Cancer Immunol Immunother (1994) 38: 16–22

Cancer Immunology Immunotherapy

Liposome-encapsulated muramyl tripeptide up-regulates monocyte chemotactic and activating factor gene expression in human monocytes at the transcriptional and post-transcriptional levels

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Received 14 April 1993/Accepted 19 August 1993

Abstract. Liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) is a novel immune modulator that is now under investigation against metastatic melanoma and osteosarcoma. We have already reported that L-MTP-PE induced monocyte-mediated tumoricidal activity and up-regulation of the tumor necrosis factor and interleukin-1 (IL-1) in vivo and in vitro. We now demonstrate that L-MTP-PE also induces monocyte chemotactic and activating factor (MCAF) mRNA expression at both the transcriptional and post-transcriptional levels. Monocyte chemotactic activity was also present in the supernatants of L-MTP-PE-stimulated cells. In monocytes, the increased expression of MCAF was induced rapidly (by 2 h) but was short-lived. By 4 h, MCAF mRNA had decreased to background level. We found no change in MCAF mRNA levels in lymphocytes exposed to L-MTP-PE. We therefore conclude that L-MTP-PE selectively up-regulates MCAF expression in monocytes and that MCAF may play a role in the tumoricidal and immune-stimulating activity of L-MTP-PE.

Key words: Liposome-encapsulated muramyl tripeptide – MCAF – mRNA, monocyte

Introduction

Muramyl tripeptide phosphatidylethanolamine (MTP-PE) is a synthesized lipophilic analog of muramyl dipeptide, the smallest component of the mycobacterium capable of stimulating the immune system [34]. MTP-PE has potent monocyte/macrophae-activating properties [17]. Liposome-encapsulated MTP-PE (L-MTP-PE) was specifically designed for in vivo targeting of macrophages by intravenous infusion [34] and is the only form of the drug available for clinical trials (CGP 19835A lipid) [19]. When administered intravenously to cancer patients, L-MTP-PE uptake was demonstrated in liver, spleen, lung, and nasopharynx [30].

The administration of L-MTP-PE to mice with B16 melanoma results in activation of alveolar macrophages to the tumoricidal state, with regression of lung and lymph node metastases [11, 12]. L-MTP-PE has also been shown to be effective in preventing lung metastases in dogs with osteosarcoma [24]. More recently, a phase II study in relapsed osteosarcoma patients with lung metastases demonstrated that a 24-week course of L-MTP-PE therapy doubled the disease-free interval compared to that experienced by historical controls who received salvage chemotherapy [21]. L-MTP-E is also being investigated for its efficacy in metastatic melanoma [14, 23]. Thus, all indications are that L-MTP-PE is a promising new agent for the treatment of at least osteosarcoma, and possibly other malignancies that metastasize to the lung.

We have previously reported histological changes in pulmonary lesions excised from osteosarcoma patients following therapy with L-MTP-PE [20]. These changes are unlike any observed following chemotherapy or surgery and consist of peripheral fibrosis surrounding the tumor, with inflammatory macrophage infiltration throughout the tumor. Lymphocyte infiltration was not significant. In an effort to understand the mechanisms by which L-MTP-PE induced such changes, we investigated the effect of L-MTP-PE on the expression of various cytokine genes in human monocytes. We demonstrated that L-MTP-PE up-regulates the expression of the genes for tumor necrosis factor (TNF) [25], interleukin-1 (IL-1) [25], interleukin-6 (IL-6) and interleukin-8 (IL-8) (T. Asano, A. McWatters, T. An, K. Matsushima, and E.S. Kleinerman, unpublished), the subsequent production of their proteins, and the activation of monocytes to the tumoricidal state [17–20, 25]. Plasma levels of all four cytokines have been demonstrated in cancer patients following the intravenous infusion of L-MTP-PE [19, 23, 30, 35]. The fibrosis seen surrounding the pulmonary tumors may be the consequence of the induction of TNF and IL-1 by L-MTP-PE, and the release of these cytokines from activated macrophages in and around

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the tumor nodules. The mechanism of monocyte/macrophage recruitment into the tumor area, however, is not explained by the induction of these cytokines.

