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Expression of monocyte chemoattractant protein 1 mRNA in human idiopathic pulmonary fibrosis

(inflammation/macrophage/monocyte chemotaxis)

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ABSTRACT **Macrophages are thought to play an important role in the pathologic changes associated with idiopathic pulmonary fibrosis (IPF). The mechanisms for increased monocyte/macrophage recruitment in IPF are unknown. Monocyte chemoattractant protein 1 (MCP-1) is the predominant monocyte chemoattractant secreted by a variety of different cell types in culture. We examined the expression of MCP-1 mRNA and its protein product *in vivo* in IPF and non-IPF lung specimens by *in situ* hybridization and immunocytochemistry. The cell types expressing MCP-1 *in vivo* were identified by immunostaining with specific antibodies. We demonstrated the expression of MCP-1 mRNA in pulmonary epithelial cells, in monocytes/macrophages, and in vascular endothelial and smooth muscle cells. Lung epithelial cells in patients with IPF strongly expressed MCP-1 mRNA and its protein product. In contrast, epithelial cells in non-IPF specimens did not express MCP-1 mRNA. Macrophages and vascular endothelial and smooth muscle cells were shown to express MCP-1 in both IPF and non-IPF lung specimens. These findings provide a basis for the understanding of the *in vivo* physiologic processes that mediate monocyte/macrophage recruitment and infiltration in the lung interstitium and the pathologic state contributing to an increased alveolar monocyte/macrophage population and inflammation in IPF.**

Idiopathic pulmonary fibrosis (IPF) represents one of the major interstitial lung diseases of unknown origin. It has a high degree of morbidity and mortality, with death usually occurring within 3–6 years after the onset of symptoms. Clinically it is characterized by a progressive reduction in the functional capacity of the lung that results in the disruption of gas exchange between the alveolus and capillaries. This progressive respiratory failure is thought to result from a disease process that is characterized by three major pathologic changes: infiltration of the interstitium by inflammatory cells, particularly lymphocytes and monocytes; increased proliferation of interstitial fibroblasts; and excessive accumulation of interstitial collagen.

Studies on the molecular events leading to pulmonary fibrosis have generally recognized that monocytes/macrophages contribute significantly to the disease process (1–3). Interstitial monocyte/macrophage infiltration is seen during the early stages of pulmonary fibrosis. In particular, monocytes accumulate before the recruitment and proliferation of mesenchymal cells such as fibroblasts. Activated monocytes/macrophages secrete growth factors including platelet-derived growth factor (PDGF), which is capable of stimulating fibroblast proliferation and collagen production, both of which are associated with pulmonary fibrosis (4, 5). Martinet *et al.* (6) have demonstrated the spontaneous production of PDGF by alveolar macrophages obtained by bronchoalveolar lavage from patients with IPF. Antoniades *et al.* (7) have shown that both pulmonary epithelial cells and macrophages in lung biopsy specimens from patients with IPF strongly express c-*sis*/PDGF-2 mRNA and produce PDGF-like proteins. Thus it appears that both alveolar macrophages and epithelial cells contribute to the fibrotic process by an excessive and sustained production of PDGF.

Increased macrophage infiltration is due, to a large extent, to the recruitment of monocytes from the peripheral vasculature (8, 9) that is induced by the local production of monocyte chemoattractants. Early studies demonstrated that alveolar macrophages secrete a protein factor that stimulates monocyte/macrophage migration (10, 11). These studies suggested that cells within the pulmonary interstitium produce monocyte chemoattractants, thereby stimulating monocyte/macrophage recruitment.

A monocyte chemoattractant produced by baboon vascular smooth muscle cells has been identified and named smooth muscle cell-derived chemotactic factor (SMC-CF) (12). SMC-CF is a small single-chain protein that stimulates chemotactic activity in monocytes but not lymphocytes or polymorphonuclear leukocytes (13). SMC-CF is identical to monocyte chemoattractant protein 1 (MCP-1), which is produced by human tumor cell lines (12–15). The successful cloning and complete nucleotide sequence of MCP-1 have been reported (14). The putative protein product of the MCP-1 gene is a single-chain polypeptide with a molecular mass of 8.7 kDa. SMC-CF/MCP-1 production seems to account for most of the monocyte chemotactic activity produced by tumor cells and nontransformed vascular endothelial and smooth muscle cells in culture (15–17).

