Inflamm. res. 50 (2001) 529–533
1023-3830/01/110529-05 $ 1.50+0.20/0

Inflammation Research

Original Research Papers

Fractalkine induces chemotaxis and actin polymerization in human dendritic cells[1]

S. Dichmann[a], Y. Herouy[a], D. Purlis[a], H. Rheinen[a], P. Gebicke-Härter[b] and J. Norgauer[a]

Departments of Dermatology[a] and Psychiatry[b], University of Freiburg, Hauptstraße 7, DE-79104 Freiburg i. Br., Germany, Fax: ++49 761 270 6720, e-mail: Norgauer@haut.ukl.uni-freiburg.de

Received 10 October 2000; returned for revision 23 February 2001; accepted by M. J. Parnham 28 June 2001

Abstract. *Objective and design:* Dendritic cells (DCs) are considered as the principle initiators of immune responses by virtue of their ability to migrate into target sites, process antigens and activate naive T cells. Here, the chemotactic activity and intracellular signaling of fractalkine was analyzed and compared to well known chemotaxins.
Methods: The mRNA-expression of G protein-coupled CX3CR1 was analyzed by RT-PCR. Chemotaxis was measured in 48-well Boyden chambers and actin polymerization by flow cytometry.
Results: The mRNA-expression of CX3CR1 in immature and mature DCs was revealed. Fractalkine elicited actin polymerization and chemotaxis in a dose-dependent manner in DCs independent of their state of maturation.
Conclusions: These results show that immature and mature DCs express mRNA for the CX3CR1 and that fractalkine induces chemotaxis and migration associated actin polymerization in immature as well as in mature DCs, contrasting with the action of other chemokines such as RANTES or MIP-3beta which act only on distinct maturation states of DCs.

Key words: Dendritic cells – Fractalkine – CX3CR1 – Migration

Introduction

Dendritic cells are antigen-presenting cells (APCs) designed for the capture of antigens for efficient presentation to lymphocytes [1, 2]. Immature DC precursors derived from the bone marrow circulate in the bloodstream. After migration to target tissues they efficiently sample the antigenic content of their environment. Thereafter dendritic cells circulate to secondary lymphoid organs in order to prime T cells [3]. At present the recruitment of DCs to different sites during their life cycle is not completely understood. Recent reports implicate involvement of different CC-chemokines such as RANTES, macrophage/monocyte chemotactic protein 1–4 (MCP1-4), and macrophage/monocyte inflammatory protein 3-β (MIP-3beta) in this recirculation process [4].

Recently, a novel chemotaxin the CX_3C-chemokine fractalkine exhibiting separation of the first two cysteins by three amino acids has been identified and cloned [5]. This molecule can exist either in a membrane-anchored form or as a shed 95 kDa glycoprotein [5]. It is produced by epithelial, endothelial or dendritic cells [5–7]. Fractalkine mediates firm cell adhesion and functions as a chemotaxin for T-cells, monocytes and microglia [5, 8]. Reconstitution experiments identified a seven transmembrane spanning protein encoded by the V28 orphan cDNA as a high affinity receptor for fractalkine [9]. Like other chemokine receptors this protein named CX_3C-R1, couples to G proteins and induces actin polymerization in microglia cells [5, 8].

Recently, CX_3C-R1 expression was revealed in murine dendritic cells [10]. The aim of our study was to identify expression of CX_3C-R1 in human dendritic cells and to characterize the effects of fractalkine on actin polymerization and chemotaxis at different maturation states of human DCs.

Materials and methods

Materials

Lysophosphatidylcholine was obtained from Sigma (Deisenhofen, Germany); anti-CD14 MicroBeads, separation columns and magnetic MultiStand from Miltenyi Biotec (Bergisch Gladbach, Germany); N-(7-nitrobenz-2-oxa-1,3-diazol-4yl)-phallacidin (NBD-phallacidin) from Becton Dickinson (Heidelberg, Germany); Interleukin-4 and granulocyte macrophage colony stimulating factor from Natutec (Frankfurt, Germany); fractalkine from R&D systems (Wiesbaden, Germany); Macrophage inflammatory protein 3-β (MIP-3beta) and RANTES from PeproTech (London, England).

Correspondence to: J. Norgauer

[1] Contract Grant sponsor: Clinical Research Group Pathomechanisms of Allergic Diseases. BMBF Contract Grant number: 01GC9701.

