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Differential regulation of C-C chemokines during fibroblast–monocyte interactions: adhesion vs. inflammatory cytokine pathways

C. Zickus,[1] S. L. Kunkel,[1] K. Simpson,[1] H. Evanoff,[1] M. Glass,[2] R. M. Strieter[2] and N. W. Lukacs[1,CA]

Departments of [1]Pathology and [2]Internal Medicine, Division of Pulmonary and Critical Care, University of Michigan Medical School, Ann Arbor, MI 48109–0602, USA

[CA]Corresponding Author
Fax: (+1) 734 764 5135
Email: nlukacs@umich.edu

THE cell-to-cell interactions during chronic inflammatory diseases likely contribute to leukocyte accumulation leading to increased pathology and organ dysfunction. In particular, there is a paucity of information relating to the maintenance of chronic fibrotic diseases. Using a lung fibroblast line and enriched monocyte populations, we have investigated the activational events which contribute to the production of two C-C chemokines, macrophage inflammatory protein-1 alpha (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1), during fibroblast-monocyte interactions. Neither the fibroblast cell line (16lu) nor isolated monocytes alone produced significant levels of MIP-1α or MCP-1. However, when isolated monocytes were layered onto 16lu fibroblast monolayers a significant increase in MIP-1α and MCP-1 production was observed. The use of fixed cell populations indicated that the MIP-1α was derived from monocytes and MCP-1 from both cell populations. To examine the molecules which were required for chemokine production during the interaction, specific antibodies were used in the co-cultures. Blocking β3-integrin interactions significantly inhibited MIP-1α production. In contrast, beta-integrin interactions had no effect on the MCP-1 production, while, neutralization of TNF significantly decreased MCP-1 production during the co-culture. These data indicate that fibroblast–monocyte interactions induce chemokine production through different mechanisms and a combination of these responses may contribute to the maintenance of the mononuclear cell accumulation during disease progression.

Key words: Chemokines, Fibroblasts, Monocytes

Introduction

The accumulation of leukocytes during infectious diseases is paramount for host protection. However, persistent leukocyte influx during chronic inflammatory diseases appears to be a driving force behind tissue pathology.[1,2] In particular, the consistent recruitment of monocytes to fibrotic tissue has been identified as a hallmark of disease progression.[3,4] The mechanisms which regulate leukocyte recruitment are quite complex and are initiated by adhesion molecules on the endothelial surface.[5,6] Once adhered to the endothelial surface the cells can then migrate into the inflamed tissue following chemotactic gradients. The chemokines appear to mediate the selective recruitment of cell populations during inflammatory diseases.[7,8] Chemokines are divided into two distinct families by sequence similarity and by function. The C-x-C family (IL-8 family or alpha chemokines) are primarily chemotactic for neutrophils, whereas the C-C chemokine family (MCP-1 family or beta chemokines) are primarily chemotactic for monocytes and lymphocytes. However, these broad divisions are now breaking down and it appears that both classes of chemokines are important mediators in both acute and chronic inflammation. One of the more potent C-C family chemokines, macrophage inflammatory protein-1 alpha (MIP-1α), is made primarily by leukocyte populations and not by stromal or structural cells. In contrast, a second member of the family, monocyte chemoattractant protein-1 (MCP-1), can be made by both immune and non-immune cells.

Once the cells have moved into the tissue, they continue to interact by cell-to-cell contact with structural cells within the tissue or organ. In earlier studies, it has been demonstrated that monocyte/macrophage interaction with various cell populations (endothelial cells, synovial fibroblasts) can drive the production of chemokines and may possibly be a mechanism for maintaining the persistent leukocyte influx observed during chronic diseases.[9–11] The

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mechanisms which govern these interactions appear to be regulated by adhesive interactions between the leukocyte and the tissue cells. These mechanisms, although intuitive, may be differential depending on the state of the inflammatory response, the cells involved, the chemokines produced, and the tissue location of the response.

In the present studies, the results demonstrate that two C-C family chemokines, MIP-1α and MCP-1, are produced during monocyte–fibroblast interactions. The two chemokines, however, are differentially regulated during this response: MIP-1α is made by only the monocytes and dependent upon β3-integrin ligation, whereas MCP-1 is made by both cell populations and the mechanism was not dependent upon beta-integrins,[1–3] but rather on cytokine (TNF) activation. These studies demonstrate novel mechanisms of regulation of C-C chemokine production.

