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Establishment and characterization of a novel cell line (HATAK) derived from natural killer cell neoplasm corresponding to the myeloid/NK progenitor

Takuji Katayama,[1] Mikio Danbara,[1] Takumi Aoki,[1] Sosei Okina,[1] Takashi Ishida,[1] Koji Eshima,[2] Koji Miyazaki,[1] Masaaki Higashihara[1]

[1] Department of Hematology, Kitasato University School of Medicine
[2] Department of Immunology, Kitasato University School of Medicine

Objective: Immature natural killer (NK) cell neoplasms are extremely rare. We established a novel cell line named HATAK from a case of NK cell lymphoblastic leukaemia/lymphoma and describe here the detailed characteristics of HATAK.
Methods: Cells were cultured in standard medium. After establishing this new cell line, we performed immunophenotyping of HATAK cells by flow cytometry-based genotyping by reverse transcription polymerase chain reaction (RT-PCR), and cytotoxity assay by the ^{51}Cr releasing method before and after interleukin (IL)-2 and IL-15 stimulation. We also investigated the activation status of lymphocyte function-associated antigen-1 (LFA-1) on chemokine stimulation.
Results: The immunophenotype was positive for CD7, CD13, CD33, and CD56, and negative for other T- and B-cell antigens. The T-cell receptor gene was in a germline configuration. Transcripts of the molecules characteristic for NK cells were detected by RT-PCR. Even under cytokine stimulation, HATAK cells exhibited neither cytotoxic activity nor IFN (interferon)-γ production. Notably, LFA-1 had already been activated in a steady state, and the stimulation from the fractalkine-CX_3CR1 axis never exerted pressure on its activation status.
Conclusion: HATAK corresponded to the myeloid/NK progenitor based on normal NK cell ontogeny. This cell line might be useful to elucidate the biological characteristics of normal NK cells as well as the pathogenesis of NK neoplasms.

Key words: natural killer cell, myeloid/NK progenitor, cell line, LFA-1

Introduction

Natural killer (NK) cells are innate immune lymphocytes that control tumors and virus-infected cells, and also produce immunoregulatory cytokines.[1,2] Freud et al.[3,4] proposed five differentiation stages in the ontogeny of NK cells. Stage 1 NK cell progenitors (pro-NK) showing $CD34^+$/c-kit $(CD117)^-/CD94^-$ immunophenotype are least mature NK lineage population within normal secondary lymphoid tissue (SLT). Stage 2 NK cell precursors (pre-NK) showing $CD34^+$/c-$kit^+/CD94^-$ are capable to differentiate into T cells or dendritic cells as well as stage 1 cells. Stage 3 immature NK (iNK) cells showing $CD34^-$/c-$kit^+/CD94^-$ also express myeloid antigen including CD33 and no longer have the differentiation potential to the multilymphoid lineage. As mature NK cells, stage 4 cells show $CD56^{bright}/CD16^{dim/-}/CD34^-$/c-$kit^{+/-}/CD94^+$, and stage 5 cells show $CD56^{dim}/CD16^+/CD34^-$/c-$kit^-/CD94^{+/-}$. Among them, two mature subsets, stage 4 cells and stage 5 cells, occupy the large part of the NK cell population. $CD56^{bright}/CD16^{dim/-}$ cells are distributed mainly in the lymphoreticular system, produce abundant cytokines, and show weak cytotoxic activity. On the other hand, $CD56^{dim}/CD16^+$ cells, distribution of which are mainly in the peripheral blood, have a high cytotoxic activity but show lower cytokine productions.[5,6] Several other studies report that $CD56^{bright}$ NK cells are precursor cells of the $CD56^{dim}$ subset, and that $CD56^{dim}/CD16^+$ NK cells are the most mature subset.[7-9]

NK cell tumors are an uncommon and heterogeneous group of disorders. The World Health Organization

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Correspondence to: Takuji Katayama, epartment of Hematology, Kitasato University School of Medicine
1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0374, Japan
E-mail: ktymtkj@med.kitasato-u.ac.jp

(WHO) classification of hematopoietic and lymphoid tissues,[10] encompasses four distinct entities: 1. NK cell lymphoblastic leukemia/lymphoma, 2. chronic lymphoproliferative disorder of NK cells, 3. aggressive NK cell leukemia (ANKL), and 4. extranodal NK/T cell lymphoma, nasal type. Three latter entities are classified as mature NK neoplasms.[10,11] NK cell lymphoblastic leukaemia/lymphoma is very difficult to define. Therefore, there has been considerable confusion in the literature. The majority of cases reported as NK leukemia on the grounds that tumor cells expressed CD56 are now recognized to represent cases of plasmacytoid dendritic cell leukemia or acute myeloid leukemia because CD56 is not specific for NK cells.[10] Especially, the $CD4^+$/$CD56^+$ subset of NK cells is considered to be derived from plasmacytoid monocytes or a plasmacytoid dendritic cell precursor rather than NK cells.[12-14] Therefore, $CD4^-$/$CD56^+$ NK cell lymphoblastic leukaemia/lymphoma include true immature NK cell neoplasms. As immature NK cell neoplasms, two clinical entities are proposed at present on the basis of detailed phenotyping; myeloid/NK precursor acute leukemia[15,16] and precursor NK-lymphoblastic leukaemia/lymphoma,[17-19] both of which are Epstein-Barr virus (EBV) negative and show unfavorable prognoses.