Recently, a novel superfamily of inflammatory mediators that regulate leukocyte motility has been identified. Monocyte chemotactic and activating factor (MCAF), a member of this family, is a specific chemoattractant for human monocytes, and a potent monocyte activator as well [22, 28]. MCAF is produced by human mononuclear phagocytes [8], and has been shown to play a role in monocyte recruitment into tumor tissue [6]. Thus, monocytes can autonomously regulate the extravasation and activation of cells from the same lineage. MCAF may contribute to inhibiting tumor growth by attracting monocytes/macrophages to the tumor site and subsequently increasing their cytostatic activity.

We therefore hypothesized that, in addition to up-regulating the TNF, IL-1, IL-6 and IL-8 genes, L-MTP-PE may also up-regulate the MCAF gene in monocytes. We studied the kinetics of MCAF mRNA expression in responses to L-MTP-PE, as well as the effects of L-MTP-PE on MCAF nuclear transcription, on the post-transcriptional stability of MCAF mRNA and on the production of chemotactic activity. The present study demonstrates that L-MTP-PE induces MCAF expression at both the transcriptional and post-transcriptional levels.

Materials and methods

Reagents and drugs. Macrophage serum-free medium (SFM) was purchased from Gibco Laboratories (Grand Island, N.Y.). Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}, and Earle's balanced salt solution (EBSS) were purchased from Whittaker Bioproducts (Walkersville, Md.). Lymphocyte separation medium was purchased from Organon Teknika Corp. (Durham, N.C.). All agents were free of endotoxin, as determined by the *Limulus* amebocyte lysate assay (sensitivity limit, 0.025 ng/ml). L-MTP-PE (Ciba Division, Ciba-Giegy Corp., Summit, N.J.), was prepared as previously described [25].

Molecular probes. The cDNA probe for human MCAF was the 350-base-pair (350-bp) *Pst*I/*Pst*I fragment [15], for human IL-8 it was the 500-bp *Eco*RI/*Eco*RI fragment [27], the chicken β-actin cDNA was the 1800-bp *Pst*I/*Pst*I fragment [7]; and the rat glyceraldehyde phosphate dehydrogenase cDNA (GAPDH) was the 1285-bp *Pst*I/*Pst*I fragment [13].

Purification of normal human mononuclear leukocytes, monocytes, and lymphocytes. Mononuclear leukocytes were separated from normal human buffy coats by density gradient centrifugation using lymphocyte separation medium as previously described [25]. The mononuclear leukocytes were then fractionated by elutriation in a Beckman J 6M (Beckman Instruments Inc., Fullerton, Calif.) to purify the monocyte and lymphocyte fractions. The purity of the elutriated monocyte and lymphocyte fractions was more than 95%, as confirmed by Diff-Quik staining (Baxter Healthcare Corp., Scientific Division, Megaw Park, Ill.) and nonspecific esterase staining. The purified monocytes and lymphocytes were cultured in SFM.

RNA extraction and northern blot analysis. The purified monocytes and lymphocytes were incubated in SFM at 37° C in Falcon 2006 polypropylene tubes (Becton Dickinson Labware, Lincoln Park, N.J.) at 5×10^6 cells/ml. A total of 2.5×10^7 cells were incubated in SFM with or without L-MTP-PE for various times. Total RNA was prepared by the acid guanidine isothiocyanate/phenol/chloroform extraction method, and size-fractionated, blotted and hybridized by a standard procedure [25]. The specific activity of the hybridization probes was between 1×10^9 and 2×10^9 cpm/μg DNA. Autoradiography was done by exposing Kodak XAR-5 film (Eastman Kodak Corp., Rochester, N.Y.) to the blotted and hybridized membranes at –70° C. The autoradiograph was scanned with a Personal Densitometer (Molecular Dynamics, Sunnyvale, Calif.) and values were normalized for differences in GAPDH scanning densities. The values for the percentage maximum expression were then calculated as follows: maximum expression (%) = (calculated density at each time point/peak density) × 100.