Materials and Methods

Mononuclear cell isolation

Peripheral blood was drawn into a heparinized syringe from healthy volunteers, diluted 1:1 in normal saline, and mononuclear cells separated by density gradient centrifugation. The recovered cells were washed three times with RPMI 1640. The PBMs were then layered onto a density gradient (1.068 g/ml) for the enrichment of monocytes (Atlanta Biologicals, Atlanta, GA). The isolated cells were then washed, cytospun onto a glass slide, stained with Diff-Quik (Baxter, McGaw, IL) and differentially counted. The purity of the monocytes from the gradient were consistently between 75 and 80% monocytes with the remainder lymphocytes.

16lu fibroblast cultures

The transformed fibroblast cell line, 16 lu, was obtained from ATCC (CCL 204) and cultured as required in Eagle's minimum essential medium with non-essential amino acids and Earle's BSS with 10% fetal calf serum. The cells were grown to near confluent monolayers in six-well tissue culture plates and fresh media applied prior to utilizing them in the assays.

Fibroblast:monocyte cocultures

Enriched monocyte populations (5×10^5 cells/ml) were layered onto unstimulated 16 lu fibroblast monolayers in six-well plates. The monocyte enriched cells were added to the fibroblasts in a total of 1 ml of media in the 60 mm culture dishes. Culture supernatants were collected at 1 to 24 h after co-culture. Peak MCP-1 and MIP-1α production was detected at 24 h of co-culture.

Blocking cellular interactions

To demonstrate that cell-to-cell interactions were required for production of the chemokines, fixed cell populations were used (4% paraformaldehyde for 5 min). Subsequently, blocking antibodies to beta-integrins (Chemicon, Temecula, CA) and/or adhesion molecules (R&D Systems, MN) were used to block adhesion of the two cell populations. The antibodies were used at a concentration of 5 μg/ml. In addition, blocking polyclonal antibodies to IL-1 and TNF were also used at a 1:200 dilution in culture to examine inflammatory cytokine networks.

MIP-1α and MCP-1 ELISA

Extracellular immunoreactive MIP-1α and MCP-1 was quantitated using a modification of a double-ligand method as previously described.[12] Standards were 1/2 log dilutions of recombinant MIP-1α and MCP-1 from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected concentrations above 10 pg/ml and did not crossreact with MIP-1β, RANTES, IL-1α/β, TNFα, ENA-78, IL-8, or NAP-2.

Statistical analysis

Data are expressed as means ± SEM. Data that appeared statistically significant were compared by ANOVA for comparing the means of multiple groups, and considered significant if P values were less than 0.05.

Results

Fibroblast: monocyte coculture induces C-C family chemokines in an adherence-dependent mechanism

To determine whether monocyte interactions with fibroblast induced an activational event, enriched monocytes were layered onto 16 lu fibroblast monolayers and cultured for 24 h at 37°C. The data demonstrates that neither monocytes nor fibroblasts by themselves produced substantial levels of MIP-1α (Fig. 1) or MCP-1 (Fig. 2). However, when the two cell populations were cultured together a synergistic increase in both chemokines was observed. In contrast, enriched lymphocyte populations (~80%) demonstrated little increase in the two chemokines when added to the fibroblasts (data not shown). To determine which cell populations were responsible for the chemokine production, one of the cell populations was fixed with 4% paraformaldehyde prior to the coculture procedure. MIP-1α production was observed only when the fibroblasts were fixed and monocytes were not, indicating that only the monocytes were responsible for the MIP-1α production (Fig. 1), following previous studies. In contrast,

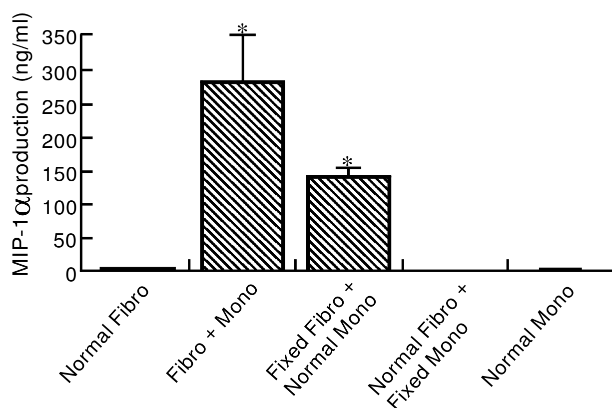

FIG. 1. MIP-1α production during fibroblast–monocyte interaction. Enriched monocyte populations were layered onto fibroblast monolayers. Either the monocytes or the fibroblast were fixed with 4% paraformaldehyde (5 min) and MIP-1α levels were determined in 2 h culture supernatants. MIP-1α was generated by monocytes but not fibroblasts. Data represents Mean ± SE from two different experiments. *$P<0.05$.

