Vol. 162, No. 2, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 31, 1989 Pages 694-700

Monocyte Chemotactic Protein Gene Expression by Cytokine-Treated Human Fibroblasts and Endothelial Cells

R.M. Strieter*, R. Wiggins**, S.H. Phan, B.L. Wharram **, H.J. Showell***, D.G. Remick, S.W. Chensue and S.L. Kunkel

*Divisions of Pulmonary and Critical Care Medicine and **Nephrology, Department of Internal Medicine, Department of Pathology, The University of Michigan Medical School Ann Arbor, Michigan

***Department of Immunology, Pfizer Central Research, Groton, Connecticut

Received June 14, 1989

A number of cytokines are active during the evolution of an inflammatory response, including tumor necrosis factor-α, interleukin-1, and novel chemotactic cytokines. This latter group of mediators belong to supergene families of inflammatory signals that play a key role in the selective recruitment of immune cells. In this presentation, we present data demonstrating, for the first time, endothelial cell expression of monocyte chemotactic protein (MCP) mRNA induced by LPS, interleukin-1 or tumor necrosis factor. Human fibroblasts were also found to express monocyte chemotactic factor mRNA in response to interleukin-1 or tumor necrosis factor, but LPS was not effective. In addition, neither primary cultures expressed MCP in response to interleukin-6. These studies demonstrate that non-immune "bystander" cells can play an active role in the recruitment of inflammatory cells via the production of novel chemotactic cytokines. © 1989 Academic Press, Inc.

The elicitation of leukocytes into various tissues constitutes a key element in the inflammatory response that is common to a variety of injurious conditions, including cancer, infection, and trauma. The events that lead to leukocyte elicitation and activation are complex, as various leukocyte populations must first adhere to the vascular endothelium and then undergo directed migration in response to a gradient of chemotactic factor(s). Interestingly, the composition of the inflammatory cell infiltrate changes as the immune response evolves. It is apparent that selective chemoattractants must be synthesized in a coordinate manner in order for the normal cascade of cellular events to occur. Recent studies have identified novel chemotactic cytokines that may participate in this process via the selective recruitment of inflammatory cells (1-7). One of these cytokines is a neutrophil/lymphocyte chemotactic factor (1-4), while another is a monocyte chemotactic protein (MCP) (5-7).

Abbreviations used in this manuscript: MCP, monocyte chemotactic protein; IL-1, interleukin-1; IL-6, interleukin-6; TNF, tumor necrosis factor; LPS, lipopolysaccharide.

0006-291X/89 $1.50
Copyright © 1989 by Academic Press, Inc.
All rights of reproduction in any form reserved.

In this manuscript, novel studies are presented demonstrating MCP gene expression by cytokine-stimulated human fibroblasts and endothelial cells. While either interleukin-1 (IL-1) or tumor necrosis factor (TNF) induced a concentration and time-dependent expression of MCP mRNA by both primary cultures, only lipopolysaccharide (LPS)-challenged endothelial cells expressed MCP activity. An additional cytokine, interleukin-6 (IL-6) was ineffective in either culture system. Thus, the expression of MCP by stromal cells and endothelial cells demonstrated stimulus specificity. These studies are important, as they identify populations of non-immune cells that possess potent effector activity with regard to the elicitation of blood monocytes.

MATERIALS AND METHODS

Cell Populations: Human endothelial cells were isolated from umbilical veins according to previously published procedures (8). The recovered endothelial cells were cultured in medium 199 supplemented with 100 ug/ml endothelial cell growth supplement, 20% fetal bovine serum, and 100 ug/ml bovine lung heparin. All cells in these studies were used prior to their fifth passage in culture. Primary human foreskin fibroblasts (4-8 passage) were grown to confluence in RPMI 1640 containing 1 mM glutamine, 25 mM HEPES, 100 units/ml penicillin, 100 ug/ml streptomycin, plus 10% fetal calf serum. On the day of use, cells were washed and either cytokines or LPS were added for the specified times and doses.