Nuclear run-on transcription assay. After various stimulations, (5–7) × 10^7 monocytes were washed with cold phosphate-buffered saline and with EBSS (Whittaker), and suspended on ice in hypotonic buffer (20 mM TRIS/HCl at pH 7.5, 5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM dithiothreitol, 0.3 M sucrose, 0.25% Nonidet P40) for 5 min. The detergent-treated monocytes were then layered onto an equal volume of isolation buffer (20 mM TRIS/HCl at pH 7.5, 5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM dithiothreitol, 0.6 M sucrose) and centrifuged at 500 *g* for 10 min. The supernatant was aspirated, and the pelletted nuclei were gently resuspended in 125 μl transcription buffer (10% glycerol, 20 mM HEPES, pH 7.8, 1 mM $MgCl_2$, 2 mM $MnCl_2$, 142 mM KCl), 0.25 μM CTP, 0.25 μM ATP, 0.25 μM GTP, 1.25 μM dithiothreitol; 0.75 μM spermidine, 1 μl RNasin (Promega Biotec, Madison, Wis.) and 100 μCi [α-^{32}P]UTP (Amersham Corp., Arlington Heights, Ill.), and incubated at 30° C for 30 min with gentle shaking. The reaction mixture was treated with 12.5 μg RQ1 RNase-free DNase (Promega Biotec) and incubated at 30° C for 5 min, and then 100 μg proteinase K, 4 μl 0.2 M EDTA, 17.5 μl 10% SDS, and 20 μg yeast tRNA were added. The mixture was incubated at 40° C for 45 min, extracted, purified and hybridized. The filters were exposed at –70° C for 7–10 days. The autoradiograph was scanned and values were normalized for differences in GAPDH scanning densities. (Relative transcriptional activity was calculated as follows: relative transcriptional activity = density of the specific probe/GAPDH density on the same filter.)

Analysis of mRNA half-life. Elutriated normal human monocytes were incubated with or without 2 μg/ml L-MTP-PE for 2 h. The monocytes were then washed, and actinomycin D (Sigma Chemical Co., St. Louis, Mo.) at 10 μg/ml in culture medium was added. At 0, 30, 60, and 120 min, total RNA was extracted, subjected to northern blot analysis, and quantified by densitometric scanning as described above.

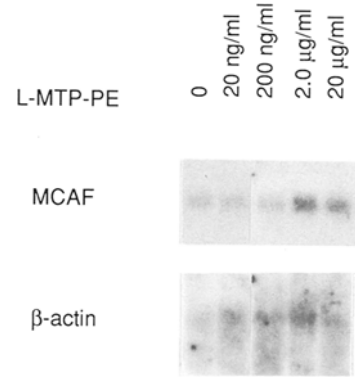


Fig. 1. Dose response of liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (*L-MTP-PE*). Human mononuclear leukocytes were incubated with various concentrations of L-MTP-PE for 2 h. RNA was extracted and analyzed by northern blot using ^{32}P-labeled monocyte chemotactic and activating factor (*MCAF*) and β-actin probes

