Establishment of human T cell clones exhibiting natural killer-like activity

Shahabuddin Alam, Yoshinori Katakura* & Sanetaka Shirahata

Graduate School of Genetic Resources Technology, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan (* Author for all correspondence, e-mail: katakura@grt.kyushu-u.ac.jp)

Received 26 February 1999; accepted 26 February 1999

Key words: human T cell, immortalization, natural killer

Abstract

We have succeeded in establishing a method to reproducibly immortalize human T cells by oncogene(s) transfection (Alam, 1997). This study was based on our previous discoveries that these immortalized T cell lines contained T cells which showed cytotoxicity against K562 cells in MHC-nonrestricted manner. Then we attempted to obtain human T cell clones exhibiting natural killer-like activity. Here, we tried to establish clones from these immortalized T cell lines by limiting dilution after stimulation with K562 cells, and then obtained 16 T cell clones. Two clones among them maintained their stability and showed vigorous growth phenotype. Thus we selected these two clones for further analysis. One is derived from the T cell line transfected with oncogenes *ras* and *fos*, the other is from the T cell line transfected with *myc* and *fos*. Both clones were demonstrated to be CD4⁺ T cells, indicating that CD4⁺ T cells were preferably expanded from T cell lines immortalized by oncogene transfection. These two clones showed cytotoxicity against K562 cells, indicating that these two T cell clones still retain a natural killer-like activity of killing target cells of K562 cells in a MHC-nonrestricted manner. The natural killer-like activity of the T cell clones was shown to be stable for more than 2 yr when cultured in the presence of IL-2, indicating that introduction of two oncogenes such as *ras/fos* or *myc/fos* resulted in the acquisition of infinite replicative life-span but not in transformational alteration of cellular function.

Introduction

Both the generation and expansion of human T cells, with well-defined functional activities, have become an important topic in immunology. The development of human T cell clones with high cytolytic potential against tumor cells may also become an attractive approach for anti-cancer therapy. Different types of cytotoxic lymphoid cells have extensively been described. They are known as the allospecific, MHC-restricted and MHC-nonrestricted cytotoxic T lymphocytes, natural killer (NK) cells, the lymphoid cells that exert antibody-dependent cellular cytotoxicity (ADCC) and lymphokine activated killer (LAK) cells that lyse a wide variety of cells only upon exposure to interleukin-2 (IL-2). Recently, IL-2 dependent cytolytic effect has been demonstrated to be triggered by tumor-infiltrating lymphocytes (TIL) (Lanier, 1987; Lotzova, 1992). All these different types of effector

cells may be involved in anti-tumor immune responses at various stages of the anti-tumor response. Here, we report on T cell clones having MHC-nonrestricted natural killer-like activity obtained from T cell lines established by transfection of oncogenes into human peripheral blood lymphocytes.

Furthermore, we characterized these T cell clones and evaluated their functionality as compared to their parental T cell lines.

Materials and methods

Cell lines

The method to reproducibly immortalize human T cells by oncogene transfection has previously been described by Alam et al. (1997). Human T cell lines used in this study were established from the peripheral blood lymphocytes of a healthy volunteer. Sev-

eral established tumor cell lines were used as target cells in the cytotoxicity assay: two Burkitt's lymphoma cell lines, RAJI and Daudi (Japanese Cancer Research Bank; JCRB, Tokyo, Japan), a lung adenocarcinoma cell line, A549 (JCRB), an erythroleukemia cell line, K562 (JCRB) and an osteosarcoma cell line, MG63 (American Tissue Culture Collection; ATCC, Rockville, MD, U.S.A.).

Cloning procedure and cell culture

Oncogene-transfected T cell lines were seeded at a density of 1.0×10^6 cells and stimulated every 7 days with 1.0×10^4 cells of mitomycin C (Sigma Chemical Co., St. Louis, MO, U.S.A.)-treated K562 cells for a month, then inoculated at a density of 1.7 cells/ml into 96-well plate (Becton Dickinson, Franklin Lakes, NJ) containing ERDF medium (Kyokuto Pharmaceutical Co., Tokyo, Japan) supplemented with 20% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA, U.S.A.). These cloned T cells have been maintained by transferring every 2–3 days for 2 months in complete medium consisting of ERDF medium supplemented with 10% FBS, 1.5 μ l/ml bone marrow extract (Alam, 1997), 1.0 μ l/ml egg yolk lipoprotein (Murakami, 1988), 5 μ g/ml insulin (Novo Nordisk, Tokyo, Japan), 20 μ g/ml transferrin, 20 mM ethanolamine, 25 nM sodium selenite, 20% of the spent medium for the T cell lines and 50 U/ml recombinant human interleukin-2 (IL-2, Genzyme, Cambridge, MA, U.S.A.).

