Up-regulation of CC chemokine ligand 20 and its receptor CCR6 in the lesional skin of early systemic sclerosis

Mononuclear cell (MNC) infiltrate is one of the earliest pathological changes in systemic sclerosis (SSc) skin. However, little is known about the recruitment of these cells into skin lesions. Recently, the role of chemokines has been suggested in the pathogenesis of SSc. Here we studied the expressions and distributions of CC chemokine CCL20 and its receptor CCR6 in early SSc skin lesions and the difference in CCL20 expression and ability to recruit MNCs of normal dermal fibroblast (NDF) and scleroderma dermal fibroblast (SSDF). We found that the expressions of CCL20 and its receptor CCR6 were obviously up-regulated in SSc in contrast to normal human skin. mRNA levels were significantly expressed in SSc lesional skins vs normal skin tissues. SSDF displayed increased constitutive expressions of CCL20 mRNA and protein. In addition, Th1 cytokines (TNF-α and IL-1β) remarkably increased the expression of CCL20 in both NDF and SSDF in a dose- and time-dependent manner. Supernatants from SSDF showed stronger chemotactic activity to PBMCs than those from NDF. Thus our findings suggest that CCL20 released from cytokine-activated SSDF plays an important role in the induction of SSc by further recruiting more MNCs to the skin.

Key words: sclerosis, systemic, chemokine, CCL20

Materials and methods

Patients

Early diffuse patients were from Department of Dermatology of Union Hospital, affiliated to TongJi Medical College and Department of Dermatology, affiliated to the Medical College of JiuJiang University. Healthy age-and sex-matched control patients were from the plastic Surgery Department, not diagnosed with any type of skin disease. Diffuse SSc patients were diagnosed according to the criteria for the classification of early systemic sclerosis [13, 14]. No patients received any systemic or topical treatment with immunosuppressive drugs for at least 4 weeks before collection of skin biopsies. Experiments were approved by the Institutional Review Board, and written informed consent was obtained from all patients and controls. The study was conducted according to the Declaration of Helsinki Protocols.
**Immunohistochemistry**

Tissue sections were de-paraffinized and hydrated through graded alcohol series. Endogenous peroxidase activity was blocked with methanol containing 3% H$_2$O$_2$. Tissue sections were then incubated in a citrate solution (pH6.0) at 95°C for 20 min and then exposed to either anti-human CCL20/MIP-3α or monoclonal anti-human CCR6 antibody (R&D Systems) overnight at 4°C. Tissue sections were then washed and incubated with biotinylated rabbit anti-goat or goat anti-mouse IgG, followed by 1 h incubation with avidin-biotin peroxidase complex, developed with diaminobenzidine, and counter stained with Mayer’s haematoxylin. Negative controls were prepared by omitting the primary antibodies.

**Real-time RT-PCR**

Cultured dermal fibroblasts were lysed directly in TRIzol, whereas skin biopsies were minced first and then placed in TRIzol and total RNA was extracted according to the manufacturer’s protocol (Invitrogen). The purity and concentration of total RNA were measured by ultraviolet spectrophotometer. ReverTraAce-α (Toyobo, Japan) was employed for cDNA synthesis and resulting cDNA was analyzed to determine CCL20 and CCR6 mRNA levels relative to β-Actin mRNA levels. cdNA was amplified using SYBR Green QPCR Master Mix (Toyobo) and the following primers: CCL20, forward CTACTCCACCTCTGCGGCG, and reverse GTTGCGT-TGCTGTCACCTTCACCGTT, and reverse GTTGCGTTGCTCAAGTGTTCACAACCTGGGAAG, and reverse GCAAGTGAAACCTCCAACCC; CCR6, forward TGCTGTCACTTCCAGCTTG, and reverse GTCGCCATTACACCCTTCTTG. Primers were annealed at 60°C and amplified using STRATAGENE Mx3000P for a total of 40 cycles.

**Dermal fibroblast culture**

Dermal fibroblasts were isolated from the leading edge of clinically involved skin of six diffuse SSc patients and six healthy controls respectively, as previously described [15]. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin mixture at 37°C in 5% CO$_2$. Cells from passage 2 through 8 were used for experiments. For exposure to cytokines, fibroblasts were grown to 50% to 80% confluence and treated with one of the following cytokines: 0.1, 1, 10, or, 100 ng/mL of TNF-α, IL-1β or IL-4 (Pepro-tech Rocky Hill, NJ, USA). At indicated time points following cytokine exposure, conditioned media and cells were collected for CCL20 protein and mRNA quantification, respectively.

**CCL20 protein determination ELISA**

The amount of CCL20 protein released by fibroblasts was analyzed by ELISA. The ELISA kit for MIP-3α/CCL20 was purchased from R&D systems (Minneapolis, MN) and used according to the manufacturer’s instructions. CCL20 production was normalized to cell count (1 × 10$^5$) in 2 mL of complete medium. The data are means of three independent experiments.