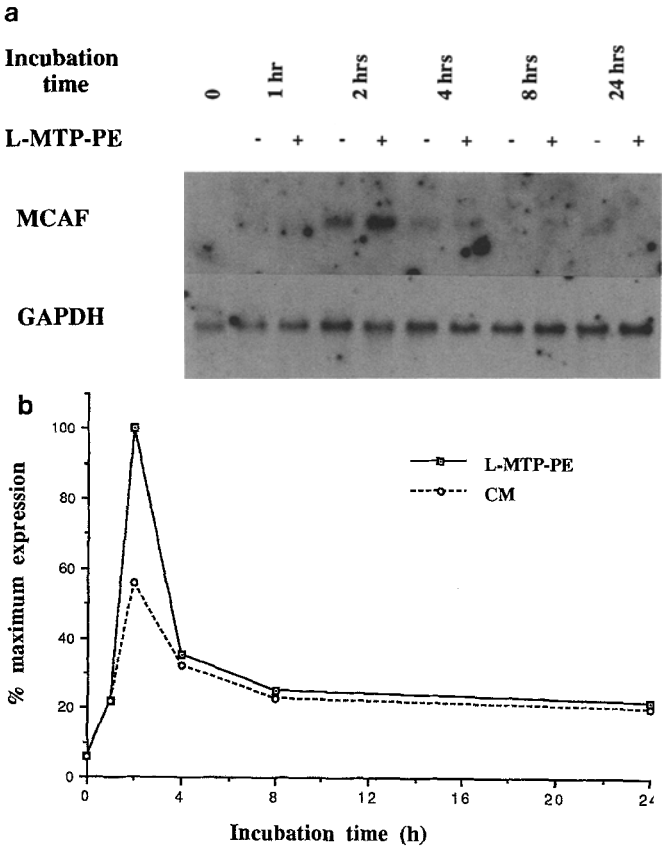


Fig. 2. a Expression of MCAF mRNA in human monocytes following incubation with or without L-MTP-PE. Monocytes were isolated from a single donor by elutriation and incubated with 2.0 μg/ml L-MTP-PE for 0–24 h. RNA was extracted and analyzed as described in Materials and methods. **b** MCAF mRNA expression kinetics in monocytes stimulated by L-MTP-PE. The density at each time point was normalized by glyceraldehyde-phosphate dehydrogenase (GAPDH) density. One representative experiment of seven. The values of percentage maximum expression were then calculated as described in Materials and methods. CM, control medium

Chemotaxis assay. Leukocyte chemotaxis was assayed by the blind-well chamber method [10]. Briefly, 200 μl test supernatant was placed in the bottom well of the chamber. The top well was filled with 0.3 ml Gey's balanced salt solution (Gibco) containing 2% bovine serum albumin (Sigma) and 8×10^4 monocytes. The two wells were separated by a polycarbonate filter with 5-μm holes (Nuclepore; Coaster Corp., Cambridge, Mass.). The chambers were incubated for 90 min at 37° C in humidified air with 5% CO_2; the filters were removed, stained by Diff-Quik stain and ten oil-immersion fields (high-power fields) were counted. Conditioned medium from unstimulated monocytes served as an additional control and was subtracted from the activity generated by the L-MTP-PE/monocyte-conditioned supernatant. Each experiment used *N*-formyl-1-methionyl-1-leucyl-1-phenylalanine (Sigma) as a reference chemoattractant at the optimal concentration of 150 nM. The statistical significance of migration toward stimulus versus medium control was assessed by Student's *t*-test.

Results

Dose response of L-MTP-PE

To determine the ideal amount of L-MTP-PE to employ in our experiments, we treated human mononuclear leukocytes with various concentrations of L-MTP-PE (Fig. 1).

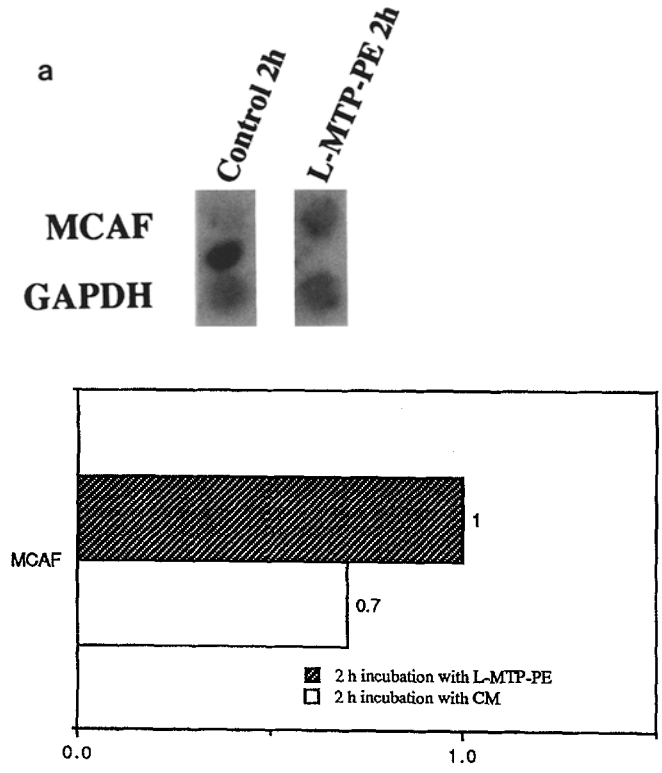


Fig. 3. a Effect of L-MTP-PE on MCAF transcription in human monocytes. Nuclei were isolated from monocytes incubated with or without L-MTP-PE for 2 h and run-on transcription assays were performed. **b** Summary of transcription assay. Relative transcriptional activity was calculated by normalization for GAPDH density. One of three experiments

Leukocytes treated with 20 ng/ml and 200 ng/ml L-MTP-PE had no greater MCAF mRNA expression than did control cells not treated with L-MTP-PE; however, cells treated with 2.0 μg/ml and 20 μg/ml had similarly increased levels of MCAF mRNA at 2 h. We, therefore, employed 2.0 μg/ml L-MTP-PE in all subsequent experiments.

