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

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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 Cx-C family (IL-8 family or alpha chemokines) are primarily chemotactic for neutrophils, whereas the CC 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 CC 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 chemotactic 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
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 popula-
tions and the mechanism was not dependent upon
beta-integrins,1–3 but rather on cytokine (TNF) activa-
tion. 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,
cytopsin 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, 16lu, 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 16lu fibroblast mono-
layers 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 superna-
tants 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-
tegrins (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 16lu fibroblast mono-
layers 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 con-
trast, enriched lymphocyte populations (~80%) dem-
onstrated little increase in the two chemokines when
added to the fibroblasts (data not shown). To deter-
mine 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 mono-
cytes were responsible for the MIP-1α production
(Fig. 1), following previous studies. In contrast,
increased MCP-1 production was also observed only when fibroblast population was fixed, indicating that viable monocytes needed to be present to incite MCP-1 production during the cell-to-cell interaction (Fig. 2). These studies established that an activation 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. 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. 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. 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.

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.
Antibodies (5 μg/ml)

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FIG. 6. Blockade of adhesion pathways do not alter production of MCP-1 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. 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 CC family chemokines, 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, MCP-1 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, but the differential regulation of the chemokines within these studies is striking. This difference of regulation may lie on the source of the chemokines. MCP-1 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 MCP-1 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. 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 activation 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. Interestingly, when monocytes were fixed prior to the addition to fibroblasts, no MCP-1 was induced, thus depicting the need for monocyte-derived factors (TNF) to induce MCP-1 production. In contrast, MIP-1α and MCP-1 production which may be utilized to maintain persistent leukocyte accumulation and cellular activation with-out 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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ACKNOWLEDGEMENTS. This work was supported in part by NIH grants, AI36302 and HL59178.

Received 26 March 1998; accepted 22 April 1998