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Synoviocyte-Derived CXCL12 Is Displayed on Endothelium and Induces Angiogenesis in Rheumatoid Arthritis

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CXCL12 (stromal cell-derived factor-1) is a potent CXC chemokine that is constitutively expressed by stromal resident cells. Although it is considered a homeostatic rather than an inflammatory chemokine, CXCL12 has been immunodetected in different inflammatory diseases, but also in normal tissues, and its potential functions and regulation in inflammation are not well known. In this study, we examined the cellular sources of CXCL12 gene expression and the mechanism and effects of its interactions with endothelial cells in rheumatoid arthritis synovium. We show that CXCL12 mRNA was not overexpressed nor induced in cultured rheumatoid synoviocytes, but it specifically accumulated in the rheumatoid hyperplastic lining layer and endothelium. CXCL12 gene expression was restricted to fibroblast-like synoviocytes, whereas endothelial cells did not express CXCL12 mRNA, but displayed the protein on heparitinase-sensitive factors. CXCL12 colocalized with the angiogenesis marker αvβ3 integrin in rheumatoid endothelium and induced angiogenesis in s.c. Matrigel plugs in mice. The angiogenic activity of rheumatoid synovial fluid in vivo was abrogated by specific immunodepletion of CXCL12. Our results indicate that synoviocyte-derived CXCL12 accumulates and is immobilized on heparan sulfate molecules of endothelial cells, where it can promote angiogenesis and inflammatory cell infiltration, supporting a multifaceted function for this chemokine in the pathogenesis of rheumatoid arthritis. The Journal of Immunology, 2003, 170: 2147–2152.
the coordinate action of multiple factors that orchestrate EC proliferation, migration, and the reorganization of the extracellular matrix and cellular elements. Among them, TNF-α, VEGF, basic fibroblast growth factor, and the chemokines IL-8, ENA-78, and fractalkine have been identified in the rheumatoid synovial environment and suggested to play a role in RA angiogenesis (25–28). We have studied the cellular sources of CXCL12 and its potential interactions with EC in RA synovium, with specific attention to its potential to induce angiogenesis. We show that CXCL12 is strongly expressed by synoviocytes in RA synovium and preferentially exposed on EC lacking CXCL12 message through heparitinase-sensitive factors. We also provide evidence demonstrating that CXCL12 induces angiogenesis in Matrigel plug assays in vivo and participates in the angiogenic activity of RA-SF.

Materials and Methods

Patients and tissue samples

Synovial tissue was obtained from 10 patients with RA and 10 patients with osteoarthritis (OA) at the time of knee prothetic replacement surgery, and from 4 healthy controls undergoing knee arthroscopic explorations due to minor traumatic lesions. Synovial tissue was embedded in OCT compound and snap frozen. Fibroblast-like synoviocyte (FLS) cultures were established from homogenized synovium in 10% FCS-DMEM medium. FLS cultures were used between passages 3 and 6. PBL were obtained from healthy blood donors by Ficoll gradient purification. HUVEC were prepared from umbilical cord by collagenase digestion and propagated in medium 199 (Life Technologies, Paisley, Scotland) with 20% FCS. Human microvascular dermal EC were obtained from Upstate Biotechnology (Lake Placid, NY) and cultured in CS-C medium (Upstate Biotechnology). Synovial fluid was obtained by arthrocentesis from 6 RA patients with active knee arthritis, and after centrifugation at 800 × g for 20 min, the supernatant was collected and frozen at −80°C until used for Matrigel assays.

Immunohistochemistry

Cryosections were fixed in cold acetone for 15 min. Endogenous peroxi-
dase was quenched in 3% H2O2 in methanol for 20 min. Staining was performed following a standard indirect avidin-biotin HRP method (ABC (avidin biotinylated enzyme complex) standard; Vector Laboratories, Burlingame, CA). Color was developed with diaminobenzidine (Vector Laboratories). We used anti-CXCL12 clone K15C at 20 μg/ml and anti-
CXCR4 clone 12G5 at 15 μg/ml. Specificity of these Abs has been previously reported (7, 29). Controls with control murine IgG instead of primary Ab were included. After immunoperoxidase development, fluorescent labeling with anti-CD51/61-fluorescein clone 23C6 (BD Biosciences, San Jose, CA) and rhodamine-Ulex Europaeus Agglutinin 1 (UEA1) at 20 μg/ml (Vector Laboratories) was performed.

Pretreatment with heparitinase I or chondroitinase ABC (Sigma-Aldrich, St. Louis, MO) was performed by incubating tissue sections with 5 μg/ml of each enzyme in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1% BSA for 1 h at 37°C.