Recently, we experienced an extremely rare case of EBV negative-NK cell lymphoblastic leukaemia/lymphoma with a $CD4^-$/$CD56^+$ immunophenotype. Furthermore, a novel cell line could be established from the tumor cells from a patient's pleural effusion and was named HATAK.

Methods

Case report

A 38-year-old man presented with complaints of fatigue, dyspnea, and sore throat was admitted to our hospital. Mass lesions in the oropharynx, right pleura, and thoracic diaphragm, swelling of abdominal lymph nodes, right hydrothorax, and ascites were revealed by serial imaging procedures. Atypical cells were not found in peripheral blood or bone marrow. The patient underwent a right pleural biopsy. The histopathological findings were medium-sized atypical lymphocytes with clear cytoplasm and pachychromatic nuclear chromatin proliferated diffusely. Small nodules had formed adjacent to adipose tissue. Immunohistochemistry of neoplasmic lymphocytes were $CD7^+$ and $CD56^+$ but negative for CD2, CD3, CD5, CD4, CD8, CD19, CD20, CD79a, granzyme-B, terminal deoxynucleotidyl transferase (TdT), and myeloperoxidase. In situ hybridization for Epstein-Barr virus-encoded RNAs was negative and MIB-1 labeling index was high. T-cell receptor (TCR) beta-chain gene rearrangement was not identified. Chromosome analysis by Giemsa banding failed to be accomplished because no mitotic cells were obtained. The patient was diagnosed as having natural killer cell lymphoblastic leukaemia/lymphoma in acute leukemias of ambiguous lineage according to the WHO classification of lymphoid neoplasm.[10] Because of CD4 negativity and histopathological inconsistency, blastic plasmacytoid dendritic cell neoplasm was excluded. Although several drainages of pleural effusion brought temporary relief from his dyspnea, he showed progressive excerbation of the disease. He was then transferred to another hospital for personal reasons to undergo chemotherapy.

Cell culture

After concentrating cells in a pleural effusion sample by centrifugation, unsorted cells were cultured in RPMI-1640 medium (Sigma Aldrich, St. Louis, MO, USA), supplemented with penicillin-streptomycin (100 U/ml penicillin and 100 μl/ml streptomycin) (Sigma Aldrich), 2 mM L-glutamine (Sigma Aldrich) and 10% fetal bovine serum (BioWest S.A.S., Nuaillé France). Half of the medium was replaced twice per week with fresh medium and incubated at 37℃ with 5% CO_2 in air.

Flow cytometric analysis

After washes with 1X PBS (phosphate buffered saline), cells were incubated with phycoerythrin- or fluorescein isothiocyanate (FITC)-conjugated antibodies for 15 minutes at room temperature. The phycoerythrin- or FITC-conjugated antibodies of CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD16, CD19, CD20, CD33, CD34, CD56, human leukocyte antigen (HLA-DR), TdT, and mouse immunoglobulin (IgG) isotype control were purchased from Beckman Coulter (Brea, CA, USA). FITC-conjugated anti-CX_3C chemokine receptor 1 (CX_3CR1) and rat IgG isotype control were purchased from BioLegend (San Diego, CA, USA). FITC-conjugated anti-CD18 (integrin β_2) and CD18 (activation epitope) were purchased from AbD Serotec (Oxford, UK). Permeabilization reagent (Intra Perp; Beckman Coulter) was used to investigate cytoplasmic antigen according to the instruction of the manufacturer. After another wash, stained cells were analyzed with a flow cytometer (Epics XL; Beckman Coulter).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cell lines with Isogen

(Nippon Gene, Tokyo). First strand complementary DNA was synthesized from 1 μg of total RNA using reverse transcriptase M-MLV (Takara Bio, Otsu), dNTP Mix (Life Technologies, Carlsbad, CA, USA), RNase inhibitor (Toyobo, Osaka), and random primer (Hokkaido System Science, Sapporo). PCR using Premix Taq (TaKaRa Taq Version 2.0, Takara Bio) and a set of primers was performed with a thermal cycler (PC-320; Astec, Fukuoka) for 30 or 40 cycles. The primers are shown in Table 1. All the primers were designed to amplify more than two consecutive exons. Each procedure was carried out according to the manufacturer's instructions. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

