Significance of chemokine receptor expression in aggressive NK cell leukemia.

Short title for running head: Chemokine receptors in ANKL

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Abstract
Natural killer (NK) cell-type lymphoproliferative diseases of granular lymphocytes can be subdivided into aggressive NK cell leukemia (ANKL) and chronic NK cell lymphocytosis (CNKL). One reason for the poor outcome in ANKL is leukemic infiltration into multiple organs. The mechanisms of cell trafficking associated with the chemokine system have been investigated in NK cells. To clarify the mechanism of systemic migration of leukemic NK cells, we enrolled 9 ANKL and 6 CNKL cases, and analyzed the expression profiles and functions of chemokine receptors by flowcytometry and chemotaxis assay. CXCR1 was detected on NK cells in all groups, and CCR5 was positive in all ANKL cells. Proliferating NK cells were simultaneously positive for CXCR1 and CCR5 in all ANKL patients examined, and NK cells with this phenotype did not expand in CNKL patients or healthy donors. ANKL cells showed enhanced chemotaxis toward the ligands of these receptors. These results indicated that the chemokine system might play an important role in the pathophysiology of ANKL and that chemokine receptor profiling might be a novel tool for discriminating ANKL cells from benign NK cells.

Key words
chemokine receptor, aggressive natural killer cell leukemia, CXCR1, CCR5
Introduction

Among natural killer (NK) cell-type lymphoproliferative diseases of granular lymphocytes (NK-LDGL), aggressive NK cell leukemia (ANKL) is diagnosed by the proliferation of large granular lymphocytes with NK-phenotype in the peripheral blood (PB), bone marrow (BM), liver, and spleen, and by a rapidly progressive clinical course with a poor outcome.\textsuperscript{1-5} Chronic NK cell lymphocytosis (CNKL) is characterized by the expansion of NK cells with a chronic indolent course,\textsuperscript{6, 7} although the morphology and the phenotypes of the NK cells are very similar to the ANKL cells.\textsuperscript{8}

The importance of chemokine receptors on the normal NK cell surface has been reported.\textsuperscript{9-12} The chemokine receptors are thought to regulate NK cell trafficking and cytotoxicity in a phenotype-specific manner.\textsuperscript{13, 14} However, the expression of chemokine receptors by human NK cells is still controversial, and little is known about the expression and function of chemokine receptors in NK cell neoplasms.

To clarify the mechanism of systemic migration of leukemic NK cells, we investigated the clinical and phenotypic features of ANKL, compared with CNKL, including the expression profiles of chemokine receptors. We also aimed to find the novel feature of ANKL cells which differ from other benign NK cells.

Patients, Materials, and Methods

Patients and healthy donors

Fifteen patients with NK-LDGL were enrolled in the present study, consisting of 9 patients of ANKL and 6 of CNKL (Table 1). The three patients of ANKL have been reported elsewhere.\textsuperscript{15-17} Diagnoses were based on the published criteria.\textsuperscript{18, 19} Six healthy volunteers were also included as controls. We collected peripheral blood from patients and volunteers including the two patients who also donated bone marrow cells (patient No.5 and No.8). This study was approved by the Shinshu University Institutional Review Board. All of the samples were obtained with informed consent according to the Declaration of Helsinki.

Flowcytometric analysis

Mononuclear cells from PB and BM were isolated by Ficoll-Hypaque density gradient centrifugation in 8 ANKL patients, 6 CNKL patients, and 6 healthy donors. The phenotype of large granular lymphocytes in patients was confirmed using the following fluorescence-conjugated antibodies: anti-CD2-allophycocyanine, anti-CD3-peridinin chlorophyll protein, anti-CD16-fluorescein isothiocyanate (FITC)
The expression levels of chemokine receptors on CD2⁺CD3⁻ cells were measured using the following antibodies: anti-CXCR1-PE, anti-CXCR2-PE, anti-CXCR3-PE, anti-CXCR4-PE, anti-CXCR5-PE, anti-CXCR6-PE, anti-CXCR1-PE, anti-CCR2-PE, anti-CCR3-PE, anti-CCR4-PE, anti-CCR5-PE, anti-CCR6-PE, anti-CCR7-PE, anti-CCR9-PE (DakoCytomation), anti-CCR4-PE, anti-CCR5-PE, anti-CCR5-FITC (BD Biosciences), and anti-CX3CR1-FITC (MBL, Nagoya, Japan). Positivity for the antigens was determined with FACSCaliber and CELLQuest software (BD Biosciences).