Reagents: Human recombinant IL-1β with specific activity of 30 U/ng was the generous gift of the Upjohn Company. Human recombinant TNF-α (20 U/ng, specific activity) was a gift of the Cetus Corporation. Human recombinant IL-6 (20 U/ul) was purchased from Genzyme Inc. Lipopolysaccharide (0111:B4) was purchased from Sigma (St. Louis, MO).

Northern Blot Analysis: Total endothelial cell RNA was extracted using a modification of the methods of Jonas *et al.* (9) and Chirgwin *et al.*(10). The adherent endothelial cells were solubilized in a solution of 25 mM Tris, pH 8.0 containing 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M beta-mercaptoethanol. After homogenization, an equal volume of 100 mM Tris, pH 8.0 containing 1.0% SDS and 10 mM EDTA was added and the RNA extracted with chloroform-phenol. The alcohol precipitated RNA was separated by formaldehyde/1% agarose gels and transblotted to nitrocellulose. The baked blots were prehybridized and then hybridized with a ^{32}P-5-end-labeled 30-mer oligonucleotide probe. The probe was complementary to nucleotides 256-285 of published cDNA sequence for human monocyte chemotactic and activating factor/monocyte chemoattractant protein (5,6). The sequence of the probe was 5'-TTG-GGT-TTG-CTT-GTC-CAG-GTG-GTC-CAT-GGA-3'. Blots were stringency washed and autoradiographs were quantitated by laser densitometry. Equivalent amounts of total RNA load per gel lane were assessed by monitoring 28s and 18s rRNA.

Monocyte Chemotactic Protein Assay: Human monocytes were prepared from peripheral blood by Ficoll-Hypaque density gradient centrifugation. Monocytes were suspended at 2 x 10^6 cells/ml in phosphate-buffered saline containing 0.2% bovine serum albumin with greater than 95% viability. Chemotaxis was performed using a modification of the method of Harvath *et al.* (11). Briefly, 30 ul of diluted supernatant specimen, 10^{-7} M formyl methionylleucylphenaylalanine (fMLP, Sigma Chemical Co., St. Louis, MO) or HBSS alone were placed in duplicate bottom well of a 48-well microchemotaxis chamber (Neuro probe, Cabin John, MD). A 5 micron pore-size polycarbonate filter (polyvinylpyrolidone-free, Nucleopore, Corp.) was placed in the assembly and 50 ul of monocyte suspension placed in each of the top wells. After incubating at 37°C in humidified 95% air/5% CO_2 for 2 hrs, the filters were removed, fixed in methanol, and stained with Diff-Quik (American Scientific Products, McGraw Park, IL) Monocytes that had migrated through the bottom of the filter were counted in 10 high power fields. Chemotactic activity was assessed as percent of the fMLP positive control.

RESULTS

MCP mRNA Expression by TNF-Treated Endothelial Cells and Fibroblasts: In initial experiments, human endothelial cells and fibroblasts were stimulated with graded doses of recombinant human TNF and assessed for the expression of MCP mRNA. As shown by the Northern blot analysis data in Figure 1, MCP mRNA was expressed over a wide concentration range of TNF. Laser densitometry of the Northern blot demonstrated that significant levels of MCP mRNA were induced with as little as 200 pg/ml TNF (Figure 1B). A plateau in MCP mRNA expression by both endothelial cells and fibroblasts was reached at 20 ng/ml TNF. The top insert in Figure 1 shows the actual Northern blot, while the bottom insert contains the corresponding 28s and 18s rRNA. From the Northern blot analysis MCP mRNA was identified as a 0.7 Kb species. Unstimulated cells did not constitutively express MCP mRNA. In parallel studies, supernatants of TNF-treated cells showed a similar dose-dependent increase in monocyte chemotaxis. Both endothelial cells and fibroblasts demonstrated maximum production of monocyte chemotactic activity at a dose of 20 ng/ml TNF.

