Metallothionein modulates lipopolysaccharide-stimulated tumour necrosis factor expression in mouse peritoneal macrophages

Masako KANEKIYO, Norio ITOH¹, Atsuko KAWASAKI, Akiko MATSUYAMA, Kimihiro MATSUDA, Tsuyoshi NAKANISHI and Keiichi TANAKA

Department of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, Japan

Metallothionein (MT) is a low-molecular-mass, cysteine-rich metal binding protein thought to be involved in the detoxification of heavy metals and scavenging of free radicals. MT is directly induced not only by heavy metals, but also by hormones and cytokines. The present study, which uses mice with genetic deletions of the MT proteins (MT^{-/-} mice), was designed to evaluate the effects of MT on the expression of pro-inflammatory cytokines in macrophages. We found that the production of tumour necrosis factor (TNF) induced by lipopolysaccharide (LPS) in peritoneal macrophages is up-regulated by MT via the modulation of nuclear factor κ B (NF- κ B) activity. This conclusion is supported by the following observations: (1) LPS stimulated the secretion of less TNF activity from MT^{-/-}

peritoneal exudate macrophages (PEMs) than from wild-type controls (MT^{+/+} mice) without a difference in the pattern of kinetics; (2) LPS-stimulated expression of TNF- α mRNA was decreased in MT^{-/-} PEMs; (3) LPS-stimulated activation of NF- κ B was decreased in MT^{-/-} PEMs; and (4) production of TNF in PEMs of MT^{-/-} mice after LPS treatment *in vivo* was decreased (compared with MT^{+/+} PEMs). Expression of other inflammatory cytokines, interleukin (IL)-1 α and IL-6 mRNA, which were modulated by NF- κ B, were also down-regulated in MT^{-/-} PEMs. Thus MT plays a key role in the LPS-induced activation of PEMs via the modulation of NF- κ B activity.

Key words: inflammatory cytokines, NF- κ B, zinc.

INTRODUCTION

Metallothionein (MT) is a low-molecular-mass, metal-binding protein that is cysteine-rich [1]. The primary functions of MT are concerned with the homeostasis of essential metals [2], the detoxification of heavy metals [3] and, possibly, the scavenging of free radicals in view of its thiol groups [4,5]. Metals associated with MT can be donated to other metalloproteins that require metal ions for their function [6,7]. These direct interactions suggest that MT regulates the activity of metalloproteins, including transcription factors such as Sp-1, TFIIIA, and members of the oestrogen receptor superfamily [8]. MT interacts with another transcription factor, nuclear factor κ B (NF- κ B) [9], and is directly induced not only by heavy metals [10], but also by hormones [11] and cytokines [12,13]. The membrane glycolipid lipopolysaccharide (LPS) also induces hepatic MT synthesis indirectly *in vivo*, but not *in vitro*. We have reported previously [14] that this induction is mediated by interleukin (IL)-6 and glucocorticoid. In addition, the inflammatory cytokines, such as IL-1 and tumour necrosis factor (TNF)- α , enhance MT gene activity [12,13,15,16]. On the other hand, LPS induces MT in a monocyte-derived cell line *in vitro* [17]. These reports suggest that MT functions not only in detoxification of heavy metals and free radicals, but also in immune responses. Macrophages play a key role in the immune response to bacterial infection. During the infective process, circulating blood monocytes migrate from the vasculature into the extravascular compartment under the influence of many factors, and they differentiate into macrophages in the tissues. Infection with Gram-negative bacteria is a leading cause of macrophage activation. This group of pathogens contain

LPS, which is known to function as an endotoxin. Activated macrophages are a major source of pro-inflammatory mediators during inflammation, infection or injury. LPS is considered to be the most potent activator of the macrophage secretory response, leading to the production of TNF- α . TNF- α is one of the major pro-inflammatory mediators secreted by macrophages when stimulated with LPS *in vivo* and *in vitro* [18,19]. TNF- α production in monocytes and macrophages constitutes between 1 and 2 % of the total secreted proteins from these cells in response to LPS [20]. Purified TNF- α induces many of the deleterious effects of LPS *in vivo* [21]; passive immunization against TNF- α protects animals from the lethal effects of LPS [22]. The intracellular events that mediate LPS-induced TNF- α secretion have been the subject of intense research. Induction of the response is via a receptor-mediated signalling pathway, and involves the action of several transcription factors. NF- κ B was found to have an important role in the regulation of TNF- α gene transcription [23,24].