Flowcytometer analysis

Surface marker antigens on immortalized human T cell lines and T cell clones were analyzed by a flowcytometer (FACS Calibur; Becton Dickinson, San Jose, CA). Simultest (CD4/CD8) (Becton Dickinson) antibodies were used to stain the immortalized human T cell lines and T cell clones.

Cytotoxicity assay

Cytotoxicity assay was performed using a non-radioactive cytotoxicity assay kit (Promega Corp., Madison, WI, U.S.A.) according to the manufacturer's protocol. This assay enables measurement of activity of lactate dehydrogenase (LDH) released from the lysed target cells into culture supernatant, and quantitative evaluation of cytotoxic activity of effector cells similarly to the radioactive ^{51}Cr release assays. Several established tumor cell lines were used as target

cells: RAJI, Daudi, A549, K562, and MG63. Immortalized T cell lines generated by oncogene transfection (Alam, 1997) and T cell clones obtained from the T cell lines by limiting dilution were used as effector cells. The cytotoxicity assay was performed in triplicate in round-bottomed 96-well plates with the effector-to-target cell (E/T) ratio being 10:1. The percentage of specific cytotoxicity was calculated using the formula:

$$\text{cytotoxicity (\%)} = 100 \times (E - S_1 - S_2) / (M - S_2)$$

where E is the experimental released LDH activity resulting from the coculture of effector cells and target cells, S is the spontaneous released LDH activity resulting from separate cultures of effector cells (S_1) and target cells (S_2), and M is the maximum (100%) LDH activity when 0.8% Triton X-100 was added to target cells.

Results and discussion

Cytotoxicity of the immortalized T cell lines

The cytotoxic activities against several tumor cell lines of immortalized T cell lines which were generated by oncogene transfection into peripheral blood lymphocytes from a healthy volunteer were shown in Figure 1. K562, RAJI, Daudi, A549 and MG63 were used as target cells in this assay. Immortalized T cell lines were shown to exert strong cytotoxic activity against NK-sensitive K562, but weak against NK-resistant Daudi. In the previous report, we showed that immortalized T cell lines were all CD3^+ cells. Furthermore, immortalized T cell lines showed enhanced proliferative responses against K562 cells in mixed lymphocyte reaction experiments (Alam, 1997). Thus we concluded that immortalized T cell lines contained T cells having NK-like activity. We then tried to clone the T cells with NK-like activity.

The number of T cell clones established

Table 1 shows the number of T cell clones established from the T cell lines immortalized by oncogene transfection. Sixteen T cell clones were generated by stimulation with K562 cells and subsequent limiting dilution. This result demonstrates that T cells immortalized by oncogene transfection could be clonally expanded in the absence of a specific antigen and antigen presenting cells, but only in the presence of the

Figure 1. Cytotoxic activity of immortalized T cells against tumor cell lines. Cytotoxicity assay was performed using a non-radioactive cytotoxicity assay kit according to the manufacturer's protocol. MG63, A549, Daudi, RAJI and K562 were used as target cells. Each effector T cells is transfected with different combinations of oncogenes; 1: *ras*, 2: *ras/myc*, 3: *ras/fos*, 4: *ras/jun*, 5: *ras/myb*, 6: *myc/fos*, 7: *myc/jun*, 8: *fos/myb*, 9: *jun/fos*, and 10: *jun/myb*. ND: Not determined.

mitogenic stimulation derived from the supplements of the medium, spent medium for T cell lines and IL-2. Two clones among them can be stably maintained and showed vigorous growth phenotype. Thus we selected

these two clones for further analysis. One is derived from the T cell line transfected with oncogenes *ras* and *fes*, the other is from the T cell line transfected with *myc* and *fes*.

Figure 2. Ratio of CD4⁺ and CD8⁺ T cells. Ratio of CD4⁺ and CD8⁺ T cells in immortalized T cell lines and T cell clones were analyzed by flowcytometer. T cells were simultaneously stained with FITC-labeled anti-CD4 antibody and PE-labeled anti-CD8 antibody. Gray bar shows the ratio of CD4⁺ T cells and white bar indicates that of CD8⁺ T cells.

Table 1. The number of T cells clones

Transfected oncogenes	The number of clones
<i>ras</i>	2
<i>ras/myc</i>	2
<i>ras/fos</i>	2
<i>ras/jun</i>	2
<i>ras/myb</i>	2
<i>myc/fos</i>	1
<i>myc/jun</i>	1
<i>fos/myb</i>	1
<i>jun/fos</i>	1
<i>jun/myb</i>	1

flowcytometer (Figure 2). In the immortalized T cell lines transfected with *ras/fos* or *myc/fos*, about 70 to 80% of the T cells were CD8⁺ T cells, indicating that CD8⁺ T cell were dominant in the population. However, T cell clones obtained from these T cell lines were shown to be CD4⁺ T cells.