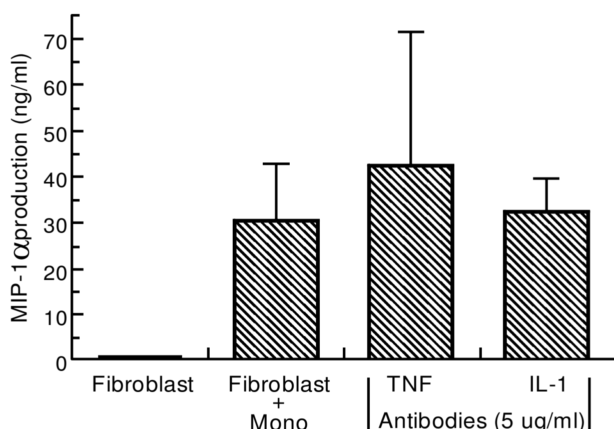

FIG. 3. MIP-1α production is not affected by antibodies to either IL-1 or TNF. Antibodies were added to fibroblast–monocyte co-culture at time 0 at a concentration of 5 μg/ml. After 24 h of co-culture the culture supernatant was harvested and MIP-1α was measured using a specific ELISA. Data represents mean ± SE of two repeat experiments. No significant decrease in MIP-1α was observed. *$P<0.05$

increased MCP-1 production was also observed only when fibroblast population was fixed, indicating that viable monocytes needed to be present to incude MCP-1 production during the cell-to-cell interaction (Fig. 2). These studies established that an activational event occurred following interaction between the two cell populations.

Differential regulation of C-C chemokines during monocyte-fibroblast interactions

To determine the mechanism of chemokine production during cell-to-cell interaction, antibodies specific for inflammatory cytokines and adhesion molecules were utilized. It has previously been demonstrated that TNF and IL-1 are strong inducers of chemokine production in multiple cell types. It was interesting that even though fibroblasts are known to produce MCP-1 and likely the main source of MCP-1, no increase was observed when the fixed monocytes were layered onto them. When antibodies to TNF and IL-1 were added into the coculture no decrease in MIP-1α was observed (Fig. 3). In contrast, when MCP-1 production was examined, treatment of the cocultures with anti-TNF significantly decreased the production of MCP-1, whereas anti-IL-1 only slightly inhibited (Fig. 4). These latter studies indicate a differential regulation of these two C-C family chemokines.

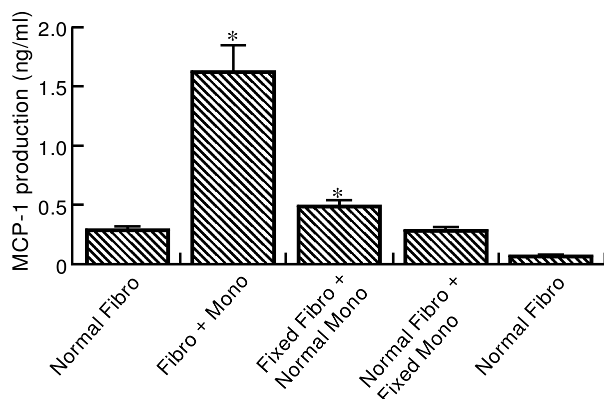

FIG. 2. MCP-1 production during fibroblast–monocyte interaction. Enriched monocyte populations were layered onto fibroblast monolayers. Either the monocytes or the fibroblast were fixed with 4% paraformaldehyde (5 min) and MCP-1 levels were determined in 2 h culture supernatants. MCP-1 was produced by both cell populations. Data represents mean ± SE from two different experiments. $P<0.05$

