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Commentary

Fibroblasts as Sentinel Cells

Synthesis of Chemokines and Regulation of Inflammation

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This commentary focuses on the central and emerging role of fibroblasts in initiating inflammation via recruiting leukocytes to the site of tissue injury. Fibroblasts are ubiquitous cells that provide much more than a source of scaffolding on which other cells function and migrate. Aside from their role as structural elements, we will highlight the fibroblast's ability to serve as a resident sentinel cell. For example, fibroblasts, when activated by substances released during tissue injury or derived from infectious microorganisms or by other environmental factors, produce chemokines that initiate the recruitment of bone-marrow-derived cells. Typically, fibroblasts had not been considered substantial producers of chemokines. This failure to recognize the broad biosynthetic repertoire of fibroblasts was due to a bias that other cell types, such as macrophages, were more attractive models for study. Our commentary emphasizes the concept that fibroblasts are key sites of chemokine synthesis, which initiates a cascade of events involved in wound healing and clearance of invading microorganisms. Moreover, fibroblasts have highly specialized roles in conditioning the cellular and cytokine environment in areas of inflammation by virtue of the complex array of factors they express.

The chemokines synthesized by fibroblasts and the pathways through which their expression is induced are only now being identified. In this issue of The American Journal of Pathology, the paper by Xia and colleagues[1] is important because it highlights two emerging issues in chemokine biology. First, it provides exciting new evidence that fibroblasts are key initial chemokine producers that act as a tissue early warning system. Second, the authors reveal fundamental differences in the molecular regulation of chemokine synthesis in fibroblasts and bone-marrow-derived cells.

In this commentary, a brief background on chemokines is provided. Following this, the mechanism by which fibroblasts become stimulated to produce certain chemokines will be discussed in terms of agents that activate the fibroblast, namely, infectious agents and factors emanating from infiltrating hematopoietic cells. A key concept to be emphasized is that fibroblasts are part of the immune system and display receptors and surface markers that are used to regulate hematopoietic cells. These determinants also provide pathways through which immune cells regulate fibroblasts and induce chemokine production. Finally, the molecular control of chemokines by the NF-κB/RelB family of transactivation proteins and their differential impact on target genes in fibroblasts and hematopoietic cells is discussed.

Chemokines: Structure and Families

The ability to recruit specific populations of leukocytes during inflammation is the role of a family of cytokines called chemokines. Chemokines are small polypeptides that range in size from 7 to 10 kd and have significant sequence identity at the amino acid level.[2] During the process of wound healing and inflammation, leukocytes infiltrate the site of infection. Chemotactic molecules such as C5a, bacterial f-met-leu-phe, leukotriene B4, and platelet-activating factor stimulate a nonspecific infiltration of cells. Most chemokines have been divided into three different groups called the CXC, CC, and C families, so designated because of a conserved amino acid

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sequence of cysteines at the NH_2-terminal end of the protein. The CXC chemokines (also called α chemokines), possess two cysteines separated by a single unconserved amino acid residue. Some CXC chemokines have an additional structural motif of E-L-R at the NH_2 terminus. This amino acid sequence is important for signal transduction involved in neutrophil chemotaxis and may also play a role in chemokine stimulation of angiogenesis.[3,4] The CXC chemokines have 20 to 50% amino acid homology, and their genes are located on human chromosome 4. The CC chemokines (also called β chemokines) have two adjacent NH_2-terminal cysteines. They exhibit 28 to 45% amino acid homology, and the encoding genes are located on the human chromosome 17. The C chemokine family contains only one member and has a single NH_2-terminal cysteine. The C chemokine gene localizes to human chromosome 1.[5]

