Abstract. Chemokine receptors are typically expressed on natural killer cells, which can be activated by membrane ligands including the membrane chemokine fractalkine (mFKN). This study investigated the function of mFKN on natural killer (NK) cell activation for interferon (IFN-γ) production and cytotoxicity against tumors. HeLa cells were transfected with a membrane human fractalkine (mhFKN)-expressing vector, and the transcription and surface expression of mhFKN in transfected HeLa cells were confirmed by RT-PCR analysis and immunofluorescence assay, respectively. After co-culture of NK-92 cells with FKN-HeLa cells, the intracellular IFN-γ in the NK-92 cells significantly increased compared to mock-HeLa cells. The concentration of IFN-γ also increased in the supernatant of the NK-92 cells stimulated with FKN-HeLa cells. Moreover, the cytolytic activity of NK-92 cells against K562 target tumor cells was significantly enhanced at each effector:target ratio in 4-h 51Cr-release assays when the NK-92 cells were pretreated with FKN-HeLa, indicating that membrane fractalkine activates the NK cells in the killing process. This study further confirms that membrane-expressed fractalkine plays a critical role in NK cell activation.

Introduction

Natural killer (NK) cells are thought to mediate immune responses against viral infection and neoplastic transformation by cytolytic activity without prior antigenic stimulation (1-8). NK cells are also believed to contribute to immunological regulation through the production of pro-inflammatory cytokines such as IFN-γ and TNF-α, which activate various types of cells including NK cells (9-11). Fractalkine (also named FKN or CX3CL1) is the only member of the CX3C subfamily, and exists in two isoforms: a membrane-anchored form and a soluble form that results from proteolytic cleavage (12-14). Membrane-bound FKN (mFKN) directly mediates the capture and firm adhesion of leukocytes expressing its receptor CX3CR1 (15,16). The expression pattern of CX3CR1 in human NK cells is associated mostly with their cytotoxic effects (17). We have previously reported (18) that FKN is expressed in a variety of human tumor cell lines, and ~90% of peripheral blood NK cells and all NK-92 cells express CX3CR1. The interaction between CX3CR1 on NK cells and FKN on tumor cells is involved in the natural cytotoxicity of NK cells against tumors. Here, we further showed that recombinant-expressed membrane human FKN (mhFKN) on HeLa cells activates NK cells against tumors and leads to a high production of IFN-γ.

Materials and methods

Antibodies and reagents. The polyclonal antibody against human fractalkine (goat IgG) used in the indirect immunofluorescence assay was purchased from R&D Systems (AF365). The secondary antibody (FITC-conjugated swine anti-goat IgG) was purchased from BD Biosciences. The mAbs against human IFN-γ and CD56 were purchased from BD Biosciences. ELISA kits for human IFN-γ were purchased from Jingmei Biotech. The kit for reverse transcription was from Invitrogen and the PCR kit from Promega.

Cell lines and cell culture. NK cell line (NK-92) was obtained from ATCC. K562, a chronic myelogenous leukemia cell line, has been kept in our laboratory for many years. HeLa, a cervix epithelial tumor cell line, was also available from our institute. NK-92 cells were maintained in α-minimum essential medium (α-MEM, Gibco BRL) without ribonucleosides and deoxyribonucleosides and supplemented with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 100 U/ml recombinant human IL-2 (PeproTech), 12.5% horse serum and 12.5% heat-inactivated fetal bovine serum (both from Hyclone), 100 U/ml penicillin and 100 U/ml streptomycin. K562 cells were
cultured in RPMI-1640 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco BRL) containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin.

Construction of recombinant membrane fractalkine-expressing vector. Firstly, the cDNA was synthesized with total RNA extracted from K562. Secondly, the complete ORF of human fractalkine (hFKN) was amplified from this total RNA using PCR with BamHI-tailed forward primer 5'-GG ATCCCCATGGCTCCGATATCTCTGT-3' and EcoRI-tailed reverse primer 5'-GAATTCAAGGAGGTTCTACACACGGGC AC-3'. Finally the 1.1-kb BamHI-EcoRI fractalkine fragment was subcloned into a cloning site of pcDNA3 vector as described previously (19). Plasmid with low endotoxin was prepared on a large scale.

Cell transfection. The HeLa cells were transfected with the mhFKN-expressing vector (pcDNA3-fractalkine) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Transfectants were selected by adding 1000 μg/ml G418 (Invitrogen) and then maintained with 500 μg/ml G418. Parent HeLa cells were also transfected with a mock vector (empty plasmid pcDNA3) as control.