Table 1. Primer list

Gene	Sequence	Gene	Sequence
GAPDH	F:5'< GAAGGTGAAGGTCGGAGT <3' R:5'< GAAGATGGTGATGGGATTTC <3'	γ_c	F:5'< TCACTTCTGGCTGTCAGTTGC <3' R:5'< CCACGGCTTCCAATGCAAACAG <3'
Granzyme B	F:5'< GCTTCCTGATACAAGACGAC <3' R:5'< CGCACAACTCAATGGTACTG <3'	CD57	F:5'< GGACTCTGCTCATCACTGTCTG <3' R:5'< GTGCAGGTGCGTGTAGTTGAG <3'
Perforin	F:5'< CTGGACGTGACTCCTAAGC <3' R:5'< GTGGATGCCTATGTTGACCTG <3'	Fas ligand	F:5'< CAGATGCACACAGCATCATCTTTG <3' R:5'< CAGTAGTGCAGTAGCTCATCATC <3'
Granulysin	F:5'< CGTGATGAGGAGAAATCCTG <3' R:5'< CTGTAGAAGGTATACACAACC <3'	c-kit	F:5'< CGTCAGGCAACGTTGACTATCAG <3' R:5'< CACGAGCCTGTCGTAAGTCAG <3'
CD94	F:5'< AACTGTTACTTCATTTCCAGTG <3' R:5'< AGCTGTTGCTTACAGATATAAC <3'	IFN-γ	F:5'< CTTGGCTGTTACTGCCAGGAC <3' R:5'< TGGGATGCTCTTCGACCTCG <3'
NKG2A/C/E	F:5'< AGTGGATTACATATTCCAACAG <3' R:5'< TCACCCATGGATGATGACTG <3'	CCR7	F:5'< CCAGAGAGCGTCATGGACC <3' R:5'< CGTAGCGGTCAATGCTGATGC <3'
KIR2DL	F:5'< TGACATGTACCATCTATCCAG <3' R:5'< GTGATTCAACTGTGTGTATGTC <3'	CXCR3	F:5'< CCACAAGCACCAAAGCAGAGG <3' R:5'< CAGCGTGTCTGCTACAGCTAG <3'
NKG2D	F:5'< CACAGCTGGGAGATGAGTG <3' R:5'< CCAATGATATGACTTCACCAG <3'	CXCR1	F:5'< GCTCCTACTGTTGGACACACC <3' R:5'< CACCTTGCACAGGAATGTGCC <3'
NKp44	F:5'< CAGACGCTAACCGTGAGATG <3' R:5'< GTCTGGGTCTGTGAAGAGAC <3'	CX3CR1	F:5'< GAAGAGCTCTCTGGCTTCTGG <3' R:5'< ATGCTGCACGGTCCGGTTG <3'
NKp46	F:5'< CCGTCTAGACACTGCAACAAG <3' R:5'< GTCTTCAGGTGCAAGGCTG <3'	CD18 (Integrin-β_2)	F:5'< TTCAGACCGAGGTCGGGAAG <3' R:5'< TTGCTGGAGTCCTCAGACAGC <3'
NKR-P1A	F:5'< CTTCACCTTCATCTCTTCCTCG <3' R:5'< GCCGTTTATCCACTTCCAGTTC <3'	CD11a (Integrin-α_L)	F:5'< GACCAAGGAGAGTCAGGAGAC <3' R:5'< CCAGCAACGAAGTCTTTTGCCG <3'
IL-2Rα	F:5'< CTGCTCACGTTCATCATGGTGC <3' R:5'< CCTGTATCCCTGGACGCACT <3'	CD11b (Integrin-α_M)	F:5'< TCACGGAACCTCAGGATCTGG <3' R:5'< TCTGGACATGGAGGCAGACTC <3'
IL-15Rα	F:5'< TCCCAGCTCAAACAACACAG <3' R:5'< TGACTTGAGGTAGCATGCCA <3'	CD11c (Integrin-α_X)	F:5'< CAAGCTGACAGACGTGGTCATC <3' R:5'< TACCAGGGTCTGCTCAGAGAC <3'
IL-2/15Rβ	F:5'< AAGCCTCTGCAAGGCGAGTTC <3' R:5'< GCCTCTATCTCCAAGGCATCC <3'	CD29 (Integrin-β_1)	F:5'< CCAACCGTAGCAAAGGAACAGC <3' R:5'< GGCTGGTGCAGTTCTGTTCAC <3'
IL-7Rα	F:5'< GTGAATGGATCGCAGCACTCAC <3' R:5'< TGGCGGTAAGCTACATCGTGC <3'	CD49d (Integrin α_4)	F:5'< CCACCTTGGTCCTCATGTCATC <3' R:5'< CCACGCCAGAGTTATCTGTGAC <3'

Note: These primers for NKG2 were prepared to be bound complementally at the sites within the consensus regions which were shared with three isoforms: NKG2A, NKG2C, and NKG2E.

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KIR2DL, killer cell immunoglobulin-like receptor 2DL; IL-2Rα, interleukin-2 receptor alpha; IL-15Rα, interleukin-15 receptor alpha; IL-2/15Rβ, interleukin-2/15 receptor beta; IL-7Rα, interleukin-7 receptor alpha; γ_c, common gamma chain; IFN-γ, interferon-gamma; CCR7, CC chemokine receptor 7; CXCR3, CXC chemokine receptor 3; CXCR1, CXC chemokine receptor 1; CX_3CR1, CX_3C chemokine receptor 1

Cytotoxicity assay

The cytotoxic activities of the HATAK cells were examined by chromium-51 radionuclide (^{51}Cr) release cytotoxicity assays using target cells. K562, Jurkat, and Daudi target cells were incubated with ^{51}Cr (PerkinElmer, Waltham, MA, USA) for 1 hour. The ^{51}Cr-labeled targets were aliquoted at a concentration of 5×10^3 cells/well. HATAK cells were mixed with plated ^{51}Cr-labeled targets at E:T (effector cells to target cells) ratios of 24:1, 12:1, 6:1, and 3:1 into 96-well, U-bottom plates and incubated for 18 hours at 37℃ in 5% CO_2. The specific release of ^{51}Cr from the target cells into the supernatant was measured with a gamma counter (ARC-370M; Hitachi Aloka Medical, Tokyo). All experiments were performed in triplicate, and the percentage of specific lysis was calculated by using the standard formula.[20]

hIL-2 and/or hIL-15 stimulation

HATAK cells were cultured in the medium described above with adding 10 ng/ml human interleukin (hIL)-2 (Shenandoah Biotechnology, Warwick, PA, USA) or 10 ng/ml hIL-15 (specific activity is $8.33-5.88 \times 10^6$ units/mg) (BioLegend, San Diego, CA, USA), or both. After 2 successive days of stimulation, RT-PCR and ^{51}Cr release cytotoxicity assays were performed.