**Natural killer cell purification**

Isolated mononuclear cells were incubated with a human NK cell isolation Kit II (Miltenyi Biotec, Auburn, CA) and loaded onto MINI-MACS magnetic cell separation columns (Miltenyi Biotec) according to the manufacturer’s instructions. Purified NK cell fractions were obtained by depletion of magnetically labeled non-NK cells. The purity of NK cell fractions was evaluated by staining with Wright-Giemsa solution as well as by analysis of the cell-surface expression of CD2 and CD3 with flowcytometry (FACSCaliber, BD Biosciences).

**Quantification of EBV DNA**

DNA was isolated from 1 to 5×10⁵ purified NK cells using a DNA Extractor WB Kit (Wako Pure Chemical Industries, Osaka, Japan). 5µl of each sample, containing approximately 500ng DNA, was amplified by quantitative real-time PCR with ABI7700 sequence detector system (Applied Biosystems, Foster City, CA) using TaqMan fluorogenic oligonucleotide probes. ABI7700 Sequence Detector version 1.6.3 software (Applied Biosystems) was used for the construction of standard curve and the analysis of results. The oligonucleotide primers for EBV DNA (GenBank #V01555) were 5’-TTCTGTCTCACCTACCGGATGA-3’ and 5’-GTAATGAAGACTTGGAACAGGC-3’ for PCR and 5’-ACCTGGAATGCAGCAGTTTGACAACAG-3’ for the TaqMan probe. The cutoff value was 10 copies/µg DNA. Values detected in the whole blood of 50 healthy donors ranged from less than 10 to 150 (median; <10) copies/µg DNA.

**Chemotaxis assay**

With cells from 3 ANKL patients and 3 healthy donors, BD BioCoat Matrigel Invasion Chambers (BD Biosciences) with a lower well volume of 500 µl were used in accordance with the manufacturer’s protocols for chemotaxis assay. Briefly, a
polycarbonate filter with a pore size of 8 µm and a thin layer of basement membrane matrix was used to separate the top and bottom chambers. Purified fresh NK cells \(5\times10^5\) were suspended in 500 µl of RPMI 1640 medium and placed in the upper compartments of the chambers. Chemokines were diluted in RPMI medium at different concentrations and were placed in the lower wells with or without goat antibodies against the corresponding chemokines. The chambers were incubated for 2 hours at 37°C in a 5% CO\(_2\) incubator. The filters were then removed, dehydrated, and stained with 15% Giemsa solution for 30 minutes and then mounted on glass slides. The cells were counted in 6 high-power fields from 3 filters and averaged for each sample. The migration index was calculated as the number of cells migrating toward the concentration gradient of chemokines divided by the number of cells migrating toward the medium without chemokines. The IL-8, RANTES, MIP-1\(\alpha\), and MIP-1\(\beta\) were purchased from R & D systems (Minneapolis, MN) and used at the following concentrations; IL-8 at 100 ng/ml, RANTES at 100 ng/ml, MIP-1\(\alpha\) at 10 ng/ml, and MIP-1\(\beta\) at 10 ng/ml. The concentrations indicated above represent the doses that elicited the highest migration index in dose-response curves with different chemokine concentrations (1, 10, 100, and 1000 ng/ml).

**Statistics**

White blood cell count, NK cell count, EBV genome load, and the chemokine receptor expression level in the patients and the healthy volunteers were analyzed statistically by the Mann-Whitney U-test, Kruskal-Wallis test or Steel-Dwass test.

**Results**

**Clinical findings**

Table 1 shows the clinical features of 15 NK-LDGL patients. Fever and hepatosplenomegaly were observed in all 9 ANKL patients, but not in the CNKL patients. All of the patients with ANKL were refractory to combined chemotherapy, and hepatic failure was a common cause of death. Patient No.6 and patient No.7 attained complete remission (CR) after cord blood cell transplantation. Although no recurrence was seen, patient No.6 died of chronic graft-versus-host disease and infection 8 months after diagnosis. The median survival time of ANKL patients was 3 months. All of the CNKL patients are currently alive with a median observation period of 53 months. Some of the CNKL patients transiently required blood transfusion, immunosuppressive therapy, or subcutaneous low dose cytarabine against concomitant hematological
abnormalities, such as pure red cell aplasia (patient No.14) or myelodysplastic syndrome (refractory anemia with excess blasts) (patient No.15).

