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Functional characterization of human T cells immortalized by oncogene transfection

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Abstract

We have succeeded in immortalizing human lymphocytes derived from the peripheral blood of a healthy donor and of an atopic patient, and from the lymph node of a cancer patient by oncogene transfection (Alam *et al.*, 1996). All immortalized human lymphocytes were shown to be CD3⁺ and CD19[–], indicating that these immortalized human lymphocytes were all T cells. We established 317, 154 and 692 individual immortalized human T cell lines derived from the healthy donor, the atopic patient and the cancer patient, respectively. The ratios of CD4⁺ and CD8⁺ subpopulations within the set containing immortalized T cells derived from the healthy donor were shown to be varied depending on the combinations of transfected oncogenes used. However, CD8⁺ cells were found to be the dominant subpopulation of immortalized T cells derived from the atopic patient and the cancer patient. These immortalized T cells showed different proliferative responses in the presence of exogenous human IL–2 depending on their origin, and was consistent with the surface expression of the IL–2 receptor. Furthermore, the cytokine secretion patterns of these immortalized T cells stimulated with mitogen were investigated. The results showed that the immortalized T cells from the healthy donor is able to secrete various kinds of cytokines such as IL–2, IL–10, γ -IFN and GM-CSF. However, immortalized T cells from the cancer patient was shown to only secrete IL–2 and GM-CSF. These results suggest that depending on the origin, the immortalized T cells came from different subsets or from cells in different activated states. Mixed lymphocytes reactions demonstrated that these immortalized T cells are able to proliferate in the presence of allogenic or xenogenic stimulator cells, suggesting that they maintain the ability to recognize specific antigens on the stimulator cells and can proliferate even after the immortalization. Furthermore, immortalized T cells derived from the healthy donor and the cancer patient strongly responded to K562 cells, suggesting that MHC-nonrestricted killer T cells were also immortalized.

Abbreviations: IL–2R – interleukin 2 receptor; MLR – mixed lymphocyte reaction

Introduction

In comparison with rodent-derived cells, the immortalization of human-derived cells has been shown to be very difficult. Several methods to immortalize human T cells have been reported, such as 1) treatment of carcinogens (Russo *et al.*, 1988), 2) irradiation of UV light or X-rays (Borek *et al.*, 1980), 3) transfection with SV40 large T antigen (Shay *et al.*, 1989), 4)

infection with specific viruses such as Epstein-Barr virus (Stevenson *et al.*, 1986) and Herpesvirus saimiri (Troidl *et al.*, 1994), and 5) stimulation with specific antigens in the presence of the antigen presenting cells (van Neerven *et al.*, 1993). However, establishment of functional human cells with the ability to overcome crisis and to acquire immortality still remains quite difficult. We have succeeded in establishing a novel method to effectively and reproducibly immortal-

ize human lymphocytes by the oncogene transfection (Alam *et al.*, 1996). Here, we characterized immortalized human lymphocytes derived from the peripheral blood of a healthy donor and an atopic patient, and from the lymph node of a cancer patient, and investigated whether the functional properties of these immortalized T cells are maintained after immortalization. We then further evaluated the phenotypical differences of these immortalized T cells with respect to their origin.

