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Fractalkine-Mediated Endothelial Cell Injury by NK Cells[1]

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Endothelial cells (ECs) are primary targets of immunological attack, and their injury can lead to vasculopathy and organ dysfunction in vascular leak syndrome and in rejection of allografts or xenografts. A newly identified CX_3C-chemokine, fractalkine, expressed on activated ECs plays an important role in leukocyte adhesion and migration. In this study we examined the functional roles of fractalkine on NK cell activity and NK cell-mediated endothelial cell injury. Freshly separated NK cells expressed the fractalkine receptor (CX_3CR1) determined by FACS analysis and efficiently adhered to immobilized full-length fractalkine, but not to the truncated forms of the chemokine domain or mucin domain, suggesting that fractalkine functions as an adhesion molecule on the interaction between NK cells and ECs. Soluble fractalkine enhanced NK cell cytolytic activity against K562 target cells in a dose- and time-dependent manner. This enhancement correlated well with increased granular exocytosis from NK cells, which was completely inhibited by the G protein inhibitor, pertussis toxin. Transfection of fractalkine cDNA into ECV304 cells or HUVECs resulted in increased adhesion of NK cells and susceptibility to NK cell-mediated cytolysis compared with control transfection. Moreover, both enhanced adhesion and susceptibility of fractalkine-transfected cells were markedly suppressed by soluble fractalkine or anti-CX_3CR1 Ab. Our results suggest that fractalkine plays an important role not only in the binding of NK cells to endothelial cells, but also in NK cell-mediated endothelium damage, which may result in vascular injury. ***The Journal of Immunology*****, 2000, 164: 4055–4062.**

Natural killer cells, which express cytolytic activity without prior antigenic stimulation, are thought to mediate immunity against viruses and surveillance against neoplastic transformation (1–3). In addition, activated NK cells are involved in mediating the therapeutic effect of IL-2 and immunotherapy with IL-2-activated cells (lymphokine-activated killer (LAK)[3] cells) in cancer (4, 5). However, both therapies are associated with a systemic toxicity known as vascular leak syndrome (VLS), reported to be mediated at least in part by IL-2-activated cells (6–10). Although IL-2 promotes the adhesion of NK cells to endothelial cells (ECs) and enhances the susceptibility of ECs to NK cell- or LAK cell-mediated cytolysis, several IL-2-inducible cytokines produced by NK cells, including TNF-α and IFN-γ, have no effect on endothelial permeability (11, 12), suggesting that cell-to-cell contact between NK or LAK cells and ECs is necessary to induce VLS. The endothelium plays an important role in the recruitment and emigration of circulating effector cells into sites of inflammation and immune responses, and endothelial cells can be the primary target of immunologic injury, which results in vasculopathy and organ dysfunction (13–17). NK cells accumulate rapidly in tissues under certain conditions, including viral infections, administration of biologic response modifiers, and transplantation of allografts or xenografts (16, 18). However, the mechanisms underlying the recruitment and extravasation of NK cells as well as NK cell-mediated EC damage under these conditions, including VLS and transplantation rejection, are not well understood.

Chemokines were first described as chemoattractant cytokines synthesized at sites of inflammation and are the major regulatory proteins for leukocyte recruitment and trafficking (19–24). Fractalkine, a recently identified chemokine, is a transmembrane protein consisting of a Cys-X-X-X-Cys chemokine domain presented on top of an extended mucin-like stalk and is expressed on the surface of activated ECs (25, 26). We have recently identified a receptor for fractalkine (CX_3CR1, V28), have demonstrated that it is expressed predominantly on NK cells as well as on $CD8^+$ T cells and $CD14^+$ monocytes, and have shown that soluble fractalkine (s-fractalkine) exhibits a potent chemoattractant activity for those cells (27). In the present study we further investigated the functional effects of fractalkine on NK cell activity and NK cell-mediated damage of ECs. Our results showed that fractalkine enhances vascular EC damage by NK cells.

Materials and Methods

Abs and reagents

Hybridomas producing mAbs against CD18 (TS1/18), CD11a (TS1/22), and CD3 (OKT3) were purchased from American Type Culture Collection (Manassas, VA), and mAbs were purified as previously described (28). Anti-CD14, anti-CD19 mAbs were purchased from Immunotech (Marseilles, France). FITC- or PE-conjugated Abs against CD3, CD14, CD16, CD21, and ICAM-1 were obtained from Becton Dickinson (Mountain

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[3] Abbreviations used in this paper: LAK, lymphokine-activated killer; VLS, vascular leak syndrome; EC, endothelial cell; s-fractalkine, soluble fractalkine; SEAP, secreted form of placental alkaline phosphatase; FRK-ECV, fractalkine-transfected ECV304 cell; PTX, pertussis toxin; VLA, very late Ag; MCP, monocyte chemoattractant protein; PTK, protein tyrosine kinase; PI 3-kinase, phosphatidylinositol 3-kinase; BLTE, N^{α}-benzyloxycarbonyl-L-lysine thiobenzyl ester.