Most of the CXC chemokines are specifically chemotactic for neutrophils. IL-8 is the most extensively studied of the CXC chemokines. It is produced by several cell types including epithelial cells, fibroblasts, macrophages, T cells, endothelial cells and neutrophils. It is mainly chemotactic for neutrophils, but also stimulates the migration of T cells and basophils.[2,5] Interleukin (IL)-8 has been implicated as the major cause of chronic lung inflammation in patients with bacterial pneumonia and cystic fibrosis.[6,7] Additional CXC chemokines include growth-related oncogene(s) (GRO-α, -β, and -γ), interferon-γ-inducible protein (IP-10), platelet factor-4 (PF-4), and epithelial neutrophil activating protein-78 (ENA-78). GRO-α, -β, and -γ and the mouse homologues KC and MIP-2 are produced constitutively by primary melanomas and are inducible in fibroblasts, monocytes, and endothelial cells after exposure to proinflammatory stimuli. These chemokines are specific for the chemotaxis and activation of neutrophils. IP-10 is secreted from interferon-γ-stimulated monocytes, fibroblasts, endothelial cells, and keratinocytes. IP-10 can activate the migration of monocytes and memory T cells to the site of infection and is thought to play a role in delayed-type hypersensitivity reactions in cutaneous inflammation. PF-4 is released during platelet degranulation and induces the chemotaxis of neutrophils and monocytes. ENA-78 is produced by activated epithelial cells and stimulates the chemotaxis of neutrophils.[8]

Unlike the CXC chemokines, the CC chemokines stimulate the chemotaxis of monocytes and lymphocytes but not neutrophils. Examples of CC chemokines include monocyte chemoattractant proteins (MCP-1, -2, and -3), macrophage inflammatory proteins (MIP-1α and -β), regulated on activation normal T cell expressed and secreted (RANTES), and I-309. MCP chemokines are produced by macrophages, lymphocytes, smooth muscle cells, epithelial cells, endothelial cells, and fibroblasts. MIP-1α and -β are expressed by stimulated macrophages, T and B cells, monocytes, neutrophils, eosinophils, epithelial cells, smooth muscle cells, and fibroblasts. They are capable of inducing the chemotaxis of macrophages, basophils, eosinophils, and subsets of lymphocytes. RANTES is produced by T lymphocytes and fibroblasts and induces the chemotaxis of monocytes, eosinophils, and subsets of lymphocytes, whereas I-309 is produced by T cells and induces chemotaxis of monocytes.[2,5,9]

Lymphotactin is the only member of the C chemokine family and is expressed by activated $CD8^+$ T cells to induce the chemotaxis of lymphocytes.[10] Other chemoattractants do not structurally fit into any of the three classic chemokine families. One example is IL-16, which induces chemotaxis of $CD4^+$ T cells, monocytes, and eosinophils. IL-16 is synthesized by T cells, eosinophils, and epithelial cells.[11] Recently, we demonstrated that, after stimulation with IL-1, primary human fibroblasts isolated from several tissues express IL-16 (unpublished data). The production of chemokines by hematopoietic and nonhematopoietic cells can be induced by diverse stimuli, including bacterial products (lipopolysaccharide (LPS)), inflammatory cytokines (IL-1, tumor necrosis factor (TNF), and interferons), and after tissue damage. Once secreted, these chemokines bind to specific cell-associated receptors where their biological effects are initiated. It is this receptor binding that confers chemokine specificity as well as promiscuity for cell recruitment.[12] The fact that there exist many chemokines suggests the biological need for redundancy to effect recruitment of immune cells and perhaps to serve other functions such as angiogenesis. Chemokines are now known to be involved in many different disease processes, including lung fibrosis, allergy, pneumonia, rheumatoid arthritis, and chronic lung inflammation.[6,8,9,13] Recent work has focused on the signals that stimulate chemokine response at the initiation of wounding and infection.

Fibroblast Activation Induces Chemokine Synthesis

Historically, the fibroblast has been defined as a mesenchymal cell that is flat and elongated, possessing an oval, flat nucleus and the machinery to produce the collagens and other connective tissue components for the tissue within which it resides.[14] Although this definition is broad, it should be noted that not all fibroblasts are mesenchymal in origin. Orbital fibroblasts, for instance, derive from neural ectoderm. Fibroblasts lack the tight junctions and keratin synthetic ability of epithelial cells and fail to produce factor VIII or display the morphological characteristics of endothelial cells. Fibroblasts were previously considered important connective tissue cells that construct a supporting lattice crucial for tissue integrity and repair. As a result, they were relegated a minor active role in the inflammatory process. Recent data from our laboratories, as well as others, have defined several new concepts concerning the function of fibroblasts. First, fibroblasts, even from a single tissue, are not composed of a homogeneous population but rather consist of subsets of cells, much like lymphocytes.[15,16] Second, fibroblasts from different anatomic regions display characteristic phenotypes.[17] Moreover, regional diversity may reflect the specialized functions of the tissue of origin. These differences among fibroblasts may be the basis for localized susceptibility to disease manifestation. Third,