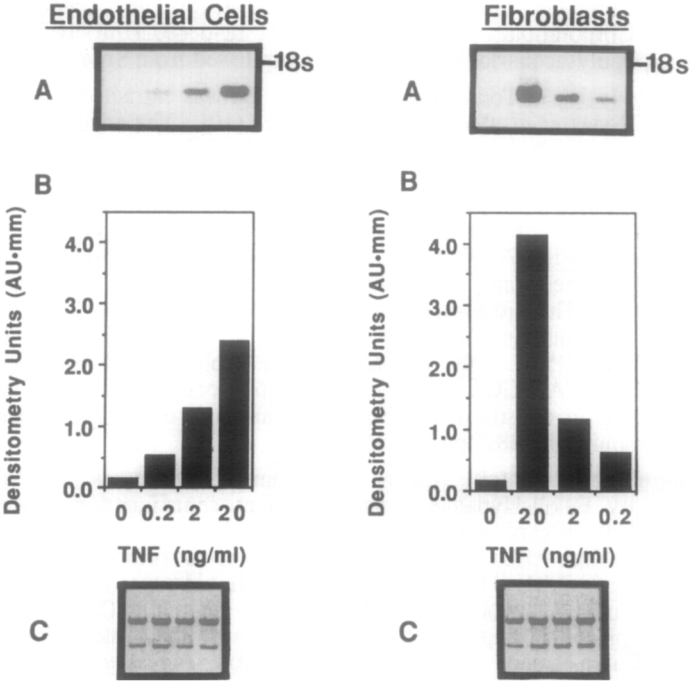

Figure 1. Expression of MCP mRNA by TNF-treated endothelial cells and fibroblasts. Primary cultures of both human cell types were treated with graded doses of recombinant TNF for 8 hours. **Insert (A)** is the Northern blot analysis of MCP mRNA which was quantitated by laser densitometry **(B)**. **Insert (C)** is the 28s and 18s rRNA found in each lane.

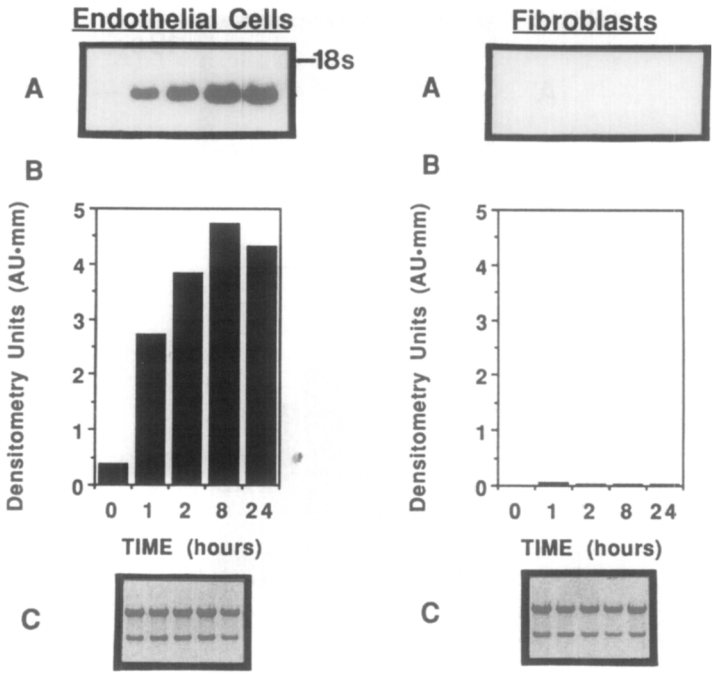

Figure 2. Stimulus specificity for MCP mRNA expression between LPS-treated human endothelial cells and fibroblasts. Northern blot analysis (**A**) demonstrated a time-dependent expression of MCP by LPS-treated endothelial cells, but not LPS-treated fibroblasts. Laser densitometry (**B**) of the MCP mRNA from LPS-stimulated endothelial cells showed a rapid expression by 1 hour and a plateau by approximately 4 hours. Equal amounts of RNA per lane is shown by the 28s and 18s rRNA (**C**).