**Laboratory data**

Table 2 summarizes the laboratory studies of NK-LDGL patients. White blood cell and NK cell counts were not significantly different between ANKL and CNKL (P = .90 and P > .99, respectively). In patient No.5, 85% of BM nuclear cells were leukemic cells, although NK cells did not increase in PB. Cholestatic type and cellular injury type of liver dysfunction were observed frequently in ANKL patients. However, these changes were rare among CNKL patients. Abnormal karyograms with G-banding analysis were observed in all of the ANKL patients examined. Three patients showed trisomy 8. On the other hand, karyotypes were normal diploid in all of the CNKL patients.

**Phenotype of NK cells**

The results of flow cytometric immunophenotyping are summarized in Table 3. All of the patients’ NK cells were positive for CD2, CD16 and/or CD56 and negative for surface CD3. None of the ANKL cells were positive for CD57, although it was expressed in 5 CNKL cells. ANKL and CNKL cells examined were negative for CD4, CD5, CD8, T-cell receptor (TCR) αβ, and TCRγδ. CD7 and cytoplasmic CD3 were positive in all of the patients examined. B-lineage markers and myelomonocytic markers were uniformly negative. CD38 was expressed in all of the ANKL patients, and in 2 CNKL patients.

**EBV genome load**

EBV DNA load in the NK cells was more than 10⁵ copies/µg DNA in seven of eight ANKL patients (Table 3). No EBV DNA was detected in the NK cells of patient No.8 (ANKL). In all of the CNKL patients and healthy donors, EBV DNA load remained less than 10³ copies/µg of DNA (Table 3 and data not shown). EBV genome increased significantly in ANKL cells in comparison to CNKL cells and NK cells from healthy donors (P < 0.01).

**Expression of chemokine receptors**

Figure 1 summarizes the positivity of chemokine receptors on CD2⁺CD3⁻ cells from ANKL patients including the EBV genome-negative patient, CNKL patients, and healthy donors, as determined by 3-color analysis. There was no difference in chemokine receptor expression profile of ANKL cells between from PB and BM (data
CXCR1 was detected on NK cells in all groups. CCR5 was positive in all ANKL cells examined, and its expression level was significantly different among the 3 groups (P < 0.05). Regardless EBV genome positivity, there was a significant difference in the expression levels of CCR5 between ANKL and CNKL (P < 0.05), however, not between CNKL and healthy volunteers.

Figure 2 shows the double expression of CXCR1 and CCR5 on NK cells as the result of 4-color analysis. CXCR1 and CCR5 were simultaneously positive on ANKL cells in all patients examined, although NK cells expressing both CXCR1 and CCR5 did not expand in CNKL patients or healthy donors (P < 0.0005). The expression of CCR5 was evident in patient No.13 (CNKL); however, the expression of CXCR1 was weak in this patient.

**Migration toward chemokines**

Migration assay was performed on freshly isolated NK cells as shown in Figure 3. The migration index for IL-8 was high in all individuals, and was significantly reduced after treatment with an inhibitory antibody (P < 0.005). The migration indexes for RANTES, MIP-1α, and MIP-1β increased in ANKL patients, but not in controls, and those chemotaxis was significantly inhibited with addition of specific antibodies (P < 0.005).

**Discussion**

The simultaneous positivity of CXCR1 and CCR5 in NK cells was unique to ANKL. ANKL cells also showed the potential of migration toward the ligands of the chemokine receptors. These results indicated that the chemokine receptors on ANKL cells could play a significant role in multi-organ involvement and that the distinct expression pattern of chemokine receptors might be a novel marker of ANKL cells.

Phenotypic analysis indicated positivity of CCR5 and CD16 on ANKL cells in the present study, although normal CD16⁺ NK cells have been reported to be negative for CCR5.13, 20, 21 Nieto et al. reported that human CD16⁺ NK cells were positive for CCR5 after activation with IL-2 and IL-15.22 Their results suggest that ANKL cells would represent a neoplasm of the activated phenotype of NK cells. In previous case series, CNKL cells expressed CXCR1 but not CCR5.23, 24 Our study using DNA microarray methods also demonstrated no significant differences in chemokine receptor mRNA expression levels between CNKL patients and healthy donors.25 These results in conjunction with our current study suggest that CNKL cells closely resemble the NK
cells of healthy donors, while ANKL cells are distinct in regards to their chemokine receptor expression profile.

Some ANKL patients have achieved CR after allogeneic stem cell transplantation (SCT). Novitzky et al. reported that, following allogeneic SCT, there is rapid proliferation of NK cells that appear to proceed directly from the transplanted hematopoietic stem cell. In the clinical course of ANKL, these NK cells should be difficult to distinguish from leukemic NK cells. In our patients who received allogeneic SCT, we were able to discriminate the reactive and leukemic NK cells by examining the surface expression of CXCR1 and CCR5 (unpublished data).

