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Pathogenic role of CCL2/MCP-1 in scleroderma

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TABLE OF CONTENTS

1. ABSTRACT

Scleroderma is a connective tissue disease with unknown etiology characterized by excessive deposition of extracellular matrix in the skin. Cellular infiltrates of certain immune cells and proinflammatory mediators are suggested to play a crucial role in cutaneous fibrosis, forming complicated networks between fibroblasts and immune cells *via* cell-cell communications. Tissue-selective trafficking of leukocytes is mediated by combinations of adhesion molecules and chemokines. Recent studies have shown that an increase in proinflammatory chemokines has been associated with the initiation and/or development of fibrotic condition, suggesting that chemokines and their receptors may be important mediators of inflammation and fibrosis in scleroderma. In particular, CCL2/monocyte chemoattractant protein-1 (MCP-1) has been suggested to play an important role in scleroderma. This review will focus on the roles of CCL2 and its receptor during the process of cutaneous sclerosis, and also provide a current insight into the potential mechanisms of scleroderma.

2. 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). In particular, type I, III collagen, fibronectin, and proteoglycans are highly produced by activated fibroblasts in the affected dermis (2). *In vitro*, activated scleroderma fibroblasts continue to synthesize increased amounts of collagen, as compared with control fibroblasts (3, 4). 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 (5, 6). A number of studies have demonstrated important roles of several fibrogenic cytokines released from immunocytes for initiating and/or leading to the sequential events of fibrosis in scleroderma, and transforming growth factor-β (TGF-β) is suggested to play a key role (7). *In vitro,* TGF-β increases the synthesis of collagen type I and type III or fibronectin by many cell types (8-10). In addition, TGF-β modulates cell-matrix

adhesion protein receptors (11, 12). TGF-β also regulates the production of proteins that can modify the ECM by proteolytic action (13-15). Furthermore, TGF-β is capable of stimulating its own synthesis by fibroblasts through autoinduction (16). *In vivo*, TGF-β induces rapid fibrosis and angiogenesis when injected subcutaneously into newborn mice (17). Thus, maintenance of increased TGF-β production may lead to the progressive deposition of ECM, resulting in fibrosis.

Chemokines are chemotactic cytokines, and play a pivotal role in leukocyte recruitment from the bloodstream in the development, differentiation, and anatomic distribution of inflammatory cells (18, 19). Monocyte chemoattractant protein-1 (MCP-1)/CCL2 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 (20). 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. Additionally, it has been suggested that CCL2 is involved in collagen turnover. CCL2 expression has been demonstrated to be upregulated in human pulmonary (21) and liver (22) fibrosis, as well as in animal models of pulmonary fibrosis (23) or crescent nephritis and interstitial kidney fibrosis (24). Current reviews have suggested important roles of CCL2 in the pathogenesis of scleroderma (25-27). In this article, recent insights of roles of CCL2 and its receptor are discussed in the pathogenesis of scleroderma.

3. INFLAMMATORY CELLS AND CHEMOKINES IN SCLERODERMA

Chemokines mediate leukocyte-endothelium interaction and transmigration at multiple steps. Leukocyte extravasation starts with the selectin-dependent process of leukocyte rolling, and thereby allows contact of leukocytes with chemokines. Chemokines bind to their respective receptors on the surface of the rolling leukocytes, which activates leukocyte integrins resulting in firm adhesion of the leukocytes to the endothelial surface, leading to migration into the tissue spaces. The triggering events in scleroderma are usually followed by inflammatory process where various type of cells release inflammatory mediators to promote the recruitment of inflammatory cells.