Kinetics of MCAF induction by L-MTP-PE

To determine how rapidly L-MTP-PE up-regulated MCAF mRNA levels, we incubated monocytes with L-MTP-PE for 0–72 h. After 2 h, monocytes incubated with L-MTP-PE had higher levels of MCAF mRNA than control cells (Fig. 2, one representative exeriment of seven). However, the increased expression of MCAF decreased after 4 h.

We were unable to detect any change in MCAF mRNA expression when purified lymphocytes were incubated with or without L-MTP-PE for up to 72 h (data not shown).

Effect of L-MTP-PE on MCAF nuclear transcription

To determine whether the increased levels of MCAF mRNA described above were related to an increase in the transcriptional activity of the MCAF gene, we compared the transcription rate of nuclei isolated from L-MTP-PE-treated and untreated monocyts. As shown in Fig. 3, the

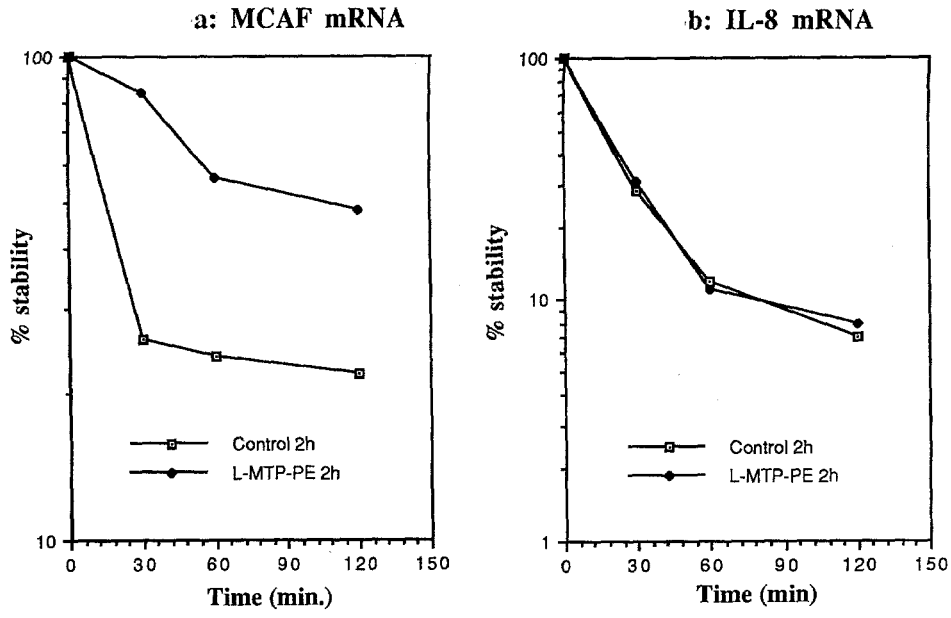
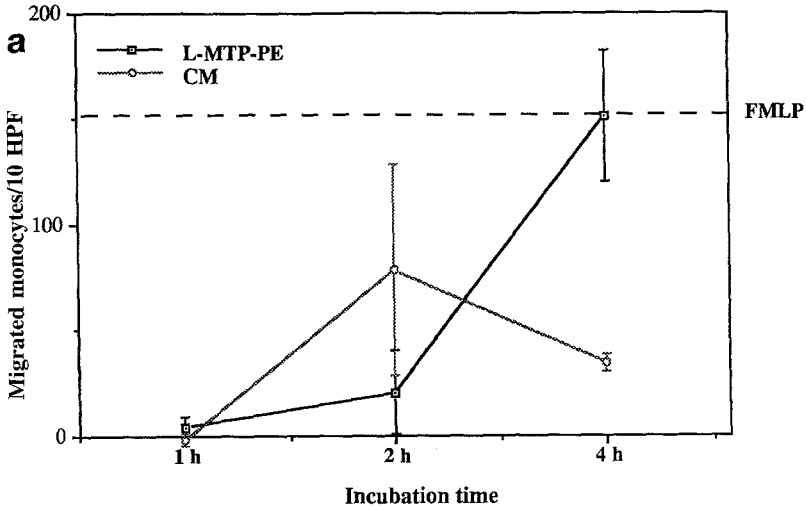


