Review

Potential roles of CCL2/monocyte chemoattractant protein-1 in the pathogenesis of cutaneous sclerosis

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ABSTRACT

Scleroderma is a connective tissue disease of unknown etiology characterized by the excessive deposition of extracellular matrix in the skin. Cellular infiltrates of certain immune cells and pro-inflammatory mediators are suggested to play a crucial role in cutaneous fibrosis, forming complicated networks between fibroblasts and immune cells and/or cell-cell communications. Tissue-selective trafficking of leukocytes is mediated by combinations of adhesion molecules and chemokines. Although chemokines and their receptors are considered to be mediators of inflammation and fibrosis in scleroderma, their pathophysiological role remains incompletely understood. Recent studies suggest that CCL2/ monocyte chemoattractant protein-1 plays an important role in the fibrotic process, including liver fibrosis, pulmonary fibrosis, and scleroderma. This review summarizes recent findings of the potential roles of CCL2 in cutaneous sclerosis in experimental animal models of scleroderma as well as human scleroderma.

Introduction

Scleroderma is a cutaneous fibrotic condition characterized by excessive production and deposition of extracellular matrix (ECM) in the skin, vascular injury and immunologic abnormalities (1). Although the initial pathogenesis is still unclear, excessive production as well as reorganization of connective tissue constituents determines the development of the disease (2). In particular, type I and III collagen, fibronectin, and proteoglycans are produced in large amounts by activated fibroblasts in the affected dermis. *In vitro* activated scleroderma fibroblasts continue to synthesize increased amounts of collagen, as compared with control fibroblasts (3). Although a number of studies have been performed to investigate the pathogenesis of scleroderma, its etiology remains still unknown. In the early stages of scleroderma, activated fibroblasts in the affected areas produce high amounts of collagen (4-6). Histological analysis of the initial stage of scleroderma reveals perivascular infiltrates of mononuclear cells in the dermis, which is associated with increased collagen synthesis in the surrounding fibroblasts (7, 8). A number of studies have demonstrated the crucial role of several fibrogenic cytokines released from immunocytes for initiating and/or leading to the sequential events of fibrosis in this disease (9-12). Recent evidence supports the concept that leukocyte activation and elicitation through cytokine-driven networks is one of the key processes in the autoimmune response (13). One of the families of effector molecules is the large group of mediators known as chemokines. Chemokines are a large family of small (7-15 kDa), structurally related heparin-binding proteins that may participate in immune and inflammatory responses through the chemoattraction and activation of leukocytes. Chemokines are subdivided into four subfamilies based on the arrangement of their N-terminal cysteine residues.

CCL2 (formerly called monocyte chemoattractant protein-1) is a chemoattractant for monocytes and T-cells, belonging to a C-C chemokine superfamily of small proteins that are important in recruiting and activating leukocytes during inflammation (14). CCL2 has also been characterized as the murine JE gene product. Previous studies showed that numerous types of cells including fibroblasts, endothelial cells, epithelial cells, mononuclear cells, and smooth muscle cells are capable of expressing CCL2 in the presence of serum or specific stimuli. Recent studies have demonstrated that CCL2

expression is upregulated in human pulmonary (15) and liver (16) fibrosis, as well as in animal models of pulmonary fibrosis (17) or crescent nephritis and interstitial kidney fibrosis (18). A recent *in vitro* study shows that CCL2 upregulates type I collagen mRNA expression in rat lung fibroblasts (19). In this article, potential roles of CCL2 in experimental scleroderma as well as human scleroderma was discussed.

Role of CCL2 in experimental mouse models of scleroderma

Bleomycin-induced scleroderma

Bleomycin is a frequently used antitumor antibiotic for various kinds of cancers. Lung fibrosis is a well-known side effect of bleomycin, and bleomycin-induced pulmonary fibrosis is an established rodent model (20-23). We confirmed that histological dermal sclerosis can be induced by repeated subcutaneous injections of bleomycin into the shaved skin of the back in various mice strains (24-28), although there is some variation among strains in the intensity and the periods required to induce dermal sclerosis. Cellular infiltrates in the lesional skin are mainly composed of CD4+ T-cells, macrophages and mast cells. Mononuclear cell infiltrates in the skin precede the induction of dermal sclerosis, which is suggested to secret cytokines stimulating ECM production.