We report here the *in vivo* expression of MCP-1 mRNA in epithelial cells, vascular endothelial cells, smooth muscle cells, and macrophages of lung biopsy specimens derived from patients with IPF. The expression of mRNA was accompanied by the production of MCP-1-like proteins. Epithelial cells in control, non-IPF lung specimens did not express MCP-1 mRNA. In contrast, both endothelial cells and macrophages expressed MCP-1 mRNA in non-IPF lung specimens. The *in vivo* findings described here may contribute to the understanding of the molecular mechanisms involved in the recruitment and increased infiltration of monocytes/macrophages in the lung tissue of patients with IPF.

MATERIALS AND METHODS

Tissue Collection. Pulmonary specimens from patients with IPF were collected by open lung biopsy. The patients exhib-

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Abbreviations: MCP-1, monocyte chemoattractant protein-1; IPF, idiopathic pulmonary fibrosis; SMC-CF, smooth muscle cell-derived chemotactic factor; PDGF, platelet-derived growth factor.

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ited clinical, radiographic, and physiologic alterations consistent with IPF. The definitive diagnosis was made by the morphological study of the tissue specimens obtained through open lung biopsy. Biopsies were performed after careful evaluation by the local medical/surgical committee for confirmation of diagnosis and for aiding the course of treatment. Non-IPF lung specimens were obtained from individuals undergoing lobectomy or wedge resection for removal of primary lung tumor. The non-IPF tissue sections used in these studies were free of pathologic evidence of malignancy. *In situ* hybridization and immunocytochemistry were performed in four lung specimens with IPF and in four specimens from non-IPF subjects. Two of the IPF specimens exhibited severe septal scarring and honeycomb formation, whereas the other two had a milder interstitial septal scarring. Three of the patients with IPF were nonsmokers and one was a smoker, all without concurrent disease. Their ages ranged from 40 to 71 years. The total lung capacities and diffusing capacities for carbon monoxide were moderately (<70% predicted) reduced in each case. The procedures and protocols described below are similar to those used previously in our investigation of the expression of the c-*sis*/PDGF-2 mRNA in lung specimens from IPF patients and non-IPF subjects (7).

***In Situ* Hybridization.** Tissues were collected intraoperatively and were immediately snap-frozen in liquid nitrogen and then embedded in optimal-cutting-temperature compound (O.C.T., Miles) for cryostat serial sectioning (10 μm). *In situ* hybridization utilizing ^{35}S-labeled cRNA probes was performed according to Höffler *et al.* (18). For each tissue section a consecutive serial section was incubated with the noncomplementary RNA probe to serve as a negative control. Triplicate sections from each tissue were hybridized with either complementary antisense or noncomplementary sense probes and were developed at weekly intervals for a period of 3 weeks. In every case a diffuse pattern of sparse silver grains typical of light "background" hybridization was noted for the noncomplementary sense probe. The cDNA clone of MCP-1 used in these studies was generously provided by E. Appella (National Cancer Institute).

***In Situ* Hybridization Combined with Immunocytochemistry.** To identify the cells expressing MCP-1 mRNA, *in situ* hybridization was combined with immunocytochemistry. Tissue sections were first hybridized with MCP-1 cRNA probe and then incubated with antibodies for specific cell types and counterstained with hematoxylin. For these combined steps the dextran sulfate was deleted from the hybridization buffer in order to avoid background staining during immunocytochemistry. To identify epithelial cells, IPF tissue sections were incubated with a polyclonal antibody to bovine keratin (K4252, Sigma). Monocytes/macrophages were identified with the use of anti-LeuM3 monoclonal antibody (Becton Dickinson), which is primarily reactive with mature monocytes and macrophages (19). Interstitial fibroblasts were stained with anti-vimentin antibody (Zymed Laboratories). For immunocytochemistry, the tissues were washed with phosphate-buffered saline, treated with 0.3% H_2O_2 in methanol to suppress endogenous peroxidase activity, incubated with the appropriate antibodies followed by reagents from the Vectastain ABC kit (Vector Laboratories). The tissues were then dehydrated, dipped in NT-B2 emulsion (Eastman Kodak), and processed as described above.

