Adoptive immunotherapy using activated T cell is recognized to be clinically effective in treating lymphoma, leukemia, and renal cell carcinoma. In addition, the efficacy of these cells in reducing the recurrence after surgical resection of hepatocellular carcinoma (HCC) has been described. Although we have conducted cell therapy using activated T cells since 1997, the identities of the effector cells used in this therapy were not elucidated. When we examined the surface markers of the cells administered in effective immunotherapies, we found an abundance of CD56+ T cells. These cells are usually present in the peripheral blood at <5%. However, they were increased five-fold after activation with immobilized CD3 and IL-2 stimulation. We assessed this cell population with respect to origine, function, and morphology. To determine the fraction from which this population is derived, we conducted the present study using clinically administered, activated T cells.

Characterization of CD8+ CD56+ T Cells Induced from CD8+ Single Positive T Cells

Yoshio Deguchi1) and Teruaki Sekine2)

Abstract: Although CD56+ T cells comprise less than 5% of human peripheral blood mononuclear cells (PBMCs), we observed that the percentage of CD56+ T cells was increased in activated T-cell populations used for adoptive immunotherapy. We hypothesized that this cell subpopulation might be an important cytotoxic effector in immunotherapy. PBMCs were obtained from both cancer patients and healthy donors. CD8+ single-positive T cells were separated with magnetic beads, and stimulated with immobilized anti-CD3 monoclonal antibody and IL-2. FACS analysis showed that the CD56 antigen was expressed more strongly on CD8 cells than on CD4 cells. The CD8+ CD56+ T cells have a tendency for more cytotoxic effects than the CD8+ CD56- T cells. SEM analysis showed that the CD56+ T cells adhered to one another, to form a cluster. Thus, T cell self-adhesion was increased by the expression of the adhesion molecule CD56. The cytotoxic CD8+ CD56+ T cells derived from CD8+ T cells in the peripheral blood are activated T cells that are distinct from natural killer T (NKT) cells.

Key words: CD8+ CD56+ T cell, N-CAM, morphology, IL-2, OKT3

Introduction

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Materials and Methods

Cell preparation and culturing

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood from normal volunteers and cancer patients using Ficoll-Paque (density 1.077; Immuno-Biological Laboratories, Japan) density gradient centrifugation at 300×g for 15 min. The cells were washed with RPMI 1640 (Nikken Bio Medical Laboratories, Japan), and 5 ml of the PBMC suspension were mixed with biomagnetic beads (Dynabeads M-450 CD8; DYNAL). The ratio of beads to target cells was 1:1, and mixture was incubated for 30 min at 4°C with gentle tilting and rotation. The rosetted cells were isolated and washed with the help of a magnet, and resuspended at a concentration of $5 \times 10^5$ / ml in RPMI 1640 medium that was supplemented with 10% heat-inactivated FBS (Cell Culture Laboratories, Ohio, U.S.A.) recombinant human IL-2 (700 U / ml; Ortho Pharmaceutical, U.S.A.), 2 mM L-glutamine, 1 mM Oxaloacetic acid, 1 mM sodium pyruvate, 0.2 U / ml insulin and antibiotics. Culture flasks (25-mm²; Sumitomo Bakelite, Japan) were coated with anti-CD3 antibody ($5 \mu$g / ml; Ortho Pharmaceutical, U.S.A.) overnight at 4°C, and then washed three times with cold PBS. Rosetted cells and 10 ml of the culture medium were added to the coated flasks and cultured in 5% CO₂ at 37°C for 7 days; the magnetic beads were removed with a magnet. The cultured cells were transferred to non-antibody-treated flasks or Gas permeable bag, so that large-scale cells were obtained.

Flow cytometric analysis

The surface phenotypes of the cells were determined using monoclonal antibodies (mAbs) in conjunction with a two-color immunofluorescence. Briefly, $5 \times 10^5$ cells were stained with the following mAbs: FITC-conjugated anti-CD3 / HLA-DR (Leu-4 / anti HLA-DR, Becton Dickinson), anti-CD4 (Leu-3a, Becton Dickinson), anti-CD8 (Leu-2a, Becton Dickinson), anti-CD16 (Leu-11a, Becton Dickinson); PE-conjugated anti-CD56 (Leu-19, Becton Dickinson), FITC-conjugated anti-TCR-α / β and PE-conjugated anti-CD8β, anti-CD158a, anti-CD158b, anti-CD94, and anti-Vα24 (Coulter, IMMUNOTECH, Marseilles, France). Cells were incubated with Abs at 4°C for 20 min. Stained cells were washed and analyzed using the FACScan (Becton Dickinson, New Jersey, USA).