Fractalkine stimulation

HATAK cells were cultured in the previously described medium by adding 20 ng/ml recombinant human fractalkine (PeproTech, Rocky Hill, NJ, USA) for a period of 10 seconds and another one for 4 hours. After formalin fixation, activated form of CD18 was analyzed by flow cytometer with FITC-conjugated anti-CD18 activation epitope (AbD Serotec, Oxford, UK).

Results

Establishment of the HATAK cell line

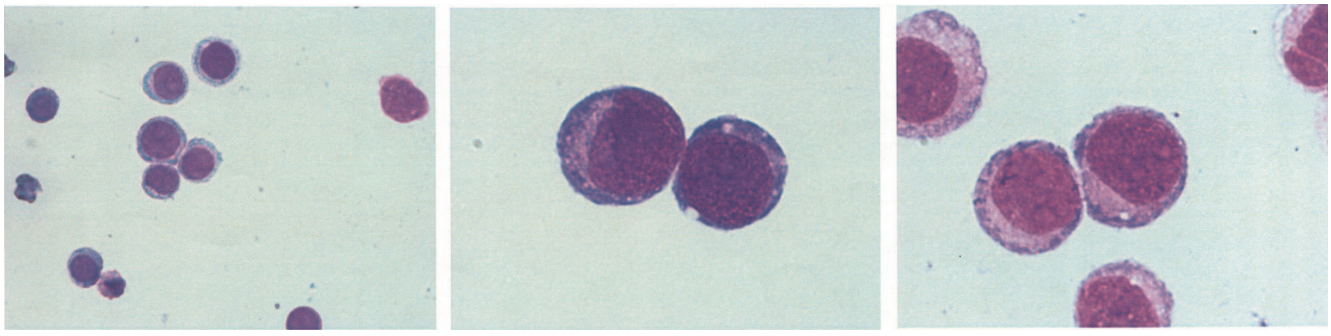
After culturing unsorted cells from the patient's pleural effusion in the standard medium described previously, mononuclear cells survived and continued to grow without the addition of any cytokines over 3 months. We confirmed the establishment of the cell line, which we named HATAK. HATAK was easy to grow because its doubling time was about 48 hours. However, repeated trials of limiting dilutions failed to clone HATAK cells.

Morphology and chromosomal analysis

HATAK cells had a small to medium-sized lymphoblast-like appearance with a coarse chromatinic nucleus bearing conspicuous nucleoli. Some of them had vacuoles in their cytoplasm, but they had no cytoplasmic granules as observed in large granular lymphocytes. They were negative for myeloperoxidase staining (Figure 1). Giemsa banding detected the complex karyotypic abnormalities including 48, XY, +Y, +add(1)(p11), add(1)(q21), add(8)(q24), del(9)(q?) × 2, add(10)(q22), -17, +21 (data not shown).

Immunophenotyping, genotyping and Southern blotting

Immunophenotype of HATAK analyzed by flow cytometry was shown in (Figure 2). HATAK showed positive for CD7, CD13, CD18, CD33, CD56, HLA-DR, and CX3CR1 but negative for CD2, and both surface and cytoplasmic CD3, CD4, CD5, CD8, CD10, CD16, CD19, CD20, CD34, and TdT. A total of 30-cycles amplification of RT-PCR detected the expression of the following transcripts: interleukin-15 receptor alpha (IL-15Rα), interleukin-2/15 receptor beta (IL-2/15Rβ), interleukin-7 receptor alpha (IL-7Rα), common gamma chain (γ_c), c-kit, CX_3CR1, integrin α_L/CD11a, integrin



A. May-Giemsa staining, low-power field **B**. May-Giemsa staining, high-power field **C**. Myloperoxidase (MPO) staining, high-power field

Figure 1. Morphology of HATAK cells

α_M/CD11b, integrin α_4/CD49d, integrin β_1/CD29, and integrin β_2/CD18. On the other hand, the two cytotoxic effector molecules, granzyme-B and granulysin, CD94, NKG2D, NKG2-A/-C/-E, killer cell immunoglobulin-like receptor 2DL (KIR2DL), NKp46, and NKR-P1A as receptors expressed on NK cells, and Fas-ligand were faintly expressed, all transcripts of which were detected only after increasing the amplification of PCR to 40 cycles (Figure 3A). Southern blotting demonstrated the reproducible results that TCR β-chain gene was in germline configuration in HATAK cells as well as primary tumor cells (Figure 4).

Flow cytometric analysis for CD56 expression suggested that HATAK was comprised of two cell populations, $CD56^+$ and $CD56^-$. To explicate whether this heterogeneity originated from the characteristics of primary tumor cells or resulted from the disappearance of CD56 antigen on the surface of HATAK cells while culturing cells, $CD56^+$ cells isolated by flow cytometric sorting were continued to be cultured instead of cloning HATAK cells. As a result, HATAK cells showed gradual declination of CD56 antigen when cultured for long periods of time, resulting in the complete disappearance accompanied with no expression of CD16 (Figure 5).

