MONOCYTE chemotactic protein (MCP-1) is a specific monocyte chemoattractant and activating factor produced by both immune cells (mononuclear phagocytes and lymphocytes) and non-immune cells (parenchymal and stromal cells). In order to define the conditions under which human monocytes express MCP-1, monocytes were exposed to IFN- γ , IL-1 β , TNF- α , IL-4 or PHA under serum free conditions. There was no significant MCP-1 production by monocytes following exposure to IL-1 β , TNF- α or IL-4. In contrast, stimulation with IFN- γ resulted in a dose dependent increase in MCP-1 protein and mRNA expression. Simultaneous stimulation with IFN- γ and IL-1 β or TNF- α resulted in no further increase in MCP-1 production. It is concluded that IFN- γ , primarily a product of T_H1 T lymphocytes, stimulates the expression of MCP-1 by monocytes.

Key words: Chemotaxins, Cytokines, Interferon- γ , Interleukin-1 β , Interleukin 4, Monocytes, Monocyte chemotactic factor-1, Phytohaemagglutinin, Tumour necrosis factor- α

Interferon- γ stimulates monocyte chemotactic protein-1 expression by monocytes

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Introduction

Monocytes are suspected of playing a key role in the development and maintenance of many acute and chronic inflammatory disease states. The recruitment of monocytes from the peripheral blood to a site of inflammation depends upon the generation of chemotactic factors by both immune cells (mononuclear phagocytes and lymphocytes) and non-immune cells (parenchymal and stromal cells). Amplification of the immune response may occur as monocytes elaborate chemotactic factors which recruit additional monocytes to the site of inflammation. Monocyte chemotactic protein-1 (MCP-1) is a potent chemoattractant and activating factor that is specific for monocytes and may play an important role in the pathogenesis of a variety of monocyte dependent disease states including atherosclerosis,¹ rheumatoid arthritis² and idiopathic pulmonary fibrosis.³ Although monocytes have previously been shown to express both peptide and mRNA for MCP-1, the conditions under which they do so are controversial.^{4,5} To date, *in vitro* studies have not consistently demonstrated that monocytes cultured in the absence of serum produce MCP-1 in response to stimuli that might be present near an inflammatory lesion.

In this study, the authors investigated the ability of adherence purified peripheral blood monocytes

cultured in serum free media to produce MCP-1 in response to several stimuli known to activate monocytes—interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ). It was also of interest to determine whether interleukin 4 (IL-4), a product of T-lymphocytes with both pro- and anti-inflammatory properties, would stimulate monocytes to produce MCP-1. The results were compared with those obtained by exposing monocytes to a known inducer of monocyte derived MCP-1, the plant lectin, phytohaemagglutinin (PHA).⁶ Although stimulation with IL-1 β , TNF- α or IL-4 did not result in the production of monocyte derived MCP-1, it was found that exposure to IFN- γ resulted in a dose dependent increase in the expression of MCP-1 protein and mRNA. Simultaneous stimulation with IFN- γ and IL-1 β or TNF- α resulted in no further increase in MCP-1 production. These findings indicate that IFN- γ , primarily a product of the T_H1 subset of T lymphocytes, stimulates the expression of MCP-1 by monocytes.

Materials and Methods

Reagents: Human recombinant IFN- γ was obtained from Genzyme Corporation (Cambridge, MA, USA) and used at a concentration of 10⁵ units/ml. Phytohaemagglutinin (PHA-P; Sigma) was dis-

solved in complete media prior to use. Human recombinant IL-1 β with a specific activity of 30 units/ng was a gift from the Upjohn Co. Human recombinant TNF- α with a specific activity of 22 units/ng was kindly provided by Genentech (San Francisco, CA, USA). Human recombinant IL-4 was purchased from R&D Systems (Minneapolis, MN, USA). Complete media consisted of RPMI 1640 (Whitaker Biomedical Products, Whitaker, CA, USA), without serum, with 1 mM glutamine, 25 mM HEPES, 100 U penicillin and 100 μ g streptomycin/ml.