Reverse transcription-polymerase chain reaction. Briefly, total RNA was isolated from HeLa cells with Trizol reagent (Invitrogen). Two micrograms of RNA were reversely transcribed with the random primer and incubated with 200 U M-MLV for 1 h at 37˚C. Single-strand cDNA was then subjected to PCR. After a 2-min incubation at 95˚C, samples were added to 2 U polymerase per 100 μl reaction medium. The mixtures were then subjected to a 30 cycle-PCR amplification program consisting of denaturing for 30 sec at 94˚C, annealing for 30 sec at a different temperature, extension for 1 min at 72˚C, with a 7-min extension after the last cycle. PCR primers for detecting transcription of human fractalkine, and β-actin were synthesized by Shanghai Sangon, Shanghai, P.R. China. Sequences of these primers were: human fractalkine (351 bp), forward 5'-AGGAGAATGCTCCGATATCTCTGT-3' and reverse primer 5'-GAATTCAAGGAGGTTCTACACACGGGC AC-3'; and human β-actin (309 bp), forward 5'-GACCTGACTACCTCATGAAGAT-3' and reverse 5'-GTCACACTTTGATGTTGAAAGG-3'.

Immunofluorescence microscopy. HeLa cells were cultured in a chamber slide (Nalge) and washed three times with ice-cold PBS. To avoid permeabilization of cell membranes, slides were fixed in PBS containing 4% paraformaldehyde before the indirect immunofluorescence staining. These cells were then stained with the primary pAb against human fractalkine (15 μg/ml) or the isotype IgG as negative control. Finally, the cells were stained with the FITC-conjugated secondary antibody (swine anti-goat IgG) (1:50 dilution).

Cell to cell stimulation. To determine the effect of membrane-bound fractalkine, NK-92 cells (5x10^5) were co-cultured with mock-HeLa cells or HeLa cells transfected with mhFKN (5x10^5) for 4 h. To measure the intracellular cytokines of the NK-92 cells, monensin (2 μM, Sigma-Aldrich) and ionomycin (1 μg/ml, Sigma-Aldrich) were added to the medium, and the cells were cultured for an additional 4 h. To measure the cytokines secreted by the NK-92 cells, the cells were cultured for another 4 h without any additions. After incubation, the cell deposits or cell-free supernatants were collected for further study.

Flow cytometric analysis. To examine the IFN-γ produced in the NK-92 cells, the cells collected from the co-culture mixture described above were exposed to a standard staining protocol of intracellular cytokine detection (Current Protocols in Immunology). The cell mixture was washed 3 times with ice-cold PBS. Then the cells were fixed and permeabilized with a Cytotox/Cytperm plus kit (BD Biosciences), and stained for intracellular IFN-γ. The mAb against human CD56 was added to identify the NK-92 cells in the mixture. The cells were analyzed by FACScalibur (BD Biosciences), and the data were analyzed using WinMDI 2.8 (free software for FACS analysis).

ELISA. To examine the IFN-γ content in the supernatants of the NK-92 cell culture, the supernatants collected from the co-culture experiments mentioned above were assessed for IFN-γ using ELISA kits according to the manufacturer’s protocol. The detectable range of each ELISA kit was 5-
1,000 pg/ml, and the optical density of each individual well was determined at 450 nm using a microplate reader (Bio-Tek Instrument Inc).

Cytotoxicity analysis. To determine the effect of mhFKN on NK-92 cell cytotoxicity, a modified 4-h 51Cr release assay was performed. The HeLa cells during the logarithmic growth phase were collected, washed and plated into 96-well round-bottomed plates in varying numbers to achieve the desired 1:1 ratio with the NK-92 cells. These cells were incubated at 37˚C in a humidified 5% CO2 incubator for 5 h for recovery. Varying numbers of NK-92 cells were then added to the plates to achieve the desired E:T ratios, and these cells were co-cultured for 4 h for cell to cell stimulation. Finally, 1x105 51Cr-labeled K562 cells [the K562 cells were collected and incubated in 100 μCi of Na51Cr (Perkin-Elmer Life Sciences) per 106 cells, at 37˚C in a humidified 5% CO2 incubator for 1 h and washed three times with media] were added into each well. After a 4-h incubation at 37˚C in a humidified 5% CO2 incubator, the supernatants were collected from each well and the radioactivity was determined using a γ-counter. Specific lysis = [(experimental cpm - spontaneous cpm)/(maximum release cpm - spontaneous cpm)] x 100%. To determine the effect of membrane fractalkine on NK-92 cell cytotoxicity against HeLa cells, a standard 4-h 51Cr release assay was performed as described (20).

Statistical analysis. The Student’s t-test for cytotoxicity was performed to determine statistical differences with p values <0.05 or p values <0.01 being considered significant or highly significant, respectively. All experiments were performed at least three times with a representative experiment being shown.

