosphorylation of IκB [4]. It was also reported that NF-κB activation was prevented by overexpression of several antioxidant enzymes including glutathione peroxidase [5], manganese superoxide dismutase [6,7] and thioredoxin peroxidase [8].

Metallothioneins (MT) are ubiquitous, low molecular weight, cysteine-rich metal binding proteins [9,10]. Of the four isoforms of MT, MT-I and MT-II are widely expressed and coregulated by many agents including metals, hormones, cytokines and alkylating agents. MT has multiple functions such as detoxification of heavy metals including cadmium or mercury and protection of cells against ROS [10,11]. MT has been observed in the nucleus and/or cytoplasm of cells [12], but little is known about its function in the nucleus. As MT has the ability to scavenge hydroxyl radicals [10], MT may control intracellular redox status and regulate the activity of NF-κB and other redox-regulated transcription factors.

Recently, transgenic mice in which the expression of MT-I and MT-II was disrupted by means of gene targeting were developed by two groups [13,14]. These mice provided a useful tool for the investigation of the roles of MT. We very recently developed a simian virus 40 (SV40)-transformed cell line from MT null mice [15]. In the present study, we examined whether MT regulates the NF-κB activity or not using the MT null embryonic cell lines.

2. Materials and methods

2.1. Cell lines

SV40-transformed embryonic cells from MT null and control mice (MT−/− and MT+/+ cells) were prepared as described previously [15]. Cells were normally maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). MT−/− cells were transfected with pcDNA3.1/Zeo plasmids (Invitrogen, Carlsbad, CA, USA) by themselves or plasmids containing sense mouse MT-I (mMT-I) cDNA [16] using the LipofectAMINE method according to the manufacturer's instructions (Life Technologies, Inc., Rockville, MD, USA). After 48 h, cells were treated with 100 ng/ml of zeocin. Zeocin-resistant clones were selected and maintained in complete medium supplemented with 100 ng/ml of zeocin.

2.2. Determination of MT

MT levels in the MT null cells and MT-expressing cells were determined by the Hg binding assay as described previously [17] with some modifications. Briefly, 2×10^5–1×10^6 cells were harvested with 1.15% KCl and sonicated. The cell lysate (1.2 ml) was incubated with 10 μl of diethylmaleate at room temperature for 15 min. After the addition of 50 μl of 10 mM cadmium chloride, the lysate was heated at 100°C for 5 min and centrifuged at $1400\times g$ for 15 min at 4°C to remove particles and high molecular weight proteins precipitated. The supernatant was incubated with 1 nmol $HgCl_2$ to saturate the metal binding sites of MT by Hg. Excess Hg was removed by the addition of ovalbumin (30 pmol) followed by acidification with 50 μl of 100% trichloroacetic acid solution and subsequent centrifugation at

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Abbreviations: NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; ROS, reactive oxygen species; MT, metallothionein; SV40, simian virus 40; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; mMT-I, mouse MT-I

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1800×*g* for 25 min at 15°C. The supernatant was collected and the amount of Hg was measured by cold vapor flameless atomic absorption spectroscopy and expressed as pmol Hg bound to MT.

2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from TNF- or H_2O_2-treated cells as described previously [18] and incubated with ^{32}P-labeled 22-mer double-stranded NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) for 30 min at room temperature. Native 4% polyacrylamide gels were used to analyze the samples. A double-stranded mutated oligonucleotide (5′-AGTTGAGatcactggga-CAGGC-3′) was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. Antibodies against p65 and p50 for supershift analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The dried gels were visualized and radioactive bands were quantitated by a Fujix Bio-Imaging analyzer BAS 2000 (Fuji Photo Film Co., Tokyo, Japan).

2.4. Transient transfection and luciferase assay

Mock-transfected Zeo cells and mMT-I-transfected cells were transiently transfected with κB site-driven luciferase reporter (3×κB-Luc) by the LipofectAMINE method. 3×κB-Luc was a kind gift from Dr. Shigeki Miyamoto and contains the luciferase gene under the control of herpes simplex thymidine kinase promoter with three NF-κB binding sites. Six hours after transfection, the cells were cultured for 24 h at 37°C, stimulated with TNF for 6 h in 0.5% FCS-containing DMEM and examined for luciferase activity as described previously [19].