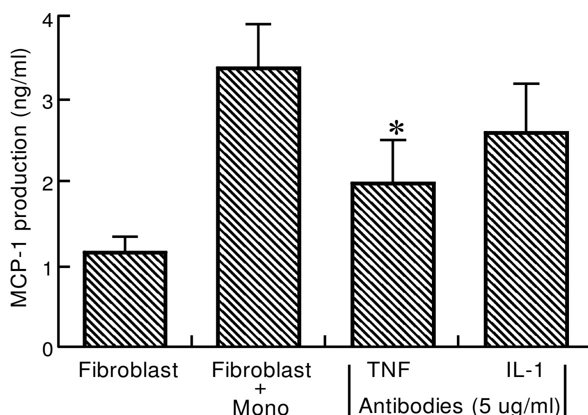

FIG. 4. MCP-1 production was decreased by antibodies to TNF and IL-1. Antibodies were added to fibroblast–monocyte co-culture at time 0 at a concentration of 5 μg/ml. After 24 h of co-culture the culture supernatant was harvested and MCP-1 was measured using a specific ELISA. Data represents mean ± SE of two repeat experiments. Statistically significant decreases in MCP-1 was observed in cultures treated with anti-TNF. *$P<0.05$

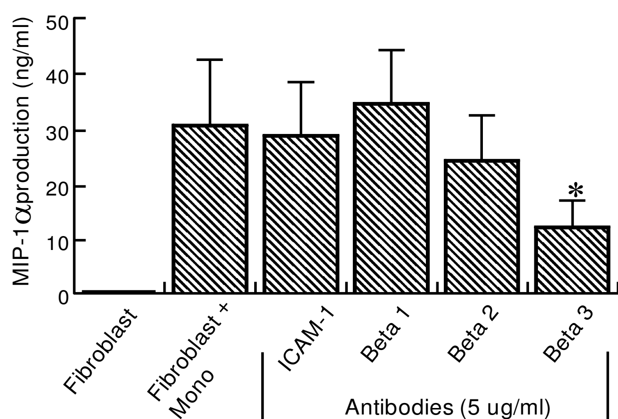

FIG. 5. Adhesion pathways mediated production of MIP-1α during fibroblast–monocyte interaction. Monoclonal antibodies to specific adhesion molecules or integrins were added to fibroblast–monocyte co-culture at time 0 at a concentration of 5 μg/ml. After 24 h of co-culture the culture supernatant was harvested and MIP-1α was measured using a specific ELISA. Data represents mean ± SE of three repeat experiments. Significant decreases in MIP-1α were observed only in cultures treated with anti-β3-integrin. *$P<0.05$.

Next, since the chemokine production was differentially inhibited by fixation of one or the other cell populations or by separation by transwells, the role of adhesion molecules was examined. Blocking antibodies to beta-integrins (β1,2,3) or adhesion molecules (ICAM-1, VCAM-1) were added to the two cell populations (5 μg/ml) for 15 min prior to combining them. The data indicated that only antibodies to β3-integrins significantly inhibited MIP-1α, while antibodies to other β-integrins (β1, β2) or adhesion molecules (ICAM-1, VCAM-1) had no significant affect on MIP-1α production (Fig. 5). In contrast, none of the antibodies to beta-integrins or adhesion molecules (ICAM-1, VCAM-1) inhibited MCP-1 (Fig. 6). Interestingly, antibodies to β1-integrins appeared to increase the production of MCP-1. Altogether, these data suggest that even though the two C-C family chemokines are produced during the adhesion event they are differentially regulated. MIP-1α appears to be dependent upon adhesion-mediated pathways, whereas MCP-1 appears to require cytokine signaling pathways for its production. Thus, even though these two C-C chemokines have overlapping functions *in vitro*, they appear to be differentially induced during the cell-to-cell interactions.

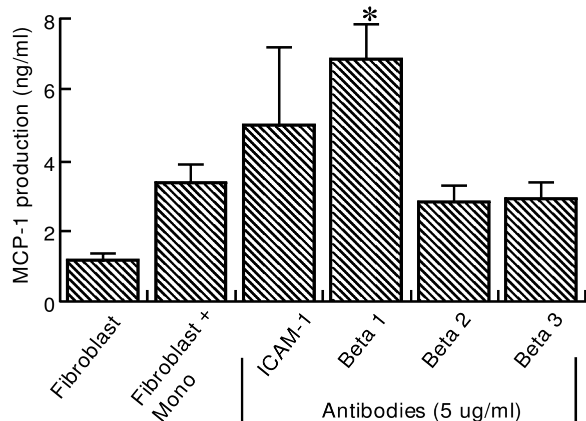

FIG. 6. Blockade of adhesion pathways do not alter production of MCP-1 production during fibroblast–monocyte interaction. Monoclonal antibodies to specific adhesion molecules or integrins were added to fibroblast–monocyte co-culture at time 0 at a concentration of 5 μg/ml. After 24 h of co-culture the culture supernatant was harvested and MCP-1 was measured using a specific ELISA. Data represents mean ± SE of three repeat experiments. *$P<0.05$.