Materials and methods

Transfection of oncogenes into human lymphocytes

Five oncogenes (human c-Ha-ras, human c-myc, human c-fos, avian v-jun and avian v-myb) were used for immortalizing human lymphocytes. The oncogene expression plasmids were constructed by inserting each oncogene downstream from the human cytomegalovirus promoter in pRc/CMV vector (Invitrogen, San Diego, CA, U.S.A.) as described elsewhere (Yano *et al.*, 1994). Transfection methods used to introduce the oncogene expression plasmids into the human lymphocytes and maintenance of these transfected lymphocytes have been described elsewhere (Alam *et al.*, 1996). Transfection with a single oncogene was previously shown to be not effective for immortalizing human lymphocytes (Okano *et al.*, 1993). Therefore, we randomly selected two oncogenes out of five for simultaneous transfection. Oncogenes were introduced into lymphocytes using the electroporation method (Okano *et al.*, 1993). Transfected human lymphocytes were maintained in the ERDF medium (Kyokuto Pharmaceutical Co., Tokyo, Japan) containing 10% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA, U.S.A.), $1.5 \mu\text{l ml}^{-1}$ of bone marrow extract (Alam *et al.*, 1996), $1.0 \mu\text{l ml}^{-1}$ of egg yolk lipoprotein (Murakami *et al.*, 1988), $5 \mu\text{g ml}^{-1}$ of insulin (Novo Nordisk, Tokyo, Japan), $20 \mu\text{g ml}^{-1}$ of transferrin, 20 mM of ethanolamine, 25 nM of sodium selenite and 20 U ml^{-1} of human interleukin 2 (hIL-2; Genzyme, Cambridge, MA, U.S.A.) at 37°C under humidified 5% CO_2 and 95% air atmosphere. Cloning of immortalized human lymphocytes is being continued. The data included in all figures and tables in this study show the results obtained by using the mixed clone cultures immortalized upon the transfection of the shown oncogene combination.

Flowcytometry analysis

Surface marker antigens on immortalized human lymphocytes were analyzed by flowcytometer (EPICS 752; COULTER ELECTRONICS INC., Hialeah, FL, U.S.A.). Simultest (CD3/CD19) (Becton Dickinson, San Jose, CA, U.S.A.), Simultest (CD4/CD8) (Becton Dickinson), FITC-labeled anti-IL-2 receptor (Becton Dickinson) antibodies were used to stain the immortalized human lymphocytes.

Proliferative responses of immortalized T cells

Proliferative responses of immortalized T cells in the presence of concanavalin A (Con A) and hIL-2 were assessed by WST-1 assay (Dojindo, Kumamoto, Japan) according to the manufacturer's manual. Briefly, the immortalized T cells were stimulated with $10 \mu\text{g ml}^{-1}$ of Con A in the presence of 10 U ml^{-1} of hIL-2 in 96-well plates and kept for 8 days at 37°C in a humidified 5% CO_2 incubator. Twenty μl of WST-1 dye solution (65 mg WST-1 (Dojindo) and 7 mg of 1-methoxy PMS (Dojindo) in 100 ml phosphate-buffered saline (PBS)) was then directly added to the culture. After the incubation for 4 h, the absorbance at 450 nm was measured.

Cytokine production of immortalized T cells

Total RNA was prepared by TRIzol reagent (LIFE TECHNOLOGIES, Gaithersburg, MD, U.S.A.) from human peripheral blood lymphocytes and immortalized T cells, both of which were stimulated with $5 \mu\text{g ml}^{-1}$ of phytohemagglutinin (PHA) and $5 \mu\text{g ml}^{-1}$ of Con A for 10 h. Five μg of total RNA was reverse-transcribed with AMV-reverse transcriptase (WAKO, Osaka, Japan) using d(T)₁₂₋₁₈ primer. One μg of the resulting synthesized cDNA was used as template for PCR with Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN, U.S.A.). PCR was initiated at 94°C for 2 min, followed by 30 cycles of 30 sec at 94°C , 30 sec at 55°C , and 1 min at 72°C .

The primers used in this study were: (1) 5'-AACAGCCTCAAGATCATCAGC-3' and 5'-CATGAGTCCTTCCACGATACC-3', which spans a 102 nt fragment corresponding to the human glyceraldehyde-3-phosphate dehydrogenase (GAPD) gene, (2) 5'-GAATGGAATTAATAATTACAAGAATCCC-3' and 5'-TGTTTCAGATCCCTTTAGTTCCAG-3', which spans a 229 nt fragment in the human IL-2 gene, (3) 5'-GTTCTTCCTGCTAGCATGTGC-3' and 5'-