2.5. Western blot analysis

Total cell extracts were prepared from TNF-treated cells and loaded on 12% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to polyvinylidene difluoride membranes, probed with rabbit polyclonal antibodies against IκB (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and detected by chemiluminescence (ECL, Amersham, Buckinghamshire, UK).

3. Results and discussion

3.1. Expression of MT inhibits TNF-induced NF-κB activation

We first examined binding activity of nuclear NF-κB to DNA in MT null cells and wild type cells (MT−/− and MT+/+ cells, respectively) by EMSA. Most studies on the function of MT had utilized MT inducers such as Zn compounds to increase the level of MT, but other effects of the inducing compounds than MT induction could not be eliminated by this approach. In fact, many kinds of MT inducers including Zn affect gene transcription and translation [20] and it had been difficult to examine the effects of MT itself on gene expression by using MT inducers. In the present study, using the MT null cells, we could examine the effect of MT on NF-κB activation without using MT inducers.

When MT−/− and MT+/+ cells were stimulated with different concentrations of TNF, NF-κB binding activities in nuclear extracts prepared from both cells increased in a dose-dependent manner. Although the basal level of DNA binding activity in MT−/− cells was lower than that in MT+/+ cells, the binding activity in MT−/− cells treated with TNF was higher than that in MT+/+ cells (Fig. 1A). Specificity of binding was confirmed by the observation that this binding was competed with unlabeled wild type probe but not with the probe having a mutated NF-κB binding site (Fig. 1B). Since antibodies against the p50 and p65 subunits of NF-κB supershifted the band on the gel shift (Fig. 1C), NF-κB activated by TNF consisted of p50 and p65 subunits.

We next tried to establish MT-expressing cells from MT−/− cells by the transfection with the plasmid containing mMT-I cDNA. After the selection with zeocin, we established several clones and measured MT levels in these clones by the Hg binding method as described in Section 2. The MT levels of parental MT−/− cells determined by this method might be due to low molecular weight Hg binding proteins other than MT. Among the Zeo-resistant clones, M1 and M4 cells ex-

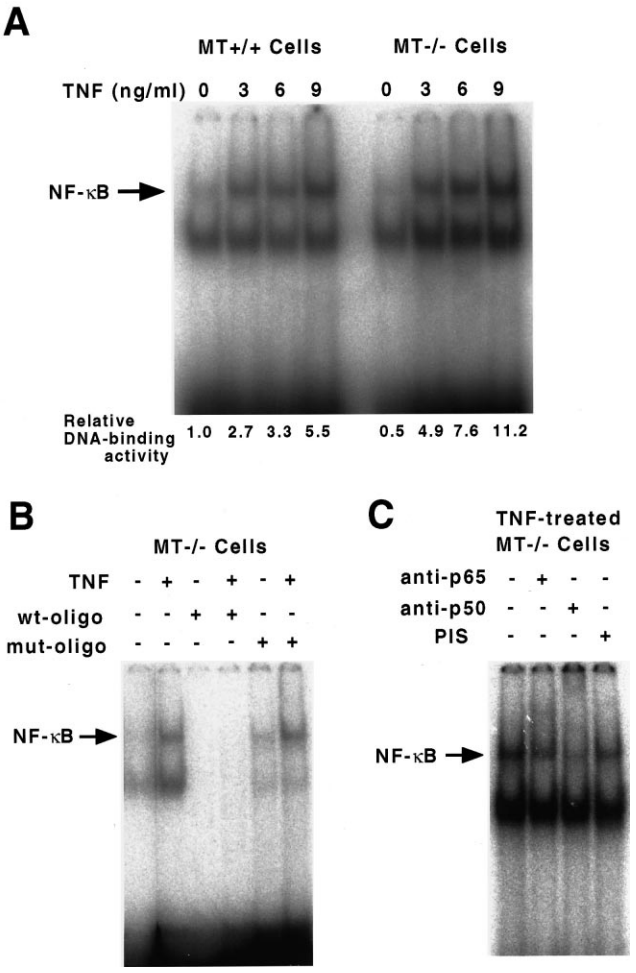

Fig. 1. Effect of expression of MT gene on TNF-dependent activation of NF-κB. A: MT+/+ cells and MT−/− cells were incubated at 37°C with different concentrations of TNF for 30 min, and then nuclear extracts were prepared and assayed for NF-κB binding activities by EMSA. Relative DNA binding activity was calculated as the ratio of the radioactivity of the NF-κB site binding band to that in non-treated MT+/+ cells. B and C: Nuclear extracts were prepared from untreated or TNF-treated MT−/− cells, incubated for 30 min with cold wild type (wt-oligo) or mutant (mut-oligo) NF-κB oligo probes (B) and rabbit preimmune serum (PIS) or anti-p65 or p50 antibodies (C), and then assayed for NF-κB binding activities.

