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Metallothionein Is Required for Zinc-Induced Expression of the Macrophage Colony Stimulating Factor Gene

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Abstract Macrophage colony stimulating factor (M-CSF) plays an important role in the proliferation and differentiation of mononuclear phagocytes. The present study investigates the effect of zinc on M-CSF expression in MC3T3-E1 and L929 cells. Zinc dose-dependently increased M-CSF mRNA levels. The time-course of zinc-induced M-CSF mRNA expression peaked at 6 h. Stability studies of mRNA using actinomycin D revealed that zinc does not affect M-CSF mRNA stability. We examined the function of the M-CSF gene promoter using a luciferase reporter assay. A construct containing the −467/+39 region of the promoter was upregulated by zinc. In the presence of cycloheximide, zinc did not induce a greater increase in the M-CSF mRNA than cycloheximide alone. To confirm the effect of MT on M-CSF mRNA expression, mouse lung fibroblasts (MLFs) were prepared from MT+/+ and MT−/− mice. Zinc induced an increase in the expression of M-CSF in MT+/+ MLFs, but this response was not evident in MT−/− MLFs. Moreover, overexpression of MT upregulated M-CSF mRNA expression as well as M-CSF secretion. Our findings suggest that MT expression mediates zinc regulation of M-CSF gene expression at the transcriptional level. J. Cell. Biochem. 86: 145–153, 2002.

Key words: macrophage colony stimulating factor; metallothionein; zinc; gene expression

Macrophage colony stimulating factor (M-CSF) controls the proliferation and differentiation of mononuclear phagocytes from determined undifferentiated precursors to fully differentiated non-dividing macrophages. Furthermore, M-CSF may be also involved in the pathogenesis of atherosclerosis [Rajavashisth et al., 1998] and in the development of osteoporosis [Kimble et al., 1996]. Besides its role, as a growth and survival factor, M-CSF functions as a chemotactic agent for monocytes and can regulate the effector functions of mature monocytes and macrophages [Wang et al., 1988]. It modulates inflammatory response by stimulating the production of several cytokines and growth factors [Warren and Ralph, 1986].

M-CSF is secreted by monocytes, macrophages, endothelial cells, smooth muscle cells, osteoblasts, fibroblasts, and other cell types. Its expression is regulated, for example, by tumor necrosis factor (TNF)-alpha, interleukin (IL)-1, endotoxin, cGMP, calcium ionophores, and minimally modified (MM)-LDL [Ku et al., 1992; Huleihel et al., 1993; Rajavashisth et al., 1995]. The M-CSF gene has been cloned, and cis-acting elements in the promoter have been identified by homology [Harrington et al., 1991]. The 5′-flanking region of the gene contains putative cis-acting sites for the transcription factors AP-1, NF-kappa B, NF-IL6, Sp1/Egr1, and PU.1 [Harrington et al., 1991]. Interaction between these regulatory transcription elements should contribute to M-CSF gene expression. The AP-1 binding site is required for basal expression of the mM-CSF gene [Harrington et al., 1997]. Rajavashisth et al. [1995] reported that NF-kappa B activation is involved in M-CSF expression by MM-LDL in murine L-cells. M-CSF expression is regulated not only by transcriptional mechanisms, but also by post-transcriptional mechanisms, such as those involved in mRNA stability [Horiguchi et al., 1988].

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Zinc is a trace element that is essential for normal cell growth and metabolism. Zinc is a constituent of various proteins and enzymes and is indispensable to catalysis, gene expression, and intracellular signaling. The cellular mechanism of zinc action stimulates proliferation and differentiation [Hashizume and Yamaguchi, 1993, 1994], as well as protein synthesis in osteoblastic cells [Hashizume and Yamaguchi, 1993, 1994]. Zinc stimulates bone formation and mineralization [Yamaguchi, 1992]. On the other hand, the integrity of the immune system and zinc status is dynamically linked. Challenging zinc-deficient mice with subacute levels of infective agents such as *Trypanosoma cruzi* resulted in death due to impaired defense [Fraker et al., 1982]. Zinc-deficient mice with half the normal number of splenocytes produced about half the number of antibody-producing cells as normal mice [Cook-Mills and Fraker, 1993]. Since it plays important roles in the osteoporosis and inflammatory response, we studied the effect of zinc on M-CSF expression.

