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Interferon-γ Stimulates the Expression of CX3CL1/Fractalkine in Cultured Human Endothelial Cells

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Imaizumi, T., Matsumiya, T., Fujimoto, K., Okamoto, K., Cui, X., Ohtaki, U., Yoshida, H. and Satoh, K. *Interferon-γ Stimulates the Expression of CX3CL1/Fractalkine in Cultured Human Endothelial Cells.* Tohoku J. Exp. Med., 2000, **192** (2) 127–139 —— CX3CL1/Fractalkine, a CX3C chemokine, is a potent agonist for the chemotaxis and adhesion of monocytes and lymphocytes. It was first identified as a membrane protein in endothelial cells activated with IL-1 or TNF-α. We have found the enhanced expression of fractalkine in human umbilical vein endothelial cells stimulated with interferon-γ (IFN-γ). Pretreatment of the cells with cycloheximide did not inhibit the expression of fractalkine mRNA. The majority of fractalkine protein was found in the cell lysate, and an antibody-blocking experiment disclosed that fractalkine contributes to the adhesion of mononuclear cells to endothelial monolayers stimulated with IFN-γ. Vascular endothelial cells produce fractalkine in response to IFN-γ, and this may play an important role in immune responses by eliciting a traffic of mononuclear cells through the vascular wall. ——— fractalkine; endothelial cells; interferon-γ © 2000 Tohoku University Medical Press

CX3CL1/Fractalkine is a chemokine with the N-terminal chemokine domain containing a unique CX3C motif (Bazan et al. 1997; Zlotnik and Yoshie 2000). It is synthesized as a membrane protein in activated endothelial cells and the cell surface-bound fractalkine promotes strong adhesion of T cells and monocytes (Bazan et al. 1997). The soluble form of this chemokine can be released from the membrane-associated molecules and has a potent chemoattractant activity for these leukocytes (Bazan et al. 1997). The cell surface receptor for fractalkine, CX3CR1, is known to be expressed in T lymphocytes and monocytes, and

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mediates adhesive and migratory functions (Imai et al. 1997; Fong et al. 1998). These facts suggest that fractalkine may constitute a part of the molecular control on leukocyte traffic through the endothelium and play a key role in inflammatory responses.

The human fractalkine gene is located on the chromosome 16 (Nomiyama et al. 1998). Fractalkine is consitutively expressed by dendritic cells and its expression is up-regulated upon dendritic cell maturation (Kanazawa et al. 1999; Papadopoulos et al. 1999). Intestinal epithelial cells can also produce fractalkine and its mRNA level is enhanced during active Crohn's disease (Muehlhoefer et al. 2000). In the nervous system, fractalkine is constitutively expressed in the olfactory bulb, cerebral cortex, hippocampus, etc. (Harrison et al. 1998; Nishiyori et al. 1998; Schwaeble et al. 1998), and its expression is not affected by experimentally-induced inflammation of the central nervous tissue (Schwaeble et al. 1998).

Interferon-γ (IFN-γ) is one of the most important proinflammatory cytokines (Young and Hardy 1995), and activates various functions of endothelial cells (Luster and Ravetch 1987; Pammer et al. 1999). This cytokine is known to upregulate the expressions of CXCL10/interferon-inducible protein-10, a CC chemokine (Luster and Ravetch 1987), and Bak, a cell death regulatory protein (Pammer et al. 1999) in endothelial cells, and to reduce the neutrophil adherence to IL-β-stimulated endothelial cells (Melrose et al. 1998). Recently, it was reported that IFN-γ stimulates the expression of fractalkine mRNA in rat aortic endothelial cells (Garcia et al. 2000). However, the details for the regulation of fractalkine expression induced by IFN-γ and its functional significance are not known. In the present study, we addressed possible induction of fractalkine in cultured human endothelial cells stimulated with IFN-γ, and the role of fractalkine in the mononuclear cell adherence to endothelial monolayers was examined.