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View, CA). The monoclonal anti-secreted form of placental alkaline phosphatase (SEAP) Ab was purchased from Genzyme (Cambridge, MA). Monoclonal anti-CD106 (VCAM-1) Ab and anti-CD54 (ICAM-1) Ab were purchased from Ancell (Bayport, MN) and Serotec (Kidlington, U.K.), respectively. Anti-fractalkine mAbs were prepared as previously described (27). Affinity-purified Ab against CX_3CR1 was provided by Dr. Yiyang Xia (Torrey Pines Biolabs, San Diego, CA). Recombinant IL-2 was provided by Shionogi (Osaka, Japan). Goat anti-mouse IgG-coupled magnetic beads (Biomag) were purchased from PerSeptive Biosystems (Framingham, MA), and calcein-AM was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Production of recombinant proteins

Soluble fractalkine (s-fractalkine), fractalkine-SEAP, CX_3C-SEAP, and mucin-SEAP fusion proteins were prepared using a baculovirus expression system as described previously (27). Briefly, to express fractalkine-SEAP, the DNA fragment encoding fractalkine was amplified from fractalkine cDNA by PCR using 5′ *Sal*I-fractalkine primer (+5′-CGCGTCGACT CAGCCATGGCTCCGATATCT-3′) and 3′ fractalkine-*Xba*I primer (−5′-CGCTCTAGAGGTGGCTGCCTGGGCGTCAGG-3′) and were subcloned into pDREF-SEAP (His)6-Hyg vector as described previously (29). CX_3C-SEAP was similarly generated using 5′ *Sal*I-fractalkine primer and 3′ fractalkine-*Xba*I-2 primer (−5′-CGCTCTAGATAGGGCAGCAGCCT GGCGGTC-3′). For generation of mucin-SEAP, the DNA fragment encoding *Sal*I-oncostatin M signal sequence *Xba*I-fractalkine lacking the chemokine domain *Nhe*I was amplified by three-step PCR using 5′ *Sal*I-OMC-fractalkine primer (+5′-CTGTTTCCATGCATGGCGAGCATGTCT3′) and 3′ fractalkine-*Nhe*I primer (−5′-CGCGCTAGCGGTGGCAGCCT GGGCGTCAGG-3′). After 3–4 days the supernatants were collected. For one-step affinity purification, supernatants were applied to 1 ml of Hisbond resin (Qiagen, Hilden, Germany). After washing, bound recombinant proteins were eluted with 100 mM imidazol. Soluble fractalkine with a tag of six histidine residues, (His)6, at its C terminus was prepared using a baculovirus expression system. The cDNA fragment encoding the extracellular domain of fractalkine was subcloned into the *Sal*I-*Xba*I sites of the modified pFastBac1 baculovirus transfer vector (Life Technologies, Gaithersburg, MD) to express fractalkine as a soluble fusion protein with Ser-Arg-Ser-Ser-Gly-(His)6. The recombinant bacmids were generated in *Escherichia coli* DH10Bac and transfected into *Spodoptera frugiperda* Sf9 cells using Lipofectin (Life Technologies) to obtain the recombinant viruses. For expression of the recombinant fractalkine-(His)6, Trichoplusia in BTI-TN-5B1–4 cells were infected with the recombinant viruses at multiplicity of infection of 10–20. The culture supernatants collected 2 days after infection were applied to a 1 ml Hisbond resin (Qiagen, Valencia, CA). After washing, bound fractalkine-(His)6 was eluted with 100 mM imidazol. Protein concentration was determined by the BCA kit (Pierce, Rockford, IL). The concentration of each recombinant protein was determined by a sandwich-type ELISA as described previously (29).

Cells and cell culture

PBMCs were isolated from samples of venous blood from consenting healthy volunteers by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation. NK cells were isolated by negative selection using a mixture of anti-CD3, anti-CD14, and anti-CD19 mAbs and immunomagnetic beads (PerSeptive Diagnostics, Cambridge, MA) to deplete T cells, monocytes and B cells, respectively, as described previously (28, 30). NK cell populations used in all experiments were >85% pure as confirmed by flow cytometric analysis for the presence of CD16 and/or CD56. K562 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and penicillin. ECV304 cells were obtained from American Type Culture Collection[4] and maintained in medium 199 supplemented with 10% FCS. For stable expression of membrane-bound fractalkine in ECV304 cells, the expression plasmid pCAGG-Neo-fractalkine-1 was transfected into ECV304 by Lipofectamine (Life Technologies). After selection with 800 μg/ml of G418 for 1–2 wk, drug-resistant cells were pooled as described previously (27). HUVECs were obtained from Iwaki (Chiba, Japan) and maintained in endothelial cell growth medium (10 ng/ml human epidermal growth factor, 1.0 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 μg/ml amphotericin B, 12 μg/ml bovine brain extract, and 2% FBS) as recommended by the manufacturer. For transient expression of membrane-bound fractalkine in HUVECs, the expression plasmid pCAGG-Neo-fractalkine-1 was transfected into HUVECs by Lipofectamine. Fractalkine- or vector-transfected cells were cultured for 16 h and used for NK cell-mediated cytotoxicity assays.

[4] After completing the experiments using ECV304 cell lines and fractalkine-transfected ECV304 cells, American Type Culture Collection announced that ECV-304 should be considered a variant of human bladder cancer line T-24 derived by cross-contamination.

Adhesion assay

Each well of 16-well glass dishes was coated with 50 μl of anti-SEAP Ab (10 μg/ml) in 50 mM Tris-HCl, pH 9.5, at 4°C overnight. SEAP fusion proteins (10 nM) were added to the wells, and plates were incubated for 2 h at room temperature and washed extensively. Fresh NK cells (4000 cells/mm^2) were added to each well in a final volume of 50 μl and incubated for 30 min at room temperature. After removing nonadherent cells, the remaining cells were fixed with 1% glutaraldehyde. All assays were performed in duplicate, and the percent adhesion of input cells was determined by counting cells in five ×400 fields (0.07 mm^2)/well (27).