***In Situ* Hybridization Combined with Immunostaining for MCP-1-Like Proteins.** For the detection of MCP-1-like proteins in lung tissue from patients with IPF, *in situ* hybridization was combined with staining with rabbit polyclonal an-

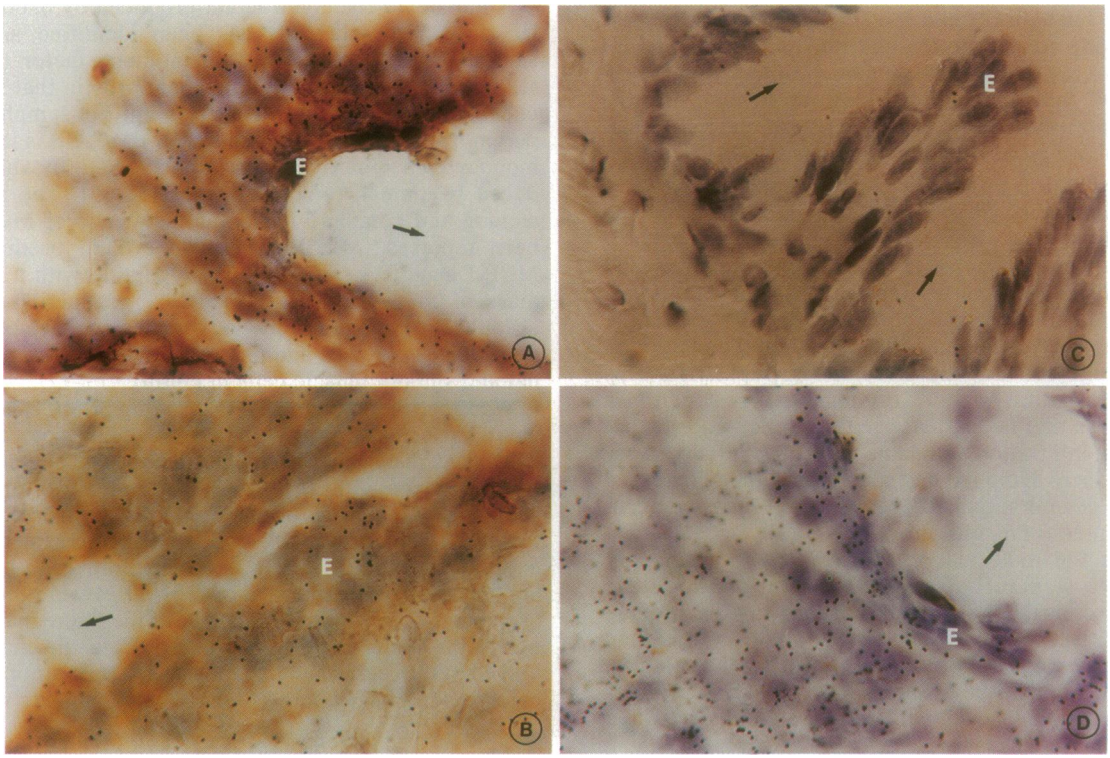

FIG. 1. Pulmonary alveolar epithelial cells from a patient with IPF demonstrate a strong expression of MCP-1 mRNA (*A*). In this study, *in situ* hybridization for MCP-1 mRNA was combined with immunostaining of the epithelial cells with anti-keratin antibody. There is no significant expression of MCP-1 mRNA in the epithelial cells of a control lung tissue specimen without IPF (*C*). Alveolar coexpression of MCP-1 mRNA and MCP-1-like proteins is seen in the epithelial cells of a patient with IPF (*B*). In this study we combined *in situ* hybridization for MCP-1 mRNA with immunostaining for MCP-1-like proteins by using a specific anti-MCP-1 antibody. *D* demonstrates the expression of MCP-1 mRNA in the epithelial cells and the lack of immunostaining for MCP-1-like proteins with control nonimmune serum. Arrows, direction of the alveolar spaces; E, alveolar epithelium. (×380.)