Table 1
MT levels in MT+/+, MT−/− and MT−/−-derived cell lines

	MT level (pmol Hg/mg protein)
MT+/+ cells	22.1 ± 3.5
MT−/− cells	7.0 ± 0.7
Mock-transfected Zeo cells	7.7 ± 2.7
mMT-I-transfected M1 cells	11.9 ± 3.1
mMT-I-transfected M4 cells	12.3 ± 4.0

MT levels were determined by the modified Hg binding assay as described in Section 2.

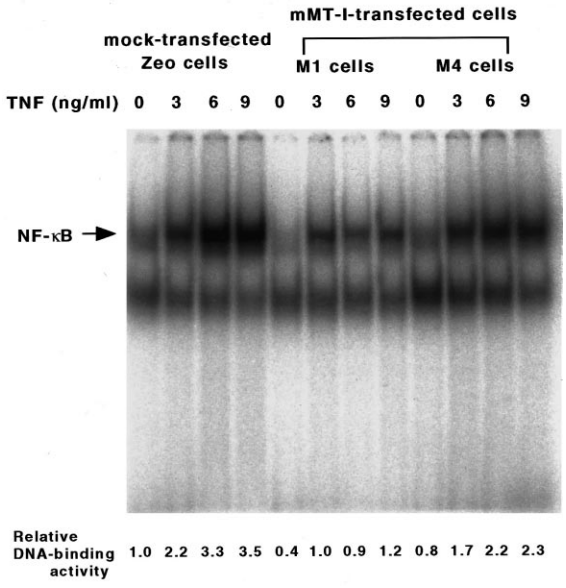

Fig. 2. Effect of transfection with mMT-I expression vector on TNF-dependent activation of NF-κB. Mock-transfected Zeo cells and mMT-I-transfected M1 and M4 cells were incubated at 37°C with different concentrations of TNF for 30 min and then nuclear extracts were prepared and assayed for NF-κB binding activities by EMSA. Relative DNA binding activity was calculated as the ratio of the radioactivity of the NF-κB site binding band to that in non-treated Zeo cells.

pressed significantly higher levels of MT than parental MT−/− cells (Table 1). On the other hand, the MT levels of mock-transfected Zeo cells were almost equivalent to that of the parental cells.

Mock-transfected Zeo cells and mMT-I-transfected M1 and M4 cells were stimulated with TNF and the DNA binding activities were examined by EMSA. As shown in Fig. 2, TNF activated NF-κB in Zeo cells as well as non-transfected MT−/− cells, whereas in both M1 and M4 cells, NF-κB activation was significantly suppressed. The binding activity of NF-κB stimulated by H_2O_2 was also suppressed in MT-transfected cells (data not shown). These results thus indicated that expression of MT inhibited NF-κB activation, suggesting that MT might function as a negative regulator of NF-κB.

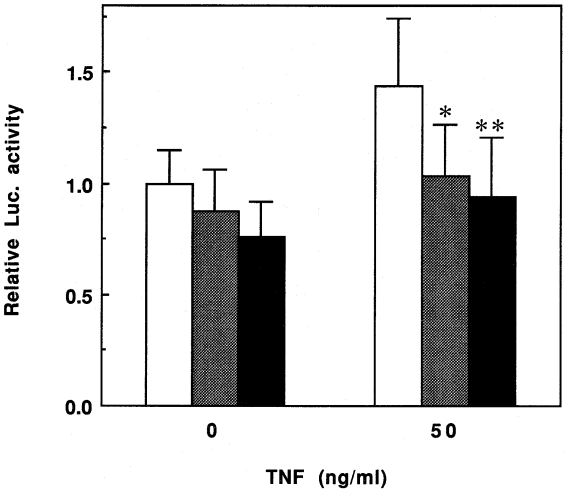

Fig. 3. Effect of transfection with mMT-I expression vector on NF-κB-dependent luciferase reporter gene expression. Mock-transfected Zeo cells (open column) and mMT-I-transfected M1 (shaded column) and M4 cells (closed column) were transiently transfected with 3×κB-Luc reporter gene, treated with 50 ng/ml of TNF for 6 h, and then assayed for luciferase activities. $*P < 0.05$ and $**P < 0.01$ vs. Zeo cells by *t*-test.