To assess NK cell adhesion to ECV304 cells (control-ECV and FRK-ECV), cells were seeded at 2×10^4 cells/well in 48-well culture dishes and cultured overnight to form confluent monolayers. Freshly separated NK cells were fluorescently labeled by incubation with calcein-AM and added to each well ($1–2 \times 10^4$ cells/well) in a final volume of 100 μl and incubated for 30 min at 37°C. After removal of nonadherent cells, fluorescence was measured using a Wallac 1420 ARVO fluoroscan (Pharmacia Biotech), using excitation and emission wavelengths of 496 and 520 nm, respectively. In blocking experiments using Abs or s-fractalkine, endothelial cells and NK cells were pretreated with saturating amounts of Abs or 10 nM of s-fractalkine for 30 min at room temperature before the assay.

Cytotoxicity and N^{α}*-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLTE) esterase assay*

For cytotoxicity assay, target cells (K562, control-ECV, FRK-ECV, and HUVECs) were labeled with calcein-AM for 30 min at 37°C. Then target cells (5000/well) and effector cells were plated onto 96-well plates at the indicated E:T cell ratio and incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO_2. In some experiments, s-fractalkine was included in the assay medium at the indicated concentrations for the indicated time intervals. In blocking experiments using Abs or s-fractalkine, NK cells were pretreated with saturating amounts of Abs or 10 nM s-fractalkine for 30 min at room temperature before the assay. After incubation, supernatants were transferred to new wells, and fluorescence was measured using a Wallac 1420 ARVO fluoroscan. Assays were performed in triplicate, and data were expressed as the percent lysis or as lytic units 20% in 10^7 effector cells (1 lytic unit containing that number of effector cells lysing 20% of the targets) (31).

For the BLTE esterase assay, purified NK cells were suspended at 7×10^6 cells/ml in phenol red-free RPMI 1640 (IBL, Fujioka, Japan) containing 0.5% FCS and the indicated concentration of s-fractalkine and were added to flat-bottom microplate wells at a final volume of 200 μl. In some experiments, NK cells were pretreated with pertussis toxin (PTX) at 500 ng/ml for 1 h at 37°C before assays. Wells were incubated at 37°C for 4 h, and 100-μl aliquots were harvested from each well for BLTE esterase assay. The BLTE esterase assay was adapted to 200 μl for use in 96-well plates as previously described (28, 32). OD was read at 405 nm on an ELISA microplate reader (Iwaki, Osaka, Japan), and the percentage of BLTE esterase activity was calculated.

FACS analysis

For assessment of membrane markers by immunofluorescence, NK cells, ECV304 cells, or HUVECs were stained directly with FITC-labeled reagents or with an unlabeled Ab and FITC-goat ($F(ab')_2$) anti-mouse IgG (Becton Dickinson). Expression of membrane-bound fractalkine on control-ECV, FRK-ECV, HUVECs, and fractalkine-transfected HUVECs was assessed by an indirect method using mAb 1D6 to the mucin domain of fractalkine as described previously (27). For analysis of CX_3CR1 expression, NK cells were incubated with 10 nM fractalkine-SEAP or control SEAP on ice for 30 min in staining buffer (1% FCS and 0.02% sodium azide in PBS). After washing, cells were incubated with biotinylated anti-SEAP Ab, washed, and incubated with FITC-conjugated streptavidin. Cells were then analyzed on a FACSCalibur (Becton Dickinson).

Statistical analysis

All data were expressed as the mean ± SEM. Differences between groups were examined for statistical significance using Student's *t* test for unpaired data and paired *t* test for paired data. A p value <0.05 denoted the presence of a statistically significant difference.

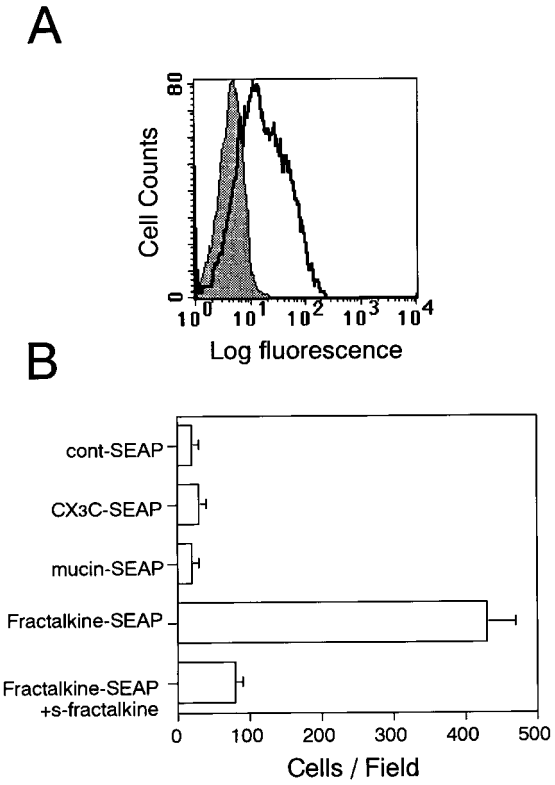

FIGURE 1. *A*, Surface expression of CX_3CR1 on freshly isolated NK cells. Negatively selected NK cells (85% pure, as based on the expression of CD16 and/or CD56) were incubated with SEAP (shaded) or fractalkine-SEAP (solid line) followed by biotinylated anti-SEAP Ab and FITC-streptavidin. After washing, cells were analyzed using a FACSCalibur. *B*, Specific adhesion of NK cells to immobilized fractalkine-SEAP. NK cells (4000 cells/mm^2) were added to wells precoated with SEAP, CX_3C-SEAP, mucin-SEAP, and fractalkine-SEAP via anti-SEAP Ab. After washing off nonadherent cells by dipping the slide gently in assay buffer, adherent cells were fixed with 1% glutaraldehyde and counted in five ×400 fields (0.07 mm^2) per well. Data represent the mean ± SEM of at least three independent experiments.