**Chemotaxis assay**

The migration of cells was assayed in order to determine the activity of the dermal fibroblast-synthesized CCL20. Chemotaxis assays were performed in 24-wells transwell (Greiner bio-one, Germany) with 8.0 μm pore size polycarbonate membranes. The lower compartment was filled with 600 μL NDF or SSDF culture supernatants. Non-conditioned medium was used as negative control. PBMCs (2 × 10$^5$ cells) in 200 μL PBS were placed on the filters and incubated for 20h at 37°C in a cell culture incubator at 37°C and 5% CO$_2$. Chemotaxis indices were calculated as the ratio of the number of cells migrating toward CCL20 divided by the number of migrating cells in the negative control [5]. Triplicate wells were prepared for each condition. Neutralization of CCL20 was performed by preincubating conditioned media with 1 μg/mL of anti-human CCL20/MIP-3α antibodies (R&D Systems) or control IgG1 for 1 hour at 37°C.

**Data analysis**

Data were analyzed with the GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) and presented as the mean±SD of three experiments carried out at least in duplicate. One representative data set from these three independent experiments is shown where appropriate. A Student’s t test was employed for statistical analysis with significant differences determined as p<0.05.

**Results**

**CCL20 and CCR6 are significantly up-regulated in patients with SSc vs normal human skin**

CCL20 and CCR6 in normal (n=6) and SSc (n=12) skin lesions were examined by immunohistochemistry. The representative results are shown in figure 1. In normal human skin, epidermal keratinocytes, sweat glands and dermis were hardly stained by anti-human CCL20/MIP-3α or CCR6 antibodies, but there was a marked and specific staining of CCL20 (figure 1A-C) and CCR6 (figure 2A-C).

**Figure 1.** Expressions of CCL20 protein in healthy donors (A, B, C) and SSc lesional skins (D, E, F). A, D) Epidermis and dermis. B, E) Hair follicles. C, F) Sweat glands (original magnification ×200).
within the basal layers and hair follicles. In contrast, both of them were widely present in SSc lesional skins (figure 1D-F, figure 2D-F), including the epidermis, hair follicles, sweat glands and dermis with lymphocytes infiltrating. CCL20 and CCR6 mRNA were both significantly expressed by seven of nine early SSc lesions versus normal human skins (n=3). An average 1.6-3.9 and 1.2-3.0 fold increase of CCL20 and CCR6 mRNA could be detected respectively (p<0.05), except that two individuals (donor 3 and 7) showed a slight increase or decrease of both CCL20 and CCR6 (figure 3). Of interest, the level of CCR6 mRNA was consistent with the CCL20 mRNA.

**Effects of Th1/Th2 cytokines on CCL20 mRNA and protein expression in dermal fibroblasts**

We first examined the expression of CCL20 mRNA and protein under basal conditions and found that the levels of CCL20 mRNA and protein were very low in NDF, but were significantly higher in SSDF than NDF (p<0.05) (figures 4, 5).

Given that skin-infiltrating MNCs take part in the pathogenesis of SSc through the production of inflammatory cytokines, some of which have been found increased in SSc skin or serum [16-19], we investigated whether the Th1 cell-derived cytokine TNF-α, monocyte-derived cytokine IL-1β and Th2 cell-derived cytokine IL-4 could enhance CCL20 production in SSDF. As shown in figure 4A, B and figure 5A, B, TNF-α and IL-1β markedly upregulated CCL20 mRNA and protein at 24 hours in a dose-dependent manner in both NDF and SSDF. Using the same standard dosages, we also found these two classic cytokines upregulated the expressions of CCL20 mRNA and protein in a time-dependent manner (figure 4D, E, figure 5D, E). CCL20 expressions in NDF and SSDF had the same time course pattern but different sensitivity, which changed more rapidly in SSDF, while only moderately in NDF treated with these cytokines. Moreover, it seemed that IL-1β was more efficient than TNF-α in CCL20 production by dermal fibroblasts (p<0.05). Unlike TNF-α and IL-1β, IL-4 had no significant effects on CCL20 mRNA and protein expression in NDF (figure 4C, F, figure 5C, F). However, we were surprised to find that in SSDF, CCL20 mRNA and protein expressions were decreased along with the increase of IL-4 concentrations and time.

**CCL20 from NDF and SSDF under basal conditions is chemotactic for PBMCs**

To evaluate whether the CCL20 protein detected in the supernatants was biologically active, we tested supernatants of NDF and SSDF cultured 24h or 48h under basal conditions (without any stimulation) by chemotaxis assays. Results were shown (figure 6) that PBMCs were migrated toward culture supernatants of NDF and SSDF in a concentration-dependent manner. Then we investigated whether CCL20 from NDF and SSDF had significant difference in chemotactic activity. We found that unconditioned media derived from both NDF and SSDF displayed strong chemotactic activity for PBMCs, while the supernatant from SSDF showed an even stronger chemotactic activity than NDF (p<0.05). The chemotactic response to supernatant from NDF cultured either at 24h or 48h was almost completely blocked by anti-human CCL20/MIP-3α antibodies. However, this chemotactic activity was inhibited by 43.98±10.48% in the supernatant from SSDF cultured 48h under basal conditions.