tibody raised against homogeneous SMC-CF produced by baboon vascular smooth muscle cells in culture. Crossreactivity of this antiserum with human MCP-1 has been established (15). The procedures for this study were identical to those described above. The specificity of anti-SMC-CF antibody in these studies was tested by comparison with a nonimmune rabbit serum (negative control) on consecutive serial tissue sections.

RESULTS

Pulmonary metaplastic epithelial cells in the alveolar septal surface of patients with IPF strongly expressed MCP-1 mRNA (Fig. 1*A*). In contrast, epithelial cells in lung tissue specimens from non-IPF subjects did not express detectable MCP-1 mRNA (Fig. 1*C*). The expression of MCP-1 mRNA in the epithelial cells of IPF specimens was accompanied by the strong expression of MCP-1-like proteins, as demonstrated by the combined steps of *in situ* hybridization for MCP-1 mRNA and immunostaining with the anti-MCP-1 antibody (Fig. 1*B*). Immunostaining with the control, nonimmune serum produced negative results (Fig. 1*D*). Negative results were also obtained when the anti-MCP-1 antibody was preincubated with excess (50 ng) purified MCP-1 (data not shown). The epithelial cells in this section were positive for expression of MCP-1 mRNA by *in situ* hybridization (Fig. 1*D*). Thus, epithelial cells in IPF appear to be a major source of MCP-1 mRNA and its protein product. In contrast, epithelial cells in control, non-IPF specimens did not express MCP-1 mRNA. The specificity of the MCP-1 mRNA expression in the epithelial cells of IPF lung sections shown in Fig. 1 was demonstrated by parallel control hybridization with noncomplementary "sense" MCP-1 probe. There was no significant expression in the epithelial cells of IPF lung sections with the noncomplementary probe (data not shown). Expression of MCP-1 mRNA was evident both in the epithelial cells lining small intrapulmonary airways involved in active inflammation and in the reactive alveolar type II cells of IPF specimens.

Expression of MCP-1 mRNA in alveolar septae macrophages was demonstrated in biopsy specimens derived from