Results

Expression of fractalkine receptor (CX_3CR1) on NK cells and specific adhesion of NK cells to immobilized fractalkine

We have previously reported the expression of fractalkine receptor, CX_3CR1 (*V28*) mRNA mainly in $CD16^+$ NK cells and low levels were also detected in $CD8^+$ T cells and $CD14^+$ monocytes, which correlated well with the pattern of surface expression (27). To assess the surface expression of CX_3CR1, freshly isolated NK cells were incubated with a fusion protein of fractalkine and SEAP (fractalkine-SEAP), reacted with biotinylated anti-SEAP Ab and FITC-conjugated streptavidin, and analyzed by flow cytometry. The mean fluorescent channel of cells treated with control SEAP was 5.17, whereas that with fractalkine-SEAP was 29.18 (Fig. 1*A*), indicating that NK cells express receptors for fractalkine.

We have previously reported that fractalkine can act as an adhesion molecule and is responsible for the adhesion of cells transfected with CX_3CR1 to fractalkine-expressing cells without involvement of other adhesion molecules (27). To examine whether the expression of CX_3CR1 on freshly isolated NK cells may induce their adhesion to fractalkine, we immobilized fractalkine-SEAP, mucin-SEAP lacking the CX_3C-chemokine domain, CX_3C-SEAP lacking the mucin domain, and control-SEAP onto glass slides using anti-SEAP Ab. As shown in Fig. 1*B*, immobilized fractalkine-SEAP, but not truncated forms (mucin-SEAP and CX_3C-SEAP) or control SEAP, supported the adhesion of NK cells. Because specific anti-fractalkine-neutralizing mAbs are not available at present, we exploited the inhibitory actions of s-fractalkine to dissect the interaction of fractalkine and CX_3CR1 in inducing cell adhesion (27). The results demonstrated that s-fractalkine efficiently inhibited the adhesion of NK cells to immobilized fractalkine. These results indicate that CX_3CR1 on NK cells recognize the intact fractalkine (i.e., CX_3C domain plus mucin domain) and that fractalkine functions as an adhesion molecule on NK cells without the involvement of other adhesion molecules.

Effects of s-fractalkine on NK cell cytotoxicity

Several chemokines, including MCP-1, -2, and -3 and RANTES, are known to modulate NK cell activities (33, 34). Therefore, we examined whether s-fractalkine enhances NK cell-mediated cytotoxicity against K562 target cells. Soluble fractalkine (10 ng/ml) or rIL-2 (2 nM) was included in a 4-h cytotoxicity assay against K562 cells at an E:T cell ratio of 20:1 for the indicated intervals. Although the effect of s-fractalkine on NK cytotoxicity was modest compared with that of IL-2, s-fractalkine significantly enhanced NK cell-mediated K562 lysis in a time-dependent manner (Fig. 2*A*). Next, we examined the effects of the s-fractalkine dose on NK cell-mediated cytotoxicity in 10 different donors, and the results were expressed as 20% lytic units. Fig. 2*B* shows that s-fractalkine enhances lysis of K562 in a dose-dependent manner, with the maximum effect, approximately a 25% increase in lysis, observed at a concentration of 10 nM ($p < 0.05$). The effect of s-fractalkine on NK cytotoxicity was also compared with those of MCP-1, RANTES, as well as IL-2 (Fig. 2*C*). Because treatment of NK cells with s-fractalkine induced no significant change in the expression of LFA-1α or -β, VLA-4 or -5, and CD2 (data not shown), this enhancement of cytotoxicity was not due to an increase in other surface adhesion receptors.

Effects of s-fractalkine on NK cell-mediated granular exocytosis

One key mechanism by which NK cells lyse target cells is through to be the granular exocytosis pathway, which primarily uses the lytic mediators, perforin and granzymes, a family of serine esterase (35, 36). To measure the effect of s-fractalkine on granule exocytosis, freshly isolated NK cells were incubated in the presence of s-fractalkine for 4 h, and supernatants were assayed for esterase activity using BLTE as a substrate. Soluble fractalkine enhanced NK cell granule exocytosis in a dose-dependent manner (Fig. 3*A*), indicating that the increased lytic activity by NK cells induced by s-fractalkine might be due at least in part to enhanced granular exocytosis. Next, we examined the effect of s-fractalkine on granular exocytosis in NK cells from nine different donors. As shown in Fig. 3*B*, s-fractalkine enhanced granular exocytosis in all donors tested.

It has been reported that chemokine receptors identified to date, including CX_3CR1, all manifest a seven-transmembrane, G protein-linked architecture and transduce signals that lead to cytoskeletal reorganization, integrin activation, and other functions, resulting in increased adhesion and migration of the cells (21–24). To examine the involvement of G protein-dependent signaling in fractalkine-mediated enhancement of granular exocytosis in NK cells, cells were pretreated with 500 ng/ml of a G_i inhibitor, PTX, for 1 h at 37°C, and then allowed granular exocytosis assays. As shown in Fig. 3*C*, enhancement of granular exocytosis induced by s-fractalkine was completely prevented by PTX.