Cytokines are important in the immune and inflammatory systems. Although a variety of cytokines and growth factors are involved in the pathogenesis of scleroderma, transforming growth factor-β (TGF-β) is suggested to play a key role (9). TGF-β, which is found in abundance in platelets and is released from activated macrophages or lymphocytes, is a strong chemoattractant for fibroblasts (29). TGF-β increases the synthesis of collagen type I and type III or fibronectin by many cell types *in vitro* (30-32). In addition, TGF-β modulates cell-matrix adhesion protein receptors (33, 34). TGF-β also regulates the production of proteins that can modify the ECM by proteolytic action, such as plasminogen activator, an inhibitor of plasminogen, or procollagenase (35-37). TGF-β is capable of stimulating its own synthesis by fibroblasts through autoinduction (38). It induces rapid fibrosis and angiogenesis when injected subcutaneously into newborn mice (39). Thus, maintenance of increased TGF-β production may lead to the progressive deposition of ECM, resulting in fibrosis.

It has been reported that bleomycin directly stimulates alveolar macrophages to secret fibroblast growth factor (40), and alveolar macrophages obtained from bleomycin-induced lung injury secret growth stimulatory factor for lung fibroblasts (41). Mononuclear cells are considered to be major sources of TGF-β. In the model of bleomycin-induced scleroderma, immunohistological analysis showed that TGF-β was detected on the infiltrating cells, which were predominantly composed of macrophages, as well as fibroblasts at sclerotic stages following bleomycin treatment. TGF-β1 and -β2 mRNA expression was also detected in the lesional skin. Blockade of TGF-β activity reduced the development of scleroderma (42). Therefore, it also plays a key role in the pathogenesis of bleomycin-induced scleroderma.

Mast cells produce a number of cytokines, growth factors, and mediators that are capable of activating fibroblasts or endothelial cells (43). Potential fibrogenic factors which are produced by mast cells include histamine (44, 45), tryptase (46), TGF-β (30-32), basic fibroblast growth factor (bFGF) (47), interleukin-4 (IL-4) (48), and platelet-derived growth factor (PDGF) (49). In the model of bleomycin-induced scleroderma, mast cells were increased in number in parallel with the induction of dermal sclerosis. In addition, a marked degranulation was found in particular in early phases, with elevated plasma histamin levels (24). On the contrary, bleomycin could induce dermal sclerosis even in genetically mast cell-deficient WBB6F1-W/W^{V} mice similarly to control littermates (25). TGF-β can be detected immunohistologically on the infiltrating cells in both WBB6F1-+/+ and WBB6F1-W/W^{V} mice (25). Mast cells may be associated with but not necessary for the induction of dermal sclerosis, implying that mast cells may be not the sole pathway to the induction of sclerosis.

Recently, excessive oxidative stress has been implicated in the pathogenesis of scleroderma (50, 51). Bleomycin is known to generate reactive oxygen species (ROS), such as superoxide and hydroxyl radicals. ROS can cause endothelial cell damage, stimulate skin fibroblast proliferation, and increase collagen production. We previously demonstrated the inhibitory effect of lecithinized superoxide dismutase (SOD) on bleomycin-induced scleroderma (52), suggesting the involvement of ROS. Oxidative stress transiently induces CCL2 mRNA and protein expression in cultured skin fibroblasts (53). Therefore, elevated levels of CCL2 in this model might be induced, in part, via ROS by bleomycin.

The biological actions of chemokines are mediated through a family of seven transmembrane G-protein-coupled receptors present on the surface of target cells. CC chemokine receptor (CCR)-2 is a major receptor of CCL2, and is expressed on monocytes, activated T-cells, B-cells, natural killer cells, fibroblasts, and mast cells. In the model of bleomycin-induced scleroderma, expression of CCL2 and CCR-2 was elevated at both protein and mRNA levels in the lesional skin following bleomycin treatment. CCL2 as well as CCR-2 were detected on the infiltrating mononuclear cells at early stages following bleomycin treatment, and also detected on the fibroblasts at later stages in the sclerotic skin (54). These findings suggest that CCL2 and CCR-2 signaling plays an important role in the pathogenesis of bleomycin-induced scleroderma. A recent report demonstrates that CCR-2-deficient mice are protected from fibrosis (55),suggesting that CCR-2 signaling promotes a profibrotic cascade.