Results

Surface expression of human recombinant membrane fractalkine on HeLa cells. The complete ORF of the human fractalkine was cloned from K562 cells, which we previously reported to express membrane FKN (mhFKN) (18). The HeLa cells were transfected with a mhFKN-expressing vector, and the transcription of mhFKN in the transfected HeLa cells was examined by RT-PCR analysis. The PCR product with the predicted length was amplified from the total RNA of HeLa cells transfected with mhFKN (FKN-HeLa), while no product was seen from those transfected with the mock vector (mock-HeLa) (Fig. 1A).

As reported, FKN exists as a membrane anchored form or as a soluble form. To determine whether the fractalkine in the transfected HeLa cells existed as the membrane-anchored form, we performed the immunofluorescence assay (Fig. 1B). Because the primary antibody reacts against the chemokine module that exists in both forms, we performed the assay following the protocol which was able to keep the integrity of the cellular membrane to ensure that the molecules we detected were those anchored to the membrane. As shown in Fig. 1B, recombinant mhFKN was detected on the surface of FKN-HeLa cells (mhFKN-HeLa), but not on the mock-HeLa cells.

Enhanced production of interferon-γ by NK-92 cells after stimulation with recombinant membrane fractalkine. The activation of NK cells is critical in the early phase of immune
performed co-culture experiments to measure the INF-γ means from at least 3 independent experiments.

Figure 4. Improved cytotoxicity of NK-92 cells against K562 cells by stimulation of mhFKN on HeLa cells. The natural cytotoxic activity of NK-92 cells against cancer cells was evaluated with the 4-h 51Cr-release assay at various effector/target ratios. (A) The natural cytotoxic activity of NK-92 cells against K562. NK-92 cells were pretreated with mock-HeLa cells or FKN-HeLa cells before the assay. (B) The natural cytotoxic activity of NK-92 cells against mock-HeLa cells or FKN-HeLa cells. Values represent the means from at least 3 independent experiments.

Discussion

In this study, we provided evidence that the FKN on tumor cells mediates antitumor activity by membrane interaction with human NK cells. This effect seems to rely solely on CX3CR1 on human NK cells. We previously reported in this journal (18) that FKN is expressed in a variety of human tumor cell lines, and ~90% of peripheral blood NK cells and almost all NK-92 cells express CX3CR1. Anti-CX3CR1 antibody strongly inhibited the cytotoxicity of NK cells against K562 cells, and pretreatment of NK cells with recombinant soluble FKN enhanced the cytolytic function on tumor cells. Here, we further show that recombinant-expressed membrane human FKN on HeLa cells enhances NK cell cytotoxicity against K562 cells and causes high production of IFN-γ. It has also been reported that FKN immobilized by transfecting endothelial cells markedly induced IFN-γ production by NK cells in a dose-dependent manner (22). In addition, transfection of FKN cDNA into ECV304 cells or HUVECs resulted in increased adhesion to NK cells and susceptibility to NK cell-mediated cytolysis (23). Moreover, both enhanced adhesion and susceptibility of fractalkine-transfected cells were markedly suppressed by anti-CX3CR1 antibody (23). These findings together demonstrate that surface FKN/CX3CR1 interaction plays an important role in the recruitment and adhesion of leukocytes including NK cells in inflammation.

Because several cytokines (IL-2, IFN-γ, and IL-12) and membrane ligands have been reported to enhance NK cytotoxicity (24,25), we examined the functional roles of membrane fractalkine on NK cell activity. The mhFKN on HeLa cells significantly enhanced NK cell-mediated cytotoxicity against K562 target cells and IFN-γ production from NK cells by both intracellular synthesis and secretion (Figs. 2 and 3). At present, the mechanisms of FKN-mediated enhancement of NK cell activity are not clear. However, the functions of NK cells, such as cytotoxicity and cytokine production, are mediated by activation of several signaling molecules such as phospholipase Cγ, protein tyrosine kinases (PTKs), and phosphatidylinositol 3-kinase (PI3-K) (25-27). Pretreatment of NK cells with the phosphatidylinositol 3-kinase inhibitor completely inhibited the production of IFN-γ induced by fractalkine (22).

As predicted by the missing self theory of immunosurveillance, initially proposed by Ljunggren and Karre (28), several families of NK inhibitory receptors which recognize allelic forms of MHC class I molecules may generate signals that inhibit NK cell-mediated cytotoxicity (29-30). Therefore, the reason why the NK-92 cells activated by mhFKN-HeLa cells secreted more IFN-γ without killing HeLa cells was because the HeLa cells kept high MHC class I molecules, unlike the K562 cells, though the NK-92 cells were totally activated (Fig. 4). The exact mechanisms underlying enhanced
cytolysis against K562 cells after mhFKN recognition are not clear, but the FKN pathway activated in NK cells after mhFKN stimulation causing more production of IFN-γ is an important finding.

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References