AGTGTCTTCTCATGGTGGC-3', which spans a 229 nt fragment in the human interleukin 4 (IL-4) gene, (4) 5'-TGAATCCTTCTCCACAAGC-3' and 5'-ATCCAGATTGGAAGCATCCA-3', which spans a 315 nt fragment in the human interleukin 6 (IL-6) gene, (5) 5'-AACCTGCCTAACATGCTTCG-3' and 5'-GGTCTTGGTTCTCAGCTTGG-3', which spans a 202 nt fragment in the human interleukin 10 (IL-10) gene, (6) 5'-TCTGCATCGTTTTGGGTTCT-3' and 5'-CAGCTTTTCGAAGTCATCTC-3', which spans a 320 nt fragment in the human interferon γ (γ -IFN) gene and (7) 5'-AGTTCTCTGGAGGATGTGGC-3' and 5'-AGCAGCAGTGTCTCTACTCAGG-3', which spans a 166 nt fragment in the human granulocyte-macrophage colony stimulating factor (GM-CSF) gene.

Mixed lymphocyte reaction of immortalized T cells

Several established tumor cell lines were used as stimulator cells: two Burkitt's lymphoma cell lines, RAJI and Daudi (Japanese Cancer Research Resources Bank; JCRB, Tokyo, Japan), an erythroleukemia cell line, K562 (JCRB), a human T cell line, Jurkat (RIKEN Cell Bank, Saitama, Japan) and a mastocytoma cell line, P-815 (JCRB). Immortalized T cells (2×10^4 cells) were cultured with mitomycin C-treated stimulator cells (2×10^4 cells) in ERDF medium containing 10% FBS and 20 U ml^{-1} hIL-2 for 7 days in 96-well plates at 37°C in a humidified 5% CO_2 incubator. Proliferative responses were assessed by WST-1 assay as described above. The stimulation index (SI) was calculated as the mean absorbance in the presence of stimulator cells divided by the mean absorbance in the absence of stimulator cells.

Results and discussions

Human lymphocytes immortalized by oncogene transfection

As shown in Table 1, 317, 154 and 692 individual immortalized human lymphocyte cell lines were established by oncogene transfection of the lymphocytes derived from the healthy donor, the atopic patient and the cancer patient respectively, demonstrating that our oncogene transfection method is effective and reproducible for the immortalization of human lymphocytes. These immortalized lymphocytes could be indefinitely propagated independent of the need for antigen and/or

Table 1. The number of T cell lines immortalized by oncogene transfection

Oncogenes	Origin		
	Healthy donor	Cancer patient	Atopic patient
<i>ras</i>	30	49	17
<i>ras+myc</i>	39	62	10
<i>ras+fos</i>	30	75	17
<i>ras+jun</i>	47	58	15
<i>ras+myb</i>	34	69	14
<i>myc+fos</i>	24	55	10
<i>myc+jun</i>	27	61	6
<i>myc+myb</i>	23	69	21
<i>fos+jun</i>	17	65	12
<i>fos+myb</i>	21	66	21
<i>jun+myb</i>	25	63	11
total	317	692	154

target cell stimulation. These results indicate that cancer patient derived human lymphocytes are easier to immortalize than lymphocytes derived from either a healthy donor or an atopic patient. This might be due to the fact that CD8^+ T cells, which were the dominant subpopulation of cancer patient derived lymphocytes, and/or that T cells showing a vigorous IL-2-dependent proliferative response which are also represented in the population of cancer patient derived lymphocytes, have an enhanced ability to be immortalized.

Surface marker antigens on immortalized human lymphocytes

Table 2 shows that the immortalized human lymphocytes, regardless where they were derived from, were all CD3^+ and CD19^- , indicating that these immortalized human lymphocytes were all T cells. The ratios of CD4^+ and CD8^+ subsets within the population of immortalized T cells derived from the healthy donor does not reflect the profile for the primary lymphocytes, rather they were shown to be variable dependant upon the oncogene they were transfected with (Table 2). Especially, healthy donor derived immortalized T cells resulting from transfection with the c-Ha-ras and v-jun combination were demonstrated to be only CD4^+ T cells. On the other hand, immortalized T cells derived from either the atopic patient or the cancer patient were shown to be dominantly CD8^+ regardless of which oncogene combination they were transfected with. CD8^+ dominancy was also seen in primary cells