During this study, we found that zinc upregulated M-CSF expression and that this process is mediated by metallothionein (MT). MTs are a group of ubiquitous, highly conserved 6-kDa proteins consisting of 61 amino acid residues including 20 cysteines that are bound to a total of seven metal ions with high affinity [Kägi and Kojima, 1987]. The present study discovered a novel relationship between M-CSF and MT. Furthermore, if M-CSF is upregulated by zinc and MT, the regulation of MT expression may affect osteoporosis and inflammatory responses.

MATERIALS AND METHODS

Mice

MT−/− mice (129S7/Sv-Mt1^{tm1Bri} Mt2^{tm1Bri}) were obtained from the Jackson Laboratory (Bar Harbor, ME), and C57BL/6CrSlc mice were obtained from Japan SLC (Hamamatsu, Japan). The MT−/− mice were originally developed on a 129S7/Sv background and back-crossed at our laboratory with C57BL/6J for one generation, then homozygous mice (MT+/+ and MT−/−) were used for experiments.

Preparation of Mouse Lung Fibroblasts (MLFs) and Cell Culture

MLFs were isolated from 1- to 2-day-old mice as described by Joyner [1993]]. Briefly, the lungs were cut into small pieces and incubated with 0.1% trypsin-EDTA at 37°C for 20–30 min. The cells were seeded in 10-cm tissue culture plates, and the attached cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. The medium was changed every 3 days. MC3T3-E1 cells were cultured with the alpha modification of MEM supplemented with 5% FCS. L929 cells were cultured in DMEM supplemented with 5% FCS. All cells were maintained at 37°C in 5% CO_2/95% air. For stable MT expression, L929 cells were cotransfected with either pBPVGRPMT or pBPVGRPTM and the plasmid pRc/CMV containing a neomycin-resistant gene as described [Kanekiyo et al., 2001].

Plasmid Constructs, Cell Transfection, and Reporter Gene Assay

Plasmids were derived from the promoterless luciferase vector, PGV-B2 (Toyo Ink Mfg. Co., Tokyo, Japan). Deletion-mutated fragments derived from the mM-CSF promoter were generated by PCR [Saiki et al., 1988] and cloned upstream from the luciferase coding sequence. All plasmids were amplified and purified by CsCl-EtBr and CsCl density gradient centrifugation. All constructs were verified by sequencing the relevant portions. Transient transfection with reporter DNA was performed by calcium phosphate precipitation [Chen and Okayama, 1988]. After an incubation with zinc for 24 h, cell were lyzed, and luciferase activity in the lysates was measured using a Luminometer (EG and G Berthold, Bad Wildbad, Germany). Transfection experiments were performed at least three times using different preparations of DNA. Luciferase activities of the cells were normalized using a luciferase vector bearing 117 bp of the 5′ flanking region of HCMV MIEP, which does not respond to zinc [Kanekiyo et al., 2001]. The mM-CSF promoter response (-fold induction) was defined as the ratio of luciferase activity in the stimulated cells to that in the unstimulated cells.

Preparation of Conditioned Medium and Murine Bone Marrow Cells Mitogenesis Assay

L929-MT and L929-TM cells were cultured at a density of 4×10^6 cells/10 ml in DMEM with 5% FCS. After 24 h, cells were washed twice with HBSS, then cultured in serum-free DMEM. After the indicated periods, conditioned media from L929-MT and L929-TM cells

were collected. M-CSF was bioassayed based on its ability to stimulate DNA synthesis in murine bone marrow macrophages [Tushinski et al., 1982]. Bone marrow cells collected from male C57BL/6CrSlc mice were seeded into 96-well cell culture plates (suspension culture treated, SUMITOMO BAKELITE, Tokyo, Japan) at a density of 1×10^5 cells per well in 50 μl RPMI 1640 medium containing 10% heat-inactivated FCS, 50 μM beta-mercaptoethanol, 100 mU/ml penicillin, and 100 μg/ml streptomycin [Suzu et al., 1997], then incubated with 150 μl aliquot of conditioned medium with or without anti-M-CSF antibody. The cells were cultured for 5 days and pulsed with 18.5 kBeq/well of [^{3}H]-thymidine (ICN, CA) for the final 6 h, then the amount of radioactivity incorporated into DNA was determined by scintillation counting. A rabbit was immunized with recombinant human M-CSF from CHO cells, which was provided by the Welfide Corporation (Osaka, Japan). The specificity of anti-M-CSF IgG purified from the rabbit antiserum was tested by Western blotting (data not shown).