Discussion

The persistent production of chemokines during an inflammatory response is likely required to maintain the constant influx of leukocytes which accompanies the development of chronic disease.[13–15] The mechanism which was examined in this study was whether the cell-to-cell interaction of monocytes with fibroblasts was sufficient to drive the production of chemokines. The production of C-C family chemokines, MIP-1α and MCP-1, may be one of the mechanisms which maintain chronic and/or fibrotic lesions. Previous studies in fibrotic human diseases have indicated that leukocyte accumulation at the site of fibrotic episodes is necessary to initiate, maintain, and progress the pathological manifestations within the affected tissue or organ. The production of the chemokines in the present study was dependent on the interaction of the two cell populations, as individual cell populations produced relatively little or no chemokine. We have also identified that chemokine production was differentially regulated. MCP-1 appeared to be partially dependent upon monocyte-derived inflammatory cytokine production, TNF, and not on cell adhesion molecules. In contrast, MIP-1α was induced by cell-to-cell interactions dependent upon β3-integrin ligation. Since β3-integrins have been shown to bind to matrix protein components, this interaction would be very appropriate within fibrotic lesions to maintain the chemokine (MIP-1α) production. These data begin to define differences in cellular interaction pathways which may allow maintenance and progression of chronic diseases. Previous studies have demonstrated chemokine production during cell-to-cell interactions,[9,10] but the differential regulation of the chemokines within these studies is striking. This difference of regulation may lie on the source of the chemokines. MIP-1α appeared to be derived primarily from the monocyte population, whereas MCP-1 can be elicited from both the fibroblast and monocyte populations. Altogether, these results may indicate why it may be so difficult to modulate disease phenotypes in chronic ailments even in the absence of any apparent inciting agent.

The induction of MIP-1α production by cell adhesion events has been previously reported by our laboratories. However, it appears that the mechanism is different depending upon the cell-type which the monocyte binds. In a previous study endothelial cells were used and the mechanism was a β2-integrin/ICAM-1-mediated mechanism.[9] In the present study, fibroblasts were utilized and it appeared that β3-integrins played a more important role during the interaction for MIP-1α production. The reason for this difference may lie in not only the cell type, but where the cells are normally located. Endothelial cells which mediated MIP-1α production via ICAM-1 interactions are the initial cell type that the monocyte contacts during inflammatory events prior to migration into the inflamed tissue. This initial activational event may set up the induction of chemokines by monocytes for production at sites of inflammation. Once at the inflamed area, stromal cells, such as fibroblasts, could then maintain the activated state of the monocyte/macrophage for continued chemokine production via cell-to-cell interaction. This latter mechanism appears to be induced by β3-integrin-mediated events. The ligand(s) for this integrin is primarily matrix proteins, a product that fibroblasts are well suited to produce.[16,17]

The induction of MCP-1 production by TNF and IL-1 has been demonstrated by many laboratories from multiple cell types.[18–24] Interestingly, TNF appears to be a major factor for inducing and maintaining fibrotic responses, even in the absence of an apparent inciting agent.[25,26] In chronic fibrotic diseases, such as idiopathic fibrosis (IPF) or liver cirrhosis, the persistent production of TNF and recruitment of mononuclear cells is maintained.[27–29] In addition, MCP-1 appears to play a role in chronic fibrotic events in animal models of fibrosis.[30] Interestingly, MCP-1 was first identified as a competence factor during fibroblast activation and has been shown to participate in collagen gene activation which can contribute to the overall pathology within fibrotic lesions.[30,31] Interestingly, when monocytes were fixed prior to the addition to fibroblast, no MCP-1 was induced, thus depicting the need for monocyte-derived factors (TNF) to induce MCP-1 production. In contrast, MIP-1α was induced via direct monocyte adherence to fixed fibroblasts. The increased production of MCP-1 via the cellular adhesion following TNF activation may represent a key mechanism in progression of chronic fibrotic diseases.

The data in the present study suggest that the interaction of fibroblasts with monocytes initiates chemokine production. The initiation of C-C family chemokines during this adhesion event appears to utilize different mechanisms of activation. Yet, the overall result is increased MIP-1α and MCP-1 production which may be utilized to maintain persistent leukocyte accumulation and cellular activation without other initiating cytokines or foreign pathogens. These mechanisms may help to contribute to the maintenance of chronic fibrotic diseases, resulting in significant pathogenic changes.

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