Materials and Methods

Reagents

Collagenase and cycloheximide were from Wako (Osaka). Cell culture medium Humedia EB-2, and its supplements were from Kurabo (Osaka). Recombinant human (r[h]) chemokine-domain and full-length fractalkine, r(h) interleukin-1α, anti-human fractalkine antibody and biotinylated anti-human fractalkine antibody were from R & D Systems (Minneapolis, MN, USA). Human serum albumin and a protease inhibitor cocktail were from Sigma (St. Louis, MO, USA). Medium 199, primer oligo(dT)$_{12-18}$ and M-Mulv reverse transcriptase were from Gibco-BRL (Gainthersburg, MD, USA). An RNeasy total RNA isolation kit and *Taq* DNA polymerase were from Qiagen (Hilden, Germany). The Oligotex-dT30⟨Super⟩ was from Takara (Otsu). R(h) IFN-γ, positively-charged nylon membranes, a digoxigenin (DIG) RNA labeling kit, a DIG nucleic acid detection kit and a DIG-labeled actin RNA probe were from Roche Boeringer Mannheim

(Mannheim, Germany). A Northern Max kit and a Lig'nScribe kit were from Ambion (Austin, TX, USA). Streptavidin-conjugated horseradish peroxisase and 3, 3′, 5, 5′-tetramethylbenzidine substrate were from Kirkegaard & Perry Laboratories, Inc. (Gainthersburg, MD, USA). Oligonucleotide primers for PCR were synthesized by Greiner Japan (Kanagawa).

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated using collagenase and cultured in 6-well plates as described (Zimmerman et al. 1990; Imaizumi et al. 2000a, b), with slight modifications. The cells were cultured in Humedia EB-2 supplemented with 2% FBS, 10 ng/ml r(h) epidermal growth factor, 1 μg/ml hydrocortisone, 5 ng/ml r(h) basic fibroblast growth factor and 10 μg/ml heparin. When the cells reached about 80% confluence, the medium containing growth factors was removed and the cells were washed twice. Then the cells were cultured in Humedia EB-2 supplemented with 20% human serum (Humedia-HS). The tightly confluent monolayers of first to fifth passages were used for the experiments. The primary culture showed $<1\%$ $CD45^+$ cells, but no $CD45^+$ cells were found after first passage. HUVEC were incubated in the Humedia-HS containing IFN-γ or IL-1α for the indicated time periods. In the experiments using cycloheximide (CHX), the cells were pretreated with 500 ng/ml CHX for 1 hour prior to the addition of IFN-γ.

RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR) and northern blot

Total RNA was isolated from the cells using an RNeasy total RNA isolation kit. Single-strand cDNA for a PCR template was synthesized from 1 μg of total RNA using a primer oligo$(dT)_{12-18}$ and the M-Mulv reverse transcriptase under the conditions indicated by the manufacturer. Specific primers were designed from cDNA sequences for fractalkine, CCL2/monocyte chemotactic protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); and each cDNA was amplified by PCR using *Taq* DNA polymerase. The sequences of the primers were as follows;

Fractalkine-F (5′-AACTCGAAATGGCGGCACCTT-3′),
Fractalkine-R (5′-ATGAATTACTACCACAGCTCCG-3′),
MCP-1-F (5′-AAACTGAAGCTCGCACTCTCGC-3′),
MCP-1-R (5′-ATTCTTGGGTTGTGGAGTGAGT-3′),
VCAM-1-F (5′-AATTTATGTGTGTGAAGGAG-3′),
VCAM-1-R (5′-GCATGTCATATTCACAGAA-3′),
ICAM-1-F (5′-CACAGTCACCTATGGCAACG-3′),
ICAM-1-R (5′-TTCTTGATCTTCCGCTGGC-3′),
GAPDH-F (5′-CCACCCATGGCAAATTCCATGGCA-3′),

and GAPDH-R (5′-TCTAGACGGCAGGTCAGGTCCACC-3′).

The reaction condition for fractalkine was 1×(94°C, 1 minute); 24 or 26×(94°C, 1 minute; 62°C, 1 minute; 72°C, 1 minute); and 1×(72°C, 10 minute). The conditions for others were 1×(94°C, 1 minute); 30×(94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute) for VCAM-1 and GAPDH, or 25×(94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute) for MCP-1 and ICAM-1; and 1×(72°C, 10 minute). The products were analyzed on a 1.2% agarose gel containing ethidium bromide. The expected sizes for the PCR products for fractalkine, MCP-1, VCAM-1, ICAM-1 and GAPDH were 887 bp, 353 bp, 742 bp, and 696 bp, respectively. Because all of these primer pairs were designed from different exons, the products with the expected size were amplified from single-strand cDNA but not from the contaminating genomic DNA. The PCR products were confirmed to be specific for each cDNA by sequencing.