3.1. T cells

Accumulation of monocytes/macrophages, lymphocytes, mast cells and occasionally eosinophils is a histological feature of the early stage of scleroderma. Since lymphocytes are not only immunological effector cells but also capable of producing a number of cytokines/growth factors, they are considered to play an important role in cutaneous fibrotic reaction. Mononuclear cell infiltrates in the skin is one of the most characteristic histological featutes in early scleroderma (2), which is suggested to secret cytokines stimulating ECM production. Moreover, infiltrating T cells, predominately CD4+, are also the major lymphocytes seen in the involved skin of scleroderma. It has been suggested that CCL2, CCL11, CCL22, and CCL17 play a role in T cell polarization, recruitment, or both into an inflammatory lesion (28, 29). CCL2 skrews T cell differentiation toward the Th2 phenotype (30). On the other hand, secretion of Th2 type cytokines result in enhanced local production of CCL1, CCL11, CCL13, CCL17, and CCL22 (31).

3.2. Macrophages

Activated macrophages appear to play an important role in fibrosis, because these cells are among the first immune cells found in increased numbers at the early stage of fibrosis, and they release a number of proinflammatory and fibrogenic mediators such as TGF-β and platelet-derived growth factor (PDGF) (32). Early scleroderma skin includes an increased CD14-positive cells (monocytes/macrophages) compared with normal skin (33). Ishikawa et al. (34) demonstrated that the ratio of infiltrating macrophages to T cells was high, suggesting an important role of cutaneous macrophages in scleroderma. Chemokines that attract macrophages to a site of inflammation include CCL2, CCL3, CCL5, CCL7, CCL8, CCL13, CCL17, and CCL22 (35).

3.3. Dendritic cells

Dendritic cells are bone marrow-derived antigen-presenting cells that function as sentinels of the immune system. Dendritic precursors in the peripheral blood migrate into peripheral tissues and differentiate to become immature dendritic cells. Upon activation, immature dendritic cells become mature dendritic cells. Immature dendritic cells can produce inflammatory chemokines including CXCL8, CXCL10, CCL3, CCL4 and CCL5, while mature dendritic cells produce CCL17, CCL18, CCL19 and CCL22 that attract T and B cells (36-38). Dermal dendritic cells are part of the dermal immune system, and suggested to play an integral part in the pathogenesis of scleroderma (39, 40).

3.4. Mast cells

Mast cells have been suggested to be one of the important initiators of scleroderma, since mast cells are increased in number in the lesional skin of its early stage (41, 42). CCL2 and CCL5 are suggested to play an important role in mast cell recruitment and activation in tissues (43). Mast cells release a number of cytokines, growth factors, and mediators that are capable of activating fibroblasts or endothelial cells. Also mast cells produce several kinds of chemokines, *i.e.* CCL2, CCL3, CCL4, CCL5, and CXCL8 (44, 45), which may play a part in the induction of fibrosis.

3.5. Eosinophils

Eosinophil infiltration in association with skin fibrosis is occasionally seen (46). A report gives evidence that eosinophils represent a primary cellular source of TGF-β (47). *In vitro* studies have shown that eosinophils readily bind to fibroblasts, which leads to the release of mitogens that augment fibroblast proliferation (48) and collagen production (49). Interleukin-5 (IL-5) is important in the differentiation, proliferation, recruitment, activation and chemotaxis of eosinophils. CCL11, CCL24, CCL26,

CCL5, CCL7, CCL13, and CCL3 have also been implicated in the recruitment of eosinophils into tissues (50). Fibroblasts can be a source of eotaxin under appropriate cytokine stimulation (51).

4. FIBROBLASTS AND CHEMOKINES

Fibroblasts are stimulated by inflammatory cells, which lead to the excessive production of ECM proteins. Additionally, recent evidence has suggested that fibroblasts themselves are also part of the immune system, not only the structural elements. A number of chemokines, such as CCL2, are produced by fibroblasts spontaneously or in the presence of stimuli. Activated fibroblasts appear to be key effector cells in the pathogenesis of fibrosis. Fibroblasts have also been shown to express functional CD40. Interaction between CD40 on fibroblasts and CD40 ligand (CD40L) on immune cells such as the T cells and mast cells can result in fibroblast activation. Expression of CD40 was increased on scleroderma fibroblasts compared to normal fibroblasts (52). Ligation of CD40 by CD40L resulted in increased production of IL-6, IL-8 and CCL2 in scleroderma fibroblasts, but not in normal fibroblasts (52). Moreover, the combination of IL-4 and ligation of CD40 on the surface of fibroblasts has synergistic effects in stimulating fibroblast proliferation (53). Fibroblasts also express CD40L. IL-13 increases the levels of detectable CD40L in human fibroblasts, while interferon-γ (IFN-γ) downregulates CD40L expression (54). Thus, the CD40-CD40L pathway may play an important role in the immune mechanisms of scleroderma.