Preparation of human dendritic cells

Peripheral blood cells were isolated from buffy coats by ficoll separation and lysis according to Dichmann et al. [11]. After centrifugation, the leukocyte-containing pellet was resuspended in 2 ml phosphate buffered saline containing 0.15% EDTA and 0.5% bovine serum albumin. The cells were separated by CD14 antibody-coated MicroBeads using Macs single use separation columns. The CD14-positive cells (purity > 96%) were suspended in RPMI 1640-medium containing 10% fetal calf serum, 1% glutamin, 50 IU/ml penicillin, 50 UG/ml streptomycin, 100 U/ml interleukin-4 and 1000 U/ml granulocyte macrophage colony stimulating factor at 37 °C in a humidified atmosphere with 5% CO_2. After 5 days cells were harvested for experiments. Differentiation of monocytes into dendritic cells was characterized by the surface markers CD14 < 5%, $CD1a_{low}$ > 95%, $CD80_{low}$ > 95%, $CD83_{low}$ > 95%, $CD86_{low}$ > 95%, $CD115_{high}$ > 95%. Further differentiation into mature DC was induced by treatment with 3 µg/ml LPS (E. coli, serotype 0111:B4) for 48 h. These DC were characterized by CD14 < 5%, $CD1a_{high}$ > 95%, $CD80_{high}$ > 95%, $CD83_{high}$ > 95%, $CD86_{high}$ > 95%, $CD115_{low}$ > 95% (antibodies and respective isotype controls: Coulter-Immunotech, Krefeld, Germany).

Actin polymerization

By flow cytometry the content of filamentous actin was analyzed with NBD-phallacidin staining [12, 13]. Briefly, aliquots (50 µl) of stimulated cell suspensions (5×10^5 DC/ml) were withdrawn at the indicated time intervals and fixed in a 7.4 % formaldehyde buffer. After 1 h cells were mixed with the staining cocktail containing 7.4% formaldehyde, 0.33 µM NBD-phallacidin and 1 mg/ml lysophosphatidylcholine. The fluorescence intensity was measured by flow cytometry with exitation and emission wavelength of 488 and 520 nm, respectively. The fluorescence intensity of NBD-phallacidin binding was measured and the relative fluorescence intensity of stimulated samples was calculated as a ratio to controls.

Migration assay

The experiments were performed using 48-well chambers (Nuclepore, Tübingen, Germany). Buffer or stimuli were filled into the wells of the lower compartment. Thereafter a polycarbonate membrane of 10 µm thickness with a pore size of 5 µm (Nuclepore) was placed over the wells. Dendritic cells (10^5 cells/well) were added in the upper compartment and incubated at 37 °C for 90 min in a humidified atmosphere. After removing the cells from the upper side of the membrane by wiping over a profiled rubber, migrated dendritic cells on the lower side of the membrane were fixed in methanol and stained with hematoxylin. For each sample dendritic cells in 5 randomly choosen high-power fields (magnification: ×400) were counted and a mean value for each sample was calculated. The chemotactic index was calculated as the ratio between stimulated cells and the medium control.

Detection of mRNA by reverse transcriptase and polymerase chain reaction

The mRNA was isolated by using QIAshredder and RNeasy kits. The cDNA was obtained using mRNA, $pd(N)_6$ primers and M-MLV reverse transcriptase. All oligonucleotides used as primers in PCR were designed to recognize a unique sequence exclusive for each target cDNA. The sequence of the specific primers was as follows:

CX3CR1: 5′-CAGCAAGAAGCCCAAGAGTGTCA-3′(sense), and
5′-CAGGGGGAGTAGGAAGCCAAGA-3′(anti-sense)

β2-microglobulin: 5′-CCTGGAGGCTATCCAGCGTA-3′ (sense), and
5′-GTTCACACGGCAGGCATACT-3′ (anti-sense)

Polymerase chain reaction consisted of 25 cycles of denaturation (94 °C, 1 min), ramped annealing (58 °C, 1 min), and extension (72 °C, 1 min). The obtained PCR products were subjected to electrophoresis on a 2% agarose gel and were visualized by ethidium bromide-staining. The identity of the generated products was proven by sequencing after cloning using pCRII vectors.