Strategies to target chemokines include inhibitors of chemokine synthesis, receptor antagonists, and chemokine toxins. Continuous application of neutralizing antibody against CCL2 reduced the development of experimental scleroderma (54). Gene therapy should also be explored in this experimental model.

Sclerodermatous graft-versus-host disease (Scl GVHD) model

In chronic graft-versus-host disease (GvHD), which occurs across minor histocompatibility barriers, severe cutaneous fibrosis is observed with loss of dermal fat, atrophy of dermal appendages, mast cell depletion, and mononuclear cell infiltration (56, 57). A recent report demonstrates that a murine sclerodermatous GvHD model for scleroderma reproduces skin thickening, lung fibrosis, and upregulation of mRNA expression of collagen and TGF-β (58). Neutralization of TGF-β prevented fibrosis in the skin as well as in the lung (58). In this model, expression of C-C chemokines including CCL2, CCL3/macrophage inflammatory protein-1α and CCL5/RANTES was increased in the lesional skin before skin thickening and infiltration of CD45+ cells (59).

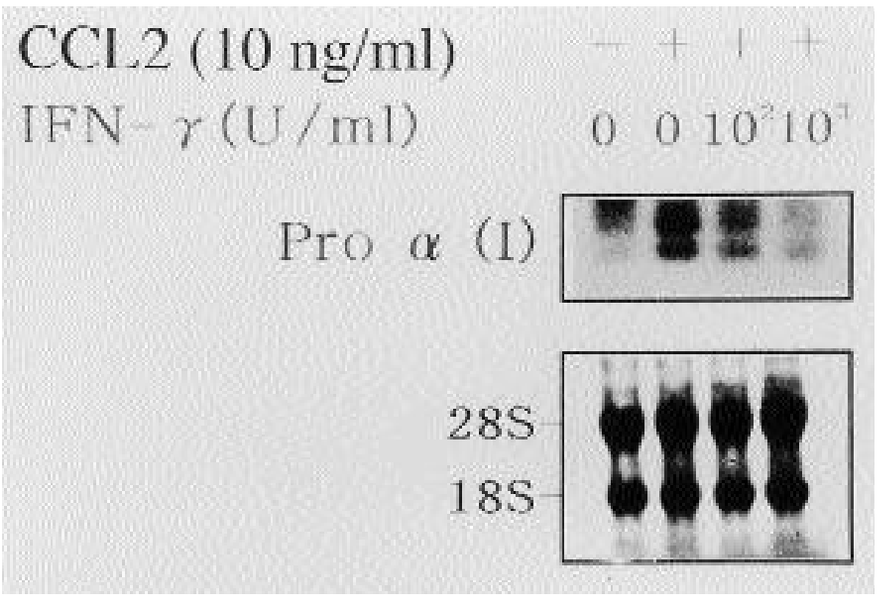

Fig. 1. Effect of CCL2 and IFN-γ on mRNA expression of β1 (I) collagen. Normal skin fibroblasts were stimulated with CCL2 (10 ng/ml) and IFN-γ (100 or 1,000 U/ml) concomitantly in the serum-free medium for 24 h. Then,total RNA was isolated and analyzed by Northern blot hybridization. The ribosomal bands stained with methylene blue confirmed equal loading of total RNA.

Tight skin mouse

TGF-β and IL-4 are suggested to play important roles in the pathogenesis of fibrosis in tight skin (Tsk) mice. Both cytokines have been shown to induce collagen synthesis by fibroblasts, and fibroblasts from Tsk mice are hyperresponsive to IL-4 and TGF-β (60). Targeted mutations in either the signaling chain of the IL-4 receptor or STAT6 prevents the cutaneous hyperplasia in Tsk mice, suggesting the importance of IL-4 (60, 61). In Tsk mice, mast cells are abundant in the thickened dermis and exhibit prominent degranulation (62). A decrease in fibrosis associated with inhibition of mast cell degranulation by cromolyn and ketotifen was also reported in the Tsk mice (63). Mast cell is one of major sources of IL-4. IL-4 has been shown to induce significant levels of CCL2 production in stromal cells (64, 65). On the contrary, CCL2 upregulates IL-4 mRNA expression and protein production (66). Thus, mutual induction of CCL2 and IL-4 has greatly been speculated. The roles of CCL2 in the fibrosis of Tsk mice should be clarified.