For northern blot, total RNA was extracted as described above and poly(A)$^+$ RNA was isolated from total RNA using an Oligotex-dT30<Super>. Poly(A)$^+$ RNA, 1 mg/lane, was analyzed by electrophoresis on a 1% agarose gel containing formaldehyde. The RNA was blotted to a positively-charged nylon membrane by capillary transfer, and probed with the DIG-labeled anti-sense RNA for fractalkine or β-actin. A T7 promoter adapter was ligated to an 887 bp PCR product specific for fractalkine using a Lig'nScribe kit, and this was used as a template for the synthesis of a DIG-labled RNA-probe. Hybridization was performed at 68°C for 16 hours with 0.5 nM probe using a NorthernMax kit. The detection was performed using a DIG nucleic acid detection kit according to the specifications of the supplier.

ELISA for fractalkine

For the measurement of fractalkine concentration in the medium or in the cell lysate, HUVEC were stimulated with IFN-γ as described above. After the incubation, the conditioned medium was collected. The cells were washed twice with cold phosphate-buffered saline (PBS), pH 7.4, and lysed using 1 ml of the cell lysis buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and a 0.01% protease inhibitor cocktail). After passing through a 23G needle, the cell debris was pelleted by centrifugation and the supernatant was collected. The concentrations of fractalkine in the medium and the cell lysate were determined by enzyme-linked immunosorbent assay (ELISA). A gout polyclonal anti-fractalkine was used as the capture antibody and a biotinylated polyclonal anti-fractalkine as the second antibody. Streptavidin-conjugated horseradish peroxidase was used for detection with 3, 3′, 5, 5′-tetramethylbenzidine as the indicator substrate. The chemokine domain or full-length r(h)fractalkine were used to generate standard curves for the measurement in culture medium or cell lysate, respectively. The detection limit of this assay was 10 pg/ml.

Adhesion Assay

Adhesion of mononuclear cells to IFN-γ-stimulated HUVEC was examined as described (Marx et al. 1999) with a slight modification. HUVEC were grown to confluence in 12-well plates, and stimulated with 10 ng/ml IFN-γ for 16 hours. The medium was removed and the cells were washed twice. Then the cells were incubated, at 37°C for 1 hour, with 15 μg/300 μl/well of an anti-fractalkine neutralizing antibody or a control antibody in Medium199 containing 0.1% human serum albumin. The medium was removed and peripheral blood mononuclear cells, isolated from a healthy volunteer using Ficoll-Paque PLUS (Böyum 1968), were added to a rinsed monolayer (6×10^5 cells/300 μl/well). It is known that functional specific receptor, CX3CR1 is expressed in mononuclear cells isolated with this method (Imai et al. 1997). After the incubation at 37°C for 15 minutes, nonadherent cells were removed by inverting the plate under rotation (63 rpm, 37°C, 20 minutes). The cells were fixed and photographed, and adherent cells in random fields were counted under a microscopy.

Results

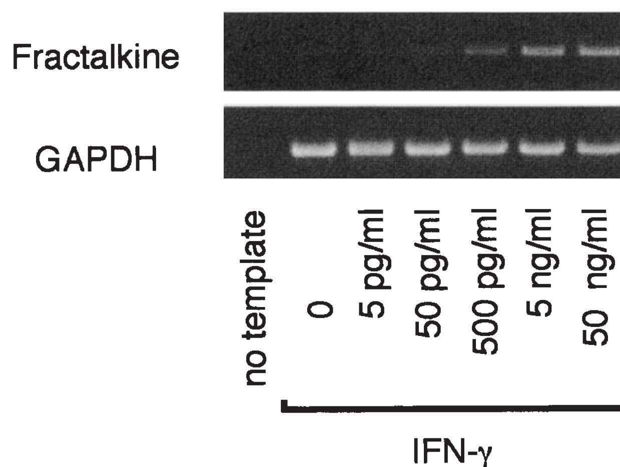
HUVEC stimulated with IFN-γ express fractalkine