Stimulus Specificity for MCP mRNA Expression:

Further studies demonstrated stimulus specificity regarding the expression of MCP mRNA by endothelial cells and fibroblasts. LPS-treated endothelial cells demonstrated a time-dependent increase in MCP mRNA levels, while human fibroblasts were not susceptible to LPS challenge (Figure 2). Endothelial cells exposed to 1 ug/ml of LPS exhibited a rapid and protracted increase in MCP mRNA. Levels were clearly observed 1 hour post-stimulation, reached a peak between 2-8 hours, and were maintained over the next 16 hours. On the contrary, MCP mRNA expression by LPS-treated fibroblasts was not detectable throughout the entire 24 hours study period.

Time-Dependent Induction of MCP mRNA by IL-1β:

In order to determine the time-dependent increase in MCP mRNA by a specific cytokine, endothelial cells were treated with 20 ng/ml of IL-1β. As shown in Figure 3, endothelial cells treated with IL-1β rapidly expressed MCP mRNA with a significant signal by 30 minutes. The laser densitometer scans (Figure 3B) of the Northern blots (Figure 3A)

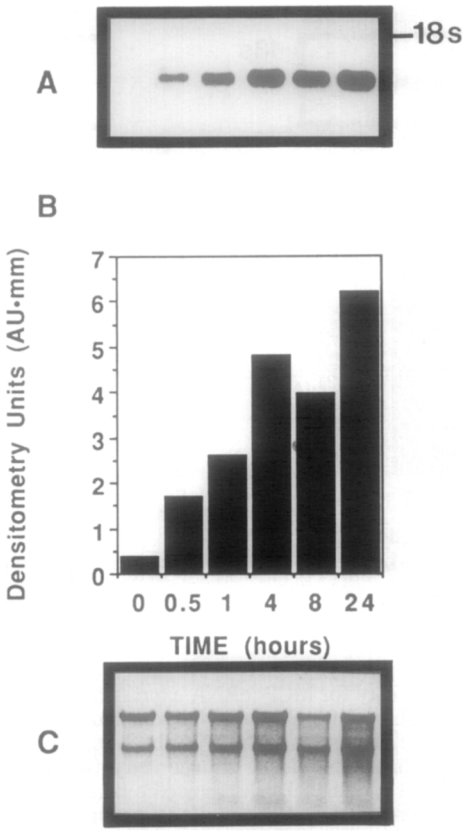

Figure 3. Time-dependent expression of MCP mRNA by IL-1β stimulated endothelial cells. Northern blot analysis **(A)** of mRNA isolated from endothelial cells treated with 20 ng/ml IL-1β, was quantitated by laser densitometry **(B)**. The bottom insert **(C)** shows equal 28s and 18s rRNA.

demonstrated that the mRNA levels plateaued at 4 hours and were maintained over the next 20 hours. The bottom insert again represents 28s and 18s rRNA of the Northern blot studies. Although the Northern blots and bioactivity data are not shown, MCP levels by IL-1β treated fibroblasts demonstrated a similar expression/production pattern.

DISCUSSION

Accumulating evidence supports the hypothesis that endothelial and stromal cells are active participants of an immune response. For example, endothelial cells are increasingly being recognized as important effector cells during inflammation. This is especially true of cytokine-stimulated human endothelial cells, which can be induced to express both surface adherence proteins (12), as well as synthesize interleukin-1 (13), chemotactic cytokines (3), hematopoietic growth factors (14), and procoagulant-like activity (15). In addition,

fibroblasts are known to undergo phenotypic alterations during inflammation and can synthesize a number of important signals, including prostaglandin E_2 (16), IL-1 (17), IL-6 (18), and colony stimulating factor (19). The synthesis of the above factors place endothelial cells and fibroblasts in a pivotal role during the initiation and maintenance of inflammation, while the production of collagen and non-collagen components of the extracellular matrix by key fibroblast and endothelial cells are important to end-stage inflammatory reactions.