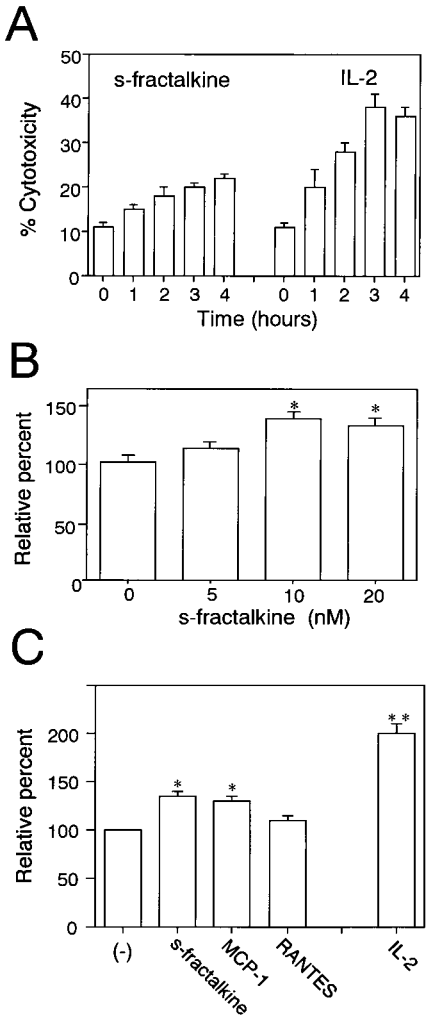

FIGURE 2. *A*, Time-dependent effects of s-fractalkine on NK cell cytotoxicity against K562 target cells. NK cells were incubated with calcein-AM labeled K562 cells at an E:T cell ratio of 20:1 for 4 h. Soluble fractalkine was included in the assay for the indicated time intervals. Supernatants were harvested, and fluorescence was measured. Data are expressed as the percent lysis. *B*, Dose-dependent effect of s-fractalkine on NK cell cytotoxicity against K562 target cells. NK cells were incubated with calcein-AM-labeled K562 cells at the titrated ratio in the presence of s-fractalkine for 4 h. Supernatants were harvested, and fluorescence was measured. Data are expressed as the mean ± SEM relative increase in 20% lytic units in 10^7 effector cells in eight independent experiments. The average increase observed was ~25%. *C*, Comparison of effect of s-fractalkine on NK cell cytotoxicity with those of MCP-1, RANTES, and IL-2. Data are expressed as the mean ± SEM for relative increase in 20% lytic units in 10^7 effector cells and are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

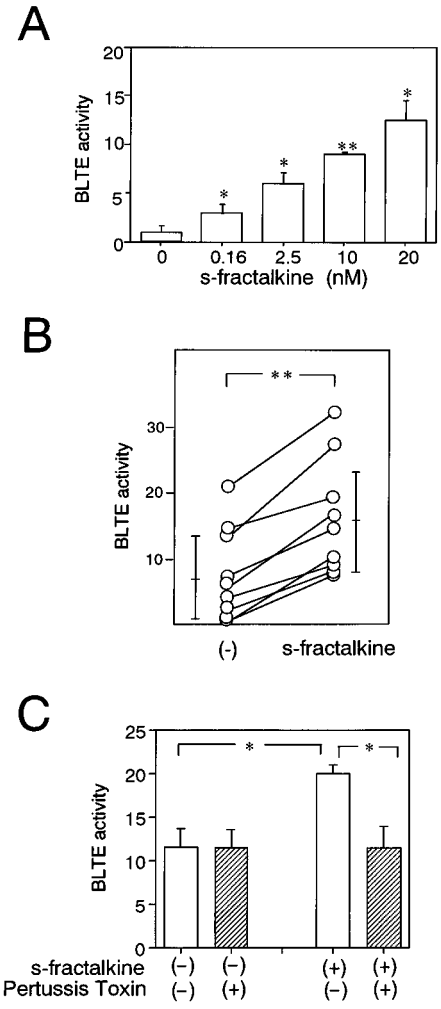

FIGURE 3. Effect of s-fractalkine on BLTE esterase release from NK cells. *A*, NK cells (1.4×10^6 cells/well) were incubated in microplate wells in the presence of the indicated concentration of s-fractalkine. BLTE esterase activity was measured and expressed as the percent release of total BLTE esterase activity. Data shown are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$. *B*, Data for each different donor are expressed as the percent release of total BLTE esterase activity. **, $p < 0.01$ (by paired *t* test). *C*, Effects of PTX on BLTE esterase release from NK cells. NK cells were pretreated with 500 ng/ml of PTX at 37°C for 1 h before assays, then incubated in microplate wells in the absence or the presence of 10 nM s-fractalkine. BLTE esterase activity was measured and expressed as the percent release of total BLTE esterase activity. Data shown are representative of three independent experiments. *, $p < 0.05$.