Expression of fractalkine mRNA was induced by the treatment of HUVEC with IFN-γ in a dose dependent manner (Fig. 1A). The expression of fractalkine mRNA reached maximal level 16 hours after the stimulation with IFN-γ (Fig. 1B). On the other hand, it reached maximal level 4 to 8 hours after the stimulation with IL-1α (Fig. 1B). IFN-γ did not induce significant change in the expression of mRNA for MCP-1, VCAM-1 or ICAM-1, while IL-1α significantly upregulated the expression of these mRNAs (Fig. 1B). The accumulation of fractalkine mRNA induced by IFN-γ was not inhibited by the pretreatment of the cells with CHX (Fig. 1C). Northern blot analysis also confirmed the induction of fractalkine transcript (3.5 kb) by IFN-γ (Fig. 1D).

Fractalkine protein levels both in the medium and in the cell lysate were enhanced by the stimulation with IFN-γ in a concentration dependent manner (Fig. 2A), and reached maximal level 16 hours after the stimulation (Fig. 2B). Time-dependent changes in fractalkine protein levels agreed with the time course of its mRNA expression. About 70-90% of the protein was found in the cell lysates, and 10-30% was secreted into the medium.

Adhesion assay

Stimulation of HUVEC with IFN-γ increased the adhesion of mononuclear cells, and pretreatment of the cells with an anti-fractalkine neutralizing antibody markedly inhibited the adhesion induced by IFN-γ (Fig. 3A). The number of adherent cells to the control and IFN-γ-stimulated HUVEC monolayers were 103 ± 4 and 286 ± 36 cells/fields, respectively ($n=4$; $p<0.01$, by t-test). Anti-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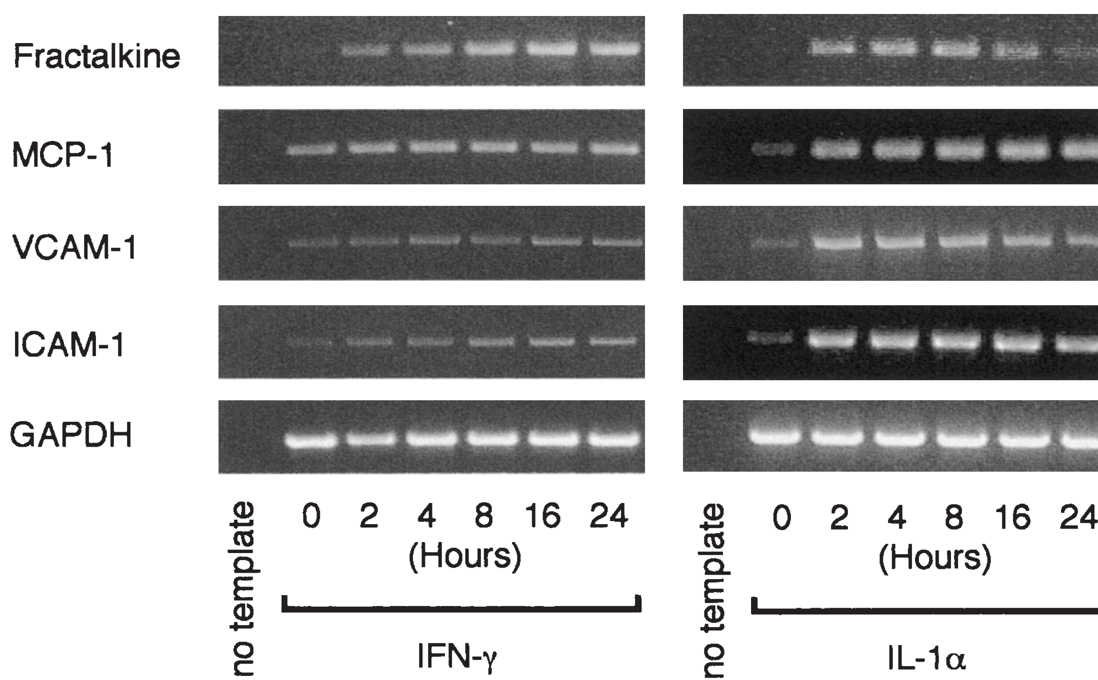
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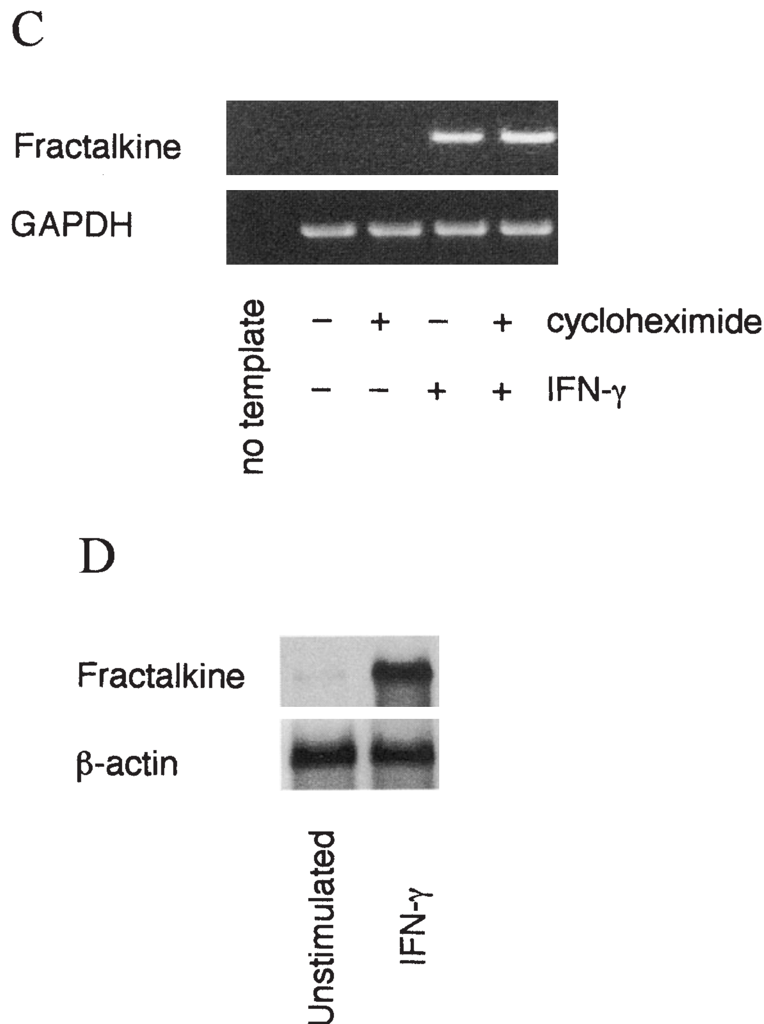


