Costimulation of Fibroblast Collagen and Transforming Growth Factor β₃ Gene Expression by Monocyte Chemoattractant Protein-1 via Specific Receptors

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Recent studies indicate potential roles of monocyte chemoattractant protein-1 (MCP-1) in recruitment of monocytes to sites of inflammation. However, their increased expression does not always correlate with monocyte influx, suggesting other possible biological activities for this member of the C-C chemokine family. In view of its potential role in regulating extracellular matrix expression in fibrotic disorders, the effects of MCP-1 on lung fibroblast collagen expression were evaluated. Isolated rat lung fibroblasts were treated with increasing doses of MCP-1 for variable periods of time and examined for effects on collagen synthesis and expression of procollagen α₁(I) mRNA expression. The results show that MCP-1 was able to stimulate collagen expression in these cells in a dose-dependent manner but required over 24 h for significant elevation to occur. In view of this delayed time course, the possibility of mediation via endogenous transforming growth factor β (TGFβ) was tested by the ability of anti-TGFβ antibody to inhibit this MCP-1 stimulation of collagen expression. Significant but incomplete inhibition by this antibody was observed. Pretreatment of the cells with antisense but not by sense or missense TGFβ₁ oligodeoxyribonucleotides caused essentially complete inhibition of this MCP-1 stimulatory effect. Furthermore, MCP-1 treatment was found to also stimulate TGFβ secretion and mRNA expression, which was also abolished by pretreatment with antisense TGFβ₁ oligodeoxyribonucleotides. The kinetics of TGFβ expression indicate that significant increase preceded that for collagen expression. Binding studies using [¹²⁵]I-labeled MCP-1 indicated the presence of specific and saturable binding sites with a dissociation constant consistent with the dose response curves for stimulation of fibroblast collagen synthesis and TGFβ activity by MCP-1. These results taken together suggest that MCP-1 stimulates fibroblast collagen expression via specific receptors and endogenous up-regulation of TGFβ expression. The latter then results in autocrine and/or juxtacrine stimulation of collagen gene expression.

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The abbreviations used are: MCP, monocyte chemoattractant protein-1; TGF, transforming growth factor; ODN oligodeoxyribonucleotide.

EXPERIMENTAL PROCEDURES

CdfS—Rat lung fibroblasts were isolated from 150–175 g male Fisher 344 rats by trypsinization of lung tissue as described previously (20). Dulbecco’s modified Eagle’s medium was supplemented with 10% fetal bovine serum and passed by trypsinization. Only cells earlier than the tenth passage from primary culture were used for these studies.
A mink lung epithelial cell line was used for TGFβ assay. These cells were obtained from the American Type Culture Collection, Rockville, MD, and were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1% antibiotics, and 2 mM L-glutamine. For preparation of rat lung fibroblast conditioned media, cells were grown to confluence and washed; then the medium was replaced with serum-free Dulbecco’s modified Eagle’s medium containing 2.5% bovine serum albumin (Bovinum reagent pure powder, Intergen Company, Purchase, NY), supplemented without or with the indicated concentrations of recombinant human (R&D Systems, Minneapolis, MN) or rat (PeproTech, Inc., Rocky Hill, NJ) MCP-1. They were incubated at 37°C in a humidified atmosphere at 5% CO2 for the indicated times, after which each indicated incubation period, the medium was collected for TGFβ assay. The conditioned medium was stored frozen at −70°C until assay and characterization. After removal of the DNA, total RNA was extracted from the cells. Some cells also were used for slide preparation for in situ hybridization. The rat and human MCP-1 gave similar responses; therefore, unless otherwise stated, all experiments described used human MCP-1.

Collagen Synthesis Assay—Rat lung fibroblasts were grown to confluence in 12-well (22-mm diameter) tissue culture dishes and treated with the indicated concentration of recombinant human MCP-1 diluted in serum-free Dulbecco’s modified Eagle’s medium containing 2 ng/ml bovine serum albumin and 50 ng/ml ascorbic acid for the indicated times. The cells were pulsed for the final 6 h with [3H]proline in the presence of bovine serum albumin and dialyzed, as described previously (39). Collagen synthesis was expressed as the collagenase-susceptible incorporated radioactivity in dpm/105 cells.