fibroblasts can be activated to display new functions important in controlling extracellular matrix synthesis and in producing cytokines and chemokines.[18,19] This feature is analogous to cells of the immune system, such as macrophages and B and T lymphocytes, which can be activated. Fourth, fibroblasts can regulate the hematopoietic cells that infiltrate a tissue that has been damaged, especially by infection.[18]

Bacterial Products Activate Fibroblasts and Other Mesenchymal Cells

Using infection as a model, several key products of bacteria, such as LPS, are now recognized as potent inducers of chemokines in fibroblasts as well as epithelial and endothelial cells. In many tissues, fibroblasts represent the dominant population of cells. Typically, there are few hematopoietic cells resident in these tissues. Therefore, the sentinel cells are likely to be the mesenchymal cells that, when properly stimulated by bacterial products such as LPS, generate chemokines capable of recruiting the inflammatory cells. The paper by Xia et al[1] in this issue of The Journal, as well as other reports,[20] focus on the ability of LPS to induce a chemokine cascade in established fibroblast lines. The chemokines MIP-2, JE/MCP-1, and KC/CINC are up-regulated with LPS stimulation. Expression of these molecules is transient although sufficient to initiate recruitment of the hematopoietic cells, which can further activate fibroblasts by secreting signals such as TNF-α. As fibroblasts are relatively long-lived, a tight control mechanism must exist to prevent overstimulation of the immune system, which could result in extensive tissue damage. As will be explored in a later section of this commentary, the transcription factor RelB has been shown to be a key brake limiting chemokine expression by fibroblasts. In part, this notion was substantiated by demonstrating that, in RelB-deficient mice, adoptive transfer of several sources of hematopoietic cells failed to correct the massive inflammation occurring in these animals.[21–23] In these RelB-deficient animals, the inflammation was often focused around areas of fibrosis. This and other evidence supported the hypothesis that fibroblasts were responsible for the dramatic dysregulation of inflammation. Thus, tissue fibroblasts, in concert with other mesenchymal cells, appear to function as key regulators of the inflammatory process in many tissues.

Induction by LPS of chemokines in mesenchymal cells represents the classic example of microorganism signaling of host cells. An exciting new family of molecules called autoinducers has recently been described that regulates the activities of bacteria and eukaryotic cells.[24,25] These autoinducers are *N*-acyl homoserine lactone-based structures that bind and activate transcriptional activator proteins leading to the induction of gene expression. Examples of target genes include those required for bioluminescence in the marine bacterium *Vibrio fischeri*[24] and in *Pseudomonas aeruginosa* the genes for elastases and exotoxin A.[26] Induction of proteolytic elastases in *Psuedomonas* permits rapid tissue destruction providing nutrients and space for the colonizing bacteria. Some autoinducer molecules were recently

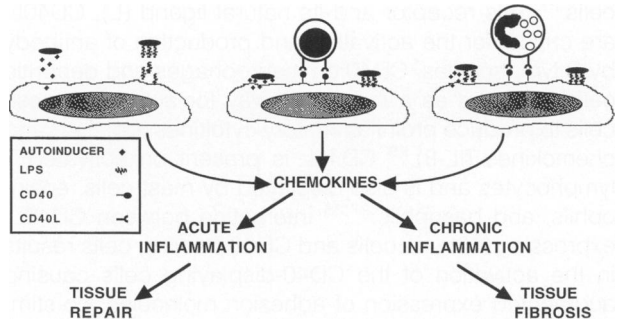

Figure 1. Activation of fibroblasts by bacterial products and by the CD40/CD40 ligand system. This figure shows how fibroblasts in a tissue are initially signaled by bacterial products such as LPS and autoinducer molecules to synthesize chemokines. This early warning system would initiate recruitment of the professional hematopoietic cells (T lymphocytes, macrophages, and granulocytes) in an attempt to resolve the infection. A second cascade of chemokine production is induced by the infiltrating immune cells, which further activate the fibroblasts through CD40/CD40 ligand interactions. Depending on the magnitude of these interactions and clearance of the invading microbe, either acute or chronic inflammation may occur with attendant tissue repair or fibrosis, respectively.