Cell preparation: Heparinized venous blood was obtained from healthy volunteers and mixed 1:1 with 0.9% saline. Mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation and adherence purified, resulting in a cell population that was >90% monocytes. Cells were plated in either 35 mm or 100 mm culture plates at a final concentration of 1×10^6 cells/ml complete media. Following stimulation, mononuclear cells and supernatants were harvested at predetermined times. Cell-free supernatants were collected and spun at 2000 rpm for 10 min. Cell pellets were extracted for total RNA.

Northern blot analysis: Total cellular RNA from mononuclear cells was isolated using a modification of the methods of Chirgwin *et al.*⁷ and Jonas *et al.*⁸ Briefly, mononuclear cell pellets were overlaid with 3 ml of a solution consisting of 25 mM Tris (pH 8.0) 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, the above suspension was added to an equal volume of 100 mM Tris (pH 8.0) containing 10 mM EDTA and 1.0% SDS. The mixture was then extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was precipitated with alcohol and the pellet dissolved in DEPC water. RNA was separated by Northern blot analysis using formaldehyde/1% agarose gels, transblotted to nitrocellulose, baked, prehybridized, and hybridized with a ³²P 5'-end labelled oligonucleotide probe. The 30-mer oligonucleotide probe was synthesized using published cDNA sequence for human-derived MCP-1.⁹ The MCP-1 probe was complementary to nucleotides 256–285 and had the sequence 5'-TTG-GGT-TTG-CTT-GTC-CAG-GTG-GTC-CAT-GGA-3'. Blots were washed and autoradiographs were quantified using a MacIntosh IIfx computer containing an Image Capture 1000 Frame Grabber (Scion Corp., Walkersville, MD, USA) and image 1.40 software (NIH Public Software, Bethesda, MD). Equivalent amounts of total RNA/gel were assessed by monitoring 28S and 18S rRNA.

MCP-1 ELISA: Antigenic MCP-1 was quantified using a modification of a double ligand method as described previously.¹⁰ Flat-bottomed 96-well microtitre plates (Nunc Immuno-Plate I 96-F) were coated with 50 μ l/well of rabbit anti-MCP-1 (1 ng/ μ l in 0.6 M NaCl, 0.26 M H₃BO₄, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C and then washed with phosphate buffered saline (PBS), pH 7.5, 0.05% Tween-20 (wash buffer). Microtitre plate nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. Supernatants (undiluted or 1:10 dilution) or standards were added and incubated for 1 h at 37°C. Plates were washed four times and 50 μ l/well of biotinylated rabbit anti-MCP-1 added, and incubated for 30 min at 37°C. Plates were washed four times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, McLean, VA, USA) added and then incubated for 30 min at 37°C. Plates were washed four times and chromogen substrate (Bio-Rad) added. The plates were incubated at room temperature to the desired extinction, and the reaction terminated with 50 μ l/well of 3 M H₂SO₄ solution. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of rMCP-1 (100 ng to 1 pg/ml). MCP-1 ELISA detected specific MCP-1 concentrations in a linear fashion greater than 50 pg/ml and did not cross-react with TNF- α , IL-1 β , IL-2, IL-4, IL-6, IL-8, INF- γ , ENA-78, MIP-1 α , MIP-1 β , IP-10, NAP-2, RANTES, MCP-2 or MCP-3.

Statistical analysis: Data were analysed by a MacIntosh II computer using the Statview II statistical software package (Abacus Concepts, Inc., Berkeley, CA, USA) and expressed as means $\pm$ S.E.M. Data that appeared statistically significant were compared by Student's *t*-test and considered significant if *p* values were <0.05.