The functions of LPS-activated macrophages are regulated by cellular redox states [25,26]. MT has many thiol groups, and all of them are bound to metals. Because of these characteristics, MT can scavenge hydroxyl radicals and donate zinc to transcription factors. We and others [9,27,28] have reported that MT modulates NF- κ B activity. Therefore we postulated that MT controls both the intracellular redox status and the zinc concentration of macrophages, and regulates these functions in immune responses. The present studies have been aimed towards whether or not MT is involved in the induction of TNF- α , and activation of NF- κ B, by LPS using peritoneal macrophages from mice with genetic deletions of the MT proteins [29].

Abbreviations used: AP-1, activator protein 1; EMSA, electrophoretic mobility shift assays; IL, interleukin; LPS, lipopolysaccharide; MT, metallothionein; NF- κ B, nuclear factor κ B; PGN, peptidoglycan; PEMs, peritoneal exudate macrophages; TNF, tumour necrosis factor.

¹ To whom correspondence should be addressed (e-mail n-itoth@phs.osaka-u.ac.jp).

MATERIALS AND METHODS

Mice

MT^{-/-} mice (129S7/Sv-Mt1^{tm1Br1}Mt2^{tm1Br1}) were obtained from The Jackson Laboratory (Bar Harbor, ME, U.S.A.) and C57BL/6CrSlc mice were obtained from Japan SLC (Hamamathu, Japan). The MT^{-/-} mice were originally developed on a genetic background of the 129 mouse strain; at our laboratory, these mice were back-crossed with C57BL/6CrSlc mice for one generation, and then homozygous male mice were used for experiments (at the age of 8–10 weeks). The mice were provided with commercial rodent chow and water *ad lib.* under conditions of controlled temperature (23–24 °C) and a 12 h light/12 h dark cycle, and were kept free from common murine pathogens.

Preparation of macrophages and cell culture

Peritoneal exudate macrophages (PEMs) were obtained by lavage 3 days after an intraperitoneal injection of 2 ml of 3% (w/v) thioglycollate medium. After plating (2 h), non-adherent cells were removed by washing. The cells were incubated in RPMI 1640 containing 10% (v/v) heat-inactivated fetal bovine serum, 50 µM 2-mercaptoethanol, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were plated at a density of 1×10^5 cells per well in 96-well tissue-culture plates to determine TNF production, and at a density of 1×10^7 cells per dish in 100 mm² tissue-culture dishes for the preparation of RNA and nuclear extracts. PEMs were treated with LPS and peptidoglycan (PGN), as described in the Results section and the Figure legends. Stock LPS (from *Escherichia coli* 026:B6; Difco Laboratories, Detroit, MI, U.S.A.) and PGN (from *Staphylococcus aureus*; Fulka, Buchs, Switzerland) were prepared at a concentration of 10 mg/ml in sterile PBS. L929 cells were cultured with Dulbecco's modified Eagle's medium supplemented with 5% (v/v) FBS. All cells were cultured at 37 °C in an air/CO₂ mixture (19:1).

TNF assay

TNF activities in supernatants were determined by the L929 cell cytotoxicity assay, as reported by Wang and co-workers [30]. Recombinant mTNF-α (Peprotech, Inc, Canton, MA, U.S.A.) was used as a standard.

Northern blotting

Total RNAs (10 µg) were resolved by electrophoresis in a 1.0% agarose/10% formaldehyde gel, then RNAs were transferred on to nylon membranes (Amersham, Little Chalfont, Bucks., U.K.) in 20 × SSC buffer. The cDNA probes were labelled with [α -³²P] dCTP (3 mCi/mmol; NEN, Boston, MA, U.S.A.) using the Klenow fragment (Toyobo, Shiga, Japan) and an appropriate primer. Membranes were hybridized with ³²P-labelled cDNA probes.

Preparation of nuclear extracts

Nuclear extracts were prepared as described by Dignam and co-workers [31]. The preparation included proteinase inhibitors (0.5 mM PMSF, 2 µg/ml leupeptin and 1 µg/ml pepstatin A).