Cytotoxic activity and the response to cytokines

Because HATAK cells expressed IL-2 receptor and IL-15 receptor along with cytotoxic molecules expression despite its faintness, their cytotoxic activity was investigated, especially to determine whether or not it would be induced by the stimulation with a cytokine or cytokines. As shown in Table 2, HATAK cells demonstrated no cytotoxic activity against three kinds of target cells, K562, Jurkat, and Daudi, in the steady state. Neither could cell cytotoxity be induced by the stimulation with a cytokine, IL-2 alone, IL-15 alone, or with both cytokines. Furthermore, these cytokines never induced the expression of interferon-gamma (IFN-γ) (Figure 3B).

Activation of LFA-1 and fractalkine stimulation on CX_3CR1

We assayed the activation status of lymphocyte function-associated antigen-1 (LFA-1) with flow cytometry using the antibody recognizing the epitope on activated CD18. The structural change of LFA-1 was so transient that cells had to be fixed with formalin immediately when stimulated by a ligand on CX_3CR1.[21,22] LFA-1 on HATAK cells had been activated in the steady state that was never influenced by the short- or long-time stimulation by the soluble form of fractalkine (Figure 6).

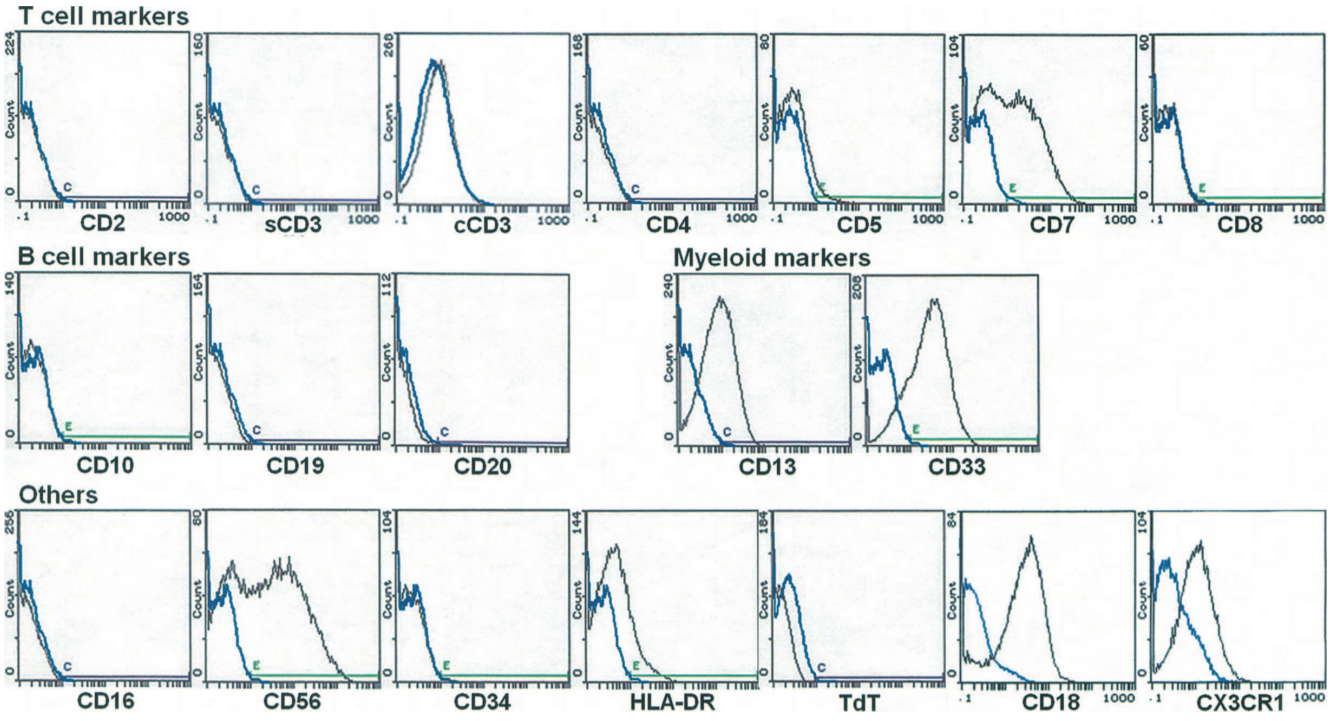
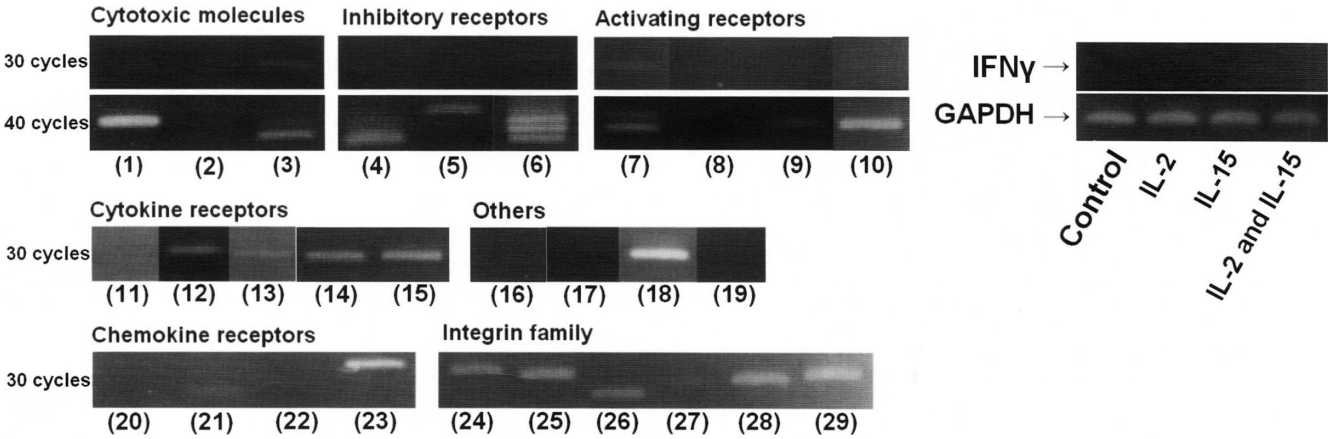


Figure 2. Immunophenotype of HATAK cells

In each histogram, the blue line indicates HATAK cells stained with isotype control antibody staining is indicated by the blue line. Cytoplasmic-CD3 (cC3) and TdT were analyzed cytoplasmic antigens. Others were analyzed surface antigens.