shown to stimulate in human epithelial cells the synthesis of the chemokine IL-8.[25] We have demonstrated that *Psuedomonas* autoinducers are powerful stimulators of IL-8 and other cytokines in several types of primary human fibroblasts (unpublished observations). These observations are exciting as most gram-negative bacteria are proposed to produce autoinducer molecules. A scenario can be established whereby secretion of autoinducers by bacteria activate the regional host mesenchymal cells to synthesize IL-8 and perhaps other chemokines, which in turn recruit immune cells to protect the tissue undergoing bacterial colonization. Although insight into the role of autoinducers is just unfolding, they add another dimension to the array of bacterial products capable of activating host defenses. Figure 1 depicts a possible scenario for bacterial products activating the resident fibroblasts and other mesenchymal cells to signal recruitment of immune cells to the site of infection. In this figure, the first signs that an intruding organism is present are derived from the invader's own products, such as the LPS from the outer cell wall and the secreted autoinducers. These bacterial products may participate in the promotion of chemokine synthesis. Continued stimulation of the fibroblasts without eradication of the infectious agent may lead to persistent colonization and chronic inflammation.

CD40 Is the Major Activation Antigen on Fibroblasts

Products of microorganisms are the initial alarm signal for resident mesenchymal cells to produce chemokines. The second defense layer for the eukaryotic host are activation receptors such as CD40 on regional mesenchymal cells. Recently, much interest has centered on CD40, a 50-kd integral membrane protein, which is a member of the TNF-α receptor superfamily originally described on B lymphocytes and subsequently on antigen-presenting hematopoietic cells such as macrophages and dendritic

cells.[27] This receptor and its natural ligand (L), CD40L, are critical for the activation and production of antibody by B lymphocytes. CD40 on macrophages and dendritic cells functions as a major pathway for activating these cells to produce proinflammatory cytokines (eg, IL-1) and chemokines (IL-8).[28] CD40L is present on activated T lymphocytes and is also displayed by mast cells, eosinophils, and basophils.[27,28] Interaction between CD40L-expressing immune cells and CD40-bearing cells results in the activation of the CD40-displaying cells causing augmented expression of adhesion molecules, co-stimulatory molecules (eg, B-7), and cytokines.[29]

Recently, human epithelial and endothelial cells and fibroblasts were also shown to be capable of displaying CD40, especially after exposure to interferon-γ.[19,30,31] Triggering of CD40 with ligands for CD40 increases expression of adhesion molecules, permitting attachment of hematopoietic cells and their entry into the tissue. Interestingly, IL-8 is one of the abundant chemokines secreted by fibroblasts and endothelial cells after CD40-mediated activation.[27,32] Recruitment of neutrophils and T lymphocytes to sites of fibroblast activation would permit further interaction between these cells. It is likely that CD40 engagement would also stimulate the synthesis of other chemokines, which could further heighten the activation of fibroblasts. Production of chemokines like eotaxin, which can recruit eosinophils potentially displaying CD40L, would serve to further activate resident tissue fibroblasts. Figure 1 shows how interactions between trafficked immune cells bearing CD40L interact with and enhance the activation of resident tissue fibroblasts, initiating mutually dependent chemokine and cytokine cascades. This is a potentially important amplification scheme that would help eradicate the invading microorganism, leading to acute inflammation and tissue repair. Alternatively, it could lead to a state of chronic inflammation, with possible fibrotic consequences, as occurs in diseases such as rheumatoid arthritis,[13] periodontal disease,[19] orbital fibrosis,[33] and idiopathic pulmonary fibrosis.[34]