Intracellular cytokine analysis

Intracellular cytokines (interferon-γ, IL-4, TNF-α) were detected using the FastImmune Assay System (Becton Dickinson, U.S.A.). Briefly, cultured cells were resuspended at a concentration of $10^6$ cells / ml in RPMI 1640 that contained 2 mM L-glutamine, and were then activated with mitogen (50 ng / ml PMA, 500 ng / ml ionomycin) in the presence of 10 μg / ml Brefeldin A for 4 h at 37°C. The cells were then centrifuged at 500×g for 5 min, and resuspended in the 2 ml of lysing buffer (FACS Lysing Solution, Becton Dickinson) to
disrupt the red cells. After incubation for 10 min at room temperature, the samples were centrifuged at 500 × g for 5 min, and the supernatant was removed. The cells were resuspended in a buffer that permeabilized cell membrane and then incubated in the dark for 30 min. Following surface staining, the cells were washed with PBS/BSA and fixed with 4% cold paraformaldehyde for cytometric analysis.

**Scanning electron microscope (SEM)**

Cultured cells were perfusion-fixed in 2.5% glutaraldehyde (pH 7.4) in PBS (pH 7.4; 0.05 mol/L) for 30 min at 4°C overnight. After rinsing, the cells were post-fixed in 1% osmium tetroxide (Euromedex, Souffelweyershem, France) in the same buffer at 4°C for 1 h.

For SEM analysis, the fixed specimens were dehydrated in a graded series of alcohol and substituted with isoamyl acetate for 1 h. After critical-point drying using CO₂ as the transitional fluid and sputter coating with gold (JEOL ION SPUTTER JFC 1100), the specimens were observed under the electron microscope.

**Cytotoxicity**

The lytic capabilities of the cultured cells were assessed using the standard 4-hour ⁵¹Cr-releasing assay at known effector to target ratios, followed by calculation of percentage specific lysis. The targets, K562 and Daudi cells were pelleted and labeled with 5 MBq of sodium chromate (ICN Biochemicals, Inc., U.S.A.) in 10 μl of PBS, and then incubated for 20 min at 37°C. After washing, the target cells were suspended in 10% FBS at 5×10⁴ cells/ml. Viable target cells were counted using the trypan blue exclusion test. Labeled cells were plated at 5×10³ cells/ml in 96-well U-bottomed plates to which effector cells had been previously added. The plates were incubated in 5% CO₂/95% air for 4 h at 37°C. After centrifugation, the supernatants were harvested from the wells and counted by Gamma counter. Spontaneous ⁵¹Cr release from the target cells was assessed after incubation in culture medium alone, and maximal lysis was assessed after incubation of the labeled cells with 1% NP-40. The level of cytotoxicity was calculated as the percentage of releasable ⁵¹Cr counts after subtraction of the value for spontaneous ⁵¹Cr release.

**PCR analysis of N-CAM gene expression**

RT-PCR analysis was performed on PBMCs and activated T cells, as well as on the separated CD⁸⁺ 56⁻ T cells and CD⁸⁺ 56⁺ T cells. One microgram of total RNA extracted from the PBMCs was incubated with 200 U Superscript reverse transcriptase (GIBCO-BRL, U.S.A.) in a 40 μl reaction volume contained 10 mM DTT, 1 mM dNTP, 2 U rRNasin (Promega, U.S.A.) and 10 μM of random hexadeoxynucleotide primers (TaKaRa, Kyoto, Japan). The cDNA from CD56⁻ T cells, CD⁸⁺ 56⁺ T cells and PBMCs (whole fraction) were subjected to PCR (25°C for 10 min, 42°C for 60 min, and 70°C for 15 min). The primer sequences (and expected sizes of the PCR products) used were:
for N-CAM (337bp); β-actin (30 bp), 5’ CCGAATTCATCCTTGTTCAAGC-3’ and 5’ TCGGGATCCCAGACTGGCTGTCTTT-3’ (313bp)\(^7\). Amplification of the N-CAM gene was performed as 40 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. The amplified fragments were subjected to electrophoresis in 2% agarose gels and stained with ethidium bromide.