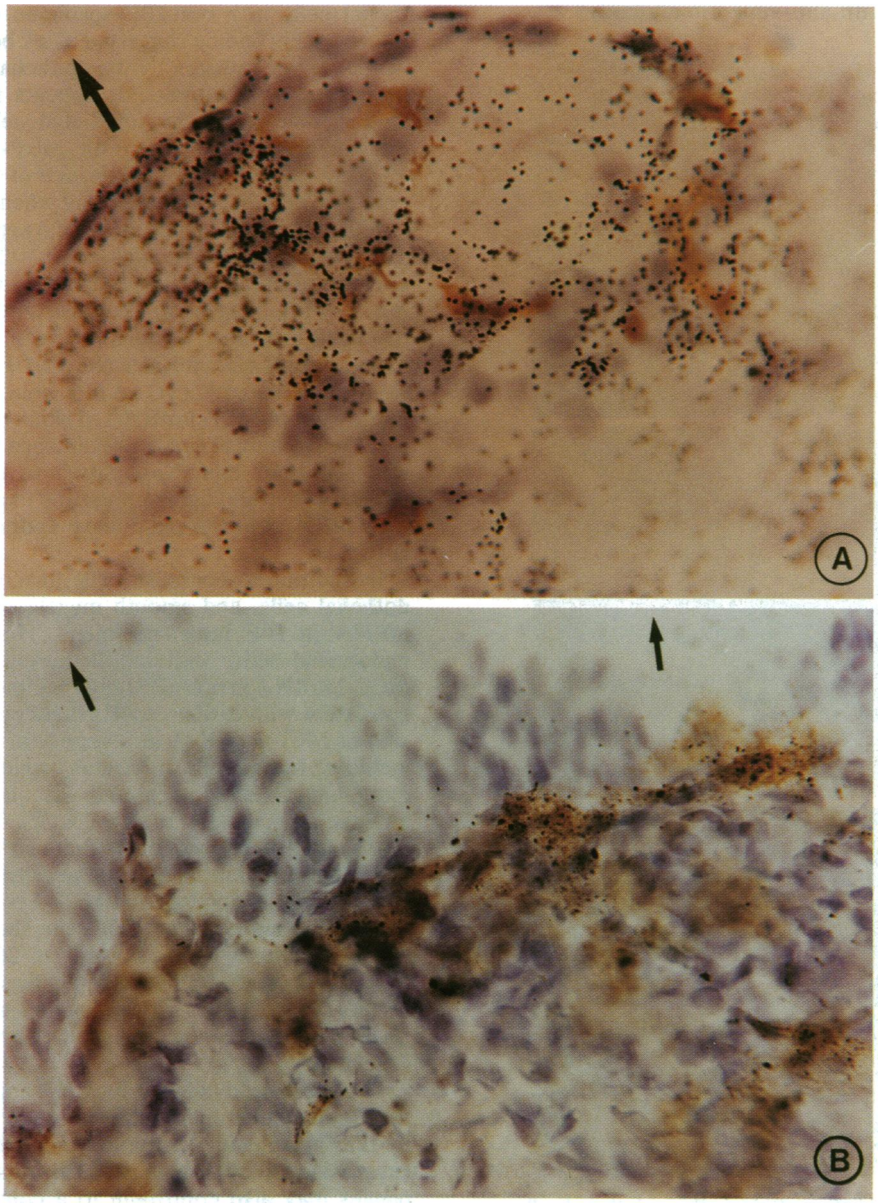

FIG. 2. Expression of MCP-1 mRNA in alveolar macrophages of lung tissues derived from a patient with IPF (*A*) and from a patient without IPF (*B*). *In situ* hybridization for MCP-1 mRNA was combined with immunostaining for macrophages with the anti-LeuM3 antibody. Arrows, direction of the alveolar spaces. (×750.)

patients with IPF (Fig. 2*A*) and without IPF (Fig. 2*B*). In these studies, *in situ* hybridization for MCP-1 mRNA was combined with immunostaining of monocytes/macrophages with the anti-LeuM3 antibody, which recognizes mature monocytes/macrophages (19). Strong expression of MCP-1 mRNA was detected in monocytes/macrophages infiltrating the alveolar septa of a patient with IPF (Fig. 2*A*). Similarly, macrophages in control, non-IPF lung tissue also demonstrated a strong expression of MCP-1 mRNA (Fig. 2*B*).

The strong expression of MCP-1 mRNA in the endothelial and smooth muscle cells of a small artery in an IPF biopsy specimen is demonstrated in Fig. 3 *A* and *B*. Exiguous expression of MCP-1 mRNA can be seen in the endothelial cells and smooth muscle cells of a small artery in a specimen from a control, non-IPF subject (Fig. 3*C*). The expression of MCP-1 mRNA in the arterial smooth muscle cells is consistent with the initial recognition of MCP-1 as a product of vascular smooth muscle cells in culture (13).

Interstitial fibroblasts in IPF specimens do not seem to express MCP-1 mRNA (Fig. 4). In this study, *in situ* hybridization for MCP-1 mRNA was combined with immunostaining of the fibroblasts with anti-vimentin antibody.