Fibroblasts Express Cyclooxygenases and Produce Prostanoids under Basal and Cytokine-Stimulated Conditions

Prostaglandin E_2 (PGE_2) is the principal prostanoid synthesized by fibroblasts. PGE_2 appears to participate in the inflammatory response in a complex manner by virtue of its effects on immunocompetent cells. The rate-limiting steps in the biosynthesis of PGE_2 and other abundant prostanoids involve prostaglandin-endoperoxide H synthases (PGHSs). PGHSs are bifunctional enzymes involved in the conversion of arachidonic acid first to PGG_2 through cyclooxygenation and then to PGH_2, which is generated from peroxidation activity. Two isoforms of PGHS have thus far been identified and both are expressed by fibroblasts. PGHS-1 is primarily a constitutively expressed enzyme[35] whereas PGHS-2, the inflammatory cyclooxygenase, is ordinarily expressed at low levels but is inducible by mitogens, cytokines, and serum.[36] Fibroblasts, irrespective of anatomical site of origin, express PGHS-1 at relatively high levels, and this activity accounts for basal PGE_2 production.[37] In contrast, the tissue of derivation appears to be a crucial determinant of the inducibility of PGHS-2 in primary human fibroblasts. To date, lung,[38] synovial,[39] and orbital fibroblasts[37] have been shown to express highly inducible PGHS-2, whereas the induction in dermal fibroblasts is not as robust. It would appear that a major mechanism for the up-regulation by cytokines of PGHS-2 in fibroblasts stems from the activation of gene transcription as well as enhanced PGHS-2 mRNA stability.[37,40] The human PGHS-2 promoter contains two identifiable NF-κB sites, which are thought to be used in the induction of PGHS-2 gene transcription. The observation that certain fibroblasts exhibit substantial prostanoid biosynthetic potential has implications with regard to the potential roles these cells play in orchestrating the cellular and cytokine milieus in sites of inflammation. For example, PGE_2 can bias the commitment of naive T lymphocytes away from the Th1 phenotype and towards Th2 and can also influence B lymphocyte behavior.[41] The prostanoid can increase IL-5 synthesis in Th2 lymphocytes, down-regulate IL-2 mRNA levels in Th1 lymphocytes,[41] and plays an important role in mast cell activation.[42] Thus, anatomic regions where fibroblasts produce high levels of PGE_2 may exhibit a characteristic immunological environment predicated on the high levels of this prostanoid.

Molecular Events Underlying the Expression of Fibroblasts of Chemokines

Historically, the fibroblast has not been considered central to immune function but has been viewed as a rather inert cell type. Owing to the inherent difficulty in studying cells *in situ*, it is not easy to assess fibroblast behavior in their natural habitat, and thus inferences made about them derive largely from studies conducted in cell culture. It is only recently that fibroblasts have been shown to be diverse with regard to the phenotypes they exhibit *in vitro*. Fibroblasts from different anatomic regions of the human body exhibit attributes that can be used to distinguish them.[17,43,44] This phenotypic heterogeneity may result in part from diversity with regard to the use of signal transduction pathways, accounting perhaps for the differential susceptibilities to target gene activation.

A particularly important and recent insight concerning the participation of fibroblasts in the inflammatory response relates to their ability to receive and respond to complex molecular cues from the pericellular cytokine and growth factor milieu. In fact, several target genes encoding proteins proximate to cell trafficking, prostanoid biosynthesis, cell adhesion, and extracellular matrix synthesis have been identified in fibroblasts. Many of these fibroblast genes are potential targets for activation through NF-κB/Rel. The NF-κB/Rel family of transcriptional regulatory proteins includes several members capable of forming either homo- or heterodimers with other family members. Components of this family include p50, p65 (RelA), p52, RelB, and c-Rel. Unlike the other members, RelB is apparently only capable of hetero-dimeriza-

tion with either p50 or p52.[45,46] The factors governing the activation of NF-κB/Rel proteins, either directly or though the inactivation of IκBα, are relevant to the function and activation of fibroblasts. An emerging concept is that members of the NF-κB family function in very different ways depending on the cell type. For instance, in nonlymphoid cells, NF-κB may function in the stimulus-provoked transactivation of genes whereas RelB could primarily mediate the constitutive expression of genes in lymphoid cells.