TGFβ Assay—This assay was performed on fibroblast conditioned medium prepared as described above using methods described previously (40–42). Samples were activated by acidification prior to assay, as described previously (40–42). Activity was expressed as ED50 units, the dose necessary to cause 50% inhibition of [3H]thymidine incorporation by the mink lung epithelial cells, which was reversible by specific anti-TGFβ antibody (mouse monoclonal antibody 1D11.6, which neutralizes TGFβ1 with weak cross-reactivity to TGFβ3) (Cetrix Pharmaceuticals, Santa Clara, CA). When compared to a standard curve generated with recombinant TGFβ1 (R&D Systems), 1 ng/ml was equivalent to 1–5 pg of the porcine protein. Given the isomeric specificity of the antibody, the activities reported by this assay were specific for TGFβ1 and TGFβ2, as noted above.

mRNA Isolation and Northern Blot Hybridization—Rat lung fibroblasts were grown in 75-cm2 tissue culture flasks to confluence as described above and treated with or without the indicated concentration of recombinant human MCP-1 for the indicated times. After removal of medium, total RNA was extracted from the cells as described previously (41–46). Briefly, the cells were lysed with 4 M guanidine isothiocyanate solution and then extracted twice with phenol:chloroform:isoamyl alcohol. Equal amounts (20 μg) of total RNA were separated by electrophoresis in formaldehyde-containing 1% agarose gels. Equal loading of RNA was judged based on the ethidium bromide-stained gels under ultraviolet light as well as by hybridization with a cytoplasmic probe. The mRNA was transblotted onto nitrocellulose membranes, baked for 2 h at 80°C in a vacuum oven, prehybridized, and hybridized with a synthetic oligonucleotide antisense probe. The 30-base oligodeoxyribonucleotide antisense probes for procollagen α1(I) and TGFβ1, and MCP-1 were synthesized by an automated DNA synthesizer (Gene Assembler) and purified by high-performance liquid chromatography before use. The sequences for these probes were: 5′-AGGGCTGACCTCCCTGCCG-3′ for procollagen α1(I) (22); 5′-GAGTTGGGATGTGAGCTTTTG-3′ for TGFβ1 (42); and 5′-GATGATGATGATGAGGTGATGATTGGG-3′ for MCP-1 (12). The probes were labeled with 32P using a 3′ end-labeling, as described previously (41). After overnight hybridization at 56°C, blots were washed as described previously (22). Autoradiograms developed from these blots were quantitated using the Ambis Optical Imaging System (Ambis, San Diego, CA) (49), and the results were expressed as random integration units. For quantitative analysis, results were normalized to the signal produced with the rat cytoplasmic probe (16).

In Situ Hybridization—In situ hybridization was undertaken using the same 30-base oligonucleotide antisense probes for rat procollagen α1(I) and TGFβ1, whereas the corresponding sense probes were used for negative control purposes. Probes were labeled with 32P-labeled dATP by 3′-end labeling and purified by electrophoresis.

Confluent rat lung fibroblasts as described above and treated with the indicated dose of MCP-1 for the indicated times were detached by brief treatment with 0.01% trypsin and 1 mM EDTA and neutralized with 1% fetal bovine serum. After cell counting, the suspension was adjusted to 5 × 105 cells/ml, and 100 μl were applied per slide for centrifugation. In situ hybridization was performed as described previously (47–49). Rat lung fibroblasts were fixed in 4% paraformaldehyde for 20 min, washed in 2× standard saline citrate, treated with 0.2% HCl, and 0.25% acetic anhydride (v/v) in triethanolamine after sequential washes in 2× standard saline citrate. Slides were prehybridized and hybridized with the indicated radiolabeled oligonucleotide probe, followed by sequential washing with various dilutions of standard saline citrate washes. After each slide was air-dried and dipped in Kodak nitro blue tetrazolium emulsion solution. The emulsion-coated slides were air-dried in the dark and stored in a desiccating chamber at 4°C for 2 days for collagen and 10 days for TGFβ and MCP-1. They were then developed in Kodak D-119 developer, washed and counterstained with hematoxylin and eosin, and coverslipped. Controls for in situ hybridization included the following: (a) hybridization using the corresponding sense probes instead of the antisense probe; (b) pretreatment of slides with 100 μg/ml of RNase at 37°C for 45 min before the addition of antisense probe; and (c) slides made from normal rat lung fibroblasts treated with interleukin 1α (2 ng/ml) for 24 h as positive control for in situ hybridization analysis of TGFβ1 and MCP-1 mRNA.

RESULTS

MCP-1 Binding Studies—[3H]-Labeled recombinant human MCP-1 (2200 Ci/mmol, DuPont NEN) was used to test the presence of MCP-1 binding sites on rat lung fibroblasts. Confluent cells in 6-well (35-mm diameter wells) tissue culture plates were pre-chilled on ice in serum-free medium supplemented with 2 ng/ml bovine serum albumin. The cells were then treated with the indicated doses and times, and then assessed for collagen synthesis as described above.