A recent report demonstrated that CCL2 induced IL-4 production in T cells. Scleroderma fibroblasts expressed IL-4 receptor, and following stimulation with IL-4 collagen production was significantly increased (55). They suggest that CCL2 contributes to fibrosis by inducing the differentiation of IL-4-producing T cells as one of its proinflammatory role.

5. ROLES OF CCL2 IN HUMAN SCLERODERMA

In vitro analysis showed that CCL2 upregulates type I collagen mRNA expression in rat lung fibroblasts, which is mediated by endogenous upregulation of TGF-β gene expression (56). 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 (57). CCL2 upregulated the mRNA levels of α1(I) collagen and decorin in normal human skin fibroblasts, whereas those of fibronectin and biglycan were not significantly altered (58). 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 (58). IFN-γ, a representative antifibrotic cytokine, abrogated the CCL2-elicited upregulation of α1(I) collagen mRNA expression in human dermal fibroblasts (26).

Increased expression of CCL2 has been demonstrated in patients with SSc (59-63). Serum levels and spontaneous production levels by peripheral blood mononuclear cells of CCL2 were elevated in SSc patients compared with normal controls, and elevated serum CCL2 levels were correlated with the presence of pulmonary fibrosis (59, 63). Immunohistochemical analysis also showed increased expression of CCL2 in scleroderma skin (59-62). Scleroderma fibroblasts express increased levels of CCL2 mRNA and protein (59-61). Distler et al. (62) 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 (64). These *in vivo* and *in vitro* results suggest an important involvement of CCL2 in the pathogenesis of scleroderma.

Excessive deposition of connective tissue is the result of an imbalance between synthesis and degradation. A number of enzymes including matrix metalloproteinases (MMPs) and inhibition of proteolytic activity by tissue inhibitor of metalloproteinases (TIMPs) are believed to play a crucial role in connective tissue remodeling. TIMP-1 is a multifunctional molecule that has the potential to modify a number of cellular activities, and important for fibrogenesis. Myofibroblasts from scleroderma skin synthesize increased amounts of TIMP-1 compared with that of normal fibroblasts (65, 66). TIMP-1 expression in fibroblasts has been shown to be regulated by several cytokines, among which TGF-β is a most important inducer. In addition, CCL2 enhances expression of MMP-1, MMP-2, as well as TIMP-1 in cultured human skin fibroblasts (67). Enhanced MMP as well as TIMP activity may contribute to the tissue remodeling.

Mast cells are suggested to be important initiators of scleroderma. Human mast cells are shown to be a rich source of chemokines, including CCL2, CCL3, CCL4 and CCL5 (44), 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 (68, 69). SCF enhances CCL2 expression in human mast cell line, HMC-1 cells (70, 71) and human lung mast cells (70). Coculture of normal fibroblasts with HMC-1 cells in monolayers showed increased expression of α1(I) collagen mRNA (71). Because CCL2 enhances type I collagen mRNA expression in skin fibroblasts, we speculate that the mutual interaction between mast cells and fibroblasts *via* CCL2/SCF may play an important role in fibrosis.

