Selective Expansion of Human Natural Killer Cells from Peripheral Blood Mononuclear Cells by the Cell Line, HFWT

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An anchorage-dependent Wilms tumor cell line HFWT was found to stimulate selective and remarkable expansion of human natural killer (NK) cells from human peripheral blood mononuclear cells (PBMC). After PBMC of healthy donors were cultured on irradiated HFWT cells for 10–21 days, the lymphocytes expanded 58- to 401-fold. This NK cell expansion required direct contact of PBMC with live, but not fixed, HFWT cells. The PBMC from an end-stage brain tumor patient also expanded 156-fold, whereas those cultured with irradiated NK-sensitive K562 grew only 30.5-fold. CD16+CD56+ NK cells accounted for more than 70% of the population expanded on HFWT cells. No essential difference in expression of NK receptors was observed in the expanded NK cells on HFWT and K562 and without feeder cells. The expanded NK cells killed not only fresh HFWT cells but, unexpectedly, also MHC class I-expressing autologous brain tumor cells at an effector/target ratio of 4 for 24 h. These results will contribute to the development of a large-scale preparation method for human NK cells, which will aid studies of NK cell biology and possible treatment of brain tumors.

Key words: Human natural killer cells — Selective expansion — Brain tumor

Despite the importance of natural killer (NK) cells in immuno-surveillance of cancer development,1,2 selective and highly efficient expansion of human NK cells from peripheral blood mononuclear cells (PBMC) of cancer patients is not necessarily sufficient for treatment. K562 cells expressing few MHC-class I molecules on the surface have been widely used for assays of NK cell activity. When NK cells in vitro kill K562 cells, an apparent growth response of NK cells follows.3 Reports suggested that human EB-virus-transformed lymphoblastoid cell lines,4,5 human malignant melanoma cells,6 and autologous monocytes7 are also useful for human NK cell expansion. Rabinowich et al.8 developed an NK expansion culture with adherent-lymphokine activated killer cells (A-LAK) in PBMC and concanavalin (Con)-A-stimulated normal T cell blasts supplied as the feeder cells. Although these authors claimed 629±±±±275-fold expansion of NK cells from adherent cells in PBMC, the adherent cell fraction consists of less than 4% of PBMC,9 so that overall expansion of NK cells from PBMC is limited to 36-fold or less. Therefore, we consider that the stimulation is not necessarily sufficient for NK expansion from PBMC of tumor-bearing patients, especially at their end-stage, which is usually accompanied with suppressed immune function. Expansion of NK cells on a large scale would contribute to precise analysis of the killer cell function and have possible application of tumor treatment. Here we report remarkable effects of a newly screened anchorage-dependent cell line HFWT,10 which induces selective and efficient expansion of NK cells from unfractionated PBMC.

MATERIALS AND METHODS

Cell lines and culture All the cell lines used in the present experiments were taken from routine cultures in RIKEN Cell Bank. Cell lines were maintained in basal medium containing 10% or 15% fetal bovine serum (FBS). TKB-17RGB cell line was established in RIKEN Cell Bank from the surgical specimen of a brain tumor patient (51-year-old male), who had suffered from a gliosarcoma and died 2 months after donation of his peripheral blood for the killer cell culture. All the peripheral blood samples were taken after having obtained informed consent.

Expansion of NK cells PBMC were prepared from heparinized peripheral blood with the conventional preparation kit “Lymphoprep” (Nycomed Pharma A.S., Oslo, Norway). The cells were washed once with calcium, magnesium-free Dulbecco’s phosphate-buffered saline (PBS(−)), then once with culture medium and centrifuged at 1400 rpm (240g) for 10 min at room temperature. Before addition of the PBMC to the NK cell expansion culture, 1×105

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feeder cells were plated into each well of 24-well tissue culture plates. After overnight incubation, these cells were irradiated with 50 Gy of X rays. The PBMC (1×10^6 cells/ml, 1 ml/well) were then cultured on the tumor cells (at this stage, the responder/stimulator ratio was adjusted to 10). RHAMTα medium supplemented with 5% autologous plasma and interleukin-2 (IL-2, 200 U/ml) was used for culture of the human lymphocytes.

In particular experiments, the feeder cells were fixed with 10% formalin, methanol-acetic acid (3:1) mixture, or by heating. Some feeder cells were submitted to freezing-thawing. Also, PBMC were separated by a cell culture insert (pore size: 0.45 µm, Nippon Becton Dickinson Co., Ltd., Tokyo) to avoid direct contact between feeder cells and PBMC. The NK expansion culture was continued with appropriate changes of the medium including the cytokine (at least half of the medium was changed every 2 days) until the adherent feeder cells disappeared. After this, the cell suspension was diluted to 5×10^5/ml and the culture was continued. Whenever the cell suspension reached 2×10^6/ml, the dilution was repeated.