The inhibition of TGFβ1 expression was also attempted by pretreatment of cells with antisense TGFβ1 ODN. Where indicated, confluent fibroblasts were pretreated for 24 h with 10 μM sense, missense, or antisense ODN in serum-free medium supplemented with 2 ng/ml bovine serum albumin. The cells were then treated with the indicated agonists, the conditioned medium was harvested, and the cells were extracted for total RNA for Northern analysis or detached for in situ hybridization analysis as described above. The ODNs used for these studies were: sense, 5′-CAGAGCCCAAAGGCTAC-3′; antisense, 5′-GATGCCCTTGGGCTG-3′; and missense, 5′-AAGATTTCAC-3′.

Preliminary studies indicated that ODN doses >10 μM did not cause significant further inhibition of TGFβ1 expression in these cells, whereas doses 50 μM caused significantly less inhibition. None of the doses tested caused detectable toxicity, as judged by trypan blue exclusion, or impaired protein synthesis, measured by radioactive proline incorporation (data not shown).

MCP-1 Expression—Both human and rat MCP-1 caused significant stimulation of rat lung fibroblast collagen synthesis. Human MCP-1 did this at doses >100 ng/ml, approaching maximal stimulation at 400 ng/ml (Fig. 1). Peak stimulation, however, required >16 h of treatment. This stimulation was also observed at the mRNA (for α1(I) procollagen) level, as shown in the Northern blots in Fig. 2. Here again,
over 24 h were required for maximal stimulation by 200 ng/ml MCP-1. In view of this delay in the time course for stimulation of collagen gene expression, the possibility that this could be mediated by an intermediate signal also stimulated by MCP-1 was explored. Since these fibroblasts are known to be inhibited by anti-TGF-β antibody at concentrations found to completely inhibit TGF-β activity in the conditioned medium. Doubling the dose of antibody did not cause further inhibition. Nonimmune IgG had no significant effects on collagen synthesis (data not shown).

Given this incomplete inhibition by anti-TGF-β antibody treatment, an alternative approach was also used to abrogate endogenous TGF-β expression. When cells were pretreated with a TGF-β1 antisense but not sense ODN, MCP-1 stimulation of collagen synthesis was completely suppressed (Fig. 3). This inhibition was also seen at the α1(I) procollagen mRNA level as well, without significant effects on cyclophilin mRNA (Fig. 4). To confirm that the TGF-β1 antisense ODN caused inhibition of MCP-1 stimulation of collagen expression was due to suppression of endogenous TGF-β expression, the antisense studies were repeated to evaluate for effects on fibroblast TGF-β production. Fig. 4 also shows that TGF-β1 antisense but not sense ODN pretreatment inhibited TGF-β1 steady-state mRNA levels.

Similar inhibitory effects on TGF-β activity secretion were seen with antisense but not sense or missense ODN (data not shown). TGF-β1 antisense ODN treatment also inhibited basal levels of collagen synthesis and mRNA expression (50–53), and in this study, the results confirm the Northern blot data (Fig. 5) and further show that the cells responded in a heterogeneous manner (Fig. 5C), with some cells showing striking increases in collagen mRNA while others barely responded.

Effects of MCP-1 on TGF-β Expression—Given the dramatic inhibitory effects of TGF-β1 antisense ODN on MCP-1 stimulation of collagen gene expression, the effects of MCP-1 on fibroblast TGF-β expression itself was examined as a way of confirming the importance of this autocrine/juxtacrine loop in the MCP-1 effects on collagen expression. Fig. 6 shows that MCP-1 also stimulated secretion of TGF-β activity by rat lung fibroblasts, while the right lane represents mRNA from control cells, while the right lane represents mRNA from MCP-1-treated cells.

Fig. 3. Effects of TGF-β1 antisense and sense ODNs on MCP-1 stimulation of fibroblast collagen synthesis. Rat lung fibroblasts were pretreated with TGF-β1 sense or antisense ODNs for 24 h. They were then treated with 200 ng/ml MCP-1 for 48 h and pulsed with [3H]proline during the final 6 h of incubation. Collagen synthesis was expressed as dpm of proline incorporated per 10^5 fibroblasts and shown as means (bars, S.E.) of triplicate samples.
blasts in a dose-dependent manner. The activities detected in the conditioned medium were in the range of 1–50 ng/ml in equivalent porcine TGFβ1 protein mass. The response, however, was more rapid compared to that for stimulation of α1(I) procollagen gene expression, with significant stimulation as early as 12 h (Fig. 7). When the kinetics were compared at the mRNA level, a similar correlation was found (Fig. 8). Basal α1(I) procollagen mRNA levels gradually increased over time in culture; however, treatment with MCP-1 caused a substantially greater stimulation in mRNA levels. Steady-state TGFβ1 mRNA levels were also stimulated by MCP-1 with a kinetics that correlated well with that for the α1(I) procollagen mRNA (Fig. 8). Cyclophilin mRNA was not significantly affected by MCP-1 treatment.