Northern Blot Analysis

Total RNAs (10 μg) were resolved by electrophoresis in 1.0% agarose/10% formaldehyde gels then transferred onto nylon membranes (Amersham Pharmacia, PLC, UK) in 20 × SSC buffer. We labeled cDNA probes with [alpha-^{32}P] dCTP (111 TBq/mmol; NEN, MA.) using Klenow fragment (TOYOBO, Shiga, Japan) and an appropriate primer, then the membranes were hybridized with the ^{32}P-labeled cDNA probe.

Statistical Analysis

Results are expressed as means ± SD. Data were analyzed by ANOVA and Fisher's PLSD test. Significance was assessed at the $P < 0.05$ level.

RESULTS

Zinc Induces M-CSF mRNA Expression in MC-3T3-E1 and L929 Cells

We initially investigated the influence of zinc on expression on the mM-CSF gene in murine MC3T3-E1 osteoblastic cells. Total RNA from untreated and zinc treated MC3T3-E1 cells were examined by Northern blotting. Levels of M-CSF mRNA (4 kb) expression were increased by 50 μM zinc (Fig. 1A). To determine whether this phenomenon is specific to osteoblasts or not, we investigated the effect of zinc on fibroblast cell line L929. Zinc (0–100 μM) increased the expression of M-CSF mRNA in a dose-dependent manner. M-CSF expression was up-regulated by zinc in both MC3T3-E1 cells and in L929 cells, we further analyzed L929 cells. The time-course in L929 cells showed that zinc increased the level of M-CSF mRNA expression within 3 h after stimulation. This level peaked at 6 h, returned to basal levels within 12 h, and increased again (Fig. 1C). Similarly, the expression of MT mRNA that is also induced by zinc, responded in the same manner as M-CSF mRNA, although to a greater extent (data not shown).

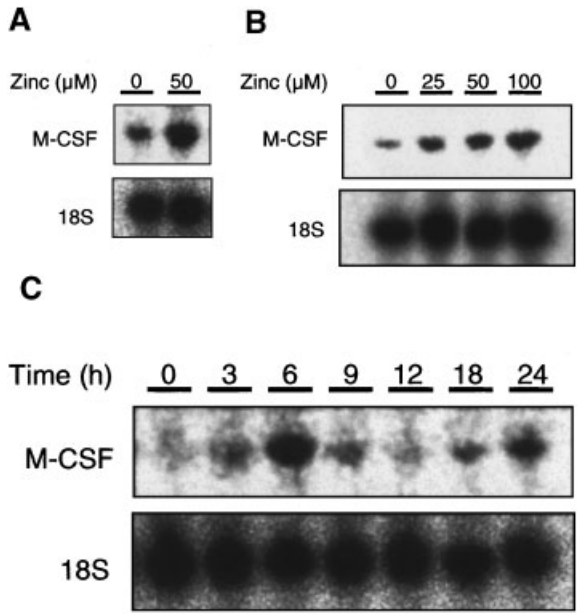

Fig. 1. Induction of M-CSF mRNA by zinc. **A**: Effect of zinc on M-CSF expression in osteoblastic MC3T3-E1 cells. MC3T3-E1 cells were incubated with 50 μM zinc in medium containing 1% FCS for 6 h. **B**: Dose response of M-CSF mRNA expression in zinc-treated L929 cells. L929 cells were incubated for 6 h with zinc at various concentrations in medium containing 1% FCS. **C**: Kinetics of M-CSF mRNA expression in zinc-treated L929 cells. L929 cells were incubated with 50 μM zinc in medium containing 1% FCS for indicated periods and were harvested for preparation of total RNA. Northern blotting performed as described in Materials and Methods. Representative data from one of two independent experiments.