Results

Gene expression of CX3CR1 in dendritic cells

The mRNA expression of CX3CR1 was analyzed by reverse transcriptase and polymerase chain reaction. These studies revealed an amplified 439 base pair product in immature and maturated DCs (Fig. 1). In addition, the comparable signal of the amplified control gene β-microglobuline in each sample indicate equal loading. Cloning confirmed that this product represents the original sequence of CX3CR1. No products

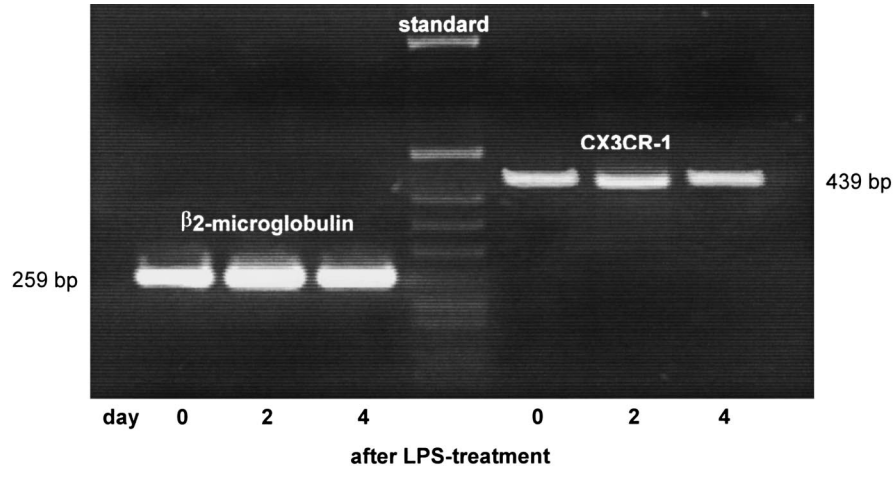

Fig. 1. Expression of mRNA of CX3CR1 in human dendritic cells. The mRNA was isolated at day 0, 2 and 4 after LPS-treatment and RT-PCR was performed as described in the Materials and methods. The experiments were repeated five times from samples of different donors with identical results.

were obtained after omitting reverse transcriptase in the reaction (data not shown).

Actin response and fractalkine

Actin reorganization is a prerequisite for migration of different types of leukocytes [12]. Here, the influence of fractalkine on the actin network in immature and LPS-differentiated dendritic cells was analyzed by flow cytometry. This chemokine caused a rapid polymerization of actin molecules within 25 sec (Fig. 2A and B). There was an increase of the f-actin content of about 50% in both types of DC. Maximal and half-maximal effects were observed at 1 µg/ml and 0.01 µg/ml fractalkine, respectively.

Fractalkine and chemotaxis of dendritic cells

Migration of immature dendritic cells to fractalkine was analyzed by Boyden chamber experiments. Fractalkine elicited a typical dose-dependent bell-shaped chemotactic response for immature and mature dendritic cells. Maximal effects were seen at a fractalkine concentration of 1 µg/ml (Fig. 3). It is known that the chemotactic activity of chemokines in DCs depends on their maturation state. Therefore, we incubated DCs with LPS and compared the chemotactic activity of fractalkine to optimal concentrations of RANTES and MIP-3beta. This study revealed that fractalkine in immature DCs provoked a similar chemotactic response as found with RANTES (Fig. 4). In contrast, MIP-3beta was not able to elicit a migration response in immature DCs. In maturated DCs after LPS-treatment the chemotaxis to RANTES was significantly reduced, whereas MIP-3beta induced a pronounced and dose dependent response. In contrast to the analyzed CC-chemokines, fractalkine acted as an effective chemotaxin in immature and mature DCs.

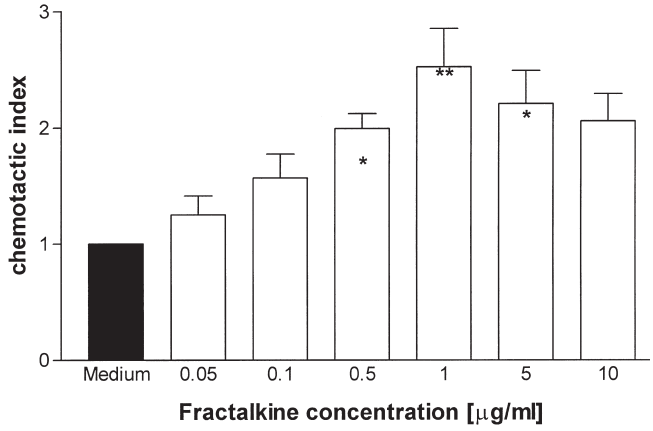

Fig. 3. Influence of fractalkine on the chemotaxis of immature dendritic cells. Dendritic cells were exposed to the indicated concentrations of fractalkine for 90 min at 37 °C in a Boyden chamber. Chemotactic index was calculated. Data are means ± SEM (n = 4 different donors). Global differences between groups: $P \leq 0.001$ (ANOVA); $P \leq 0.001$ (**) compared with medium control (Tukey test); $P \leq 0.01$ (*) compared with medium control.