A. The numbers in parentheses under the lanes indicate molecules: (1) Granzyme B, (2) Perforin, (3) Granulysin, (4) CD94, (5) NKG2A/C/E, (6) KIR2DL, (7) NKG2D, (8) NKp44, (9) NKp46, (10) NKR-P1A, (11) IL-2Rα, (12) IL-15Rα, (13) IL-2/15Rβ, (14) IL-7Rα, (15) γ_c, (16) CD57, (17) Fas ligand, (18) c-kit, (19) IFN-γ, (20) CCR7, (21) CXCR3, (22) CXCR1, (23) CX_3CR1, (24) CD18, (25) CD11a, (26) CD11b, (27) CD11c, (28) CD29, (29) CD49d. From 1 to 9, the upper lanes, are amplifications for 30 cycles; and the lower lanes are amplifications for 40 cycles. GAPDH was used as an internal control (data not shown).

B. IL-2 and/or IL-15 stimulation assay in HATAK cells. With IL-2 alone, IL-15 alone, or both cytokines stimulation never induced the expression of IFN-γ.

Figure 3. Expression of molecules in HATAK cells

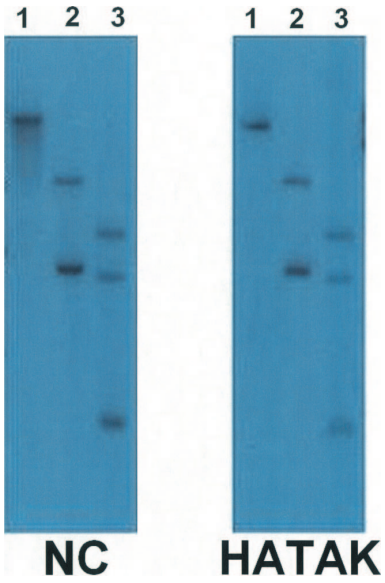
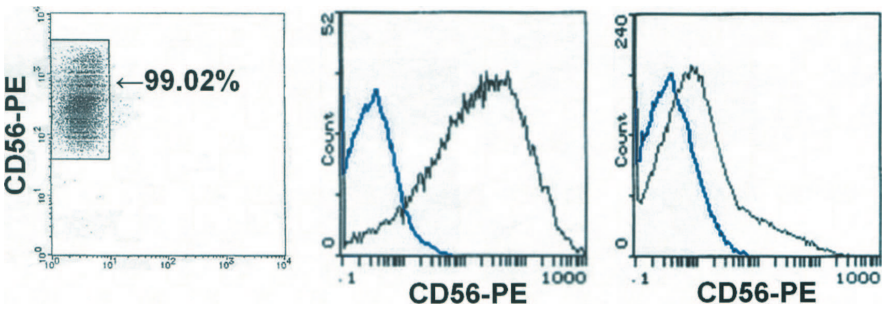


Figure 4. Southern blot analysis

Human placenta DNA was used as a germline control (NC). DNA was digested by BamHI (lane 1), EcoRV (lane 2), and HindIII (lane 3), and hybridized with approximately 3 kb TCR-Cβ1 specific probe using standard protocol (SRL, Tokyo). TCR β-chain gene was in a germline configuration in HATAK cells.



A. Immediately after cell sorting, the cells in gated regions were sorted.

B. 1 month after cell sorting

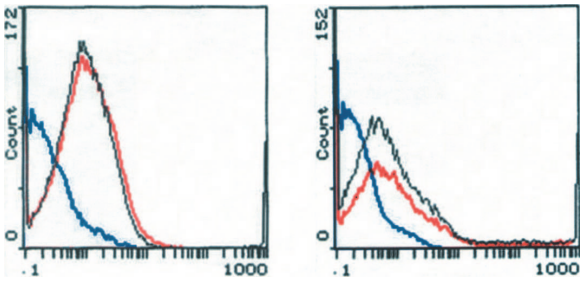
C. 3 months after cell sorting

Figure 5. Gradual declination of CD56 expression in HATAK (The blue lines indicates HATAK cells stained with an isotype control antibody.)

Table 2. Cytotoxity of HATAK cells

Target cells	% Cytotoxity at E:T ratio			
	48:1	24:1	12:1	6:1
K562	3.31	2.57	1.184	0.054
Daudi	-2.54	-0.46	0.91	-1.95
Jurkat	0.90	-2.23	-1.57	-0.35
K562[a]	2.03	1.52	0.90	-0.76
K562[b]	1.91	1.79	1.27	0.015
K562[c]	1.71	1.64	1.64	0.51

% Cytotoxity is the mean value of triplicate.
[a]HATAK cells were stimulated by IL-2.
[b]HATAK cells were stimulated by IL-15.
[c]HATAK cells were stimulated by IL-2 and IL-15.