Resting and activated NK cells generally express CXCR1. The results of expression and functional analyses of CXCR1 and IL-8 indicate that circulating ANKL cells might migrate toward IL-8 in vivo. Our results concur with Campbell’s report that IL-8 is a chemoattractant for NK cells. CCR5 plays a significant role in lymphocyte trafficking in various disorders and NK cells generally have the potential to produce RANTES, MIP-1α, and MIP-1β. The results of experiments with DNA chips also suggested high levels of expression of MIP-1α and MIP-1β in patients with EBV-positive NK cell lymphoma, as compared with patients of nonspecific lymphadenitis. MIP-1α might cause the inflammatory cell recruitment into tissues in patients with nasal NK/T cell lymphoma and chronic EBV infection. Further investigation about the expression of the CXCR1- and CCR5-ligands might be required for clarifying the migration mechanism in ANKL patients.

CCR5 ligands have also been shown to promote cytolytic activity of CCR5 positive NK cells by promoting cytotoxic granule release. Interaction of CCR5 and its ligands may strengthen the cytolytic ability of infiltrating ANKL cells, which would cause organ injury.

The migration of ANKL cells was inhibited by neutralizing antibodies against corresponding chemokines. The results suggest that the inhibition of the interaction between these chemokine receptors and their ligands would be a treatment option for ANKL. CCR5 antagonists, anti-CCR5 antibodies, and RANTES antagonists have been shown to reduce the cell infiltration into the tissue in several animal models. Further studies are required to determine methods capable of inhibiting chemokine systems in ANKL.

**Conclusion**

We examined chemokine receptor profile on ANKL and CNKL cells. The expression of
CXCR1 and CCR5 is unique to ANKL cells and the migration of the cells is regulated, in part, through their ligands.

Acknowledgments

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Figure Legends

Figure 1. Expression of chemokine receptors on NK cells. CD2⁺CD3⁻ fractions of peripheral blood or bone marrow mononuclear cells were gated, and the positive percentages of chemokine receptors were measured by 3-color analysis in (●) ANKL patients, (▲) CNKL patients, and (♦) healthy donors. Asterisks indicate statistically significant differences in chemokine receptor expression level (P < 0.05).

Figure 2. Simultaneous expression of CXCR1 and CCR5 on NK cells. (a) CD2⁺CD3⁻ fractions of peripheral blood or bone marrow mononuclear cells were gated, and CXCR1 and CCR5 expression were evaluated by 4-color analysis. (b) The positive percentages of double positive NK cells were measured in (●) ANKL patients, (▲) CNKL patients, and (♦) healthy donors. Asterisks indicate statistically significant differences in chemokine receptor expression levels (P < 0.0005).

Figure 3. Effects of chemokines on NK cell migration.

Purified NK cells (5×10⁵) were placed in the upper compartments of the chambers, while (a) IL-8, (b) RANTES, (c) MIP-1α or (d) MIP-1β was added to the lower wells with or without the corresponding antibody. The chambers were incubated for 2 hours at 37°C in a 5% CO₂ incubator. The migration index was calculated as the number of cells migrating toward the concentration gradient of chemokines divided by the number of cells migrating toward the medium without chemokines. The data represents means ± SEM from three experiments. Asterisks indicate statistically significant differences in the migration index (P < 0.005).
Figure 3

(a) Migration index

(b) Migration index

(c) Migration index

(d) Migration index
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<tr>
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<th>Age(y.o.)</th>
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<td>F</td>
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<td>32</td>
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<td>M</td>
<td>+</td>
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<td>F</td>
<td>+</td>
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<td>M</td>
<td>-</td>
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ANKL; aggressive natural killer cell leukemia, CNKL; chronic natural killer cell lymphocytosis,
CBT; cord blood transplantation, M (in outcome); month, D; day, *; therapy for the concomitant myelodysplastic syndrome
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<th>NK cell (/µl)</th>
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<th>ALT (&lt; 45) (U/l)</th>
<th>ALP (&lt; 367) (U/l)</th>
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T. Bil; total bilirubin, ALT; alanine transaminase, ALP; alkaline phosphatase, NE; not examined

*; Neutrophil count was over 1000 / µl in all the CNKL patients except for the patients with myelodysplastic syndrome (No. 15).

**; Trisomy 8 was observed with interleukin 2-stimulated analysis.

***; Inv(9)(p11;q13) was a constitutional anomaly.
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NE; not examined, sCD3; surface CD3