Interaction between NK cells and fractalkine-expressing ECV cells

Because the membrane-bound form of fractalkine is reported to be expressed on EC membranes following stimulation by TNF-α or IL-1 (25), we hypothesized that fractalkine might be involved in NK cell-mediated endothelial cell damage. To examine this possibility, we used the endothelial cell line, ECV304 (ECV), to establish a fractalkine-transfected subline (FRK-ECV) that constitutively expressed membrane-bound fractalkine without significant changes in the expression of other adhesion molecules (Fig. 4). We then used this subline to examine whether NK cells adhere to membrane-bound fractalkine. As shown in Fig. 5, NK cells adhered to FRK-ECV more efficiently than to control-ECV. Soluble fractalkine, used as a competitor, markedly reduced the adhesion between NK cells and FRK-ECV to the basal levels of adhesion between NK cells and control-ECV, whereas mAbs against ICAM-1 and VCAM-1 only partially inhibited NK cell adhesion to FRK-ECV. The enhanced adhesion of NK cells to FRK-ECV could not be attributed to increased expression of surface receptors usually thought to be involved in adhesion by NK cells, since there was no significant difference in the expression of ICAM-1, VCAM-1, or class I molecules between FRK-ECV and control ECV (Fig. 4). These findings support the conclusion that physical interaction between the membrane-bound fractalkine on ECs and CX_3CR1 on NK cells can directly mediate adhesion between ECs and NK cells independent of integrins.

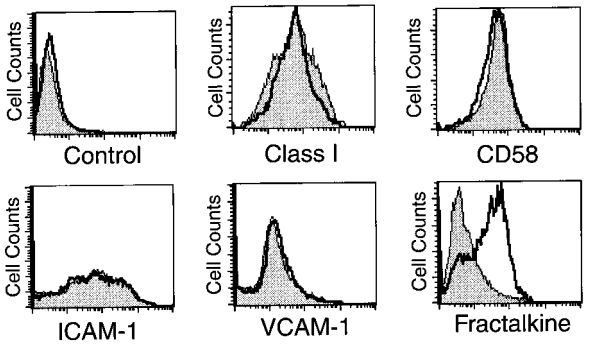

FIGURE 4. Fractalkine expression on FRK-ECV. Surface receptor expression was analyzed in control ECV (shaded) and FRK-ECV (solid line) using a FACSCaliber.

Increased susceptibility of FRK-ECV to NK-mediated cytolysis

Although endothelial cells are protected from proteolytic attacks by extracellular matrix, heparan sulfate, or other glycosaminoglycans under normal conditions (37), it has been reported that NK cells or LAK cells show enhanced adhesion to the endothelium and increased cytotoxicity against endothelial cells through the interaction of LFA-1/ICAM-1 or VLA-4/VCAM-1 (38–41). The endothelium is a major site for chemokine action in leukocyte activation and for transmigration of leukocytes through vessels (14, 16, 42). Because fractalkine functions not only as an adhesion molecule but also as a regulator for NK cell lytic activity (Figs. 1–3), we analyzed the sensitivity of FRK-ECV to lysis by NK cells. As shown in Fig. 6*A*, FRK-ECV showed increased susceptibility to NK cell-mediated cytolysis compared with control-ECV in repeated tests using cells from eight different donors, although some variability was observed among individuals. Moreover, we examined whether the increased susceptibility of FRK-ECV to NK lysis was due to the enhanced interaction between membrane-bound fractalkine and CX_3CR1 on NK cells. As shown in Fig. 6*B*, s-fractalkine, which efficiently blocked adhesion of NK cells to FRK-ECV (Fig. 5), significantly inhibited NK cell-mediated FRK-ECV lysis in all experiments in different donors.

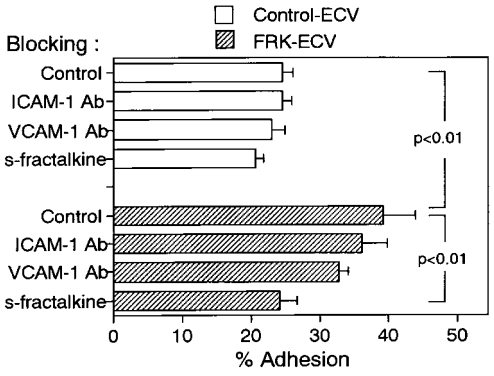

FIGURE 5. Adhesion of NK cells to control-ECV and FRK-ECV. Control ECV or FRK-ECV were cultured in a 48-well plate overnight to form confluent monolayers. Calcein-AM-labeled NK cells (1×10^4/well) were added to each well in the presence of Abs against ICAM-1 and VCAM-1 or 10 nM s-fractalkine. After removal of nonadherent cells, the fluorescence of the bound cells was measured using Wallac 1420 ARVO fluoroscan. Data are expressed as the percent adhesion relative to the total cells added and are representative of three independent experiments. Bars across the top indicate significant differences ($p < 0.01$, by Student's t test).

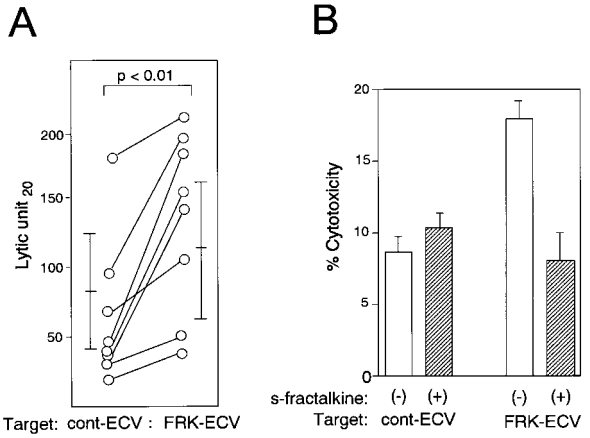

FIGURE 6. Susceptibility of FRK-ECV to NK cell-mediated cytolysis. *A*, Calcein-AM-labeled control-ECV and FRK-ECV were used as target cells for cytotoxicity mediated by purified NK cells. Data for each donor are expressed as 20% lytic units in 10^7 effector cells against control-ECV and FRK-ECV ($p < 0.01$, by paired t test). *B*, Effect of s-fractalkine on NK cell-mediated cytolysis of FRK-ECV. Calcein-AM-labeled control-ECV and FRK-ECV were cultured with NK cells for 4 h at an E:T cell ratio of 1:50. Supernatants were harvested, and fluorescence was measured. Data are expressed as the percent lysis of independent experiments, each using NK cells obtained from a different donor ($p < 0.05$, by Student's t test).