Fig. 1. Expression of fractalkine mRNA in HUVEC stimulated with IFN-γ. HUVEC were stimulated with IFN-γ. After the incubation, the medium was removed and the cells were subjected to total RNA extraction. Single-strand cDNA was synthesized from 1 μg of total RNA, and the specific cDNAs for fractalkine and GAPDH were amplified by PCR. (A) Concentration-dependence of the fractalkine mRNA expression in HUVEC stimulated with IFN-γ. (B) Time course of the expression of mRNAs for fractalkine, MCP-1, VCAM-1, ICAM-1 in HUVEC stimulated with 10 ng/ml IFN-γ or 10 ng/ml IL-1α for up to 24 hours. (C) Effect of the pretreatment with CHX on the expression of mRNAs for fractalkine and GAPDH in HUVEC stimulated with IFN-γ. HUVEC were preincubated with 500 ng/ml CHX for 1 hour before the stimulation with 10 ng/ml IFN-γ for 16 hours. (D) Northern blot analysis of mRNAs for fractalkine and β-actin in HUVEC. HUVEC were treated with 10 ng/ml IFN-γ for 16 hours. Poly(A)$^+$RNA was isolated and subjected to Northern blot analysis.

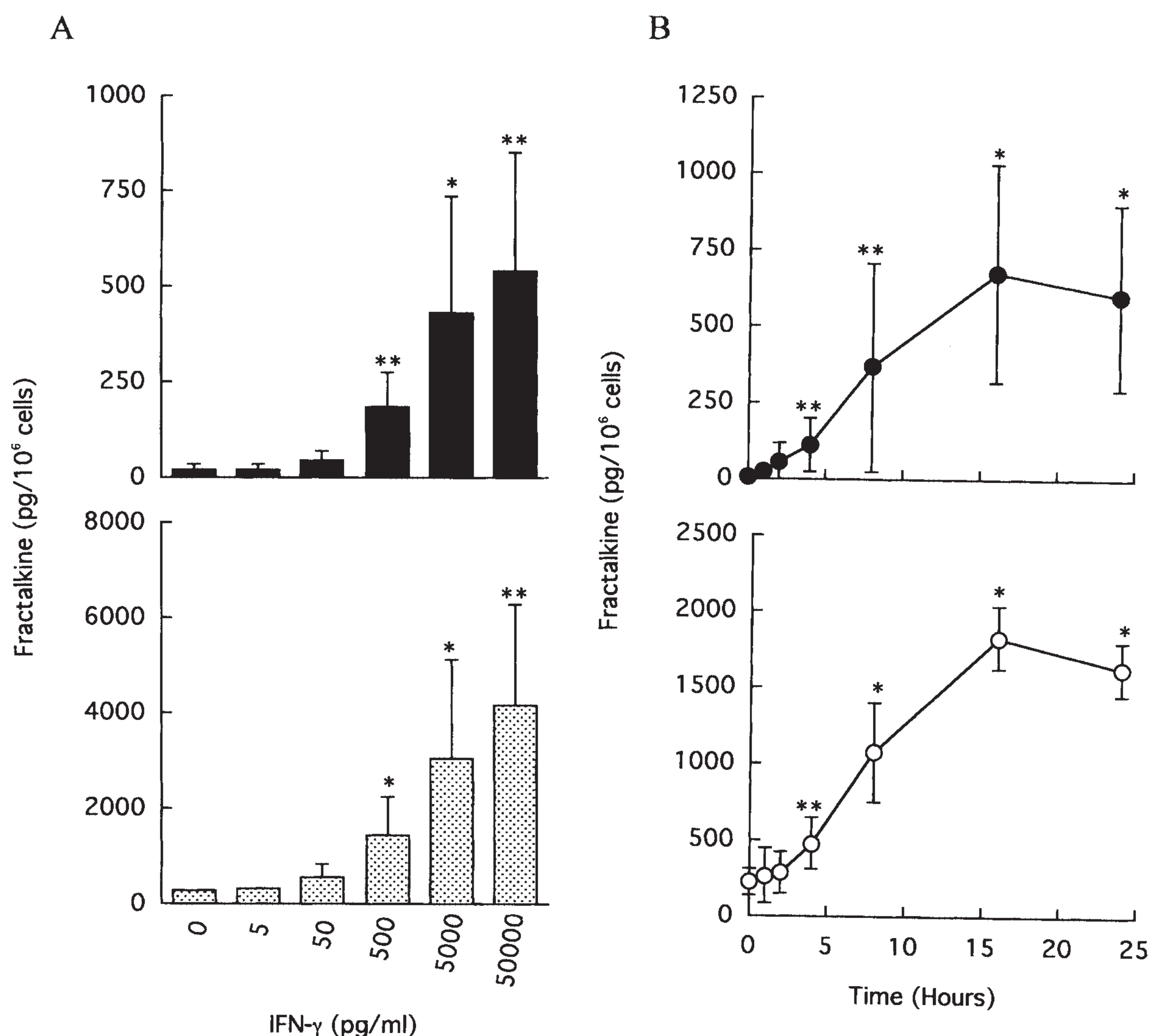
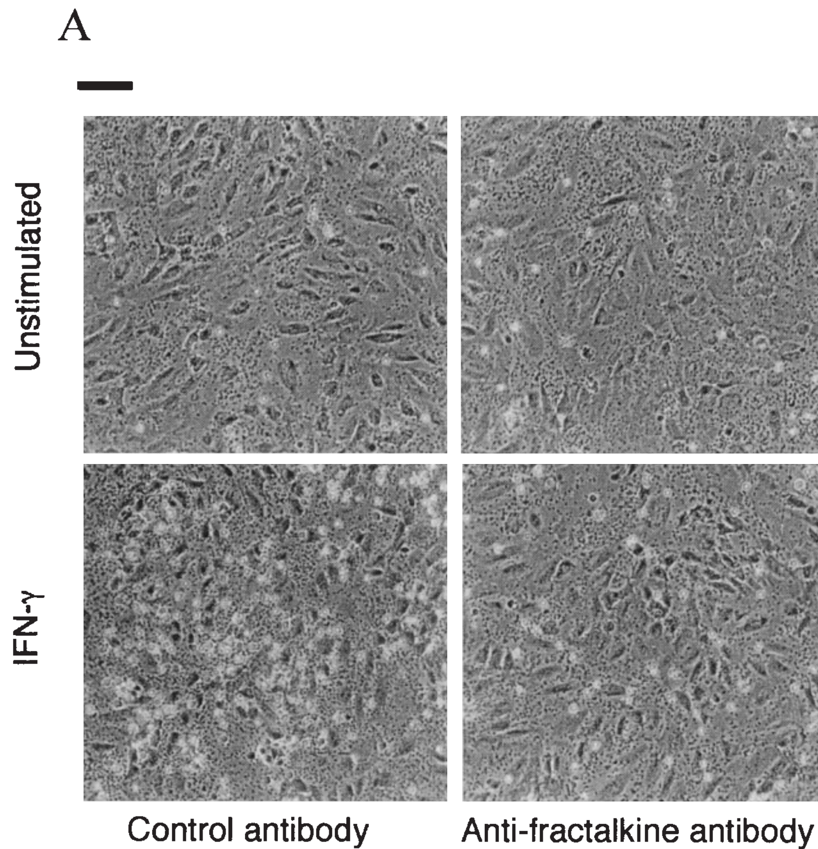