Zinc Does Not Affect the Stability of M-CSF mRNA in L929 Cells

Taylor and Blackshear [1995] reported that zinc inhibits the turnover of tristetraprolin and c-fos mRNA. To examine whether post-transcriptional regulation is involved in the

zinc-induced increase in steady-state M-CSF mRNA levels or not, L929 cells were incubated with zinc or vehicle alone for 4 h, followed by the transcription inhibitor, actinomycin D. The decline in accumulation of these transcripts was determined by Northern blotting (Fig. 2A). Figure 2B shows the amount of M-CSF mRNA generated at each time point after correction according to the amount of GAPDH mRNA. These results indicated that zinc does not influence the decay rate of M-CSF mRNA.

Luciferase Activity Induced by M-CSF Promoter Fragments in Untreated and Zinc-Treated L929 Cells

To understand whether promoter activation is involved in zinc-induced M-CSF gene transcription or not, we examined the function of three 5′ deleted mM-CSF gene promoters (bp-756/+39, −467/+39, and −281/+39) on the luciferase reporter gene in L929 cells (Fig. 3). Zinc doubled the luciferase activity in cells transfected with the construct containing the −467 to +39 region of the promoter. However, zinc did not increase luciferase activity in cells transfected with the construct containing the −756 to +39 and −281 to +39 regions.

Zinc-Induced M-CSF mRNA Expression Is Dependent on *De Novo* Synthesis of Protein

To examine whether zinc-induced increase in M-CSF expression is dependent on *de novo* synthesis of protein or not, L929 cells were incubated with cycloheximide for 30 min, followed by 6-h co-treatment with zinc (Fig. 4). Cycloheximide alone caused a marked increase in the M-CSF mRNA expression as reported by Horiguchi et al. [1988]. In the presence of cycloheximide, zinc decreased M-CSF mRNA expression rather than increased compared with cycroheximede alone. On the other hand, MT mRNA expression was upregulated by zinc in the presence of cycloheximide. These results indicated that M-CSF mRNA expression was not regulated by zinc in the same manner as MT mRNA, which is directly upregulated by metal-responsive transcriptional activator (MTF)-1.

Zinc Fails to Induce M-CSF mRNA Expression in MLFs From MT−/− Mice

Figure 4 shows that de novo protein synthesis is necessary for the M-CSF expression induced by zinc, which induces MT. We recently found that zinc upregulates HCMV MIE promoter activity through MT expression [Kanekiyo et al., 2001]. To determine the requirement of MT for zinc-induced M-CSF expression in L929 cells, primary MLFs were prepared from MT+/+ and MT−/− mice. Total RNA isolated from untreated and zinc-treated MLFs was examined by Northern analysis. Although MLFs from MT+/+ and MT−/− mice constitutively expressed similar amounts of M-CSF mRNA, and although zinc induced an increase in