**Discussion**

Here we demonstrated by immunohistochemistry that both CCL20 and CCR6 were significantly increased and widely localized in the epidermis, hair follicles, sweat glands and dermis in SSc skin. However, in normal skin tissues, CCL20 and CCR6 were distributed within the basal layers and hair follicles. Notably, the location of CCL20 was totally consistent with CCR6, indicating the inseparable roles of the ligand/receptor pair in SSc. The expressions of CCL20 and CCR6 in normal skin basal layers and hair follicles indicated that the CCL20/CCR6 pair, except for its prominent features of lymphocyte chemotaxis, might also possess other biological activities [20, 21] such as cell proliferation and differentiation, organ development, angiogenesis/angiostasis, etc. Furthermore, we also found an increase of CCL20 and CCR6 mRNA in SSc compared to normal skin and also showed a positive relationship between the expression of CCL20 and its receptor CCR6 in SSc.

**Figure 2.** Expressions of CCR6 protein in healthy donors (A, B, C) and SSc lesional skins (D, E, F). A, D) Epidermis and dermis. B, E) Hair follicles. C, F) Sweat glands (original magnification ×200).

**Figure 3.** The levels of CCL20 and CCR6 mRNA in early SSc lesional skins vs healthy donors. The expression level for each healthy donor was arbitrarily assigned a value of 1, and the final results were expressed as a fold number compared with healthy donors. * p<0.05. Bars show the mean and SD.
Figure 4. Effects of TNF-α, IL-1β, and IL-4 on CCL20 mRNA expression in NDF and SSDF. Cells were treated with the indicated concentration of cytokines for 24 hours (A, B, C). Cells were treated with the optimal cytokine concentrations for 6, 24, or 48 hours (D, E, F). The mRNA expression level for each control sample (0h) was arbitrarily assigned a value of 1, and the final results were expressed as fold numbers compared with healthy donors. * p<0.05. Bars show the mean and SD.

So these results demonstrate that the increase of CCL20 and CCR6 protein in SSc was caused by their mRNA increase. However, we also found there were some variations in the levels of CCL20 and CCR6 among the SSc patients, which probably represented either heterogeneity in the patient population, either from the genetic background or, alternatively, at different stages of the disease. Thohgaki et al observed that, as one of the three SSc-specific genes, CCL20 were upregulated in peripheral monocytes of patients with early systemic sclerosis, and CCL20 was

Figure 5. Effects of TNF-α, IL-1β and IL-4 on CCL20 protein expression in NDF and SSDF. Cells were treated with the indicated concentration of cytokines for 24 hours (A, B, C). Cells were treated with the optimal cytokine concentrations for 6, 24, or 48 hours (D, E, F). CCL20 protein levels were quantified by ELISA. * p<0.05. Bars show the mean and SD.
and time. These might be explained by the character of Th2 cytokines, defined as anti-inflammatory cytokines on the basis of their inhibitory effect on the production of TNF-α, IL-1β, IL-6, and IL-8 by monocytes, and similar effects were also reported in bleomycin-induced pulmonary inflammation and fibrosis [26-29]. However, some studies reported IL-4 could stimulate fibroblast proliferation, collagen and fibronectin synthesis [17, 30], contributing to dermal and pulmonary fibrosis [29, 31-33]. These may support the hypothesis that an imbalance exists between the Th1 and Th2 cytokine response in the pathogenesis of scleroderma.

In fact, it has been confirmed that cultured primary keratinocytes as dermal fibroblasts took part in CCL20 production in the skin [5, 34]; moreover, it has been proved that TNF-α markedly increased the expression of CCL20 in normal human keratinocytes [34], so we presume that these proinflammatory cytokines also affected SSc keratinocytes to produce CCL20 and tried to separate keratinocytes from SSc skin, but in vain, due to the low viability of the epidermal cells and/or by the too small biopsy tissues. Lastly, we assessed the chemotactic activities of supernatants from NDF and SSDF in the absence of exogenous stimuli, which showed stronger chemotactic bioactivities than those from NDF in attracting PBMs. In addition, the chemotaxis of supernatants from SSDF could not be completely blocked by anti-human CCL20/MIP-3α antibodies, suggesting that there must be some other chemokines participating in the migration of MNCs, such as monocyte chemoattractant protein-1 (MCP-1/CCL2). MCP-1 is considered to play a central role in the chemotactic activity for MNCs that dermal fibroblasts exhibit in vitro [1], so our study was complementary to the mechanism study of MNCs migration.

In general, our study suggested that cytokines derived from infiltrating cells activate fibroblasts to produce more chemokines (CCL20), which might be involved in the process of inflammation of SSc by further recruiting more MNCs into the skin.

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References