The role of CCL2 in human scleroderma

In vitro analysis showed that CCL2 upregulates type I collagen mRNA expression in rat lung fibroblasts, which is indirectly mediated by endogenous upregulation of TGF-β gene expression (19). TGF-β production by fibrotic fibroblasts was dependent on endogenous CCL2 synthesis because the presence of CCL2 antisense oligonucleotides markedly reduced TGF-β levels in the mouse (67). Also our recent data show that CCL2 upregulates the mRNA levels of α1(I) collagen and decorin in normal human skin fibroblasts, whereas those of fibronectin and biglycan were not significantly altered (54). Biglycan/ PG-I and decorin/PG-II are two small proteoglycans with a core protein of similar size (42 kDa), containing most often two and one chondroitin/dermatan sulphate glycosaminoglycan side chains, respectively. Although these molecules display a high degree of structural similarities, differential regulation of these molecules by CCL2 was suggested. Interferon-γ (IFN-γ), a representative antifibrotic cytokine, abrogated the CCL2-elicited upregulation of α1(I) collagen mRNA expression in human dermal fibroblasts (Fig. 1). Whether CCL2 is directly or indirectly involved in the development of fibrosis should be further clarified.

Current studies have demonstrated increased expression of CCL2 in patients with systemic sclerosis (SSc) (68-71). Hasegawa *et al*. (69) demonstrated that serum levels and spontaneous production levels by peripheral blood mononuclear cells of CCL2 were elevated in SSc patients compared with normal controls. Elevated serum CCL2 levels were correlated with pulmonary fibrosis. Immunohistochemical analysis also showed increased expression of CCL2 in scleroderma skin (68-70). Scleroderma fibroblasts express increased levels of CCL2 mRNA and protein (68-70). Distler *et al*. (71) reported that stimulation with PDGF resulted in a significant increase in CCL2 mRNA and protein. Furthermore, we demonstrated the autoinduction of CCL2 in scleroderma fibroblasts (72). These *in vivo* and *in vitro* results suggest a significant involvement of CCL2 in the pathogenesis of scleroderma. In addition, we previously reported that CCL2 enhances expression of matrix metalloproteinase-1 (MMP-1), MMP-2, as well as tissue inhibitor of metalloproteinase-1 (TIMP-1) in cultured human skin fibroblasts (73). Enhanced MMP as well as TIMP activity may contribute to the tissue remodeling.