In further studies, we now demonstrate that both human primary cultured endothelial cells and fibroblasts can express a novel chemotactic cytokine with specificity for monocytes (monocyte chemotactic protein). Both IL-1 and TNF were effective in inducing the expression of MCP mRNA and bioactivity from fibroblasts and endothelial cells. The expression of MCP by these primary cultures was not a global response to all cytokines, as interleukin-6 was inactive in this system. Interestingly, LPS or cytokine-treated monocytes themselves did not produce MCP. These studies are important as they demonstrate that non-immune "bystander" cells synthesize key mediators that participate in the orchestration of an immune response. The production of novel chemotactic cytokines by fibroblasts and endothelial cells, is especially noteworthy, as coordinated communication circuits between the interstitium and vascular bed may dictate the initiation and maintenance of inflammation.

Acknowledgments: These studies were supported in part by National Institutes of Health grants DK38149, HL31963, and HL35276. Dr. Kunkel is an Established Investigator of the American Heart Association. The authors thank Dr. Glen Andrews for the synthesis of the oligonucleotide and Mrs. Kathleen Atkins for excellent secretarial support.

REFERENCES

1. Waltz, A., Peveri, P, Aschauer, H., Baggiolini, M. (1987) Biochem. Biophys. Res. Commun. 149, 77-761.
2. Yoshimura T., Matsushima, K., Tanaka, S., Robinson, E.A., Appella, E., Oppenheim, J.J., Leonard, E.J. (1987) Proc. Natl. Acad. Sci. USA 84, 9233-9237.
3. Strieter R.M., Kunkel, S.L., Showell, H.J., Remick, D.G., Phan, S.H., Ward, P.A., Marks, R.M. (1989) Science 243, 1467-1469.
4. Larsen, C., Anderson, A.O., Appella, E., Oppenheim, J.J., and Matsushima, K. (1989) Science 243, 1464-1466.
5. Furutani, Y., Nomura H., Notake, M., Dyamada, Y., Fukui, T., Yamada, M., Larsen, C.G., Oppenheim, J.J., Matsushima, K. (1989) Biochem. Biophys. Res. Commun. 159, 249-255.
6. Yoshimura, T., Yuhki, N., Moore, S.K., Appella, E., Lerman, M., Leonard, E.J. (1989) FEBS Ltr 244, 487-493.
7. Larsen, C.G., Zachariae, Oppenheim, J.J., Matsushima, K., (1989) Biochem. Biophys. Res. Commun. 160:1403-1408.

8. Jaffe, E.S. (1984) in, Biology of Endothelial Cell (Jaffe, E.A., editor) Martines Nijhoff, The Hague pp. 1-29.
9. Jonas, E., Sargent, T.D., David, I.B. (1985) Proc. Natl. Acad. Sci. USA 82, 5413-5417.
10. Chirgwin, J.M., Przbyla, E.A., MacDonald, R.J., and Rutter, W.J. (1979) Biochem. 18,5294-5299.
11. Harvath, L., Falk, W., Leonard, E.J. (1980) J. Immunol. Meth. 37, 39-45.
12. Pober, J.S., Gimbone, M.A., Lapierre, L.A., Mendrick, D.L., Fiers, W., Rothelein, R., and Springer, T.A. (1986) J. Immunol. 137, 1893-1905.
13. Nawroth, P.P., Bank, I., Handley, D., Cassimeris, J., Chess, L., and Stern, D. (1987) J. exp. Med. 163, 1363-1374.
14. Broudy, V.C., Kaushansky, K., Segal, G.M., Harlan, J.M., Adamson, J.W. (1986) Proc. Natl. Acad. Sci. USA 83, 7467-7474.
15. Bevilacqua, M.P., Pober, J.S., Majeau, G.R., Fiers, W., Cotran, R.S., and Gimbrone, M.A. (1986) Proc. Natl. Acad. Sci. USA 83, 4533-4541.
16. Elias, J.A., Gustilo, K., Baeder, W., Freundlich, B. (1987) J. Immunol. 138, 3812-3816.
17. Le, J., Weinstein, D., Gubler, V., Vilcek, J. (1987) J. Immunol. 138, 2137-2142.
18. Kohase, M., Henriksen-DeStefano, D., Hay, L.T., Vilcek, J. (1986) Cell 45, 659-633.
19. Zucali, J.R., Broxmeyer, H.E., Gross, M.A., Dinerallo, C.A. (1988) J. Immunol. 140, 840-851.