**Flow cytometry** To screen MHC-class I expression, cultured anchorage-dependent cell lines were stained with phycoerythrin (PE)-labeled anti-MHC-class I (HLA-ABC) monoclonal antibodies (mAbs; W6/32, which is IgG2a, DAKO Japan, Kyoto). Lymphocytes were stained with mAbs, i.e., FITC-labeled anti-CD3 (UCHT1, IgG1) and PE-labeled anti-CD56 mAbs (MOC-1, IgG1) purchased from DAKO Japan; FITC-labeled anti-CD3 (UCHT1, IgG1), -CD16 (3G8, IgG1), -CD94 (HP-3D9, IgG1) and -CD161 (VI NK12, IgG1), PE-labeled anti-CD56 (V NK75, IgG1), -CD158a (VI NK14, IgM) and -CD158b (VI NK8, IgG2b) purchased from BD PharMingen (San Diego, CA); PC5-labeled anti-CD3 (UCHT1, IgG1) and -CD56 (NKH-1, IgG1) purchased from Beckman Coulter (Tokyo). Isotype-matched control mAbs were used as negative controls. Cells were stained with these mAbs for 30 min at 4°C. After washing, these cells were immediately analyzed by a FACSscan flow cytometer (Becton Dickinson, San Jose, CA).

**Cytotoxicity assay** The non-radioisotopic crystal violet (CV) staining assay was adopted for ecological reasons in our laboratory and has been used for the determination of killing activity of cytotoxic T lymphocytes (CTL) specific to anchorage-dependent target tumor cells as described previously. This assay is compatible with the standard ^51^Cr-release assay at an effector/target (E/T) ratio of 10 or lower (see Fig. 1b in ref. 12).

### RESULTS

**Expansion of NK cells from PBMC**

Seeking a new cell line with a potent effect on NK expansion from PBMC, we have screened 259 human anchorage-dependent cell lines in RIKEN Cell Bank. Eleven cell lines scarcely expressed surface MHC-class I molecules. Among them, a Wilms tumor cell line, HFWT, induced remarkable expansion of CD3^−^CD56^+^ lymphocytes in coculture with PBMC taken from healthy subjects (Table I). The resulting lymphocyte population was found to contain more than 77% of CD3^−^CD56^+^ NK cells, while other cell lines induced less than 51%, usually around 20%, of NK cells in coculture for 8–13 days. So far, we have not been able to induce such a high proportion of NK cells from PBMC cultured on an irradiated lymphoblastoid cell line, Daudi, (data not shown).4, 5)

When cocultured for 10–21 days on irradiated HFWT cells, the lymphocytes grew 58-, 229-, and 401-fold from

| Cell line | Origin | Proportion of CD3^−^CD56^+^ cells (%) |
|-----------|--------|--------------------------------------|
| HFWT      | Wilms tumor | 85.6, 77.4 |
| HHUA      | Uterine endometrium tumor | 51.5, 25.8 |
| HMV-II    | Melanoma | 18.9 |
| HuH-6     | Hepatoblastoma | 18.2, 11.4 |
| Lu-130    | Lung small cell carcinoma, classic type | 21.4 |
| Lu-134-A  | Lung small cell carcinoma, classic type | 20.9 |
| NB19      | Neuroblastoma | 13.2 |
| NB69      | Neuroblastoma | 15.5 |
| NEC14     | Embryonal carcinoma from testis | 44.4, 23.2 |
| TCO-2     | Cervix carcinoma | 18.5 |
| TNB1      | Bone marrow-metastated neuroblastoma | 14.0 |

The anchorage-dependent cell lines were X-ray-irradiated and served as feeder cells of PBMC. After 8–13 days in the coculture, percentages of CD3^−^CD56^+^ cells in the floating lymphocyte fraction were determined by flow cytometry. Data from one or two experiments are shown.
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unfractionated PBMC taken from 3 healthy subjects. Also, irradiated HFWT cells stimulated NK expansion from PBMC of an end-stage brain tumor patient (51-year-old male) by 79±37-fold in five repeated experiments. Representative results from the end-stage brain tumor patient, who has been suffering from recurrent gliosarcoma, are shown in Fig. 1. When his PBMC were submitted to co-culture with irradiated feeder cells, the lymphocytes proliferated 156-fold on HFWT cells in 21 days, whereas those cocultured with K562 cells grew only 30.5-fold in 16 days.