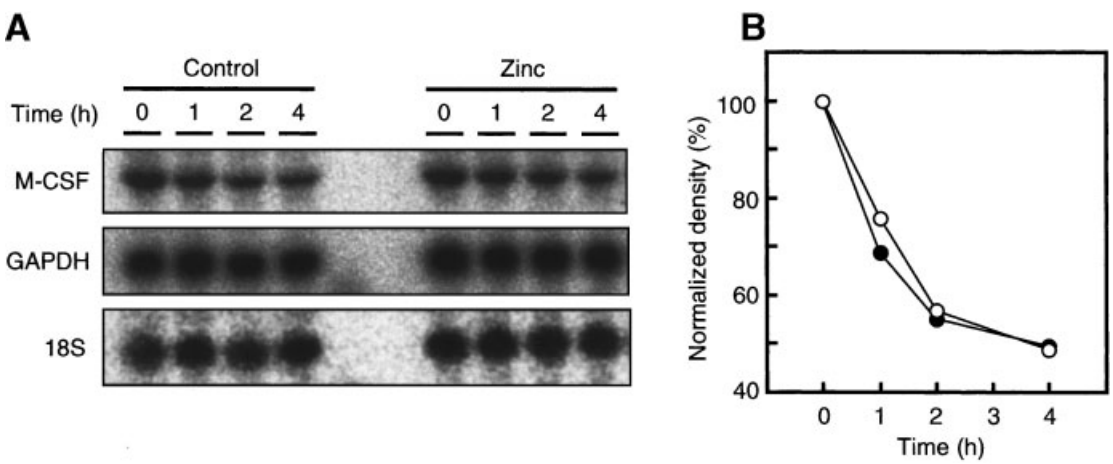

Fig. 2. Effect of zinc on stability of M-CSF mRNA in L929 cells. **A**: L929 cells were incubated with 50 μM zinc or vehicle for 3 h in the medium containing 1% FCS, then 5 μg/ml actinomycin D was added for indicated periods. Cells were harvested for Northern blot analysis. **B**: Densitometric analysis of bands on Northern blots as shown in (A) was obtained by radioimaging analysis. Normalizing each band value over GAPDH mRNA hybridization. The amount of mRNA at time 0 of actinomycin D exposure was taken as 100%. Solid circles, 50 μM zinc; open circles, control. Representative data from one of two independent experiments.

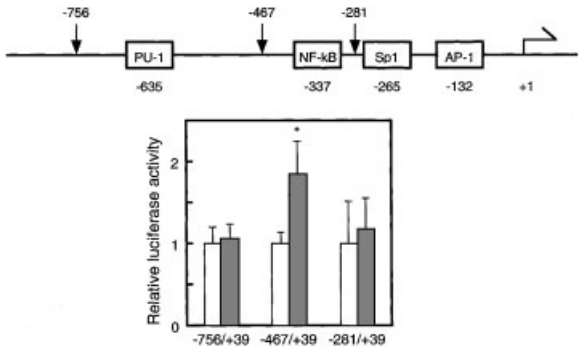

Fig. 3. Zinc induces expression of M-CSF promoter-luciferase constructs in L929 cells. Three deletion mutants of promoter regions upstream of +39 were connected to the luciferase reporter gene. L929 cells transiently transfected with 1.5 μg of luciferase reporter plasmid in six-well tissue culture plates were stimulated with (solid column) or without (open column) 50 μM zinc in medium containing 2.5% zinc-free FCS. Cell lysates were prepared 24 h after stimulation, and luciferase activities were assayed. Data are represented as relative values of luciferase activities against activities in untreated cells. This experiment was independently repeated four times, and the average ± SD of three experiments are shown. $*P < 0.01$ compared with control value.

M-CSF expression in MT+/+ MLFs, this response was absent in MT−/−MLFs (Fig. 5). However, wild type and mutant MT mRNA expression was increased in both MLFs.

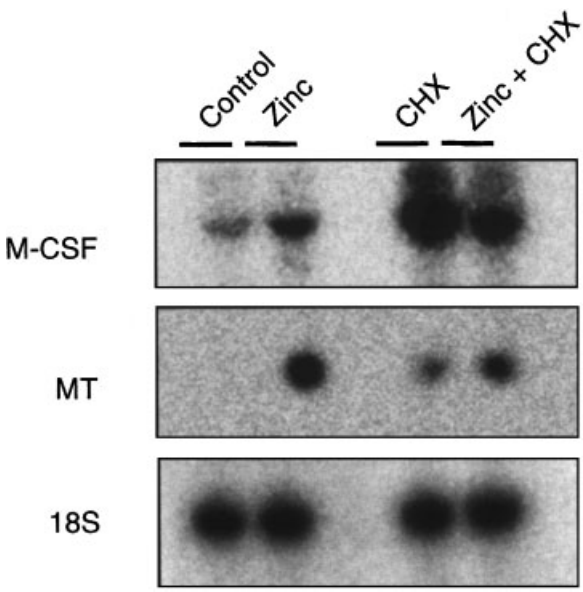

Fig. 4. Effect of cycloheximide (CHX) on M-CSF expression in untreated and zinc-treated L929 cells. Cells incubated with 10 μg/ml CHX or vehicle for 30 min were exposed to 50 μM zinc or vehicle for 6 h in medium containing 1% FCS. Cells were then harvested for Northern blotting. Representative data from one of three independent experiments.