A. Short stimulation (10 seconds)

B. Long stimulation (4 hours)

Figure 6. Activation of CD18 on HATAK cells with or without fractalkine stimulation (The blue line indicates HATAK cells stained with an isotype control antibody. The red line indicates nonstimulating HATAK cells stained with an anti-CD18 antibody against activating epitope. The black line indicates fractalkine-stimulating HATAK cells stained with an anti-CD18 antibody against activating epitope.)

Table 3. HATAK cell's immunophenotype and normal NK cell differentiation[4]

Surface antigen	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	HATAK
	pro-NK	pre-NK	iNK	$CD56^{bright}$ NK	$CD56^{dim}$ NK	
CD2	−	+/−	+/(−)	+/(−)	+/(−)	−
CD3	−	−	−	−	−	−
CD5	−	−	−	−	−	−
CD7	+/−	+/−	+/−	+/(−)	+/(−)	+
CD10	+	−/(+)	−	−	−	−
CD13	N	N	N	N	N	+
CD16	−	−	−	−	+	−
CD33	+	+	+	−/(+)	−/(+)	+
CD34	+	+	−	−	−	−
CD56	−	−/(+)	+/−	+	+	+
HLA-DR	+/−	+	−	−	−	+
CD94	−	−	−	+	+/−	w+*
c-kit	−	+	+	+/−	−	+*
NKG2A	−	−	−	+	+/−	w+*
NKG2D	−	−	−	+	+	w+*
NKp44	−	−	+/−	−/(+)	−/(+)	−*
NKp46	−	−	−	+	+	w+*
IL-2Rα	+	+	+/−	−/(+)	−	−*
IL-15Rα	N	N	N	N	N	w+*
IL-2/15Rβ	−	−	−	+	+	w+*

HATAK, new cell line; NK, natural killer; iNK, immature natural killer; N, not listed
+ All cells are positive.
− All cells are negative.
+/− variable expression
+/(−) A majority of cells are positive.
−/(+) A majority of cells were negative.
+* A molecule expression was detected by 30 cycles of RT-PCR.
w+* A molecule expression was detected by 40 cycles of RT-PCR.
−* A molecule expression was not detected by RT-PCR.

Discussion

NK cell neoplasms are rare, representing less than 1% of non-Hodgkin lymphoma.[23] Among them, NK cell lymphoblastic leukaemia/lymphoma, thought to be immature NK cell neoplasm, is extremely rare and not yet defined well. The WHO categorizes it into acute leukemia of ambiguous lineage rather than NK cell neoplasms. This ambiguity is attributed in large part to the fact that NK cells express many shared molecules with other lineage cells, including CD2, CD11b, CD16, CD56, C-type lectin-like receptors, and the effector molecules, perforin and granzyme B. To verify that tumor cells are of bona fide NK-cell origin, analyses of TCR gene rearrangement and immunoglobulin gene rearrangement, detection of transcripts expressed in NK cells when necessary, and thorough immunophenotyping with a panel of antibodies were performed.

We successfully established a novel cell line, named HATAK, from a patient with NK cell lymphoblastic leukaemia/lymphoma. Dissimilar to many other established NK-cell cell lines showing IL-2-dependent cell growth, HATAK cells could continue to grow over 3 months without particular support by any cytokines. They showed morphological immaturity, unlike mature NK cells such as large granular lymphocytes (Figure 1). Extensive phenotyping revealed that HATAK cells expressed cytotoxic molecules, both activating and inhibitory receptors expressed on NK cells, the expressions of which were faint but detectable by PCR. However, they lacked numerous antigens associated with T-cell and B-cell lineages. With the TCR-β gene not being rearranged, we concluded that HATAK cells were NK-lineage cells. Notably, they showed the distinct expression of CD13 and CD33, myeloid-lineage specific antigens. It is unreasonable to construe this finding as an aberrant expression that occurred contingently in the process of cell immortalization and irrelevant to the phenotypic transition through normal NK cell ontogeny on the grounds that the expressions of more than one molecule specific for myeloid lineages were definitely detected at the same time by flow cytometry. Whereas neoplasms showing a biphenotype like HATAK cells of myeloid/NK lineages are categorized as myeloid/NK cell precursor acute leukemia, according to the aforementioned classification,[15,16] the existence of a bona fide myeloid-like NK cell progenitor has been debated in the normal NK cell development.[24,25] However, in 2011, Grzywacz et al.[26] demonstrated that myeloid progenitor cells derived from $CD34^+$ hematopoietic progenitors, and still more differentiated cells to the myeloid lineage with the expression of CD13 could increasingly differentiate into NK cells in the presence of cytokines (IL-7, IL-15, stem cell factor, and Fms-like tyrosine kinase-3 ligand), stromal cells, using fetal liver stromal cell lines, and hydrocortisone. These findings warrant the existence of poorly differentiated NK cell populations possessing bi-lineage potential in the model of *in vivo* NK cell development proposed by Freud et al.[3] Applying HATAK cells to this model, it is thought to be immature NK cells corresponding to the transitional stage from stages 3 to 4 (Table 3).