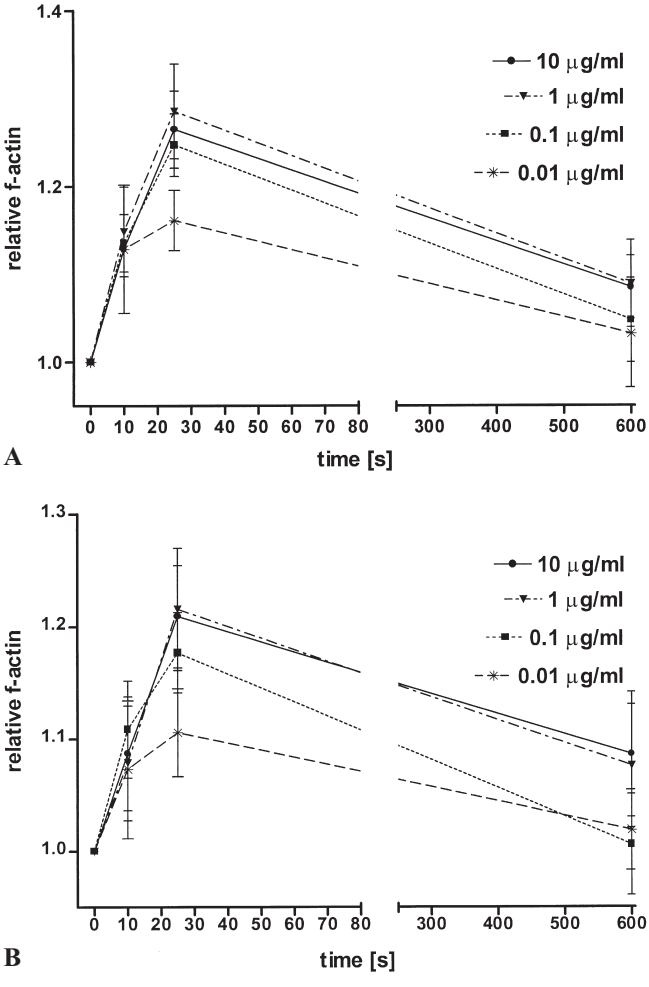

Fig. 2. Fractalkine and actin polymerization of immature (**A**) and LPS-differentiated (**B**) dendritic cells. Cells were stimulated with 10 µg/ml (●), 1 µg/ml (▼), 0,1 µg/ml (■) and 0,01 µg/ml (✳) of fractalkine. The relative f-actin content was determined at the indicated time points by flow cytometry. Data are means ± SEM (n = 5 different donors).

Discussion

The CC-chemokines RANTES, monocyte chemotactic protein 1–4 and macrophage inflammatory protein-3β are well characterized chemotaxins for dendritic cells [4, 14]. It can be assumed that all these agents are involved at different stages during the recirculation process of dendritic cells from the bone marrow via the blood stream and target sites in the skin to secondary lymphoid organs [15]. Recently, a novel chemokine exhibiting separation of the first two cysteins by three amino acids has been identified and cloned [5]. This glycoprotein is produced by epithelial, endothelial or dendritic cells [5–7]. After binding to a G protein coupled receptor it induces migration of T-cells, monocytes and microglia as well as it stimulates actin reorganization in microglia [5, 8]. To improve our understanding of the biological activities of fractalkine, we analyzed chemotaxis, actin polymerization and CX3CR1 mRNA-expression in dendritic cells.