Results

Monocyte derived antigenic MCP-1 production: It was of interest to learn what types of stimuli resulted in the production of antigenic MCP-1 by Ficoll isolated, adherence purified peripheral blood monocytes cultured under serum free conditions. Monocytes were exposed to either PHA (10 μ g/ml), IL-1 β (2.0 ng/ml), TNF- α (2.0 ng/ml) or IFN- γ (10 000 units/ml) overnight. Cell-free supernatants were examined for the presence of antigenic MCP-1 using a specific MCP-1 ELISA. As shown in Fig. 1, unstimulated monocytes and monocytes stimulated with IL-1 β or TNF- α , did not produce antigenic MCP-1. Similarly, monocytes failed to produce MCP-1 following exposure to IL-4 (10 ng/ml, *n* = 4, data not shown). Monocytes incubated in the presence of IFN- γ produced

FIG. 1. Monocyte derived antigenic MCP-1 expression post-stimulation by PHA, IL-1 β , TNF- α , IFN- γ as determined by ELISA. *Indicates $p < 0.05$ compared with control monocytes at comparable time point (experimental $n = 7$).

1.56 ± 0.21 ng/ml MCP-1, which was significantly increased compared with unstimulated monocytes. By comparison, monocytes stimulated with the plant lectin, PHA, produced 10.78 ± 1.48 ng/ml antigenic MCP-1, which was seven-fold greater than that generated by IFN- γ .

Dose dependent monocyte derived antigenic MCP-1 production: Because IL-1 β and TNF- α are important components of the inflammatory response, an attempt was made to determine whether co-stimulation with IFN- γ and either IL-1 β or TNF- α affected MCP-1 production by monocytes. Monocytes were exposed to varying doses of IFN- γ (1 to 10 000 units/ml) alone or in combination with either IL-1 β (2 ng/ml) or TNF- α (2 ng/ml). MCP-1 production increased in a dose dependent fashion following stimulation with IFN- γ alone (Fig. 2). There was no significant change in the amount of antigenic MCP-1 generated from these cells when they were incubated with IFN- γ and IL-1 β or TNF- α compared with IFN- γ alone.

Expression of monocyte derived MCP-1 mRNA: Northern blot analysis was performed on mRNA obtained from monocytes stimulated with varying concentrations of IFN- γ . As shown in Fig. 3, there was a dose dependent increase in MCP-1 mRNA following stimulation with IFN- γ (10 units/ml to 10 000 units/ml). There was no detectable increase in MCP-1 mRNA following exposure to the lowest dose of IFN- γ tested, 10 units/ml. Of interest was the finding that even at the maximum dose of IFN- γ used (10 000 unit/ml), the amount of steady-state mRNA was only 60% of that generated by

FIG. 2. Dose dependent monocyte derived antigenic MCP-1 expression post-stimulation by IFN- γ , either alone or in combination with IL-1 β , TNF- α , as determined by ELISA. *Indicates $p < 0.05$ compared with control monocytes at comparable time point (experimental $n = 7$).

FIG. 3. Northern blot of monocyte derived MCP-1 post-stimulation by varying doses of IFN- γ , as indicated. Cells were incubated in conditioned media for 24 h. (A) Representative Northern blot of monocytes. (B) Results of densitometry of Northern blot. (C) Corresponding 18S and 28S rRNA for the Northern blot in panel A, demonstrating equivalent loading of RNA (experimental $n = 2$).

FIG. 4. Northern blot of monocyte derived MCP-1 mRNA stimulated by PHA, IL-1 β , or TNF- α , as indicated. Cells were incubated in conditioned media for 24 h. (A) Representative Northern blot of monocytes. (B) Results of densitometry of Northern blot. (C) Corresponding 18S and 28S rRNA for the Northern blot in panel A, demonstrating equivalent loading of RNA (experimental $n = 2$).

stimulation using PHA (100 μ g/ml). There was no apparent expression of MCP-1 mRNA following stimulation with IL-1 β (2 ng/ml) or TNF- α (2 ng/ml) as shown in Fig. 4. MCP-1 mRNA expression was also not detectable following stimulation with IL-4 (10 ng/ml, data not shown).