The gradual declination of CD56 antigen on the HATAK cells in proportion to the long-term culture intrigues us. The mechanism of regulating CD56 expression in the NK-cell differentiation remains to be elucidated. $CD56^{bright}$ NK cells isolated from human peripheral blood were turned into $CD56^{dim}$ cells when cultured in the presence of synovial fibroblasts,[7] or inversely, the stimulation of IL-12 with 4-1BB ligand made a conversion of $CD56^{dim}$ NK cells to $CD56^{bright}$ phenotype.[27] Regarding HATAK, it is not thought that this phenotypic change is caused by the terminal differentiation of cells to $CD56^{dim}$ phenotype with no extrinsic stimulation, because unsorted cells containing $CD56^{dim}$ cells did not exhibit cytotoxic activity, and the declination of CD56 antigen did not accompany the expression of CD16. An intrinsic factor or factors and/or epigenetic changes would play a pivotal role in this spontaneous down-regulation of CD56 expression. It is noteworthy that the other NK cell line, NKL, was also reported to have shown the declination of CD56 together with CD16 and CD57 antigens when cultured for a prolonged period of time.[28]

It is well known that numerous cytokines are involved in the differentiation, proliferation, and function of NK cells. Among them, both IL-2 and IL-15 stimulate proliferation, survival, and functional activities of NK cells.[2,29] They are especially able to support the generation of $CD3^-CD56^{bright}$ NK cells from $CD34^+$ hematopoietic progenitor cell populations in the absence of additional cytokines or stroma.[30] Herein, we investigated the response of HATAK cells to these cytokines. The lack of the transcript of perforin in addition to the trace expression of other cytotoxic molecules made us expect that HATAK cells had no cytotoxic activity at steady state. As expected, the HATAK cells never demonstrated cytotoxic activity against any target cells with no stimulation by cytokines (Table 2). Furthermore, neither the independent stimulation by IL-2 and IL-15 nor the combined stimulation by both cytokines induced cytotoxic activity and the expression of IFN-γ (Figure

3B). Unfortunately, it remains to be unveiled to whom the naive characteristic of HATAK cells showed is attributed. Because cytokines were naturally dispensable for the robust growth of HATAK cells, they might never have been involved in the proliferative activity of HATAK cells. Acquired mutation in the molecules downstream to IL-2 and IL-15 receptor complexes such as Jak3 may force HATAK cells not to respond to these cytokines as observed in the cases of severe combined immunodeficiencies with impaired NK cell development.[31] Or the lack of the additional cytokine or cytokines critical for releasing these immature NK cells from the state of maturation arrest may hamper the acquisition of functional activities that the mature NK cells possessed. Certainly, the stimulation from stromal cells, in addition to various cytokines including IL-15, was required to induce the differentiation of myeloid/NK progenitor into mature NK cells showing cytotoxic activity in the aforementioned study by Grzywacz et al.[26] Moreover, several lines of evidence have also demonstrated that although soluble IL-15 or IL-2 could differentiate precursor NK cells presenting $CD34^+$/$CD45RA^+$/$c\text{-}kit^+$/$CD94^-$ immunophenotype into immature NK cells corresponding to stage 3 NK cells in the absence of other cytokines or stroma,[3] stage 3 NK cells could not show further differentiation when cultured in high-dose IL-15 alone3 or IL-2 alone, but the addition of IL-12 made it possible to induce NK cell maturation from these cells.[32]

The clinical findings that primary tumor cells demonstrated an outstanding preference to infiltrate into biomembranes such as pleura and peritoneum to proliferate influenced us to hypothesize that the activation of certain adhesion molecules on tumor cells would play a pivotal role in cell-to-membrane adhesion. Intriguingly, HATAK cells expressed not merely various integrin family molecules but also CX_3CR1, a chemokine receptor to its ligand, CX_3CL1 (fractalkine). Soluble fractalkine is reported to stimulate the development of a high-affinity state in the LFA-1 constituted of two subunits, integrin α_L/CD11a and integrin β_2/CD18.[33] Accordingly, we focused our interest on LFA-1 and investigated the activation status thereof before and after the stimulation by soluble fractalkine. It was remarkable that LFA-1 on HATAK cells had already been activated in steady state. Moreover, the stimulation from CX_3CR1-fractalkine axis never exerted pressure on the activation status of LFA-1 (Figure 6). This autonomic activation of LFA-1 found in HATAK cells was possibly the aberrancy inherited from primary tumor cells. Chemokine receptors are G-protein-coupled receptors. G-protein α-subunit-mediating signals activate several different signal-transduction cascades resulting in integrin activation. On the other hand, small GTPase Ras homolog H (RhoH) functions as a negative regulator of LFA-1 avidity.[34] Provided that molecules responsible for signal-transduction are mutated to be activated constitutively, or the negative regulator is dysfunctional, LFA-1 would be activated irrespective of the ligand-triggered stimulation. T-cell clones expressing RhoH engineered by insertional mutagenesis demonstrates constitutive activation of LFA-1 and bound spontaneously to ICAM-1 (intercellular adhesion molecule-1).[35] Further investigation might identify the responsible molecule or molecules for the autonomic activation of LFA-1 in HATAK cells.

To date, several established NK cell lines have been reported; the majority of them are, however, not qualified completely as NK cell origin.[36] Among them, the following eight cell lines were confirmed to be derived from true NK cells: HANK,[37] KHYG-1,[38] NK-92,[39] NKL,[28] NK-YS,[40] YT,[41,42] SNK-6,[43] and NKG.[44] To our knowledge, HATAK is a unique NK cell line presenting immature, myeloid/NK progenitor phenotype. This valuable cell line might be useful to gain an understanding of the mechanism involved in the differentiation, adhesion, and proliferation, cell cytotoxity of normal NK cells as well as NK cell neoplasms.

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