Electrophoretic mobility shift assays (EMSAs)

To test NF-κB binding to DNA, 6.25 µl of nuclear extracts containing 20 µg of protein were mixed with 6.25 µl of 20 mM Tris/HCl, pH 7.5, 10% (v/v) glycerol, 2 mM dithiothreitol and 20 mM EDTA, with 2 µg poly(dI-dC) and 10 fmol of radio-

labelled κB probe. The nucleotide sequences of the κB probe (wild-type and mutant) were as follows: wild-type upper strand, 5'-agttgaggggactttcccaggc-3'; and mutant-type upper strand, 5'-agttgagggcagctttcccaggc-3' (where the mutated nucleotide is shown in bold).

Binding reactions were allowed to proceed for 30 min at room temperature. Reaction mixtures were then separated by electrophoresis on non-denaturing polyacrylamide gels at 4 °C. Thereafter, the gel was fixed with 10% methanol/10% acetic acid (v/v) before autoradiography.

Experiments *in vivo*

Functions of PEMs *in vivo* were determined as described by Wollenberg and co-workers [32]. After an intraperitoneal injection of thioglycollate medium (3 days), each mouse was injected intraperitoneally with either 5 µg of LPS/0.5 ml of RPMI 1640 medium or vehicle. Then, after 1 h, 1 ml of RPMI 1640 medium was added to the peritoneal cavity, mixed with the resident ascites fluid to ensure complete removal of ascites fluid, and aspirated. After centrifugation at 4 °C to remove cells, the ascites fluid was mixed 1:1 with RPMI 1640 medium, before filter-sterilization.

Statistical analysis

Results are expressed as means ± S.D. Data were analysed by ANOVA and Fisher's protected least-squares difference test. Significance was assessed at the $P < 0.05$ level.

RESULTS

PEMs from MT^{-/-} mice secrete less of TNF than MT^{+/+} mice by LPS stimulation

Macrophages stimulated by LPS secrete large quantities of TNF [18,19], and this process is influenced by the redox state of cells [25,26]. PEMs obtained from MT^{+/+} or MT^{-/-} mice were used to investigate whether or not these proteins are involved in TNF production. We measured TNF activities in culture media collected 0 to 12 h after LPS stimulation (Figure 1; concentrations of LPS used were 100 ng/ml and 1 µg/ml for panels 1A and 1B respectively). Maximal TNF levels in the MT^{+/+} PEM culture media were equivalent to 73.7 ± 4.7 units/ml and 325.2 ± 116.2 units/ml 6 h after LPS stimulation (for additions of LPS of 100 ng/ml and 1 µg/ml respectively). The kinetics of TNF production from MT^{+/+} PEMs compared with MT^{-/-} PEMs did not differ, but the TNF levels in the MT^{-/-} PEM culture media were significantly lower at all times (maximal levels of 25.4 ± 8.6 units/ml and 35.4 ± 2.7 units/ml respectively). The down-regulation of TNF production from MT^{-/-} PEMs was investigated by incubation for 6 h with the concentrations of LPS shown in Figure 1(C), and was more pronounced at a higher concentration of LPS (Figure 1C). To determine whether the down-regulation of TNF production from MT^{-/-} PEMs was specific for LPS or not, we examined TNF production from PEMs by PGN, another stimulator of NF-κB. After stimulation with 1 µg/ml PGN (6 h), the level of TNF in the MT^{+/+} PEM culture media was 166.5 ± 15.3 units/ml; that in the MT^{-/-} PEM culture media was 62.4 ± 8.7 units/ml.

Lower levels of TNF secretion by MT^{-/-} PEMs results from down-regulation of TNF-α mRNA synthesis

To determine whether or not the down-regulation of TNF production from MT^{-/-} PEMs results from the down-regulation

Figure 1 Comparison of the LPS-stimulated production of bioactive TNF- α by PEMs from MT^{+/+} and MT^{-/-} mice

PEMs, from MT^{+/+} (●) and MT^{-/-} (○) mice (cells were pooled from five mice), were incubated in cultures prepared in triplicate in 200 μ l of complete medium with 100 ng/ml (A) or 1 μ g/ml (B) LPS for indicated periods, or with the concentrations of LPS indicated for 6 h (C). Cell-free supernatants were harvested and assayed using the L929 cell cytotoxicity assay. Data are representative from one of three independent experiments; data from triplicate measurements are expressed as means $\pm$ SD. * P < 0.0001 compared with respective control value; † P < 0.001; ‡ P < 0.0001 compared with MT^{-/-} PEMs incubated under the same conditions.