MCP-1 Binding Studies—In view of the above functional responses to MCP-1, rat lung fibroblasts were evaluated for the presence of MCP-1 receptors. Incubation of these cells with increasing doses of radiolabeled MCP-1 showed dose-dependent increases in specific binding that approached saturation at 20 nM (Fig. 9A). Scatchard analysis revealed Kd = 5.0 ± 1.3 × 10−10 M and Bmax = 7.4 ± 1.1 × 10−11 M (Fig. 9B), which translates to 2.23 × 104 binding sites per cell. Binding at this temperature showed a lag time of about 30 min, before it rapidly approached peak values at 60 min (Fig. 10).
binding kinetics suggest that MCP-1 stimulated fibroblast collagen and TGFβ expression via these receptors.

**DISCUSSION**

MCP-1 is a specific and potent chemoattractant for monocytes and has been implicated in a variety of inflammatory and fibrotic diseases, the pathogenesis of which is known to involve infiltration and activation of monocytes (12). Up-regulation of MCP-1 production has been shown in pulmonary fibrosis in animal models (12). A number of cell types, including macrophages, lymphocytes, and fibroblasts, have been shown to both produce and/or be modulated by MCP-1 and another C-C chemokine macrophage inflammatory protein-1α (21). In this context, the specific roles of MCP-1 in these diseases have not been definitively explored. Thus, the basis for increased MCP-1, TGFβ collagen synthesis and deposition in pulmonary fibrosis remains unclear. In this study, we have examined the possibility that MCP-1 may be involved in the production of mediators or cytokines with fibrogenic properties. To test this hypothesis, we examined first the effect of MCP-1 on rat lung fibroblast collagen synthesis. The results show that MCP-1 increased fibroblast collagen synthesis, with highest responses observed at a dose of MCP-1 ≥ 200 ng/ml. These doses of MCP-1 are comparable to those found to stimulate monocyte chemotaxis (54, 55) and basophil (56–58) and monocyte activation (59). Since mixed monocyte and lymphocyte cultures easily secrete a minimum of 40 nM (approximately 400 ng/ml) MCP-1 into their conditioned medium (60), it is not inconceivable that these effective doses of MCP-1 could be encountered in vivo.

On the basis of the selective stimulatory effect on collagenous protein synthesis, the activity could be mediated by costimulation of TGFβ expression. This possibility was supported by the following findings. (a) TGFβ activity was significantly elevated in conditioned medium of MCP-1-stimulated fibroblasts with a time course which correlated with the increases in collagen synthetic rate. (b) Fibroblasts stimulated with MCP-1 contained significantly higher steady-state levels of TGFβ1 mRNA, which again preceded the peak increase in procollagen α1(I) gene expression. (c) The levels of TGFβ activity in conditioned medium of MCP-1-stimulated cells were in the range known to stimulate collagen synthesis in these cells (42). (d) MCP-1-induced increases in collagen and TGFβ1 gene expression were inhibited by both anti-TGFβ antibody and TGFβ1 antisense ODN. (e) The presence of specific MCP-1 binding sites with comparable kinetics as that for the observed functional effects confirm these new biological activities of MCP-1. Taken together, these results suggest that MCP-1 stimulates collagen synthesis indirectly via endogenous up-regulation of TGFβ gene expression, which could then stimulate collagen gene expression via an autocrine and/or juxtacrine loops. These effects appear to be mediated by specific receptors expressed by these cells. These novel findings indicate potentially important new roles for this C-C chemokine in the pathogenesis of a number of fibrotic diseases and in tissue repair.