Recent hypotheses have indicated that imbalance exists between the Th1 and Th2 cytokine response in the pathogenesis of scleroderma, which is suggested to be a Th2 disorder. Th2 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 (72-74). 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 (75-77). Serum in the majority of SSc patients showed elevated levels of CD30 (78), which is expressed on activated Th2 type cells. Most CD4+ T cell clones generated from scleroderma skin biopsies exhibited Th2 cytokine profiles, and increased expression of IL-4 and decreased IFN-γ levels were shown in the lesional skin of SSc (78). IL-13 has also been implicated in the pathogenesis of fibrotic conditions. *In vitro* studies demonstrated that IL-13 is a potent stimulator of fibroblast proliferation and collagen production (79-81). Additionally, IL-13 is a potent inducer of CCL2 *in vivo* (82). In addition, a functional single nucleotide polymorphism in the promoter region of CCL2 gene has recently been reported to be associated with SSc (83). Taken together, 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.

CCR2 is a major receptor of CCL2. CCR2 is expressed on monocytes, activated T cells, B cells, natural killer cells, fibroblasts, and mast cells. Very recently, CCR2 upregulation on vascular structures, perivascular inflammatory infiltrates, and fibroblasts has been demonstrated in SSc (84). In particular, myofibroblasts in early-stage diffuse SSc, along with overexpression of α-smooth muscle actin (α-SMA), connective tissue growth factor (CTGF) and CCL2 (84). Their results suggest potential autocrine regulation of key fibrotic properties *via* a CCL2/CCR2 loop in early phase of scleroderma.

6. ROLES OF CCL2 IN ANIMAL MODELS OF SCLERODERMA

6.1. Bleomycin-induced scleroderma

Dermal sclerosis can be histologically induced by repeated subcutaneous injections of bleomycin in various mice strains (85-90). 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. *In vitro*, bleomycin upregulates mRNA levels of ECM, TGF-β, and CTGF in dermal fibroblasts (91), as well as stimulates lung fibroblasts to release chemotactic activity including CXCL8 and CCL2 (92).

In the model of bleomycin-induced scleroderma, expression of CCL2 and CCR2 was elevated at both protein and mRNA levels in the lesional skin following bleomycin treatment (58). CCL2 as well as CCR2 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. These findings suggest that CCL2 and CCR2 signaling plays an important role in the pathogenesis of bleomycin-induced scleroderma.

Excessive oxidative stress has been implicated in the pathogenesis of scleroderma (93, 94). 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. The inhibitory effect of lecithinized superoxide dismutase (SOD) on bleomycin-induced scleroderma was previously demonstrated (95), suggesting the involvement of ROS in this experimental model. Oxidative stress transiently induces CCL2 mRNA and protein expression in cultured skin fibroblasts (96). Therefore, elevated levels of CCL2 in this model might be induced, in part, *via* ROS by bleomycin.

6.2. Sclerodermatous graft-versus-host disease (Scl GvHD) model

In the 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 (97, 98). 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 (99). Neutralization of TGF-β prevented fibrosis in the skin as well as in the lung (100). In this model, expression of CC chemokines including CCL2, CCL3 and CCL5 was increased in the lesional skin before skin thickening and infiltration of CD45+ T cells.

6.3. Tight skin mouse

TGF-β and IL-4 are suggested to play important roles in the pathogenesis of fibrosis in tight skin (Tsk) mice. Fibroblasts from Tsk mice are hyperresponsive to IL-4 and TGF-β (101). Smad2 and Smad3 are considered to be the primary signaling molecules involved in the TGF-β signaling transduction pathway. Recent studies suggest that Tsk fibroblasts have elevated Smad3 transcriptional activity compared with normal fibroblasts (102), providing a potential explanation that Tsk fibroblasts are more responsive to TGF-β stimulation. Previous studies concerning TGF-β mRNA expression in Tsk mice are inconsistent; one group showed increased expression in the skin of Tsk mice (103, 104), while another detected expression in only in the skin of neonate Tsk mice (105). 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 (101, 102). CD4+ T cells have been shown to be required for the excessive accumulation of dermal collagen in Tsk mice (106). In Tsk mice, mast cells are abundant in the thickened dermis and exhibit prominent degranulation (107). A decrease in fibrosis associated with inhibition of mast cell degranulation by cromolyn and ketotifen was also reported in the Tsk mice (108). 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 (109, 110). On the contrary, CCL2 upregulates IL-4 mRNA expression and protein production (111). Thus, mutual induction of CCL2 and IL-4 has greatly been speculated. Roles of CCL2 in the fibrosis of Tsk mice should be clarified. Recent studies show that CCL7/MCP-3 was highly overexpressed by neonatal Tsk fibroblasts (112). Increased CCL7 protein