In this study we revealed by RT-PCR experiments the mRNA expression of CX3CR1 in immature as well as in LPS-maturated DCs. The stable expression of CX3CR1 dur-

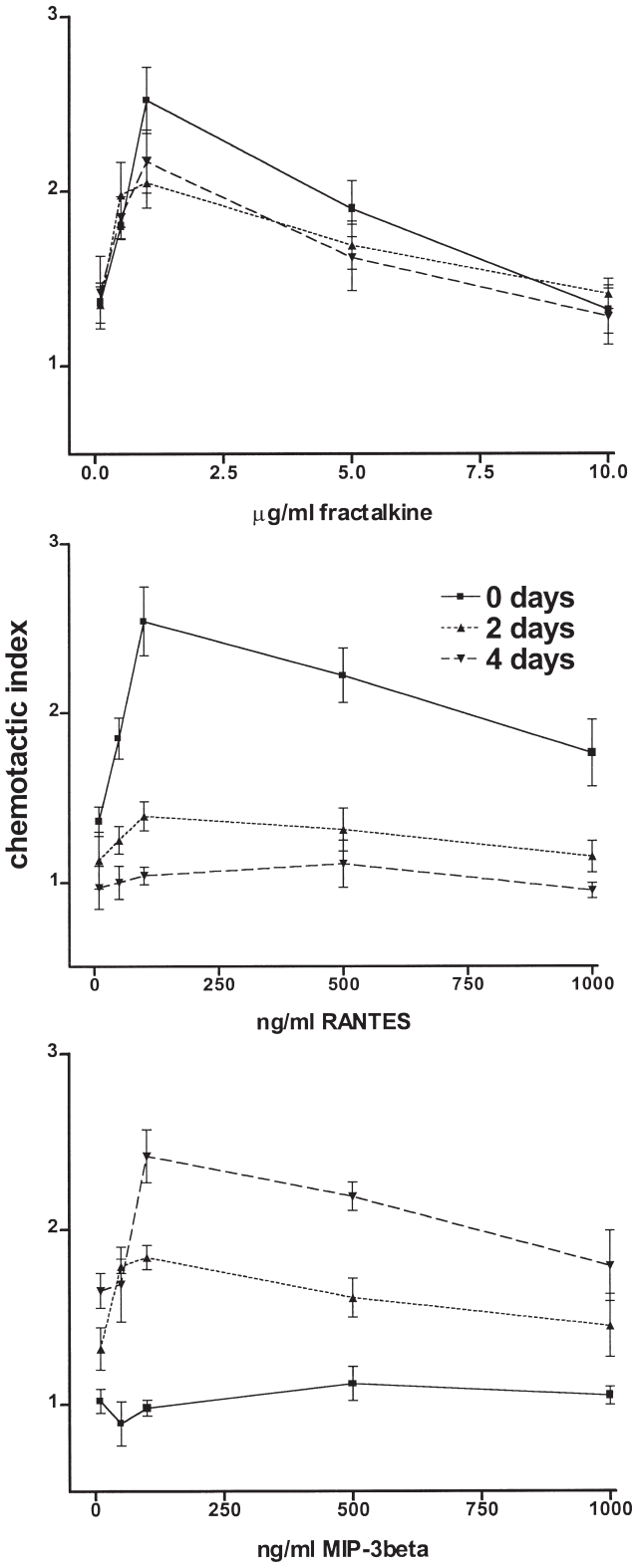

Fig. 4. Maturation-dependent chemotaxis of dendritic cells. Dendritic cells were stimulated with 3 µg/ml LPS for 0, 2 and 4 days. Thereafter cells were stimulated with the indicated concentrations of Fractalkine, RANTES- and MIP-3beta. Data are means ± SEM (n = 4 different donors)

ing maturation process is in contrast to the maturation state-dependent expression of the CC-chemokine receptors 1, 5 and 7. It has been suggested that expression and function of CCR-1 and 5 in immature DCs might regulate the traffic of these cells from blood to target sites, whereas MIP-3beta via CCR-7 regulates trafficking of maturated DCs to lymphoid organs [16]. The constant expression of CX3CR1 during different maturation states of DCs might allow function of fractalkine during migration from blood to target sites as well as on the way back to secondary lymphoid organs.

In order to demonstrate functional CX3CR1 expression on the cell surface of dendritic cells, chemotaxis and actin polymerization were analyzed. These assays showed that fractalkine has chemotactic activity for DCs and stimulates migration associated intracellular signal pathways. In addition, the analysis of chemotaxis at different stages of maturation suggest that fractalkine might be involed in trafficking of DCs at different points of time during their life cycle. Since fractalkine is upregulated and secreted during maturation one can speculate that this chemokine acts as a paracrine chemotaxin for DCs amplifying trafficking to inflammatory sites as well as to lymphoid organs.

The present results indicate that fractalkine has chemotactic activity and stimulates intracellular signal pathways in immature and mature DCs. These findings suggest a role of this novel chemokine and CX3CR1 in the recruitment of DCs.

Acknowledgements. The authors gratefully acknowledge the assistance of A. Komann and G. Grüninger.

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