**Results**

*Phenotypic profiling*

Analyses of surface phenotypes of lymphocytes subjected to activating culturing revealed that the mean percentage of CD8\(^+\) was 87.4%. The percentage was much higher than that of CD4\(^+\) cells. The CD56 antigen was expressed more strongly on CD8\(^+\) cells. The percentage of CD8\(^+\) CD56\(^+\) lymphocytes induced from PBMCs by IL-2 and immobilized anti-CD3 mAb was 19.4%, whereas the corresponding percentage of CD4\(^+\) CD56\(^+\) was 4.6%. Thus, the percentage of CD8\(^+\) CD56\(^+\) cells was more than four-fold higher than the percentage of CD4\(^+\) CD56\(^+\) cells (Fig. 1).

![Fig. 1. Increase in the population of the CD56\(^+\) T cells after activation with IL-2 and immobilized CD3.](image)
Analysis of Activated T Cells Induced from PBMCs

Increase in the population of CD8<sup>+</sup>CD56<sup>+</sup> T cells

The expression of CD56 on CD8<sup>+</sup> T cells scarcely increased during the 7 days of stimulation with immobilized anti-CD3 mAb, which was conducted in accordance with the method reported previously<sup>8</sup>. In the absence of immobilized anti-CD3 mAb, the proportion of CD56-expressing cells increased gradually, reaching 40.6% on day 21 (Fig. 2).

Other surface marker profiles

Using mAb directed against NK receptors, surface markers were analyzed with FACS. The expression levels of CD158a and CD158b were within the normal ranges, and showed no correlations with the levels of CD56. The expression of CD94 was high even before activation, and was not changed after activation. The Fcγ receptors found on NK cells were also analyzed using mAbs against CD16, CD32, CD64. The results were negative, and the cells lacked NK cell-like characteristics. For mouse NKT cells, Vα14 chain of the T cell receptor has been used selectively, and its human equivalent is considered to be Vα24. Staining of the cells with an anti-Vα24 mAb gave a negative outcome (Table 1).

![Graph showing the increase in CD56 expression over time](image)

**Fig. 2.** Expansion of CD8<sup>+</sup>CD56<sup>+</sup> T cells accordance with the way reported by Sekine et al.

**Table 1. Phenotypic characteristics of CD8<sup>+</sup>CD56<sup>+</sup> T cells**

<table>
<thead>
<tr>
<th>T cell</th>
<th>TCR</th>
<th>NKR</th>
<th>FCγR</th>
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</thead>
<tbody>
<tr>
<td>CD3</td>
<td>+</td>
<td>Valpha24</td>
<td>–</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD158a</td>
<td>–</td>
<td>CD16</td>
<td>–</td>
</tr>
<tr>
<td>CD158b</td>
<td>–</td>
<td>CD32</td>
<td>–</td>
</tr>
<tr>
<td>CD94</td>
<td>+/-</td>
<td>CD64</td>
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CD8⁺ CD56⁺ cells produce IFN-γ

The intracellular cytokine assays revealed that the CD8⁺ CD56⁺ T cells produced mainly IFN-γ, with negligible production of IL-4. Thus, activation predominantly induced Th1-type cells (Table 2).

**Cytotoxicity of CD8⁺ CD56⁺ T cells**

The cytotoxic activities of CD8⁺ 56⁺ T cell subpopulations separated by positive selection for CD56 were measured using Daudi and K562 cells as the target cells. CD56⁻ fraction served as the control. Both the CD8⁺ 56⁺ and CD8⁺ 56⁻ T cells exerted cytotoxic effects on the Daudi and K562 cells. The CD8⁺ 56⁺ T cells had slightly higher cytotoxic activities, although there was no significant difference in this respect between the two subclasses of CD8⁺ T cells (Table 3).

**Morphologic analysis**

Following the activation of CD8⁺ T cells with IL-2 and solid-phase OKT3, morphologic changes in CD8⁺ T cells of the PBMC population were recognized (Fig.3). The CD8⁺ T cells were sphere-shaped and barely discernible from the CD4⁺ T cells. In parallel with activation, the CD8⁺ T cells became polymorphous. During culturing in a flask, the CD8⁺ 56⁺ T cells extended in a bipolarily humped shape, and adhered to the bottom of the flask. SEM of the CD8⁺ 56⁺ T cells revealed that the CD56⁺ T cells adhered to one another, thereby forming a cluster. Concomitant with the expression of CD56, mutual adhesion of T cells increased.