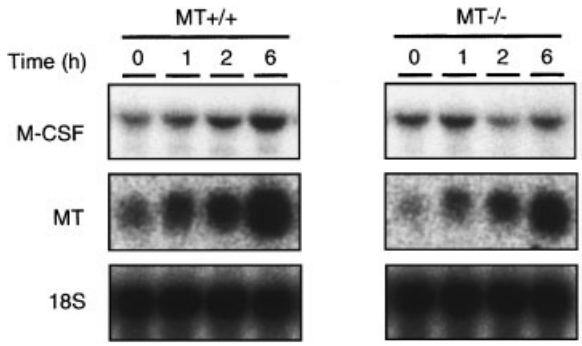

Fig. 5. Zinc affects M-CSF expression in MLFs from MT+/+ and MT−/− mice. MLFs were prepared as described in Materials and Methods. Cells incubated with 50 μM zinc in medium containing 1% FCS for various periods were harvested for Northern blotting. Representative data from one of two independent experiments.

Overexpression of MT Increases M-CSF mRNA Expression in L929 Cells

To further examine the role of MT in M-CSF expression, a plasmid-containing mouse MT cDNA was stably transfected into L929 cells as described [Kanekiyo et al., 2001]. The level of MT in L929-MT cells that overexpress MT was tripled compared with control (L929-TM) cells [Kanekiyo et al., 2001]. Total RNA isolated from L929-MT and L929-TM cells was examined by Northern blotting. As Figure 6 shows, MT mRNA expression was increased in L929-MT

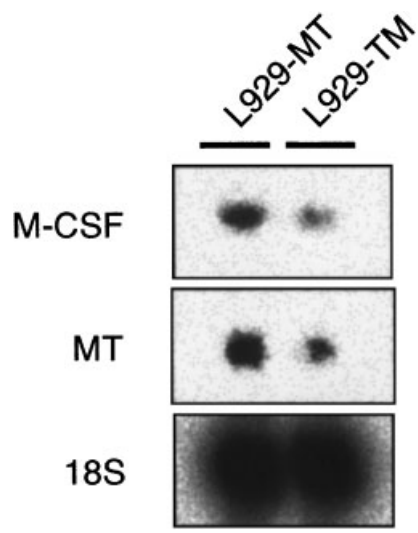

Fig. 6. Effect of MT overexpression on M-CSF expression in L929 cells. L929 cells were stably transfected with pBPVGRPMT (L929-MT) and pBPVGRPTM (L929-TM). Northern blotting showed M-CSF mRNA expression. Representative data from one of two independent experiments.

cells, and M-CSF mRNA expression was also upregulated in L929-MT cells.

Overexpression of MT Increases M-CSF Protein Expression in L929 Cells

The effect of MT on M-CSF protein was studied using murine bone marrow cell mitogenesis assay (Fig. 7). Consistent with the Northern blot findings, the content of M-CSF protein was significantly increased in the conditioned medium from L929-MT cells compared with that from control cells. The proliferation rates of these two cell lines did not differ (data not shown). Conditioned medium from L929-TM cells induced six and eight fold increase in the proliferation of murine bone marrow cells (after 24 and 48 h, respectively). On the other hand, conditioned medium from L929-MT cells induced a 15- and 22-fold increase in the proliferation of murine bone marrow cells (after 24 and 48 h, respectively). To determine whether the mitogenic activity of the murine bone marrow cells is due to M-CSF or not, conditioned media from L929-MT and L929-TM cells were examined in the presence of a polyclonal antibody against M-CSF. The antibody inhibited the mitogenic activity of murine bone marrow cells in conditioned medium from L929 cells in a dose-dependent fashion (59.8 ± 16.1 and $80.4 \pm 3.8\%$ reduction with 10 and 100 μg/ml of anti-M-CSF antibody, respectively). Under the same conditions, inhibition by normal IgG did not have so much effect on mitogenic activity (16.4 ± 12.7 and $21.3 \pm 3.2\%$ reduction by 10 and 100 μg/ml of normal IgG, respectively). The anti-M-CSF antibody (100 μg/ml) reduced mitogenic activity of conditioned medium from L929-MT and L929-TM cells by 87.9 ± 2.2 and $89.2 \pm 1.2\%$, respectively. These results indicate that MT-overexpression increases cellular M-CSF production.