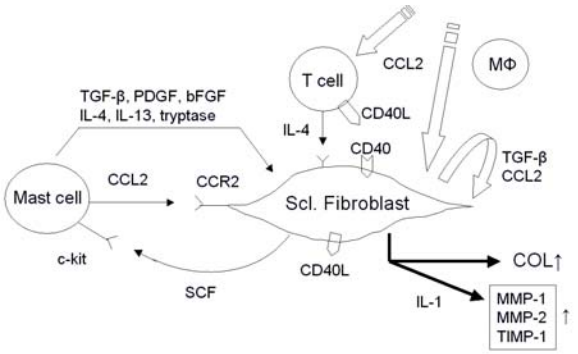

Figure 1. Schematic design of the role of CCL2 in the pathogenesis of scleroderma.

secretion by Tsk fibroblasts was observed. Immunohistochemistry revealed that CCL7 was detected abundantly in the dermis of Tsk mice at 10 days and 3 weeks old.

6.4. Skin fibrosis by exogenous injection of TGF-β and CTGF

Takehara's group showed that TGF-β-induced subcutaneous fibrosis and subsequent CTGF or basic fibroblast growth factor (bFGF) application caused persistent fibrosis (113, 114). They suggest that TGF-β plays an important role in inducing granulation and fibrotic tissue formation, and CTGF and bFGF are important in maintaining fibrosis (115). Mast cell number was significantly but transiently increased at the early phase, whereas the number of macrophages continued to rise (116). In the lesional skin, serial injections of CTGF after TGF-β increase CCL2 mRNA expression up to 8-folds, as compared with those after the single injection of TGF-β or CTGF (116).

7. THERAPEUTIC STRATEGIES

Strategies for fibrogenesis to target chemokines include inhibitors of chemokine synthesis, receptor antagonists, and chemokine toxins, and several studies have been published using experimental animal models. Blocking CCL2 attenuated bleomycin-induced dermal sclerosis (58), as well as pulmonary fibrosis (117) in mice. Recently, it has been shown a modest yet measurable dermal thickening following bleomycin treatment in CCL2 -/- mice, demonstrating a diminished response to fibrotic stimuli in the absence of CCL2 (118). They show that the loss of CCL2 significantly alters the assembly and accumulation of collagen. Another study showed that intraperitoneal administration of SKL-2841, an antagonist of CCL2, reduced the infiltration of inflammatory mononuclear cells and polymorphonuclear cells and also significantly suppressed fibrillization in bleomycin-induced scleroderma (119). These studies suggest that blockade of CCL2 may abrogate skin sclerosis, which might lead to the therapeutic potential. Also, other studies have demonstrated that CCR2-deficient mice were protected from fibrosis (120-122), suggesting that CCR2 signaling promotes a profibrotic cascade. Regulation of chemokine and/or their receptors may be one of therapeutic approach in modulating fibrosis.

8. CONCLUSION

Pathogenic role of CCL2 in the induction of scleroderma is schematically proposed in Figure 1. 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. Prior to the onset of dermal sclerosis, inflammation usually precedes. Chemokines derived from infiltrating cells in the dermis may further enhance cellular infiltrates and release of proinflammatory or fibrogenic cytokines, leading to fibroblasts activation. Among them, CCL2 may play a crucial role in attracting cellular infiltrates and also upregulating gene levels of ECM proteins in fibroblasts. Activated fibroblasts further produce CCL2, which may participate in fibrotic process *via* autocrine mechanisms.

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