A number of chemokines have been found to be inducible in primary human dermal fibroblasts. IL-8 is expressed in dermal fibroblasts treated with IL-1 and TNF-α.[47] Leukoregulin, a 50-kd lymphocyte-derived cytokine, can induce IL-8 expression in dermal fibroblasts. This action of leukoregulin is mediated through the activation of NF-κB.[48] Lung fibroblasts have also been shown to express IL-8 after stimulation with macrophage-derived cytokines[49] or after the interaction of CD40 with CD40L.[18]

In a recent report, Olashaw demonstrated that RelB can function in mouse fibroblast lines as a mitogen-inducible protein.[50] It would appear from these studies that quiescent fibroblasts express low levels of RelB and that the protein is bound to IκBα and is therefore inactive. Platelet-derived growth factor (PDGF), TNF-α, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and serum were demonstrated to up-regulate DNA binding of NF-κB and RelB in the fibroblast lines. Using supershift assays, PDGF was shown to increase the binding of both p65/p50 NF-κB and RelB/p50 heterodimers in AKR, BALB/c 3T3, and Swiss 3T3 fibroblasts. In contrast, RelB was not up-regulated by TPA in HeLa cells despite the activation of DNA binding to complexes containing p65. An important question remaining relates to whether the pattern of increase in RelB activity observed in established fibroblast lines will prove relevant to the biology of primary fibroblasts.

The report by Xia et al[1] brings to light a potentially important departure for RelB function in mouse kidney fibroblasts as compared with certain bone-marrow-derived cells. Whereas RelB appears to exert considerable support for transcriptional activity in lymphoid cells, in fibroblasts, this protein apparently attenuates the transcriptional up-regulation of NF-κB-driven genes. In their study, Xia et al[1] examined the expression of several chemokines. In RelB-deficient fibroblasts, chemokine expression was dysregulated in a generalized manner. A series of seven chemokines, including MIP-1α, MIP-1β, MIP-2, IP-10, JE/MCP-1, RANTES, and KC/CINC were found to be massively and persistently overexpressed after treatment with LPS in RelB-deficient fibroblasts when compared with the responses observed in the wild-type fibroblasts. Chemokine induction in normal fibroblasts was of a considerably lower magnitude and was transient. Transfection of the RelB-deficient fibroblasts with RelB cDNA attenuated the overinduction of the chemokines elicited by LPS. In a set of *in vivo* experiments, the authors injected LPS-prestimulated wild-type and RelB-deficient fibroblasts and assessed the granulocyte reaction elicited. They demonstrated that fibroblasts without adequate RelB expression were incapable of modulating the infiltration of inflammatory cells when compared with the wild-type fibroblasts. Thus, it would appear that the observations concerning disordered chemokine expression in cultured fibroblasts with interrupted RelB expression may well have direct relevance to the performance of these cells *in situ*.

Summary and Conclusion

In this commentary, we have suggested that the fibroblast should be considered a sentinel cell. This concept is based on the fibroblast's ability to function both as a structural element and as a vital immunoregulatory cell. In some tissues, these capabilities may be ascribable to subsets of fibroblasts, rather than to some of the general fibroblast populations. The pioneering research of Xia et al,[1] as well as that of others, highlights the need to explore the importance of fibroblasts as playing critical roles in disease. Emerging concepts regarding tissue-specific fibroblasts and fibroblast heterogeneity need to be considered in studies of their biosynthetic capabilities. Of special importance is the recent insight that the NFκB/RelB family of transcription proteins have apparently different regulatory roles in fibroblasts and hematopoietic cells. Therefore, with regard to therapeutic strategies targeting molecules such as RelB, caution should be exercised as their interruption may have very different consequences in macrophages compared with fibroblasts. For example, inhibition of RelB in macrophages may well prevent enhanced chemokine expression, whereas in fibroblasts, a critical governor for preventing chemokine expression would be lost. Overall, this could lead to exacerbation of inflammation rather than to an attenuation of the process.

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