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REFERENCES

1. Rollins, B. J. (1992) Am. J. Respir. Cell. Mol. Biol. 7, 126–127
2. Strowiak, R. M., and Kunkel, S. L. (1993) in The Chemokines (Lindley, I. J. D., Westwick, J., and Kunkel, S. L., eds) pp. 19–28, Plenum Publishing Corp., New York
3. Oppenheim, J. J., Zachariae, C. O., Mukaida, N., and Matsuhashi, K. (1991) Annu. Rev. Immunol. 9, 617–648
4. Strieter, R. M., Wiggins, R., Phan, S. H., Wharram, B. L., Showell, H. J., Remick, D. G., Chenese, S. W., and Kunkel, S. L. (1989) Biochem. Biophys. Res. Commun. 162, 694–700
5. Carr, M. W., Roth, S. J., Luther, E., Rose, S. S., and Springer, T. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 91, 1652–1656
6. Yoshimura, T., Robinson, E. A., Tanaka, S., Appelia, E., and Leonard, E. J. (1989) J. Immunol. 142, 1956–1962
7. Yoshimura, T., and Leonard, E. J. (1990) J. Immunol. 144, 2377–2383
8. Yoshimura, T., and Leonard, E. J. (1990) J. Immunol. 144, 2377–2383
9. Standiford, T. J., Kunkel, S. L., Phan, S. H., Rollins, R. J., and Strieter, R. M. (1991) J. Clin. Invest. 86, 148–154
10. Standing, T., Vialle-Valentin, C., Chenese, S. W., and Fantone, J. C. (1993) in J. Biol. Chem. 268, 9912–9918
11. Alexanian, H., Neill-Golden, J., Galanopoulos, T., Kradein, R. L., Walz, A., and Kunkel, S. L. (1989) Immunol. Today 10, 724–742
12. Breen, E., Shull, S., Burns, S., Absher, M., Kelley, L., Phan, S. H., and Cutruno, K. (1992) Am. J. Respir. Cell. Mol. Biol. 9, 300–305
13. Lukacs, N. W., Kunkel, S. L., Strieter, R. M., Waromking, K., and Chenese, S. W. (1989) J. Exp. Med. 177, 1531–1539
14. Rollins, R. J., Walz, A., and Baggiolini, M. (1991) J. Immunol. 146, 130–138
15. Adamiak, H. H., Neville-Golden, J., Galanopoulos, T., Kradein, R. L., Valente, A. J., and Graves, D. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5371–5375
16. Phan, S. H., Varani, J., and Smith, D. (1985) J. Clin. Invest. 76, 241–247
17. Smith, R. E., Strieter, R. M., Zhang, K., Phan, S. H., and Kunkel, S. L. (1995) J. Exp. Med. 177, 185–198
18. Thrall, R. S., Phan, S. H., and Rollins, R. J., and Ward, P. A. (1981) Am. J. Pathol. 105, 76–81
19. Schier, D. J., Phan, S. H., and Mccormick, B. (1983) Am. Rev. Respir. Dis. 127, 614–617
20. Hoyt, D. G., and Lazo, J. S. (1988) J. Pharmacol. Exp. Ther. 246, 765–771
21. Piquet, F. P., Collart, M. A., Grau, G. E., Kapand, Y., and Vassalli, P. (1989) J. Exp. Med. 170, 655–664
22. Raghuv, R., Irish, P., and Kang, A. H. (1989) J. Clin. Invest. 84, 1836–1842
23. Phan, S. H., and Kunkel, S. L. (1992) Exp. Lung Res. 18, 29–43
24. Sutoh, A., Takahashi, K., Yusui, S., Arali, S., and Sendi, V. (1988) Am. J. Pathol. 132, 512–520
25. Elias, J. A., and Freundlich, B. (1990) Chest 97, 1439–1445
26. Mauve, V., Leyton, L., Bhatnagar, R., Penforsn, H., Laurent, M., Hartmann, K., and Rehner, J. L. (1991) J. Immunol. 146, 245–247
27. Jalliffe, M. (1991) Am. J. Respir. Cell. Mol. Biol. 4, 126–127
28. Spoon, M. B., Roberts, R. B., Wakefield, L. M., and Assaian, R. K. (1986) Science 233, 352–354
29. Ignasz, R. A., and Massague, J. (1986) J. Biol. Chem. 261, 4337–4345
30. Roberts, A. B., Spoon, M. B., Assaian, R. K., Smith, M. J., Roche, N. S., and Wakefield, L. M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4167–4172
31. Peterofsky, B., and Diegelman, R. (1971) J. Immunol. 107, 300–305
32. Piguet, P. F., Collart, M. A., Grau, G. E., Sappono, A. P., and Vassalli, P. (1990) Nature 344, 245–247
33. Leonard, E. J., Skeed, A., and Yoshimura, T. (1991) in Chemotatic Cytokines (Westwick, J., ed) pp. 57–64, Plenum Publishing Corp., New York