In scleroderma skin, mast cells are

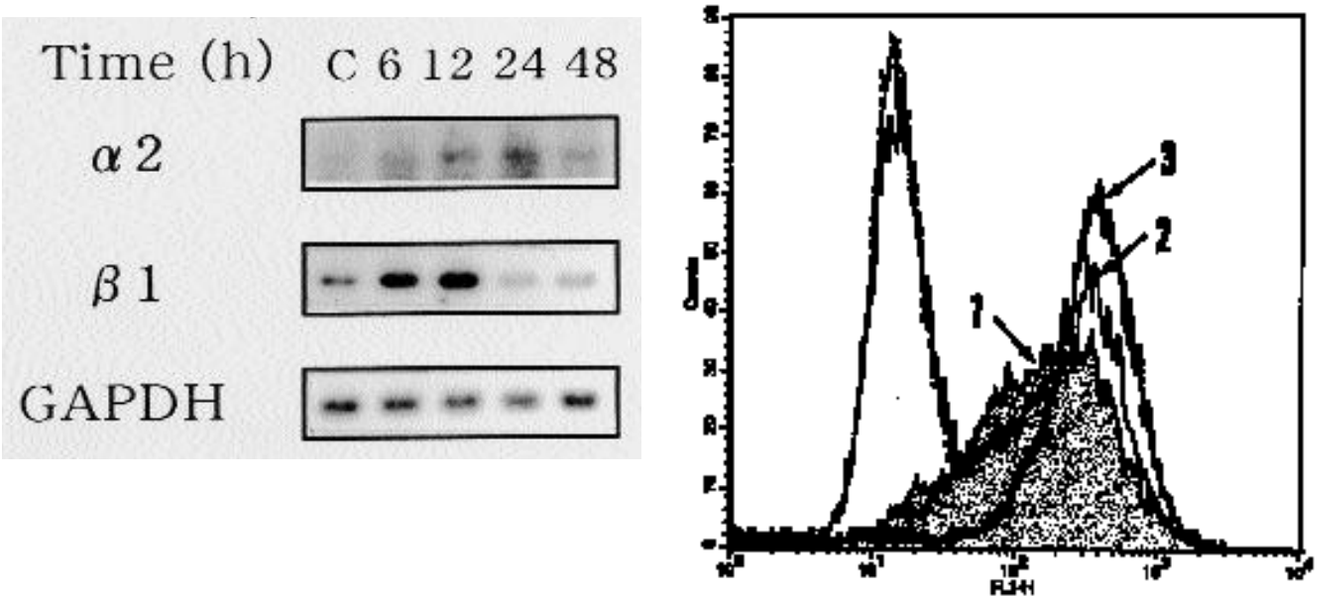

Fig. 2. Expression of β1 integrin following CCL2 stimulation.
(**A**) Time course of the expression of β1 integrin mRNA steady-state levels in transformed Wi-26/SV-40 fibroblasts after stimulation with CCL2. Cells were incubated with 10 ng/ml CCL2 for 6 to 48 h in serum-free medium. As a control (C),unstimulated cells were cultivated in serum-free medium for 48 h. Total RNA was analyzed by Northern blot hybridization with cDNA probes.
(**B**) Cell surface expression of β1 integrin after CCL2 stimulation. Cells were incubated with or without 10 ng/ml CCL2 for 24 and 48 h, and analyzed by flow-cytometry. The dotted lines are isotype-matched control. 1 (shaded histogram); unstimulated, 2; stimulated with CCL2 for 24 h, 3; stimulated with CCL2 for 48 h.

increased in number (74, 75), and with degranulated mast cells as an activated form (76), suggesting that mast cells are important initiators of scleroderma. Human mast cells are recently shown to be a rich source of chemokines, including CCL2, CCL3, CCL4/ macrophage inflammatory protein-1β, and CCL5 (77), as well as a number of cytokines, growth factors and mediators. Expression of stem cell factor (SCF), a mast cell growth factor, is upregulated in scleroderma fibroblasts (78, 79). SCF enhances CCL2 expression in human mast cell line, HMC-1 cells (80, 81) and human lung mast cells (80). Co-culture of normal fibroblasts with HMC-1 cells in monolayers showed increased expression of α1(I) collagen mRNA (81). Because CCL2 enhances type I collagen mRNA expression in skin fibroblasts, we speculate the mutual interaction between mast cells and fibroblasts via CCL2/ SCF, which may play an important role in fibrosis.

Recent hypotheses have indicated that imbalance exists between the type 1 and type 2 cytokine response in the pathogenesis of scleroderma. Type 2 cytokines include IL-4, IL-5, IL-10, IL-13 and CCL2. IL-4, which is produced by activated memory T-cells and mast cells, is known to promote fibroblast proliferation, collagen gene expression, and collagen synthesis (82-84). Plasma levels of IFN-γ are decreased in SSc patients, while those of IL-4, IL-10 and IL-13 are increased, compared to normal controls (85-87). Serum in the majority of SSc patients showed elevated levels of CD30 (88), which is expressed on activated type 2 cells. A recent report shows that most CD4+ T-cell clones generated from scleroderma skin biopsies exhibited type 2 cytokine profiles, and increased expression of IL-4 and decreased IFN-γ levels are shown in the lesional skin of SSc (88).