Fig. 4. Effect of L-MTP-PE on MCAF (**a**) and interleukin-8 (IL-8) (**b**) mRNA stability in human monocytes. Monocytes were isolated by elutriation and incubated for 2 h with 2 μg/ml L-MTP-PE, or without L-MTP-PE. Actinomycin D (10 μg/ml) was then added. Total RNA was extracted at various times and analyzed by northern blot, and autoradiographs were scanned. The mRNA level is expressed as a percentage of the mRNA level at time 0, after L-MTP-PE incubation but immediately before actinomycin D was added. One of three experiments



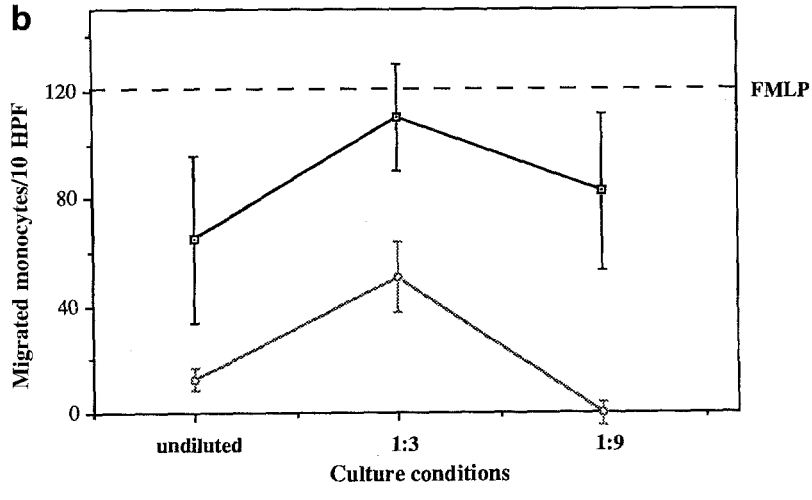


Fig. 5 a, b. Culture supernatants from L-MTP-PE-stimulated monocytes contain chemotactic activity. **a** Human monocytes were incubated with or without L-MTP-PE (2 μg/ml) for the indicated time. The supernatants were harvested and then assayed for chemotactic activity. Results were presented as the average number of migrated monocytes in ten oil-immersion fields (high-power fields). Triplicate determinations were made at each time point. The value obtained from control supernatants was subtracted from that obtained with the L-MTP-PE-stimulated supernatants. **b** Human monocytes were incubated with L-MTP-PE for 4 h. Supernatants were undiluted or diluted with serum-free medium and assayed for chemotactic activity as described in **a**. *FMLP*, *N*-formyl-1-methionyl-1-leucyl-1-phenylalanine

transcription rate of MCAF was greater in monocytes incubated with L-MTP-PE for 2 h than in control monocytes incubated with medium for 2 h. We therefore concluded that the up-regulation of MCAF expression stimulated by L-MTP-PE involved increased transcription of the MCAF gene. The transcription of GAPDH did not change when monocytes were incubated with L-MTP-PE.

Effect of L-MTP-PE on MCAF mRNA and IL-8 stability

To determine if the increase in MCAF mRNA accumulation seen following L-MTP-PE stimulation was in part also due to an increase in the stability of MCAF mRNA, we performed mRNA stability assays. Purified monocytes were first incubated with or wthout L-MTP-PE for 2 h, and then 10 μg/ml actinomycin D was added. Total RNA was extracted at different times following actinomycin D treatment, subjected to northern blot analysis, and quantified by densitometric scanning (Fig. 4). L-MTP-PE treatment increased the half-life of MCAF mRNA at the 2-h time point compared with the control (Fig. 4a). As demonstrated in Fig. 4b, the stability IL-8 mRNA was unaltered by L-MTP-PE. The stability of β-actin mRNA was also not modified by L-MTP-PE treatment (data not shown).

Effect of L-MTP-PE on monocyte chemotaxis

To determine if L-MTP-PE was inducing production of MCAF protein, conditioned supernatants from L-MTP-PE-stimulated monocytes were assayed for chemotactic activity. As demonstrated in Fig. 5, conditioned supernatants from L-MTP-PE-treated monocytes contained significant chemotactic activity as compared to medium alone or supernatants from unstimulated monocytes.