3.2. Expression of MT suppresses NF-κB-dependent reporter gene expression induced by TNF

We next examined whether expression of MT suppresses NF-κB-dependent gene expression or not. NF-κB-dependent luciferase reporter gene was transiently transfected into Zeo cells and mMT-I-transfected M1 and M4 cells, and then the cells were stimulated with TNF. We first treated the cells with 10 ng/ml of TNF for 30 min, 2 h and 6 h, but TNF-induced increment of luciferase activity was not observed in these MT null mice-derived SV40-transformed embryonic cells. Thus, we stimulated the reporter gene-transfected cells with 50 ng/ml of TNF for 6 h. As shown in Fig. 3, 50 ng/ml of TNF activated luciferase activity by about 1.5-fold in Zeo cells. On the other hand, no increase in luciferase activity over the basal level was observed in both M1 and M4 cells. These results suggested that the expression of MT suppressed TNF-induced NF-κB-dependent gene expression. Very recently, Abdel-Mageed et al. reported that transfection with human MT-II cDNA into MCF-7 cells caused transactivation of NF-κB [21]. However, we could not observe differences of luciferase activities between Zeo cells and mMT-I-transfected cells without the treatment of TNF.

3.3. MT inhibits TNF-dependent degradation of IκB

Association with the inhibitory IκB proteins tightly regulates the activity of NF-κB. Activation of NF-κB is triggered by signal-dependent phosphorylation and subsequent degradation of IκB through the activation of IκB kinase [1–3]. The observations that both treatment of antioxidants and overexpression of antioxidant enzymes [5,6] inhibited IκB degradation indicated the involvement of ROS in the signal-induced IκB kinase activation [2]. To determine whether MT also inhibits TNF-induced IκB degradation as an antioxidant, the levels of cellular IκB proteins after treatment of cells with TNF for different time periods were examined by Western blot analysis. IκB was degraded after 20–30 min of treatment with TNF, and then retranscribed as a result of NF-κB acti-

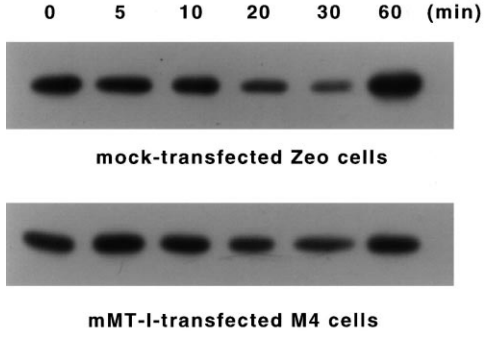

Fig. 4. Effect of transfection with mMT-I expression vector on TNF-induced degradation of IκBα. Mock-transfected Zeo cells or mMT-I-transfected M4 cells were incubated for different time periods with TNF (10 ng/ml) and then assayed for IκBα in cell lysates by Western blot analysis.

vation and reappeared at 60 min in Zeo cells (Fig. 4). In mMT-I-transfected M4 cells, however, a lesser extent of IκB degradation was observed in response to TNF. These results suggested that MT, as well as other antioxidants, might regulate IκB degradation through its ability to scavenge ROS in the cytoplasm of cells.

MT has been observed in both the nucleus and cytoplasm [12], but little is known about its function in the nucleus. Although we have shown that MT suppresses TNF-induced NF-κB activation by the inhibition of IκB degradation in the cytoplasm, the possibility still remains that MT may regulate NF-κB activity in the nucleus. It was recently shown that MT directly interacted with NF-κB by EMSA supershift analysis using anti-MT antibody [21], although we could not observe an interaction between NF-κB and MT when we used commercial anti-MT antibody (data not shown). MT has an ability to bind Zn and regulate Zn homeostasis in the cells [9] and Zn is an essential component of NF-κB for its DNA binding activity [22]. It was also reported that apoMT could remove Zn from Zn finger containing factors Sp1 and TFIIIA and modulate their activities to bind to DNA and RNA [23,24].

In summary, using MT null cells, we found that MT may function as a negative regulator of NF-κB activity. It was suggested that MT might suppress NF-κB-dependent gene expression through the inhibition of IκB degradation. However, further study is needed to clarify the precise mechanism by which MT regulates NF-κB activity.

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