Table 2. Surface markers on immortalized human lymphocytes

Origin	Transfected oncogenes	CD3	CD19	CD4 ⁺ /CD8 ⁻	CD4 ⁻ /CD8 ⁺	IL-2R
Healthy donor	<i>ras</i>	+++	-	+	+++	-
	<i>ras+myc</i>	+++	-	++	++	-
	<i>ras+fos</i>	+++	-	+	+++	-
	<i>ras+jun</i>	+++	-	+++	-	-
	<i>ras+myb</i>	+++	-	+	+++	-
	<i>myc+fos</i>	+++	-	+	+++	-
	<i>myc+jun</i>	+++	-	+++	+	-
	<i>myc+myb</i>	+++	-	+	+++	-
	<i>fos+jun</i>	+++	-	++	++	-
	<i>fos+myb</i>	+++	-	+++	+	-
	<i>jun+myb</i>	+++	-	+++	+	-
	primary	+++	-	++	++	ND*
Cancer patient	<i>ras</i>	+++	-	+	+++	++
	<i>ras+myc</i>	+++	-	+	+++	+
	<i>ras+fos</i>	+++	-	+	+++	++
	<i>ras+jun</i>	+++	-	+	+++	+
	<i>ras+myb</i>	+++	-	++	++	-
	<i>myc+fos</i>	+++	-	+	+++	+
	<i>myc+jun</i>	+++	-	+	+++	-
	<i>myc+myb</i>	+++	-	+	+++	++
	<i>fos+jun</i>	+++	-	+	+++	+
	<i>fos+myb</i>	+++	-	+	+++	+
	<i>jun+myb</i>	+++	-	+	+++	+
	primary	+++	-	+	+++	++
Atopic patient	<i>ras</i>	+++	-	+	+++	ND
	<i>ras+myc</i>	+++	-	+	+++	ND
	<i>ras+fos</i>	+++	-	+	+++	ND
	<i>ras+jun</i>	+++	-	+	+++	ND
	<i>ras+myb</i>	+++	-	+	+++	ND
	<i>myc+fos</i>	+++	-	ND	ND	ND
	<i>myc+jun</i>	+++	-	ND	ND	ND
	<i>myc+myb</i>	+++	-	+	+++	ND
	<i>fos+jun</i>	+++	-	ND	ND	ND
	<i>fos+myb</i>	+++	-	+	+++	ND
	<i>jun+myb</i>	+++	-	ND	ND	ND
	primary	+++	-	ND	ND	ND

* ND: Not determined.

Relative amounts of human lymphocytes with specific surface marker antigens were evaluated by flowcytometry analysis and the results are shown as follows: +++ = >70%; ++ = 20~70%; + = <20%; - = Not detected.

taken from the cancer patient. These results indicate that the CD4⁺ and CD8⁺ ratios of immortalized T cells derived from the cancer patient may reflect the T cell ratio in primary culture.

Furthermore, analysis of surface expression of the IL-2 receptor (IL-2R) demonstrated that IL-2R⁺ T cell subpopulations could be detected only in the immortalized T cells derived from the cancer patient (Table 2). These results could explain the IL-2-

Figure 1. Proliferative responses of immortalized human T cells. Proliferations of the immortalized T lymphocytes derived from the healthy donor (A), the cancer patient (B) and the atopic patient (C) in the absence (white bar) or presence (hatched bar) of exogenous hIL-2 were assessed by WST-1 assay. (ND: not determined).

dependent proliferation of the immortalized T cells derived from the cancer patient as discussed below.