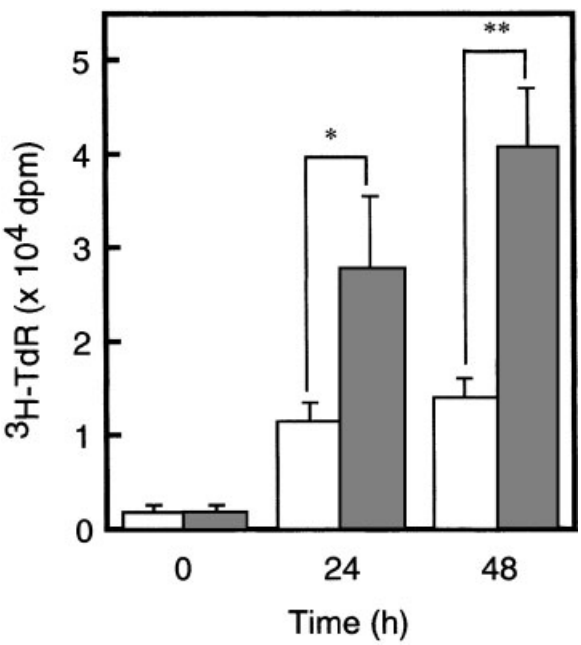

Fig. 7. Effect of MT overexpression on M-CSF release from L929 cells. L929-MT (solid column) and L929-TM cells (open column) were incubated in serum-free medium for various periods. Medium was harvested and bioassayed for M-CSF based upon ability to stimulate DNA synthesis in murine bone marrow cells. $*P < 0.005$; $**P < 0.0001$ compared with L929-TM cells incubated under identical conditions. Representative data from one of four independent experiments.

DISCUSSION

The present study describes novel functions of zinc and MT in M-CSF expression. Several independent observations at various stages of M-CSF induction support the contention that MT is involved in the induction of M-CSF by zinc.

Several reports have described the regulation of M-CSF expression. TNF-alpha increases M-CSF transcription, as well as the half-life of its mRNA and protein [Sherman et al., 1990]. Dexamethasone also increases the half-life of M-CSF protein in bone cells [Rubin et al., 1998]. IL-3 and granulocyte-macrophage (GM)-CSF increase M-CSF mRNA via a post-transcriptional mechanism in human blood monocytes [Ernst et al., 1989]. IL-1, endotoxin, cGMP, calcium ionophores, and MM-LDL also affect M-CSF expression [Ku et al., 1992; Heuchel et al., 1994; Rajavashisth et al., 1995]. However, the effect of metals on M-CSF expression has not been reported.

Zinc plays various roles in mammalian systems, and it is essential for the growth of human and many other species of animals [Hurley et al., 1969; Burch et al., 1975]. Zinc is mechanistically involved in the processes of genetic stability and gene expression from many aspects including the structure of chromatin, the replication of DNA, and transcription of RNA through the activity of transcription factors and RNA and DNA polymerases, and it plays roles in DNA repair and programmed cell death [Fraker et al., 1982]. For example, zinc upregulates MTF-1 activity and increases the expressions of genes such as gamma-glutamylcysteine synthetase,

MT, and zinc-transporter through the metal responsive element (MRE)-MTF system [Searle, 1990]. Zinc upregulates intercellular adhesion molecule (ICAM)-1 mRNA expression due to a rapid increase in the transcriptional rate via an unknown mechanism [Martinotti et al., 1995]. On the other hand, Taylor and Blackshear [1995] reported that zinc upregulates tristetraprolin and c-fos mRNA accumulation by increasing the half-lives of the mRNA. These effects of zinc do not depend on *de novo* protein synthesis. The present study found that unlike tristetraprolin and c-fos mRNA, the stability of M-CSF mRNA in L-929 cells is not influenced by zinc. Furthermore, cycloheximide inhibited the upregulation of M-CSF mRNA but not that of MT mRNA, and zinc did not induce M-CSF mRNA directly through MRE but was mediated by *de novo* protein synthesis. An examination of effects of cycloheximide on untreated L929 cells revealed M-CSF mRNA upregulation. This phenomenon has been investigated in other cell types, and the notion that expression of M-CSF mRNA is regulated by a labile protein that enhances the decay rate or inhibits the transcription rate of M-CSF has been proposed [Horiguchi et al., 1988].