Figure 2 Comparison of TNF- α mRNA expression by PEMs from MT^{+/+} and MT^{-/-} mice

Kinetics of TNF- α mRNA and 18 S rRNA expression are shown as a composite autoradiogram. Northern blots are of RNA harvested from freshly isolated PEMs incubated with 100 ng/ml (A) or 1 μ g/ml (B) LPS for indicated periods. (C and D) Densitometric analysis of bands on Northern blots, as in (A) and (B), was performed by radio-image analysis, normalizing each band value over 18 S rRNA hybridization.

of mRNA synthesis, TNF- α mRNA levels were compared in PEMs harvested 0–6 h after LPS exposure. Northern blots revealed that the stimulation of MT^{+/+} PEMs with different doses (100 ng/ml and 1 μ g/ml) of LPS induced the production of high levels of TNF- α mRNA (Figure 2). In contrast, lower levels of TNF- α mRNA production were induced in MT^{-/-} PEMs, although the kinetics were the same as those for MT^{+/+} PEMs. The down-regulation of mRNA was more pronounced

at higher LPS concentrations (1 μ g/ml), a finding that correlates well with the results of the TNF bioassay.

MT regulates DNA binding activities of NF- κ B

We and others [9,27] have reported that MT up-regulates the activation of NF- κ B, an essential process that precedes LPS-induced TNF- α transcription and translation. To determine

Figure 3 Analysis of NF- κ B binding activity induced in PEMs from MT^{+/+} and MT^{-/-} mice

(A) Nuclear extracts from fresh PEMs that were incubated with 1 μ g/ml LPS for 0 to 20 min were assayed for DNA binding activity of NF- κ B by EMSA. (B) Competitive assays were performed in the presence of a molar excess ($\times 100$) of unlabelled κ B probes (wild- and mutant-type) added to nuclear extracts from MT^{+/+} PEMs that were incubated with 1 μ g/ml LPS for 20 min. The arrowhead indicates specific NF- κ B bands.

Figure 4 Comparison of the LPS-stimulated production of bioactive TNF by PEMs from MT^{+/+} and MT^{-/-} mice *in vivo*

Three days after intraperitoneal injection of thioglycollate medium, mice were injected intraperitoneally with 5 μ g of either LPS or vehicle, before TNF activity in ascites fluid was assayed (see the Materials and methods section). Representative data are from one of two independent experiments; data from three mice are expressed as means $\pm$ S.D. * $P < 0.05$ compared with MT^{-/-} PEMs incubated under the same conditions.

whether or not the down-regulation of TNF- α expression in MT^{-/-} PEMs is mediated by decreased NF- κ B activation, we determined the DNA-binding activities of this transcription factor by EMSA, with nuclear extracts of PEMs harvested 0 and 20 min after exposure to LPS (1 μ g/ml). NF- κ B was substantially activated in MT^{+/+} PEMs, but activity was only slightly increased in MT^{-/-} PEMs (Figure 3A). Binding specificity was confirmed by the competitive effect exerted by the unlabelled wild-type, without any competition afforded by the mutant probe (Figure 3B), and by the supershift elicited by an antibody against p65, a subunit of NF- κ B (results not shown). No difference was shown in the DNA-binding activities of activator protein 1 (AP-1) by EMSA, with nuclear extracts of MT^{+/+} and MT^{-/-} PEMs

Figure 5 Comparison of IL-1 α and IL-6 mRNA expression in PEMs from MT^{+/+} and MT^{-/-} mice

The kinetics of IL-1 α and IL-6 mRNA and 18 S rRNA expression are shown as a composite autoradiogram. The Northern blot shows RNA harvested from isolated PEMs that were incubated with 100 ng/ml LPS for the periods indicated. Densitometric analysis: bands were normalized against band value over 18 S rRNA hybridization. Relative density (% of maximum) is described under each band.

harvested 0 and 60 min after exposure to LPS (1 μ g/ml) (results not shown).