Fig. 2. Concentration of fractalkine protein in the medium and cell lysate from the HUVEC. The cells were incubated with IFN-γ as described in Fig. 1. After the incubation, the medium was collected. The cells were washed twice with cold PBS and lysed. The concentration of fractalkine in the medium and cell lysates were determined by ELISA. (A) Concentration-dependence of the fractalkine preoten expression in HUVEC stimulated with IFN-γ. ■, medium; ▩, cell lysate. Mean±s.d. of six experiments are shown. $*p<0.01$, $**p<0.05$ vs. unstimulated cells based on Welch's t-test. (B) Time course of the expression of fractalkine protein. —●—, medium; —○—, cell lysate. Mean±s.d. of six experiments are shown. $*p<0.01$, $**p<0.05$ vs. Time 0 based on Welch's t-test.

fractalkine antibody reduced the number of adherent cells to 130±23 cells/fields ($n=4$; $p<0.01$ vs. IFN-γ-stimulated cells) (Fig. 3B).

Discussion

The vascular endothelial cells play an important role in inflammatory responses (Zimmerman et al. 1992; 1996; McIntyre et al. 1997). When endothelial cells are stimulated with LPS or IL-1, blood cell tethering molecules such as



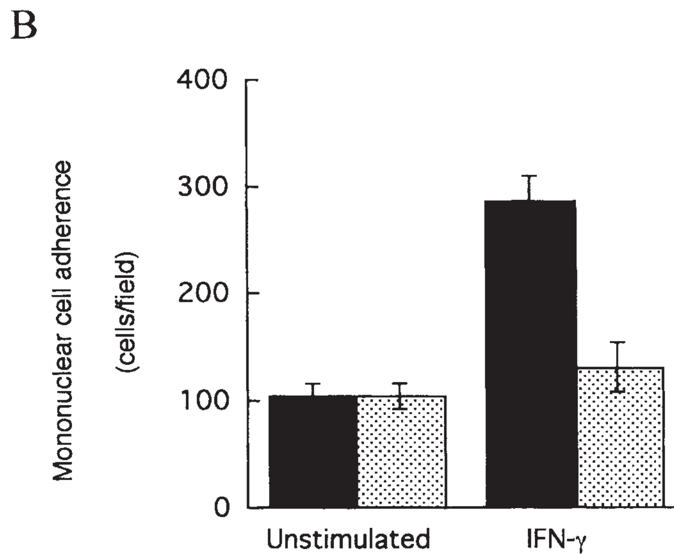


Fig. 3. Adhesion of mononuclear cells to HUVEC. HUVEC were stimulated with 10 ng/ml IFN-γ for 16 hours. The cells were preincubated with an anti-fractalkine neutralizing antibody (50 μg/ml) or a control antibody, and human peripheral blood mononuclear cells (6×10^5 cells/300 μl/well) were added to a rinsed monolayer. After the incubation at 37°C for 15 minutes, nonadherent cells were removed by inverting the plate under rotation (63 rpm, 37°C, 20 minutes). (A) The cells were fixed and photographed; Bar = 30 mm. (B) Adherent cells in random low power fields were counted under a microscope. The value represent Mean ± S.D. from four different fields. ■, control antibody; ▨, anti-fractalkine antibody.