Discussion

The present study demonstrated that L-MTP-PE increased human monocyte expression of MCAF. MCAF mRNA expression was rapidly up-regulated (Fig. 2), but this stimulation persisted for only a short time. By 4 h, MCAF mRNA was at background levels in L-MTP-PE-treated monocytes and no additional changes were observed over 72 h. The up-regulation of this gene appears to be at the transcriptional *and* post-transcriptional levels since both the transcription rate of the gene, as measured by nuclear run-on assays (Fig. 3), and MCAF mRNA half-life (Fig. 4) were elevated following the monocytes' exposure to L-MTP-PE. We were unable to detect any MCAF mRNA in lymphocytes incubated with or without L-MTP-PE. We, therefore, conclude that L-MTP-PE selectively induces MCAF expression in monocytes.

The kinetics of MCAF induction by L-MTP-PE were different from those previously observed for IL-1, IL-6 and IL-8 [25]. While MCAF expression increased at 2 h for only a short period, IL-1, IL-6 and IL-8 mRNA expression increased for up to 72 h with L-MTP-PE stimulation. It is particularly intriguing that IL-8, a cytokine that belongs to the same proinflammatory supergene family and has some homology to MCAF [31], shows such different expression kinetics. In addition, while both MCAF and IL-8 expression are regulated at the transcriptional level (Fig. 3, and T. Asano, A. McWatters, T. An, K. Matsushima, E. S. Kleinerman, unpublished data), IL-8 post-transcriptional mRNA stability was not regulated as it was for MCAF (Fig. 4). Other investigators have also shown different regulation of these two genes [8].

Monocytes/macrophages play an important role in host defense, and they have been identified as a part of the cellular infiltrate in tumors [9, 26, 29]. The presence of inflammatory macrophages in tumors results from the migration of monocytes from the peripheral circulation into the tumor tissue. MCAF is a cytokine that stimulates the chemotaxis of peripheral blood monocytes, activates their oxidative burst, and increases their cytostatic capacity [28, 33, 36]. MCAF has also been shown to regulate the expression of cell-surface adhesion molecules in the β-2 integrin subfamily [16], proteins intimately involved in the movement of leukocytes through tissue and in the binding of monocytes to target cells. Several investigators have demonstrated a correlation between the number of macrophages in a tumor and the production of chemotactic factor by the tumor [2–5]. Transfecting the MCAF gene into melanoma tumor cells has resulted in an increase in tumor-associated macrophages and a slower growth rate of the tumor following inoculation into either syngeneic or nude mice [6]. MCAF-producing Chinese hamster ovary cells are less tumorigenic in vivo and also showed an increase in leukocyte infiltration [32]. Thus, MCAF may contribute to the control of tumor growth both by attracting monocytes to the tumor site and by enhancing their tumoricidal capacity once they arrive.

We have observed peripheral fibrosis with inflammatory macrophages in lung lesions removed from osteosarcoma patients following L-MTP-PE therapy [20]. These findings were unique to L-MTP-PE-treated patients [20]. Since L-MTP-PE is taken up by lung macrophages [30] and can induce MCAF expression, this cytokine may be responsible for the increased macrophage infiltration seen in and around the tumors after L-MTP-PE therapy. MCAF may also have contributed to the fibrosis formation seen in these excised tumors. Both MCAF mRNA and protein are prominent in lung epithelial cells of patients with idiopathic pulmonary fibrosis [1]. One mechanism for this fibrosis is thought to be the persistent monocyte/macrophage infiltration in the lung. Therefore, in addition to the induction of IL-1 and TNF, the up-regulation of MCAF may contribute to the ability of L-MTP-PE to control tumor growth, through the recruitment of monocytes into the tumor area.

In summary, we have demonstrated that liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) up-regulated the expression of the monocyte chemotactic and activating factor (MCAF) gene by increasing both its transcription rate and its post-transcriptional mRNA stability in human monocytes. By attracting and activating monocytes/macrophages, the cytokine MCAF may be an important mediator in the ability of L-MTP-PE to treat metastatic tumors of the lung.

Acknowledgements. This work was supported by grant CA 42992 from the National Cancer Institute and by the Cancer Center Core Grant CA 16672 from the National Cancer Institute, National Institute of Health. We thank Taeha An for technical assistance and Dahliah Garza for preparation of manuscript.

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