This indicates that CD4⁺ T cells were preferably expanded from T cell lines immortalized by oncogene transfection. This possibly suggests that CD4⁺ T cells transfected with oncogenes can be expanded in the absence of a specific antigen and antigen presenting cells, but only in the presence of mitogenic stimulation.

Cytotoxic activity of T cell clones

Then we measured cytotoxic activity of T cell lines and T cell clones. Immortalized T cell lines transfected with oncogenes showed strong cytotoxic activity against K562, indicating that immortalized T cell lines contained T cells having NK-like activity.

Surface marker antigen expression on T cell clones

Surface marker antigen expression on immortalized human T cell lines and T cell clones were analyzed by

Figure 3. Cytotoxic activity of immortalized T cell lines and T cell clones against K562 cells. Cytotoxic assay was performed as described in Figure 1. K562, NK-sensitive tumor cells was used as target cells.

Here we further analyzed cytotoxic activity against K562 of T cell clones obtained after stimulation with K562 for 7 days and control T cell clones established by direct limiting dilution without stimulation with K562. Figure 3 showed that stimulation with K562 enabled the establishment of T cell lines having higher NK-like activity. T cell lines obtained after stimulation with K562 showed almost the same NK-like activity as parental T cell lines. This study reveals that stimulation with K562 may promise efficient establishment of T cells having NK-like activity, and further demonstrates that oncogene transfection into human T cells can vest infinite replicative life-span without altering their functionality even in the absence of a specific antigen and antigen presenting cells.

Conclusions

In this study, we succeeded in cloning human T cell lines immortalized by oncogene transfection and demonstrated their surface marker expression and cytotoxic activity. Important step in our cloning step is to stimulate T cell lines with K562, a target cell of natural killer cells, before limiting dilution procedure. After this stimulation, we obtained 16 T cell

clones by limiting dilution. Two of these T cell clones showed CD4⁺ and strong cytotoxic activity against K562, demonstrating that CD4⁺ T cells having NK-like activity can be preferentially expanded from the T cell population. Generally, T cells recognize antigen presenting cells via T cell receptor either with CD3 and CD4 and/or CD3 with CD8, thus antigen stimulation is thought to be needed for cloning (Glasebrook, 1981; Chesnut, 1982). However in our case, infinite proliferative potential of T cells is likely compensated by transfected oncogenes, thus T cell clones obtained in this study can be maintained for more than 2 yr. Furthermore, T cell clones obtained in this study showed almost the same level of cytotoxic activity against K562 after 2 yr culturing, indicating that the transfected oncogenes do not alter the functionality of T cells, but only vest the infinite replicative life-span of T cells in the absence of specific stimulation via specific antigens and antigen presenting cells. The further novel point in our cloning protocol is the complete medium for propagation of T cells. Combination of medium supplements described in the Section 'Materials and methods', T cell spent medium and IL-2 is thought to be best suited for T cell propagation.

References

- Alam S, Katakura Y, Yoshida H, Kim E-H and Shirahata S (1997) Functional characterization of human T cells immortalized by oncogene transfection. *Cytotechnology* 23(1–3): 185–192.
- Chesnut RW, Colon SM and Grey HM (1982) Antigen presentation by normal B cells, B cell tumors, and macrophages: functional and bio-chemical comparison. *J Immunol* 128: 1764–1768.
- Glasebrook AL, Sarmiento M, Loken MR, Dialynas DP, Quintans J, Eisenberg L, Lutz CT, Wilde D and Fitch FW (1981) Murine T lymphocyte clones with distinct immunological functions. *Immunol Rev* 54: 225–266.
- Lanier LL, Le AM, Ding A, Evans EL, Krensky AM, Clayberger C and Phillips JH (1987) Expression of Leu-19 (NKH-1) antigen on IL-2 dependent cytotoxic and non-cytotoxic T cell lines. *J Immunol* 138: 2019–2023.
- Lotzova E (1992) Role of interleukin-2 activated MHC-nonrestricted lymphocytes in antileukemia activity and therapy. *Leukemia and Lymphoma* 7: 15–28.
- Murakami H, Okazaki Y, Yamada K and Omura H (1988) Egg yolk lipoprotein, a new supplement for the growth of mammalian cells in serum-free medium. *Cytotechnology* 1: 159–169.