In situ hybridization

Synovial sections were fixed in cold 4% paraformaldehyde in PBS for 15 min. After treatment with 200 μg/ml pronase E, sections were prehybridized in 5× SSC buffer, 50% formamide, and 1× Denhardt’s solution, and hybridized in the same solution containing 0.8 mg/ml of digoxigenin-UTP-labeled riboprobe for 12 h at 50°C. Sections were washed in SSC up to a final concentration of 0.1× SSC at 55°C. Hybridized probe was detected by incubation with 1/500 alkaline phosphatase-conjugated anti-digoxigenin Ab (Roche Diagnostics, Mannheim, Germany) and color developed with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate. Digoxigenin-UTP-labeled sense and antisense CXCL12-β riboprobes were synthesized by in vitro transcription of the full-length CXCL12-β cDNA (0.3 kb) cloned in pcDNA3 plasmid (7). Linearized plasmids were transcribed in vitro, using SP6 and T7 polymerases, according to the manufacturer’s protocol (Roche Diagnostics).

Northern blot

Total RNA was extracted from cultured FLS from OA and RA synovium, HUVEC, human microvascular dermal EC, and PBL, using Ultraspec reagent (Biotex, Houston, TX). Total RNA (10 μg) was electrophoresed on 1.2% formaldehyde agarose and transferred to nylon membranes. Membranes were hybridized with [α-32P]UTP-labeled full-length CXCL12 or GAPDH cDNA probes cloned on pcDNA3 vector, as previously described (7).

Cells were pretreated for 6–24 h with 50 U/ml human TNF-α (Genzyme, Cambridge, MA), 100 ng/ml human VEGF, 10 ng/ml human TGF-β1 (R&D Systems, Abingdon, U.K.), or 10 μg/ml LPS (Difco Laboratories, Detroit, MI), or cultured under hypoxic atmosphere (3% O2), where indicated.

Angiogenesis assays

Pooled SF supernatants from 6 RA patients were diluted at 1/4 in PBS and preincubated for 1 h with either anti-CXCL12 K15C mAb or murine IgG2a (Sigma-Aldrich) at a final concentration of 30 μg/ml. SF preincubated with K15C mAb or IgG2a were further diluted at 1/10 in ice-cold Matrigel (BD Biosciences). In additional experiments, we incorporated 100 ng/ml human VEGF or 100 ng/ml human CXCL12 (R&D Systems) to Matrigel implants. A total of 500 μl of Matrigel containing SF, VEGF, or CXCL12 was s.c. injected in the abdominal skin of female 8–12-wk-old BALB/c mice. After 10 days, Matrigel plugs were removed and processed for hematoxylin staining and histological study or weighted and homogenized in 500 μl of dH2O for hemoglobin determination. Hemoglobin was determined using Drabkin’s reagent (Sigma-Aldrich).

Results

CXCL12 and CXCR4 expression in synovial cells and tissues

In all groups of synovial sections, healthy, OA, and RA, CXCL12 was detected by peroxidase immunohistochemistry (Fig. 1a). Healthy synovial membrane showed weak immunostaining restricted to the single cell layer of synoviocytes that constitutes the lining. RA sections showed a strongly increased immunostaining of the hyperplastic lining that extended to the nearby extracellular matrix and perivascular areas of the sublining. The endothelium of blood vessels was frequently, but not uniformly immunostained, contrasting with healthy synovium in which immunostaining was not detected in blood vessels. Identification of CXCL12-immuno-
stained EC was confirmed by double UEA fluorescent labeling (Fig. 1a). RA inflammatory cell infiltrates were not immunostained, but contained interspersed immunostained cells with fibroblastic or large mononuclear cell morphology. In some OA synovial sections (4 of 10 examined) showing abundant inflammatory cell infiltrates, increased immunostaining of lining synoviocytes and detectable immunostaining of blood vessels were observed (Fig. 1a).

To evaluate CXCL12 gene expression in the different cell lineages, CXCL12 mRNA was analyzed by Northern blot in cultured cells. Cultured FLS from OA and RA synovium displayed CXCL12 mRNA expression (Fig. 2a). Gross differences between individual FLS lines were not observed. To analyze whether CXCL12 can be induced by proinflammatory or angiogenic factors in FLS, we stimulated cultured FLS with LPS, TNF-α, TGF-β, and VEGF, and they were cultured under hypoxic conditions (3% O2). None of these stimuli significantly increased CXCL12 mRNA expression neither in RA nor in OA FLS (Fig. 2b). Contrarily, a decrease was observed after TNF-α or VEGF treatment. In contrast with FLS, CXCL12 mRNA was undetectable in HUVEC, microvascular endothelial cells (MVEC), and PBL. Hypoxia or VEGF treatment of HUVEC or MVEC did not induce CXCL12 mRNA expression.