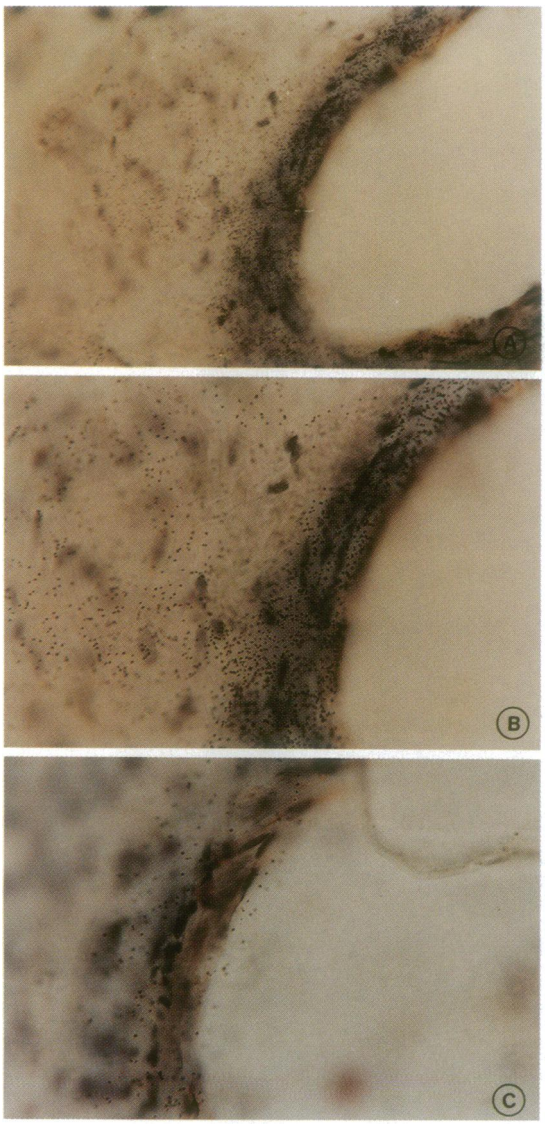

FIG. 3. Expression of MCP-1 mRNA in the endothelial cells and smooth muscle cells of a small artery in a lung tissue specimen from a patient with IPF (*A* and *B*) and from a non-IPF subject (*C*). (*A*, ×240; *B* and *C*, ×380.)

The results presented in Figs. 1–4 were uniform and representative in the four IPF and non-IPF tissue sections examined. This includes the IPF specimens with severe septal scarring and honeycomb formation as well as those with milder interstitial septal scarring. The staining and recognition of distinct cellular features was more difficult in the sections with severe scarring.

DISCUSSION

The studies presented here demonstrate the *in vivo* expression of MCP-1 mRNA and its protein product in lung biopsy specimens of patients with IPF and of non-IPF subjects. The important finding is that pulmonary epithelial cells of patients with IPF were shown to strongly express MCP-1 at both the mRNA and the protein level. In contrast, epithelial cells in lung specimens from non-IPF subjects did not express MCP-1 mRNA. In a previous study, we reported similar differences in the expression of PDGF between IPF and non-IPF lung specimen (7). In that study, we reported that the pulmonary epithelial cells in an IPF specimen strongly expressed both c-*sis*/PDGF-2 mRNA and PDGF-like proteins, whereas epithelial cells in a non-IPF specimen did not express c-*sis* mRNA. This finding suggested a link between the expression of PDGF-like mitogen by the epithelial cells and the fibrotic process in IPF patients. It appears from these combined studies that pulmonary epithelial cells in IPF specimens express inappropriately both MCP-1 mRNA and c-*sis*/PDGF-2 mRNA. The significance of this expression in the epithelial cells becomes apparent when one considers the fact that the lung "epithelial surface (is) approximately two thirds the area of a singles tennis court" (20). The inappropriate expression in the epithelial cells may result from "chronic" injury. This suggestion is consistent with recent findings demonstrating that acute injury can induce the reversible expression of c-*sis* mRNA and PDGF receptor b mRNA in skin epithelial cells (21). Acute injury can also induce the expression of MCP-1 mRNA in skin epithelial cells (H.N.A., T.G., and Samuel Lynch, unpublished data). It is conceivable that "chronic" injury, in contrast to acute injury, can cause the inappropriate, nonsuppressible expression of MCP-1 and c-*sis* mRNAs in the pulmonary epithelium of patients with IPF.