MTs are a group of ubiquitous, highly conserved 6-kDa proteins consisting of 61 amino acid residues including 20 cysteines that are bound to a total of seven metal ions with high affinity [Kägi and Kojima, 1987]. The primary functions of MT are involvement in the homeostasis of essential metals [Cousins, 1985; Dunn et al., 1987], the detoxification of heavy metals [Yoshikawa, 1970; Webb and Verschoyle, 1976; Goering and Klaassen, 1984; Min et al., 1987], and perhaps the scavenging of free radicals because of its sulfhydryl groups [Thornalley and Vasak, 1985; Thomas et al., 1986]. Zinc is a well-known inducer of MT [Heuchel et al., 1994]. The molecular mechanism of zinc-induced transcription of the MT gene is thoroughly documented. The MT promoter contains MREs that are activated by MTF-1 that contains bound zinc [Searle, 1990]. MT appears to play important roles in intracellular zinc homeostasis [Vallee, 1995]. Apo MT can remove zinc atoms from the zinc-finger transcription factor, Sp1 in cell-free systems. The removal of zinc reduces DNA-binding and transcriptional activity of Sp1 [Zeng et al., 1991]. The present study found that in using MT−/− cells and MT-overexpressing cells, M-CSF gene expression induced by zinc is mediated by MT expression. Therefore, zinc may not directly activate M-CSF promoter, but MT induced by zinc may be involved in transcriptional activation.

The −467/+39 region of the M-CSF promoter was upregulated by zinc, indicating that the zinc-induced upregulation of M-CSF expression is mediated by activation of the M-CSF promoter. However, the activities of other deletion mutants (−756/+39 and −281/+39) were not influenced by zinc. These results indicated that elements involved in zinc-upregulation are located between positions −467 to −281 upstream of the transcription start site. Additionally a repressor element may be situated between positions −756 to −467. Similar phenomena occur in the human M-CSF promoter; TNF-induces activation of the reporter gene in a plasmid containing the −406 to +10 region of the hM-CSF promoter, but not the −343 to +100 and −490 to +10 regions [Yao et al., 2000]. The kappa B motif is located at positions −467 to −281 of the mM-CSF promoter [Harrington et al., 1991]. We and others recently reported that zinc or the overexpression of MT upregulates the expression of genes with kappa B sites in their promoter [Abdel-Mageed and Agrawal, 1998]. We discovered by EMSA and promoter analysis that zinc or MT overexpression upregulates the DNA binding activity of NF-kappa B [Kanekiyo et al., 2001] and that the activity of AP-1 was not affected by MT overexpression (data not shown). However, Rubin et al. [2000] reported that the NF-kappa B response element (−369/−378) in the mM-CSF promoter is not necessary for M-CSF-expression by TNF-alpha. On the other hand, Yao et al. [2000] reported that M-CSF mRNA expression induced by TNF-alpha is largely eliminated in osteoblasts from NF-kappa B p50 knockout mice [Zeng et al., 1991]. Rajavashisth et al. [1995] reported that NF-kappa B activation is involved in M-CSF expression by MM-LDL in murine L-cells. These two reports indicate that NF-kappa B is critical for M-CSF expression. Therefore, more experiments are required to clarify the region of the promoter, including the kappa B site that is involved in activation by zinc.

The present study demonstrated that zinc and MT modulate M-CSF expression. MT is induced by cytokines and xenobiotics [Friedman and Stark, 1985; Cousins and Leinart, 1988; De et al., 1990; Liu et al., 1991; Min et al., 1991; Coyle et al., 1993; Kasutani et al., 1998],

so they may affect on osteoporosis and inflammatory responses, through the regulation of M-CSF expression by MT. Moreover, M-CSF is involved in many diseases. Therefore, we are interested in whether MT and MT inducing (or reducing) agents participate in these diseases or not.

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