These data suggested that EC immobilize, but do not produce CXCL12 protein. To exclude inflammation-related or tissue-specific expression of CXCL12 by RA-EC, we specifically evaluated whether the endothelium of rheumatoid synovium expresses CXCL12 mRNA. In situ hybridization studies were performed in parallel sections of rheumatoid synovial tissues previously shown to display abundant CXCL12 protein by immunohistochemistry. In all cases, CXCL12 mRNA was detected in lining synoviocytes
and scattered perivascular cells of the sublining, but not in EC (Fig. 3).

Because CXCL12 mRNA was not detected in synovial tissue EC, where we had detected the protein, we studied whether the presence of CXCL12 on EC could be due to its accumulation bound to HS molecules. We analyzed CXCL12 immunostaining in serial sections from RA synovial membrane after removal of HS by heparitinase digestion. Heparitinase pretreatment removed most immunodetectable CXCL12 from endothelium (Fig. 1c), whereas immunostaining of lining synoviocytes remained intact after this treatment. CXCL12 protein seems specifically associated to HS, because chondroitinase treatment did not modify CXCL12 immunostaining.

CXCR4 expression was detected by peroxidase immunohistochemistry in all groups of synovial sections from healthy, OA, and RA individuals. Expression was widespread in most cell types present in the synovial membrane. Specifically, expression of CXCR4 was detected in synoviocytes, inflammatory infiltrates, and EC in all groups of synovial sections (Fig. 1b). To evaluate whether exogenous CXCL12 protein accumulated on CXCR4-expressing EC is associated with angiogenesis, we studied its association with angiogenesis markers. RA endothelium was not uniformly immunostained for CXCL12, allowing us to study the relationship between the presence of CXCL12 in EC and angiogenesis by performing triple labeling with anti-CXCL12, rhodamine-UEA as a marker of EC, and fluorescein anti-CD51/61 (integrin αβ3) as a marker of angiogenic EC. CXCL12 colocalized to CD51/61-labeled endothelium and most vessels lacking CXCL12 protein did not show CD51/61 expression (Fig. 1a).

**RA-SF-induced angiogenesis**

To further study the potential effects of synovial CXCL12 on angiogenesis, we examined the contribution of CXCL12 to the angiogenic activity of rheumatoid SF in Matrigel in vivo assays. Matrigel plugs containing CXCL12, PBS as negative control, or VEGF as positive control were implanted s.c. in the abdominal skin of mice. After 10 days, large vascular structures invading the plug were similarly observed in plugs containing VEGF, CXCL12, or RA-SF, whereas PBS controls did not show vascular formation (Fig. 4a). Because CXCL12 is expressed by lining cells in direct contact with SF and high levels of CXCL12 have been demonstrated by rheumatoid SF (9), we next studied the neutralizing effect of anti-CXCL12 mAb on the angiogenic activity of SF. We used K15C mAb, which has been shown to neutralize CXCL12 binding to CXCR4 receptor in vitro at concentrations similar to what was used in this study (16). Matrigel plugs containing RA-SF and anti-CXCL12 mAb did not display angiogenic activity, whereas in controls containing murine IgG2a, RA-SF-induced angiogenesis was not inhibited. The hemoglobin content of the Matrigel plugs was determined as a marker of the number of blood

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**FIGURE 1.** Expression of CXCL12 and CXCR4 by synovial tissues. **a.** Upper panels, Show immunoperoxidase staining of CXCL12 in healthy, OA, and RA synovial sections (hematoxylin counterstaining). Middle panels, Show double labeling of CXCL12 (brown immunoperoxidase staining) and EC identified as red fluorescent cells by rhodamine-UEA in an RA section. Lower panels, Show triple labeling of CXCL12 and EC (as above) and the angiogenesis marker integrin αβ3 (green fluorescence). Small vessels lacking CXCL12 and integrin αβ3 are marked by arrows. Integrin αβ3 is also expressed by lining synoviocytes. **b.** CXCR4 immunoperoxidase staining of healthy, OA, and RA synovial sections. c. CXCL12 immunodetection in parallel sections of the same RA synovium pretreated with chondroitinase ABC (left) or heparitinase (right). Images are representative of 10 RA and 10 OA samples.
vessels. Hemoglobin content increased in plugs treated with VEGF, CXCL12, and RA-SF compared with controls (PBS). In plugs treated with CXCL12-neutralizing mAb, the hemoglobin concentration was significantly reduced compared with those treated with control IgG2a (Fig. 4b).

**Discussion**

The participation of CXCL12 in the pathogenesis of RA has been previously suggested by several studies that demonstrate expression and a potential role of CXCL12 in the homing of leukocytes to rheumatoid synovium (9–12). CXCL12 produced by cultured rheumatoid FLS supports the migration and survival of B and T cells in vitro (10, 11, 30), and injected into RA synovium engrafted in SCID mice it induces a dose-dependent increase in the chemotraction of myelomonocytic cells (12). In addition, CXCR4 antagonist does not prevent, but ameliorates arthritis in the collagen-contraction of myelomonocytic cells (12). CXCL12 is not a cytokine-inducible gene, and therefore, CXCL12 expression can also play a role in the pathogenesis of the inflammatory process associated to OA (9, 32).