Fractalkine transfection increased susceptibility of HUVECs to NK cell-mediated cytolysis

Finally, we examined the involvement of membrane-bound fractalkine in NK cell-mediated endothelial cell damage in a more native setting using primary HUVECs. Although treatment of HUVECs with TNF-α, IL-1, and IFN-γ markedly induced membrane-bound fractalkine on the surface as reported by Bazan et al. (25), expression of ICAM-1 as well as that of VCAM-1 were also strongly enhanced on HUVECs. Therefore, to demonstrate the involvement of fractalkine in primary endothelial cell damage by NK cells, we exploited transfection of fractalkine cDNA in HUVECs for the transient expression of membrane-bound fractalkine without up-regulation of ICAM-1 and VCAM-1 expression (Fig. 7). We examined their susceptibility to NK cell-mediated lysis and found that transfection of fractalkine cDNA markedly

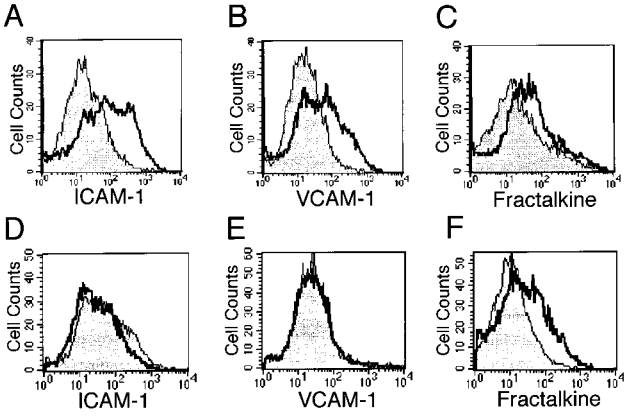

FIGURE 7. Fractalkine expression on TNF-α-activated and fractalkine-transfected HUVECs. HUVECs were cultured in the absence (shaded) or the presence of 100 ng/ml of TNF-α for 16 h (solid line; *A–C*). The expression plasmid (solid line) or vector (shaded) were transfected into HUVECs by Lipofectamine and cultured for 16 h (*D–F*).

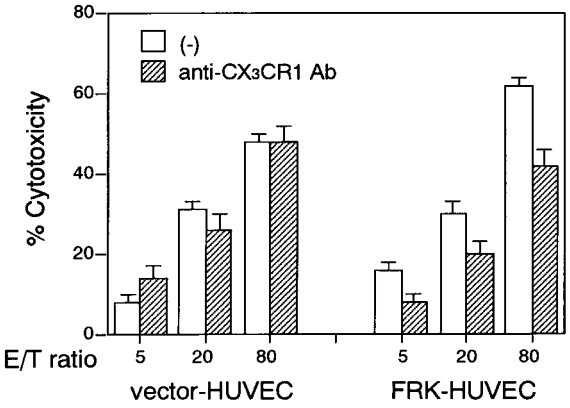

FIGURE 8. Effect of fractalkine cDNA transfection in HUVECs on NK cell-mediated cytolysis. The expression plasmid or vector was transfected into HUVECs by Lipofectamine and cultured for 16 h. Cells were labeled with calcein-AM and used as target cells for cytotoxicity assays in the absence or the presence of affinity-purified Ab against CX_3CR1. Data are representative of three independent experiments.

increased the susceptibility of HUVECs to NK cell-mediated cytolysis compared with vector transfectants. Moreover, affinity-purified Ab against CX_3CR1 strongly decreased NK cell cytotoxicity against fractalkine-transfected HUVECs, whereas no effect of anti-CX_3CR1 Ab was observed on cytolysis of control transfectants (Fig. 8).

Discussion

Leukocyte-endothelial cell adhesion is thought to be mediated predominantly by interaction of integrins with their ligands, i.e., LFA-1/ICAM-1 and VLA-4/VCAM-1 (13–17). Furthermore, integrin activation by selectins or soluble chemokines immobilized on the endothelial surface proteoglycan is believed to be required for firm cell-to-cell adhesion (43). On the other hand, we and others have reported that fractalkine is expressed in a membrane-bound form on activated ECs and promotes strong adhesion of leukocytes (25–27). In this report we demonstrated that NK cells expressed CX_3CR1 (Fig. 1*A*) and adhered to intact fractalkine immobilized on glass slides or membrane-bound fractalkine expressed on ECs, and that such adherence did not require the participation of other adhesion molecules, such as integrins (Figs. 1*B* and 5). Moreover, both types of adhesion were effectively inhibited by s-fractalkine, used as a competitor to dissect the interaction of fractalkine and CX_3CR1 on NK cells (Figs. 1*B* and 5), indicating that fractalkine functions as an adhesion molecule for NK cells. Our results are consistent with the recent studies by Fong et al. (44) and Haskell et al. (45) that fractalkine-mediated adhesion under physiologic flow is integrin independent.