Fig. 1. Growth responses and killing activities of lymphocytes from a brain tumor patient cultured with two target cell lines. Irradiated HFWT cells or irradiated K562 cells were used as indicated. The NK expansion medium contained IL-2 (200 U/ml). A, Growth responses. PBMC were taken from a patient (51-year-old male) who was at the end-stage of recurrent malignant gliosarcoma. ○ HFWT, △ K562. B, Phenotypes of the lymphocyte populations from this brain tumor patient after 13 days in the NK expansion culture with irradiated HFWT or K562 cells. C, Killing activities of the patient’s lymphocyte population expanded on irradiated HFWT cells in 24-h CV assay (see ref. 12). The target TKB-17RGB cells, which express MHC-class I molecules (see Fig. 2), are autologous tumor cells derived from the brain tumor patient. ● HFWT, ○ TKB-17RGB.

Fig. 2. Examination of MHC-class I surface molecules on cell lines. Flow cytometric patterns of K562, HFWT, and TKB-17RGB are shown (filled peaks). Open peaks represent control staining with an isotype-matched control antibody. As in the well known leukemia cell line, K562, slight expression of MHC-class I molecules was observed in HFWT cells. TKB-17RGB cells that expressed MHC-class I molecules were established from a brain tumor patient (51-year-old male).
and then ceased proliferation (Fig. 1A). The lymphocyte population stimulated with HFWT cells and K562 cells contained 74.1% and 67.7% of CD56+CD16+ NK cells, respectively (Fig. 1B).

The NK cells expanded on the irradiated HFWT cells almost completely killed fresh HFWT cells at an E/T ratio of 2 for 24 h (Fig. 1C), as would be expected for NK cells from healthy subjects (data not shown). Also, the NK cells killed 90% of the fresh autologous gliosarcoma cell line, TKB-17RGB, at an E/T ratio of 4 for 24 h (Fig. 1C). This was an unexpected result, since TKB-17RGB cells strongly express MHC-class I molecules on their surface, whereas, like K562 cells, HFWT cells weakly express MHC-class I molecules (Fig. 2).

Expression of NK cell markers on expanded lymphocytes

Expression of NK cell markers, i.e., CD16, CD94, CD161, CD158a and CD158b, was investigated on the surface of CD3−CD56+ NK cells expanded on HFWT cells and K562 cells, or those cultured without feeder cells. CD16, CD94 and CD161 were expressed on almost all the NK cells, while CD158a and CD158b were expressed on approximately half of NK cells. Although a difference was observed in expanded number of NK cells in these three populations, no essential difference were found in the expression pattern or percentage of NK marker-positive cells in these NK cell populations (Fig. 3).

Requirement of direct contact of PBMC with live HFWT cells

To investigate factors influencing the NK cell expansion, we examined the effect of fixation or physical separation of HFWT cells in the coculture with PBMC. Although the percentage of CD3−CD56+ cells reached 94.1% by day 13 in the coculture on irradiated but live HFWT cells, the percentage did not increase on those HFWT cells prefixed with 10% formalin, methanol-acetic acid (3:1) mixture, or heated (Fig. 4). After freezing and thawing, the HFWT cell residue did not stimulate any NK cell expansion.

We separated PBMC from HFWT cells by means of a cell culture insert with a 0.45 µm pore-size membrane to determine whether or not the NK cell expansion is depen-

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**Fig. 3.** Expression of NK cell markers on CD3−CD56+ cells expanded on feeder HFWT cells and K562 cells and without feeder cells. NK markers tested on day 8 were A, CD16; B, CD94; C, CD161; D, CD158a; E, CD158b. Fine-dotted line high peaks (——) at the left extreme in each figure show lymphocytes stained with isotype-matched control antibody. The peaks locating in middle of each figure correspond NK cells cocultured with HFWT cells (solid lines ——), NK cells cocultured with K562 cells (fine-dotted bold lines ••••) and NK cells cultured without feeder cells (coarse-dotted lines ----).
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dent on soluble factors. After 10 days, percentages of CD3−CD56+ NK cells in the culture were 5.3% and 78.6% for non-contacted and contacted PBMC, respectively (Fig. 5, A and B). The PBMC which did not contact on HFWT feeder cells turned to be mainly CD3+ T cells (Fig. 5A).

Virus contamination was not detected in spent medium of HFWT cells tested for Epstein-Barr virus and hepatitis type-C virus with anti-sera, and for Parvo virus B19, cytomegalovirus, human T cell leukemia virus type 1, and hepatitis type-B virus by PCR amplification (tested by SRL Laboratories, Tokyo).