The generation of chemokine gradients or their accumulation and immobilization on HS molecules of the EC membrane facilitates the firm adhesion and extravascular migration of leukocytes. We show that EC do not synthesize CXCL12, but in inﬂamed synovium they display CXCL12 protein, facilitating its presentation to inﬁltrating leukocytes and EC that express CXCR4 receptor. Binding of chemokines to surface HS on EC has been suggested as an important mechanism that prolongs their $\tau_{1/2}$ and efﬁciently increases their presentation to speciﬁc receptors in circulating leukocytes (33, 34). Our observation of extracellular and EC-bound CXCL12 protein in RA synovial membrane and the removal of EC-CXCL12 by heparitinase treatment suggests that most CXCL12 is bound to HS molecules on the EC membrane. Two mechanisms can regulate this phenomenon in rheumatoid endothelium: first, the local accumulation of CXCL12 by hyperplastic FLS, and second, the specific regulation of HS-proteoglycan expression by RA-EC. CXCL12 is not a cytokine-inducible gene, and the mechanism for CXCL12 overexpression in RA seems different from other chemokines that are strongly induced by TNF-α in resident synoviocytes. CXCL12 shows a constitutive and cell-specific pattern of expression, and proinflammatory cytokines such as TNF-α or factors involved in angiogenesis such as TGF-β, VEGF, or hypoxia did not increase its expression. In a previous study in gingival fibroblasts, TNF-α and IL-1 also inhibited CXCL12 expression (35). Because most CXCL12 protein in RA synovium is expressed by hyperplastic FLS in the lining and sublining, expansion of these cells can explain CXCL12 accumulation in RA synovium. Enhanced presentation of CXCL12 bound to EC-HS in inﬂamed tissues can provide an alternative, but not mutually exclusive mechanism of functional up-regulation of this constitutive chemokine. EC are heterogeneous regarding HS expression, and consistently, cultured cells lose their ability to present surface chemokines (17, 36). Our unpublished observations also indicate that cultured human EC do not bind exogenous...
CXCL12. High endothelial venules in rheumatoid synovium display a specific pattern of HS-proteoglycan expression that correlates with their enhanced ability to immobilize MIP-1β and to induce adhesion of T cells (37). A similar mechanism might be involved in the presentation of CXCL12 by endothelium of RA or hemopoietic tissues in which CXCL12 is also present, but not expressed by EC (5, 6).

In addition to its direct effects on leukocyte recruitment, the accumulation of chemokines on surface HS proteoglycans of EC may have autocrine effects by facilitating its interaction with functional receptors in the nearby membrane of EC, as demonstrated for heparin-binding EC growth factors (38). Migration of EC in response to several chemokines has been demonstrated, but importantly, CXCR4 receptor seems the most abundant and functionally prominent in EC (18–20). The potent proangiogenic effect of CXCL12 has been demonstrated on aortic rings, Matrigel, rat cornea, or murine skin (14, 21, 22). Our data confirm the presence of CXCR4 receptor on synovial EC and colocalize its ligand to angiogenic vessels, providing support to the hypothesis of CXCL12 as an angiogenic factor in RA. The Matrigel in vivo system has been demonstrated as a sensitive method to test the proangiogenic activity of different factors present in the synovial environment (27). We demonstrate that immunodepletion of CXCL12 decreases the proangiogenic activity of RA-SF in vivo. Several studies have previously shown the potent antiangiogenic effects of the depletion of a single factor from the complex medium provided by RA-SF (26–28). This suggests that proangiogenic factors in the RA-SF are not functionally redundant, and that the cooperative action of multiple factors is required to permit its full proangiogenic effects. In contrast, single factors such as VEGF or CXCL12 induce potent proangiogenic effect in different angiogenesis models. A potential explanation is the observed up-regulation of both angiogenic and angiostatic factors such as thrombospondin-1 or IL-12 in RA with a net angiogenic balance (25). In this setting, a partial loss of the proangiogenic factors can shift the balance toward angiogenesis inhibition, and this may have important therapeutic implications because partial influences in this balance can have profound effects on angiogenesis.

In summary, these data demonstrate that increased production of CXCL12 by RA synovium leads to its accumulation and presentation on heparitinase-sensitive factors of EC, and that this factor participates in the angiogenesis associated to chronic inflammation. The multifunctional effects of CXCL12 in the pathogenesis of RA suggest its potential as a target for pharmacological interventions. Importantly, CXCL12-CXCR4 system seems independent of the chemotactic axis CXCR4/stromal cell-derived factor-1 in the inflammatory component of allergic airway disease. J. Immunol. 165,499.