Discussion

MCP-1 is a secreted 76-amino acid peptide with potent monocyte chemotactic and activating properties.⁶ MCP-1 is a member of the C-C chemotactic cytokine supergene family of pro-inflammatory peptides which includes RANTES,¹¹ MIP-1,¹² MCP-2¹³ and MCP-3.¹³ A variety of cultured cells are able to synthesize MCP-1, including endothelial cells,^{14,15} fibroblasts,¹⁶ vascular smooth muscle cells,¹⁷ keratinocytes,¹⁸ synovial cells,¹⁹ pulmonary alveolar type II-like cells,²⁰ various tumour cells,^{21,22} and peripheral blood mononuclear cells in the presence of serum.^{4,23,24} A serine/threonine protein kinase signalling pathway is thought to mediate the effects of MCP-1 induced monocyte chemotaxis.²⁵

It has been suggested that the inappropriate expression of MCP-1 by immune and non-immune cells may be associated with the pathophysiology of various diseases mediated by monocytes/macrophages.²⁶ Investigators have found MCP-1 protein and mRNA in the abnormal joints of patients with rheumatoid arthritis,² in lung tissue from patients with idiopathic pulmonary fibrosis,³ and in atheromatous plaques from patients with atherosclerosis.^{1,27,28} Increased antigenic MCP-1 was demonstrated in supernatants of cultured pulmonary alveolar macrophages from rats with immune complex-mediated acute lung injury,²⁹ and in bronchoalveolar lavage fluid from rats with glucan induced pulmonary granulomatosis.³⁰ Of interest, monocytes/macrophages were identified as expressing MCP-1 protein or mRNA in each of the above human or experimental diseases. The production of monocyte/macrophage-derived MCP-1 may be an important factor in the initiation or maintenance of these inflammatory states. However, the regulation of monocyte/macrophage-derived MCP-1 is as yet unclear.

In this study the authors attempted to learn under what conditions monocytes cultured in serum free media express MCP-1 mRNA and protein. Although monocyte derived MCP-1 expression was induced by PHA, it was found that monocytes did not generate MCP-1 in response to IL-1 β or TNF- α . In contrast, other investigators have found that IL-1 β and TNF- α both stimulate MCP-1 production from endothelial cells,¹⁵ fibroblasts,³¹ epithelial cells,⁵ vascular smooth muscle cells¹⁷ and mesangial cells.³² IL-4, an immunoregulatory peptide produced primarily by the T_H2 subset of T lymphocytes³³ also did not induce the production of MCP-1. It was found, however, that stimulation with IFN- γ resulted in a dose dependent increase in MCP-1 mRNA and protein. Simultaneous stimulation with IFN- γ and IL-1 β or TNF- α did not alter the response to IFN- γ alone.

IFN- γ has previously been identified as a potent regulator of monocyte and macrophage functions including enhanced monocyte MHC class and II antigen expression,³⁴ increased antimicrobial activity,³⁵ and stimulation of the respiratory burst.³⁶ IFN- γ is largely a product of the T_H1 cell type CD4⁺ T lymphocytes.^{37,38} This subset of helper T cells is predominately involved in delayed type hypersensitivity responses (DTH).³⁸ IFN- γ has been found in many DTH type diseases such as sarcoidosis,³⁹ tuberculoid lesions of leprosy⁴⁰ and experimental *Schistosoma mansoni* infection.⁴¹ Thus, IFN- γ is likely to be present in several types of inflammatory conditions and may be capable of stimulating monocyte/macrophage derived MCP-1 production *in vivo*. It was noted that the IFN- γ stimulated MCP-1 production by monocytes was

modest compared with that of the nonspecific monocyte activator, PHA. It is possible that factors other than IFN- γ may stimulate MCP-1 production by monocytes/macrophages or may act synergistically with IFN- γ . These factors may arise from T lymphocytes or from other cells in the inflammatory milieu. However, the identity of such factors is as yet unknown.

In summary, a dose dependent increase in monocyte derived MCP-1 production was observed following stimulation with IFN- γ under serum free conditions. Monocytes did not produce MCP-1 in response to stimulation with IL-1 β , TNF- α or IL-4. In addition, no change in MCP-1 production was found when cells were stimulated with IFN- γ in the presence of IL-1 β or TNF- α compared with that seen with IFN- γ alone. These findings suggest that the generation of monocyte derived MCP-1 production is signal specific. It is concluded that IFN- γ , primarily a product of T_H1 lymphocytes, is a primary stimulus for the production of monocyte derived MCP-1.

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