Because several cytokines (IL-2, IFN-γ, and IL-12) and CC-chemokines (macrophage inflammatory protein-1α; IFN-γ-inducible protein-10; MCP-1, -2, and -3; and RANTES) have been reported to enhance NK cytotoxicity and granular exocytosis (33, 46, 47), we examined the functional roles of fractalkine on NK cell activity. Although the effects of fractalkine were modest compared with those of rIL-2, s-fractalkine significantly enhanced NK cell-mediated cytotoxicity against K562 target cells and granular exocytosis from NK cells in a dose- and time-dependent manner (Figs. 2 and 3). At present, the mechanisms of fractalkine-mediated enhancement of NK cell activity are not clear. However, NK cell functions, such as cytotoxicity and granular exocytosis, are mediated by activation of phospholipase Cγ, protein tyrosine kinases (PTKs), and phosphatidylinositol 3-kinase (PI 3-kinase) (28, 32), and it has been reported that the chemokine receptors identified to date, including CX_3CR1, all manifest a seven-transmembrane, G protein-linked architecture (20, 21, 24, 27, 48) and transduce signals for PTKs and PI 3-kinase activation that lead to increased adhesion and migration of the cells (49–52). One possible explanation for enhancement of NK cell cytotoxicity is G protein-mediated activation of PTKs and PI 3-kinase by fractalkine, despite the fact that fractalkine-mediated cell adhesion is independent of G proteins (27, 45). In support of this idea, we found that pertussis toxin, an inhibitor of G_i, markedly inhibited the fractalkine-mediated granular exocytosis in NK cells (Fig. 3*C*).

Systemic therapy with IL-2 or with adoptively transferred LAK cells has exhibited significant systemic toxicity and multiorgan dysfunction in human. These effects have been attributed to VLS, in which proteinaceous vascular fluid leaks across the endothelial barrier into the interstitium of various organs, including the lungs, liver, and spleen, and also into the interstitial space of connective tissues (6–10). Although IL-2 promotes the adhesion of NK cells to ECs and enhances the susceptibility of ECs to NK cell- or LAK cell-mediated cytolysis, several IL-2-inducible cytokines, including TNF-α, IL-1β, and IFN-γ do not show any direct effect on endothelial permeability (11, 12). Therefore, direct cell-to-cell contact between NK or LAK cells and ECs is thought to be necessary to cause VLS. Because fractalkine is expressed on EC membranes after stimulation by TNF-α or IL-1 and promotes strong adhesion of NK cells to ECs, we postulate that fractalkine is likely to be involved in NK cell-mediated vascular damage. However, the cytokine-activated HUVECs express high levels of ICAM-1 and VCAM-1 as well as exhibit de novo expression of fractalkine (Fig. 7), producing a highly complex pattern of adhesion pathways between NK cells and activated HUVECs. To assess the role of membrane-bound fractalkine in the adhesion of NK cells to endothelial cells and NK cell-mediated endothelial cell damage in a nonconfounded system, we have established a fractalkine-transfected subline (FRK-ECV) that constitutively expresses membrane-bound fractalkine without significant changes in the expression of other adhesion molecules (Fig. 4). We have clearly demonstrated that FRK-ECV expressing the membrane-bound fractalkine showed increased adhesion of NK cells and susceptibility to NK cell-mediated cytolysis compared with control-ECV (Figs. 5 and 6*A*). Moreover, s-fractalkine, which efficiently dissects the interaction of membrane-bound fractalkine on ECs and CX_3CR1 on NK cells (Fig. 5), significantly inhibited NK cell-mediated FRK-ECV lysis (Fig. 6*B*). Finally, we also examined the involvement of membrane-bound fractalkine in NK cell-mediated endothelial cell damage in a more native setting by transfection of fractalkine cDNA in primary HUVECs (Fig. 7). The results indicated that fractalkine-transfected HUVECs exhibited increased susceptibility against NK cell-mediated cytolysis compared with vector transfectants (Fig. 8). Although our data show that fractalkine-mediated adhesion and susceptibility of endothelial cells do not necessitate the participation of other adhesion molecules, it is possible that cellular interactions stabilized through other adhesion molecules, e.g., LFA-1/ICAM-1 and VLA-4/VCAM-1, which are induced on ECs by TNF-α and IL-1, may be involved in vascular damage in vivo.

As predicted by the missing self theory of immunosurveillance, initially proposed by Ljunggren and Karre (53), several families of NK inhibitory receptors that recognize allelic forms of MHC class I molecules generate signals that inhibit NK cell-mediated cytotoxicity (3). Therefore, autologous endothelial cells are considered to be protected from NK cell-mediated lytic attack by expression of MHC class I molecules. However, viruses, bacteria and their products, ischemia-reperfusion, or IL-2 administration directly or

indirectly activate ECs and induce cytokine production, such as IL-1, TNF-α, and IL-6 (13–16), resulting in induction of fractalkine as well as ICAM-1 and VCAM-1 on the EC membrane. Although the mechanisms underlying endothelial damage in various pathological states are not clear, NK cells may be able to damage the vascular endothelium despite the presence of autologous MHC in these conditions, where fractalkine on NK cells acts not only as an adhesion molecule, but also as a regulator in cooperation with other adhesion molecules and cytokines. Recently, Chen et al. (54) have reported that a viral protein, vMIP-II, encoded by human herpesvirus 8, has antagonistic activity for CC, CXC, and CX_3C chemokine receptors and anti-inflammatory activity in an experimental glomerulonephritis in rat model system. Although additional studies are needed, these findings suggest that the use of fractalkine antagonists might be therapeutically useful for endothelial injury in various pathological states, including acute inflammatory reaction, graft-vs-host disease, and VLS.

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