Proliferative responses of immortalized T cells

Proliferative responses of immortalized T cells in the absence or presence of hIL-2 were assessed by WST-1 assay (Fig. 1). Proliferative responses of these immortalized T cells against exogenous hIL-2 were varied, depending upon their origins. Immortalized T cells derived from the healthy donor showed vigorous propagation both in the absence and presence of exogenous hIL-2. However, immortalized T cells derived from the cancer patient showed high responses only in the presence of exogenous hIL-2. The IL-2-dependent proliferative responses of the cancer patient derived immortalized T cells are consistent with their surface expression of IL-2R, and might be related to the high expression of CD8 antigen. The CD8⁺ T cells are thought not to be able to produce sufficient IL-2 for self proliferation (Schwartz *et al.*, 1992), but can proliferate in response to increased concentrations of IL-2 or the presence of other cytokines (Zupo *et al.*, 1993). The requirement of exogenous hIL-2 for the spontaneous growth of these immortalized T cells suggest that these cells retain the phenotypical function of the primary CD8⁺ T cells which exhibit cytokine-

dependent growth (Karasuyama *et al.*, 1989). This IL-2-dependency of immortalized T cells derived from the cancer patient might explain their high immortalization efficiency, because oncogene transfected lymphocytes were cultured in the presence of exogenous hIL-2 until they were shown to be immortalized. On the other hand, the immortalized T cells derived from the atopic patient showed low responses both in the absence and presence of exogenous hIL-2. These results suggest that the immortalized T cells were in different subsets or in different activated states depending upon their origins when immortalized.

Primary T cells and immortalized T cells from the healthy donor showed a slightly higher response in the presence of exogenous hIL-2 when compared to exogenous hIL-2 negative conditions, although individual immortalized T cell proliferative response varied depending upon the oncogene combination used. Interestingly, this oncogene combination dependent variance could not be observed in immortalized T cells derived from the cancer patient or from the atopic patient. These results suggest that the transfected oncogene combinations do not always influence the proliferative responses of immortalized T cells.

Table 3. Cytokine production patterns of immortalized human T cells

Origin	Transfected oncogenes	GAPD	IL-2	IL-4	IL-6	IL-10	γ -IFN	GM-CSF
Healthy donor	<i>ras</i>	+++	+++	-	-	-	+	++
	<i>ras+myc</i>	+++	+++	-	-	-	-	-
	<i>ras+fos</i>	+++	++	-	-	-	++	+
	<i>ras+jun</i>	+++	+++	-	-	-	+++	-
	<i>ras+myb</i>	+++	+++	-	+	+	++	++
	<i>myc+fos</i>	+++	+++	-	-	-	-	-
	<i>myc+jun</i>	+++	+++	-	-	-	-	-
	<i>myc+myb</i>	+++	-	-	-	-	-	-
	<i>fos+jun</i>	+++	-	-	-	-	-	-
	<i>fos+myb</i>	+++	+++	-	-	-	-	+
	<i>jun+myb</i>	+++	+++	-	+	+	+	++
	primary	+++	+++	-	++	++	+	++
Cancer patient	<i>ras</i>	+++	-	-	-	-	-	-
	<i>ras+myc</i>	+++	+	-	-	-	-	+
	<i>ras+fos</i>	+++	-	-	-	-	-	-
	<i>ras+jun</i>	+++	-	-	-	-	-	-
	<i>ras+myb</i>	+++	-	-	-	-	-	-
	<i>myc+fos</i>	+++	+	-	-	-	-	+
	<i>myc+jun</i>	+++	-	-	-	-	-	-
	<i>myc+myb</i>	+++	-	-	-	-	-	+
	<i>fos+jun</i>	+++	+	-	-	-	-	+
	<i>fos+myb</i>	+++	++	-	-	-	++	-
	<i>jun+myb</i>	+++	-	-	-	-	-	+
	primary	+++	+++	+	++	+	+	++

Relative amount of each cytokine mRNA was assessed by the strength of the band corresponding to each PT-PCR product. +++ = Strong band similar as GAPD; ++ = thin but clear band; + = faint band; - = Not detected.