E-selectin, ICAM-1 and VCAM-1 are expressed (Zimmerman et al. 1992; Collins et al. 1995), and this is associated with the expression of CXC chemokines such as CXCL8/interleukin-8 and CXCL5/epithelial neutrophil activating peptide-78 (Imaizumi et al. 1997) as well as CC-chemokines such as CCL2/MCP-1 (Sica et al. 1990) and CCL11/eotaxin (Rothenberg et al. 1995). The regulation of these chemokines and adhesion molecules has been well characterized: in most cases the NF-κB system plays an important role in the induction of these molecules (Zimmerman et al. 1992; Collins et al. 1995). It has been shown that TNF-α and IL-1 upregulate fractalkine expression in endothelial cells (Bazan et al. 1997; Garcia et al. 2000), and the NF-κB system may be also involved in the fractalkine expression (Garcia et al. 2000). However, there is only limited information available about the mechanisms of the regulation of fractalkine production by endothelial cells and its functional significance.

IFN-γ is another potent agonist for vascular endothelial cells and activates endothelial cells through a different signaling pathway from that of TNF-α and IL-1 (Young et al. 1995). In the present study, we found a concentration-dependent stimulation of fractalkine expression by IFN-γ. The accumulation of fractalkine mRNA reached maximum level 16 hours after the stimulation with IFN-γ. On the other hand, IFN-γ did not induce significant changes in mRNA levels of MCP-1, VCAM-1 or ICAM-1. In contrast, IL-1α upregulated the expression of all of these mRNAs. This suggests that fractalkine expression is differentially regulated with other chemokines and adhesion molecules. RT-PCR analysis is a "semi-" quantitave method, and is not effective in determining the size of the mRNA. Therefore, we also performed the northern blot analysis, and confirmed that IFN-γ induced the expression of a 3.5 kb fractalkine transcript in HUVEC.

When the cells were pretreated with CHX, the IFN-γ-induced accumulation of fractalkine mRNA was not inhibited. This suggests that induction of fractalkine mRNA was due to the direct effect of IFN-γ, and the secondary de novo protein synthesis is not required. The analysis of the promoter region of fractalkine gene is now under investigation, and future studies will clarify the detailed mechanisms of transcriptional regulation of the fractalkine expression.

IFN-γ also induced the expression of fractalkine protein in HUVEC in concentration- and time-dependent manners. About 70–90% of fractalkine protein was detected in the cell lysate, with a small amount of secretion into the medium. This suggests that the role of fractalkine as an adhesion molecule may be more important than that as a chemotactic factor. Therefore, next we examined if fractalkine expressed on HUVEC has an ability to induce the adherence of mononuclear cells. The adhesion of mononuclear cells to HUVEC was markedly enhanced by IFN-γ, and the adhesion was inhibited by the pretreatment of the cells with an anti-fractalkine neutralizing antibody.

It is reported that fractalkine mRNA is expressed in most human organs

including the heart, brain, lung, kidney, intestines and skeltal muscles (Bazan et al. 1997). Therefore, fractalkine may play a role in the interactions between mononuclear cells and endothelial cells in those tissues. Fractalkine is expressed in dendritic cells which play a role in antigen-presentation to T cells (Kanazawa et al. 1999; Papadopoulos et al. 1999), and also in the small intestinal epithelial cells which is important in host-defense mechanisms (Muehlhoefer et al. 2000). Taken together, these facts suggest that fractalkine expressed in IFN-γ-stimulated endothelial cells may be involved in the immune response by recruiting mononuclear cells.

We conclude that functional fractalkine protein is produced by endothelial cells stimulated with IFN-γ. Fractalkine may be involved in the interaction between mononuclear cells and endothelial cells, and mediate a part of immune responses in endothelial cells elicited by IFN-γ.

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