In addition to epithelial cells, macrophages, vascular endothelial cells, and smooth muscle cells expressed MCP-1 mRNA in IPF lung specimens. However, macrophages, endothelial cells, and smooth muscle cells also expressed MCP-1 mRNA in non-IPF lung specimens. These findings are consistent with earlier reports that demonstrated the expression of MCP-1 mRNA in a variety of cultured human cells, including tumor cells of various origins (14, 15), fibroblasts, mononuclear leukocytes, and vascular smooth muscle and endothelial cells (14, 16, 22, 23, 27). In several normal cell types, MCP-1 expression is induced by cytokines such as PDGF, interleukin, and tissue necrosis factor α (14, 22–24). Interestingly, it appears that MCP-1 is the human homologue of the JE gene (25), the first gene shown to be induced by PDGF in cultured mouse fibroblasts (26). However, evidence for expression of MCP-1 mRNA in fibroblasts is not provided by this *in vivo* study. There was no significant expression of MCP-1 mRNA in the interstitial fibroblasts of IPF tissue specimens.

The expression of MCP-1 mRNA and its protein product in the lung vascular endothelial cells may provide a mechanism for the physiologic recruitment of circulating monocytes. The expression of MCP-1 in smooth muscle cells and macrophages may also contribute to a continuous basal level of monocyte infiltration in the pulmonary interstitium. In contrast, the inappropriate strong expression of MCP-1 in the lung epithelium of patients with IPF may cause increased and

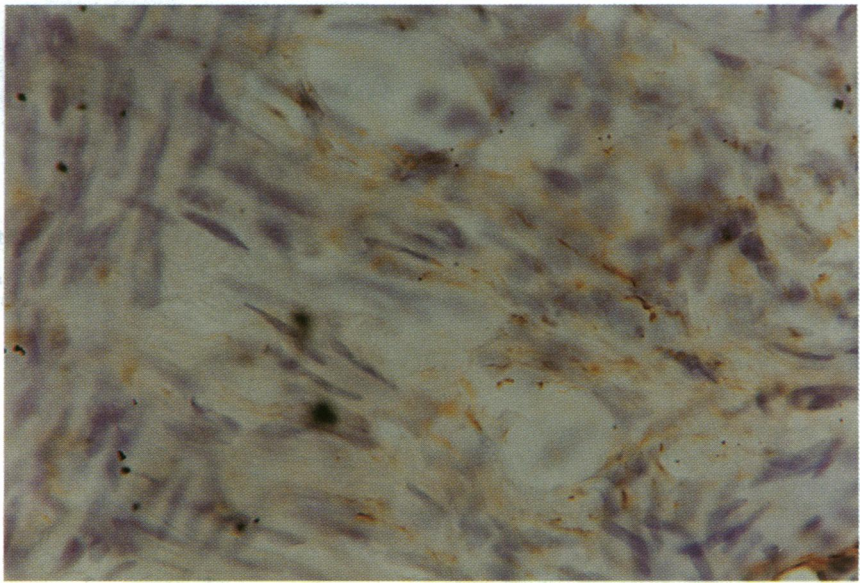

FIG. 4. Lack of significant expression of MCP-1 mRNA in the interstitial fibroblasts of a tissue section from an IPF patient. *In situ* hybridization for MCP-1 mRNA was combined with immunostaining for fibroblasts with anti-vimentin antibody. (×475.)

persistent monocyte/macrophage infiltration of the alveolar spaces and chronic inflammation, events characteristic of this disease. Accumulation of these inflammatory cells in the lungs of patients with IPF may in turn contribute to the fibrotic process by the spontaneous production of cytokines such as PDGF, a potent mitogen for mesenchymal cells and a stimulator of collagen synthesis by fibroblasts (6, 7).

The *in vivo* studies described here may serve as a model for the investigation of the molecular mechanisms on monocyte/macrophage infiltration involved in other inflammatory disorders such as rheumatoid arthritis and atherosclerosis.

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