The regulation of ECM deposition is a key event in cutaneous sclerosis. The contact of fibroblasts with the surrounding ECM exerts a direct profound influence on cellular functions by inducing expression of ECM proteins and proteases involved in tissue remodeling. This interaction is mediated by specific receptors of which the β1 integrin family has been characterized in most detail. On fibroblasts, α1β1 and α2β1 integrins are the major structures of recognition of collagen and for relaying signals which direct synthesis and degradation of collagen. β1 integrins have also been shown to be present in scleroderma skin samples in areas of perivascular lymphoid cell accumulation and between collagen bundles, in association with resident fibroblasts (89). We observed that CCL2 upregulates β1 integrin gene transcription as well as cell surface expression in human transformed fibroblasts (Fig. 2). Although similar experiments should be performed using primary normal as well as scleroderma fibroblasts in the future, there is a possibility that CCL2 plays a role in cutaneous fibrosis/sclerosis in part by modulating β1 integrin receptor.

Conclusion

Recent evidence has suggested that fibroblasts are not only the structural elements but also part of the immune system. Fibroblasts can be activated to display new functions important in controlling ECM synthesis and in producing cytokines and chemokines. The interaction between immunocytes and

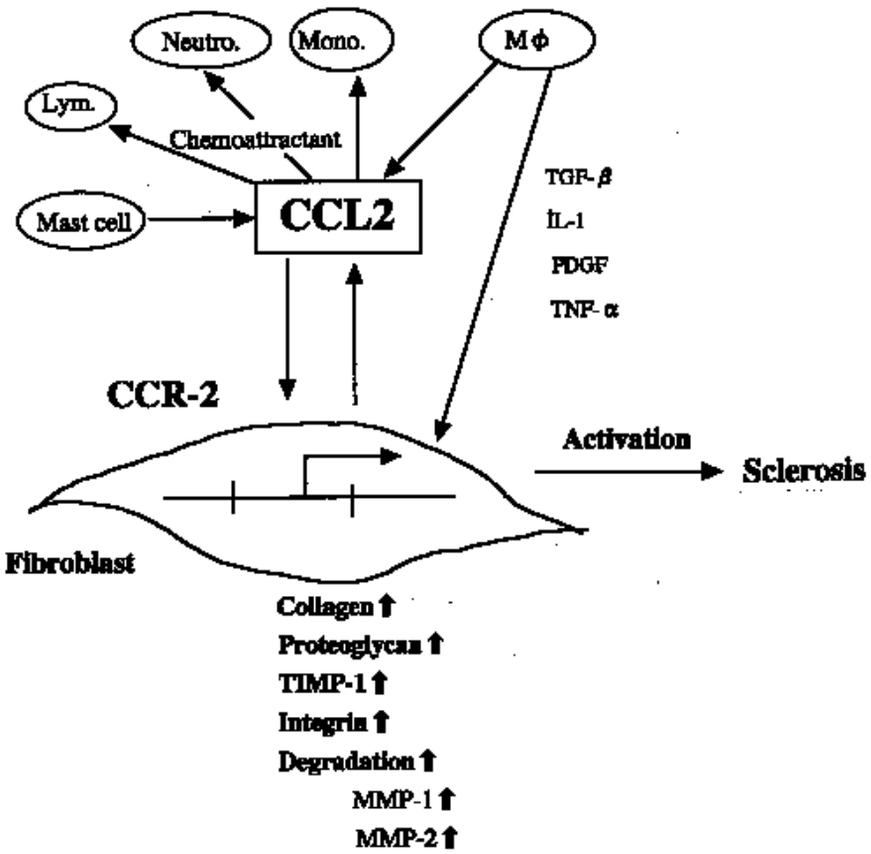

Fig. 3. Proposed roles of CCL2 in the induction of skin sclerosis.

fibroblasts via CCL2 is proposed in Figure 3. Prior to the onset of dermal sclerosis, inflammation usually precedes. CCL2 derived from infiltrating cells in the dermis may further enhance cellular infiltrates and release of proinflammatory or fibrogenic cytokines, leading to fibroblasts activation. Activated fibroblasts produce ECM proteins and also CCL2, which may participate in an autocrine fashion in fibrotic process. Enhanced MMP and TIMP activity may also contribute to regulate matrix degradation and remodeling in fibrotic process. CCL2 may play an important role in the induction of cutaneous sclerosis via its direct effect of upregulation of mRNA expression of ECM proteins, as well as indirect effect mediated by a number of cytokines released from immunocytes recruited into the lesional skin.

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