Thioglycollate-primed MT^{-/-} mice secrete less TNF into the peritoneal cavity upon LPS stimulation

To determine whether the production of TNF in PEMs of MT^{-/-} mice given LPS *in vivo* is decreased similarly to that found *in vitro*, TNF activity was quantified in cell-free ascites fluid harvested 1 h after thioglycollate-primed (PEMs-induced) mice

were injected intraperitoneally with LPS (5 µg/mouse). Under these conditions, TNF levels peaked after 1 h [32]. As found *in vitro*, PEMs of MT^{-/-} mice secreted less TNF than those of MT^{+/+} mice after LPS stimulation *in vivo* (Figure 4).

PEMs from MT^{-/-} mice express low levels of IL-1α and IL-6 mRNA upon LPS stimulation

Like TNF-α, IL-1α and IL-6 are expressed at high levels in PEMs upon LPS stimulation, via NF-κB activation [33]. To determine the effect of MT, we compared the levels of these cytokine mRNAs in PEMs harvested 0–6 h after LPS (100 ng/ml) stimulation. Northern hybridization revealed that LPS induced high levels of IL-1α and IL-6 mRNA synthesis in MT^{+/+} PEMs (Figure 5). In contrast, lower levels of these mRNAs were induced in MT^{-/-} PEMs than in MT^{+/+} PEMs, although the kinetics of induction followed an identical pattern.

DISCUSSION

The present study describes novel functions of MT in PEMs. Several independent observations at different stages of TNF induction support the supposition that MT is involved in the induction of TNF-α via the modulation of LPS-induced NF-κB activation.

Several reports have described previously the functions of endogenous MT in immunological activation. Using an antisense MT mRNA expression vector in human monocyte cell-line THP-1 cells, Leibbrandt and co-workers [34] found that diminished MT levels affect broad-ranging processes associated with LPS-activated monocyte functions, such as H₂O₂ production and cell adherence. On the other hand, Crowthers and co-workers [35] reported that the humoral response to challenge with ovalbumin and the proliferation of splenocytes induced by LPS and concanavalin A are increased in MT^{-/-} mice. That report states that NF-κB activity is up-regulated in stimulated splenocytes in MT^{-/-} mice. However, whether or not the level of NF-κB activity per cell or per a number of cells is up-regulated, and the question of in which cells the NF-κB activity was up-regulated, were not resolved, since whole splenocytes were used in their studies. These reports suggest that MT is involved in immunological activation. However, there has been to date no report about the function of MT in terms of protein and mRNA expression during the immune response. The present study is the first to reveal the involvement of MT in cytokine expression at the protein and mRNA level.

When macrophages are exposed to LPS, LPS binds to receptors on the surface, activating several signal transducers. The macrophages then express pro-inflammatory mediators, such as TNF-α. The LPS signalling cascade leading to TNF-α production bifurcates to control both transcription of the TNF-α gene and post-transcription of TNF-α mRNA [36]. Post-transcriptional regulation of TNF-α mRNA is mediated by a short AU-rich sequence, which is important for the destabilization of TNF-α mRNA. This sequence is conserved among various cytokines and oncogenes, and is located within the 3'-untranslated regions of such mRNAs [37]. On the other hand, transcriptional regulation of the TNF-α gene is quite complex and differs among species and cell types. Regulation of TNF-α gene transcription is conferred by transcription-factor-binding sites presented within the TNF-α promoter, including the Y box, the SP-1 site and the κB site, which are conserved among species [38], as well as the AP-1 site in the human TNF-α promoter [39]. LPS-induced TNF-α promoter activity is dependent on the NF-κB binding site. LPS stimulates the nuclear localization of NF-κB in macro-

phages [23]. The activity of NF-κB is regulated by an inhibitor, IκB, which forms a complex with NF-κB in the cytoplasm and inhibits the nuclear localization [40]. When cells receive signals that activate NF-κB, IκBα is phosphorylated and degraded through a ubiquitin-proteasome pathway [41].