Cytokine secretion patterns for immortalized T cells

The cytokine production patterns of the immortalized T cells derived from the healthy donor and the cancer patient were investigated by RT-PCR (Table 3). IL-2 mRNA from immortalized T cells derived from the healthy donor could be detected in 10 out of 11 oncogene combinations. IL-6 and IL-10 mRNAs were detected in 2 oncogene combinations. GM-CSF mRNA could also be detected in 5 oncogene combinations, and a higher amount of IFN- γ mRNA was detected in 5 oncogene combinations. The immortalized T cells transfected with the *c-Ha-ras/v-myb* and the *v-jun/v-myb* combinations produced 5 out of 6 cytokines tested. Immortalized T cells transfected with *c-Ha-ras* only or *c-Ha-ras* in combination with other oncogenes were shown to be high producers of cytokines as compared to other oncogene combinations. These results sug-

gest that the oncogene products of *c-Ha-ras* and/or *v-myb* might function in the signaling network which is important for cytokine secretion.

Overall cytokine production of immortalized T cells derived from the cancer patient was very low as compared to those from the healthy donor. Indeed, although low amounts GM-CSF mRNA was detected in 5 out of 11 oncogene combinations, other cytokines tested could hardly be detected. This biased expression of the GM-CSF mRNA and the decreased expression of IL-2 and IFN- γ might have some relationship to the occurrence of cancer. In fact, Pisa *et al.* (1992) reported the selective expression of GM-CSF mRNA in ovarian tumor tissue as compared to normal ovaries. Furthermore, when stimulated with mitogens, the cancer patient derived immortalized T cells produced low levels of cytokines. These T cells however vigorously proliferated in response to the exogenous hIL-2. The

Figure 2. Mixed lymphocyte reactions of immortalized T cells in the presence of allogenic and xenogenic stimulator cells. Proliferative responses of immortalized T cells derived from the healthy donor (grey bar) and the cancer patient (white bar) in the presence of stimulator cells (1: primary, 2: Daudi, 3: K562, 4: Jurkat, 5: RAJI and 6: P-815) were assessed by WST-1 assay. The stimulation index (S.I.) was calculated as the mean absorbance in the presence of stimulator cells divided by the mean absorbance in the absence of stimulator cells.

fact that proliferative responses against mitogen does not correlate with cytokine production in the immortalized T cells derived from the cancer patient might have some relation to the fact that $CD8^+$ T cells were the dominant subpopulation.

Mixed lymphocyte reactions of immortalized T cells

We examined the responses of oncogene-transfected T cells to allogenic or xenogenic stimulator cells (Fig. 2). Immortalized T cells from the cancer patient responded preferentially to K562 cells, which were used as target cells in a natural killer assay. Furthermore, immortalized T cells from the healthy donor responded preferentially to K562, Jurkat and RAJI cells, but only slightly to Daudi cells. These results indicate that the immortalized T cells maintained the ability to recognize specific antigens. Furthermore, the immortalized T cells from both donors were shown to contain

K562 responsive cells, suggesting that T cells having MHC-nonrestricted killer activity might be effectively immortalized using our procedure.

In 8 out of 11 oncogene combinations, proliferative responses of immortalized T cells from the healthy donor were shown to be higher than the immortalized T cells from the cancer patient, indicating that the immortalized T cells from the healthy donor have an increased reactivity to allogenic and xenogenic stimulator cells. The low responses against stimulator cells observed in the immortalized T cells derived from the cancer patient might be explained in part by the fact that these T cells are derived from a cancer patient whose immune system was generally considered to be suppressed and that $CD8^+$ T cells were the dominant subpopulation. We speculate that the immortalized T cells derived from the cancer patient were generally unresponsive against stimulation with specific anti-

gens, although these T cells maintained the ability to proliferate in the presence of hIL-2.

In this study, we demonstrated that by transfecting human lymphocytes with various oncogene combinations, we easily and reproducibly obtained immortalized functional human T cells. Infection of human lymphocytes with Herpesvirus saimiri (HVS) was also considered to be an effective method to immortalize human T cells (Biesinger *et al.* 1992). However, the immortalized T cells obtained by the infection with HVS were reported to have a partially altered functionality as compared to the parental T cells (De Carli *et al.* 1993). In this respect, we must further investigate whether our immortalized T cells maintain functions similar to the primary T cells. However, high immortalization efficiency demonstrated by our method can be used to establish novel functional human T cells for further understanding the immune system *in vivo*.

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