**Detection of the N-CAM (CD56) gene in the N-CAM-positive fraction and N-CAM-negative fractions**

<table>
<thead>
<tr>
<th>Table 2. Intracellular cytokine production within the CD8⁺ 56⁺ subset</th>
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<tbody>
<tr>
<td>% Cytokine positive cells</td>
</tr>
<tr>
<td>IFN-gamma</td>
</tr>
<tr>
<td>IL-4</td>
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<tr>
<td>TNF-alpha</td>
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<table>
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<tr>
<th>Table 3. Comparison of cytotoxic activity between CD56 positive fraction and negative fraction</th>
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<td></td>
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<tr>
<td>CD8⁺ 56⁺</td>
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<td>CD8⁺ 56⁻</td>
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(Percentage lysis of target cells)
Analysis of Activated T Cells Induced from PBMCs

N-CAM gene expression in the CD8$^+$ 56$^+$ T cell fraction was stronger than in PBMCs or CD8$^+$ CD56 fractions. A low-level expression of N-CAM was detected in the CD56-depleted fraction and in the PBMCs (whole fraction). As seen in electrophoresis using the β-actin gene as the control, N-CAM expression was weak, even though the concentration of cDNA was higher (Fig. 4).

Discussion

We investigated the mechanism by which CD56$^+$ T cells appear in the peripheral blood, and examined whether CD8$^+$ CD56$^+$ T cells are derived from CD8$^+$ T cells. In many cases of clinically effective immunotherapy, abundant CD56$^+$ T cells have been found among the administered cells. Previously, it was reported that when peripheral blood cells were stimulated with IL-2 and cultured in vitro, CD56$^+$ T cells were induced. We investigated the development of CD8$^+$ CD56$^+$ T cells from CD8$^+$ T cells using stimulation with IL-2 and solid-phase OKT3 before large-scale culture. The proliferation of pre-existing CD8$^+$ CD56$^+$ T cells seems unlikely, since it was confirmed that CD8$^+$ CD56$^+$ T cells could be induced by activation from CD8$^+$ T cells from which the CD56 antigen had been removed using magnetic beads prior to culture. These results suggest that activating stimuli induced the expression of CD56 on CD8$^+$ 56$^-$ T cells, as well as the subsequent proliferation of the CD8$^+$ 56$^+$ T cells.

Morphologic changes were noted for the activated T cells that expressed CD56. CD56
is primarily an adhesion molecule. It is expressed not only on NK cells and a proportion of T cells, but also in the brain, smooth muscle and neural crest-derived epithelium. It is a surface antigen that mediates homotypic adhesion. During culturing in a falk, the lymphocytes adhered to the bottom of the flask. Under light microscopy, the lymphocytes exhibited changes in shape, and electron microscopy revealed mutual lymphocyte adhesion.

RT-PCR with the N-CAM-specific primer revealed strong expression of CD56 (N-CAM) mRNA following activation. There was slight expression of CD56 gene in PBMCs, although gene expression associated with activation was considered to be enhanced.

There have been some reports on NKT cells\(^9,^{10}\). The CD8\(^+\) CD56\(^+\) T cells expressed both the T-cell receptor and NK surface antigen markers. However, the CD8\(^+\) CD56\(^+\) T cells differ from the so-called NKT cells with regard to the expression of CD4, CD8 and \(\text{V}\alpha 24\). The fact that they scarcely express the FC receptor (FCR) or NK receptor (NKR) strongly suggests that the CD8\(^+\) CD56\(^+\) T cells that have been activated. Thus, these cells are considered to represent a population that is induced at the stage of differentiation seen in the peripheral blood.

In the present study, the origin and functions of CD8\(^+\) CD56\(^+\) T cells were studied.
CD56+ T cells, which are often seen in cases of clinical efficacy, are considered to be effector cells in cell therapies\(^{11}\). In the present *in vitro* experiments, we identified CD8+ 56+ T cells as immunocompetent cells which more potent cytotoxic activities than the CD8+ T cells found in the peripheral blood of patients. Moreover, we showed that the CD8+ CD56+ T cells are induced from precursor CD8+ T cells.

Reference


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