Several reports have indicated that the redox balance can substantially influence macrophage responses to LPS both *in vitro* and *in vivo* [25,26,42]. When cellular glutathione levels are increased by cysteine or glutathione pro-activatory drugs, LPS-induced TNF-α release is inhibited through a block of NF-κB activation in Kupffer cells [25]. On the other hand, Wang and co-workers [26] reported that TNF-α release induced by LPS is eliminated in mice administered with a glutathione-depleting agent or an inhibitor of glutathione synthesis. Glutathione plays a central role in maintaining intracellular redox balance [43]. However, MT depletion has no effect on levels of cellular glutathione and its related enzymes [44,45]. Consequently, the effect of MT on TNF-α expression is not mediated by a change in glutathione levels, and we did not therefore analyse a contribution of glutathione in MT^{-/-} PEMs in the present study.

MT is involved in the homeostasis of essential metals [2]. Metals associated with MT can be donated to other metallo-proteins that require metal ions for their functions [6,7]. Therefore it was unclear as to whether MT exerts a specific effect on NF-κB transcription, or whether the apparent effects of MT are mediated indirectly, as a result of the perturbation of metal homeostasis. We investigated the basal activity of copper/zinc superoxide dismutase, which requires metal ions for its function, in PEMs by the cytochrome *c* method [46], but there was no difference between the activities of MT^{+/+} and MT^{-/-} PEMs (4.89 ± 0.39 and 4.81 ± 0.42 units · mg protein⁻¹ respectively). On the other hand, zinc has been shown previously to promote responses of peripheral blood mononuclear cells to LPS [47]. We investigated whether the addition of zinc to the MT^{-/-} PEMs results in the recovery of TNF production by LPS. Addition of zinc at non-toxic concentrations (0–25 µM) alone up-regulated TNF production in MT^{+/+} and MT^{-/-} PEMs; however, zinc showed no effect on LPS-induced TNF production in either cell type in our experimental conditions (results not shown). Therefore MT cannot affect LPS-induced TNF production via an indirect influence on the maintenance of metal homeostasis, but might affect LPS-induced TNF production directly due to the interaction of MT with NF-κB.

We and others [9,27,28,35] have described the interaction of MT with NF-κB. Abdel-Mageed and Agrawal [9] reported that MT directly interacts with NF-κB, as demonstrated by EMSA analysis. In their experiments, AP-1 activity was not affected by MT, in agreement with the results in the present study. On the other hand, Sakurai and co-workers [28] reported that MT inhibits TNF-induced IκB degradation and suppresses NF-κB-dependent gene expression. These reports appear to be self-contradictory, but this discrepancy could be explained on the basis of different functions of MT in the nucleus and in the cytoplasm. Thioredoxin suppresses NF-κB activity in the cytoplasm, but enhances it in the nucleus [48]. MT has been identified in the nucleus and/or cytoplasm of cells [49], but little is known about its function in the nucleus. As for thioredoxin, MT might function positively in the nucleus and negatively in the cytoplasm in terms of up-regulating NF-κB activity. For example, when cells are stimulated with NF-κB activators, such as TNF-α, several signal transducers are activated, and when IκB is finally degraded, MT might act as inhibitor of this reaction. Thereafter, NF-κB translocates from the cytoplasm to the nucleus [50]. Nuclear translocation of thioredoxin is also induced by TNF [50]. In the nucleus, disulphide bonds in NF-κB are reduced to

thiol residues by thioredoxin. At that time, the cysteine residues of thioredoxin must interact with those of NF- κ B. To bind to DNA, the cysteine residues in NF- κ B must therefore be freed from thioredoxin in order to bind zinc [51], although the mechanisms involved in this process remain enigmatic. However, zinc channels are located in the nuclear envelope, and the level of free zinc in the nucleus is very low compared with extranuclear levels. Therefore, in the nucleus, MT could conceivably provide zinc to NF- κ B in the nucleus. We surmise that MT acts as a redox factor in the same way as thioredoxin, and thus also as a zinc donor.

To summarize, the major implications of our observations are that: (i) MT is an important regulatory protein in PEMs; and (ii) MT is involved in the regulation of cytokine expression, which might be important in overcoming Gram-negative infection.

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