DISCUSSION

Although it is well known that MHC-class I negative cells can be targets of NK cells, MHC-class I-negative/dim cells do not necessarily show high potential for expansion of NK cells (Table I). Of the eleven cell lines found in the extensive screening, only the HFWT cell line potently and selectively stimulated expansion of NK cells and/or NK precursor cells in PBMC (Fig. 1, A and B). This effect was the strongest we have ever found.4–9 It was dependent on direct contact of NK cells/NK precursors with live, but not fixed, HFWT cells (Figs. 4 and 5), and appeared to be unrelated virus infection. We therefore assume that this effect may be attributable to very fragile surface molecules on HFWT cells, i.e., molecules that may be extremely unstable to the treatments adopted in the fixation experiment (Fig. 4), although Pierson et al. reported that ethanol/acetic acid-fixed M2-10B4 cells, a murine fibroblast cell line, could expand human NK cells.14

K562 cells are known to stimulate expansion of NK cells cultured in medium containing IL-2 from PBMC (Fig. 1A).3, 15 In this process, costimulatory signals are required after the direct cell-cell contact.15–17 The costimulation does not involve CD16 or costimulatory molecules of T cells, such as CD2, CD27, CD28, CD29 or LFA-1. NK cells express various NK receptors which act in the activation and inhibition of NK cells.18, 19 Duchler et al. reported that NKG2-C, an activating receptor on NK cells, specifically recognizes K562 cells.20 On the other hand, CD94, a subunit of NK receptors, does not necessarily participate in the costimulation of NK cells via K562 cells,21 but does participate in the inhibition of NK activity when complexed with NKG2A molecules.22 Since we have no other Wilms tumor cell line which does not express MHC-class I molecules, it is unclear whether the Wilms tumor gene, WT-1, plays an essential role in this stimulation. Also, the possibility is not excluded that metabolic cooperation which may have happened after direct contact of NK cells/NK precursors on live feeder HFWT cells contributes to the NK cell expansion.23 Further studies are required to clarify the molecular mechanisms of the feeder cell-dependent NK cell expansion.

Fig. 4. Failure of NK cell expansion from PBMC cocultured on pretreated HFWT cells. PBMC were cocultured with HFWT cells prefixed with 10% formalin (○), with methanol-acetic acid (3:1) mixture (▲), or by heating (△), or cocultured with residues of HFWT cells after freezing-thawing (×). Control HFWT cells (●) were previously X-ray-irradiated. ○ lymphocytes cultured without feeder cells in the medium containing IL-2.

Fig. 5. Requirement of direct contact of PBMC with live HFWT cells. A cell-culture insert (pore size: 0.45 µm) was used in the NK-cell expansion culture as illustrated on the right side. A, CD3+CD56− T cells but not NK cells expanded from PBMC separated from irradiated HFWT cells. B, CD3−CD56+ NK cells expanded from PBMC directly layered on HFWT cells. FACS analysis was performed on day 10.
NK cells have killer inhibitory receptors (KIR). The ligands, MHC-class I molecules on the target cells, should bind to KIR and inactivate the NK cells. However, TKB-17RGB cells which strongly express MHC-class I molecules (Fig. 2) such as HLA-A24, -B35, -B53 and -Cw3 (data not shown) were killed by autologous NK cells (Fig. 1C). Therefore, in the present experiment, the MHC-class I molecules on TKB-17RGB cells may have not contained the KIR ligand subfraction, or contained it at insufficient levels. Otherwise, the expanded NK cells were strongly activated, sufficiently to overcome the inhibitory signal transduced through KIR. Others have reported that NK cells killed autologous target cells expressing MHC-class I molecules. For example, autologous NK cells killed monocyte-derived immature dendritic cells.

Spaggiari et al. reported that autologous antigen presenting cells (APC) which expressed MHC-class I were killed by IL-2-activated NK cells, in that NKp-30 and NKp-46, which are known as natural cytotoxic receptors (NCR), are involved in this cytotoxicity. Although the ligands for these NCRs are unknown at present, the autologous brain tumor cells might express these ligands on their surface.

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Although the contrasting properties of CTL, which kill MHC-class I positive and tumor-associated antigen-presenting target cells, and NK cells, which kill MHC-class I negative target cells, have been expected to be complementary in the immune cytotoxic response to tumor tissues that may contain both MHC-class I-positive and -negative tumor cells, generation of human CTL is not necessarily an easy process, especially against autologous tumor cells. The present results, however, suggest that expansion of NK cells on irradiated HFWT cells may not only allow large-scale preparation of human NK cells for studies of NK cell biology, but also provide a method for adoptive-immunotherapy of brain tumors without the simultaneous presence of CTL.

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