Ectopic Human Telomerase Catalytic Subunit Expression Maintains Telomere Length But Is Not Sufficient for CD8+ T Lymphocyte Immortalization

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Ectopic Human Telomerase Catalytic Subunit Expression Maintains Telomere Length But Is Not Sufficient for CD8+ T Lymphocyte Immortalization

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Like most somatic human cells, T lymphocytes have a limited replicative life span. This phenomenon, called senescence, presents serious barriers to clinical applications that require large numbers of Ag-specific T cells such as adoptive transfer therapy. Ectopic expression of hTERT, the human catalytic subunit of the enzyme telomerase, permits fibroblasts and endothelial cells to avoid senescence and to become immortal. In an attempt to immortalize normal human CD8+ T lymphocytes, we infected bulk cultures or clones of these cells with a retrovirus transducing an hTERT cDNA clone. More than 90% of transduced cells expressed the transgene, and the cell populations contained high levels of telomerase activity. Measuring the content of total telomere repeats in individual cells (by flowFISH) we found that ectopic hTERT expression reversed the gradual loss of telomeric DNA observed in control populations during long term culture. Telomere length in transduced cells reached the levels observed in freshly isolated normal CD8+ T lymphocytes. Nevertheless, all hTERT-transduced populations stopped to divide at the same time as nontransduced or vector-transduced control cells. When kept in IL-2 the arrested cells remained alive. Our results indicate that hTERT may be required but is not sufficient to immortalize human T lymphocytes. The Journal of Immunology, 2000, 165: 4978–4984.

The impossibility to propagate T lymphocytes indefinitely limits their use for clinical applications that require large cell numbers such as adoptive transfer therapy of infections and malignancies. Why cultured T cells stop proliferating at a certain point remains unclear. In fibroblasts, telomere shortening during cell division is the molecular clock that triggers the entry of cells into senescence (7, 8). Expression of the enzyme telomerase allows germline cells and tumor cells to maintain telomere length during proliferation. Ectopic expression of human telomerase catalytic subunit (hTERT)3 is sufficient to permit some cell types, such as fibroblasts, retinal pigment epithelial cells, and endothelial cells (7, 8), to avoid senescence and to proliferate indefinitely. These immortalized cells continue to display normal cellular functions and do not undergo changes characteristic of malignant transformation (9, 10). Other cell types require alterations in cell cycle regulatory elements, besides telomerase expression, for immortalization. In particular, blocking of the Rb/p16 pathway together with telomerase expression are required to immortalize human keratinocytes and breast epithelial cells (11).

Telomerase expression can be detected in hematopoietic stem cells, where it is transiently up-regulated during in vitro cytokine-driven expansion (12, 13). Telomerase activity is also detectable in different stages of T lymphocyte differentiation (14). In vitro, mature T cells transiently express telomerase activity in response to stimulation with specific Ag (15), mitogens, or anti-CD3/anti-CD28 Abs (14, 16, 17). However, if the same cultures are subjected to repeated cycles of stimulation, the peak of telomerase activity becomes progressively lower (16). The ability of lymphocytes to express telomerase activity is exceptional among normal differentiated human cells. It has been proposed that up-regulation

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3 Abbreviations used in this paper: hTERT, human telomerase catalytic subunit; hTER, human telomerase RNA template subunit; CFSE, 5-carboxyfluorescein diacetate-succinimidyl ester; MSCV, mouse stem cell virus; TRAP, telomerase repeat amplification protocol.
of telomerase allows T lymphocytes to preserve their replicative potential during clonal expansion so that memory cells with a strong capacity for expansion can be generated (18). However, a negative correlation between telomere length and donor age has been observed in CD4+ T lymphocytes, and peripheral blood T lymphocytes with a memory phenotype have shorter telomeres than naive cells (19, 20). Telomeres also shorten in vitro cultured lymphocytes (19, 20), and both telomere length and CD28 expression decline with the number of cell divisions (5, 21). To investigate whether ectopic hTERT expression leads to immortalization of normal human CTL we infected bulk cultures or clones of human CD8+ lymphocytes with a retrovirus transducing an hTERT cDNA clone. We report that although the transduced cells express high levels of telomerase and maintain telomere length, they do not have a significantly longer life span.

Materials and Methods

Purification and culture of CD8+ T lymphocytes

PBMC were obtained from healthy donors by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and CD8+ cells were purified by magnetic cell sorting using a miniMACS device (Miltenyi Biotec, Auburn, CA). The CD8+ CTL clones NH 55 and LAU 203.0.3/3 were obtained by limiting dilution culture in the presence of PHA, irradiated allogeneic feeder cells, and IL-2 as previously described (22). Clone NH 55 was obtained from peptide-stimulated PBMC of normal donor NM (23) and clone LAU 203.0.3/3 from tumor-infiltrating lymph node cells of melanoma patient LAU 203 (24).

Purified fresh CD8+ T cells and CD8+ CTL clones were plated at 10^6 cells/well in Iscove’s medium (Life Technologies, Basel, Switzerland) supplemented with 10% human serum, asparagine, arginine, and glutamine in the presence of 150 U/ml recombinant human IL-2 (a gift from Glaxo, Geneva, Switzerland) and stimulated with 1 μg/ml PHA (Life Technologies) plus irradiated allogeneic PBMC (3000 rad) as feeder cells, as described elsewhere (22). In the case of freshly isolated CD8+ T cells, additional cycles of stimulation were performed at intervals of 2 or 3 wk. Population doublings were determined by weekly counts of viable cells. To estimate the mean number of cell divisions vs time, we stained cell samples with the diacetate form of the carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) once or twice per week, and analyzed them by flow cytometry 6 h, as well as 4 and 7 days after staining (25, 26). A senescent population of clone NH 55 was stained at the same times and used as a standard. Living cells were gated and peaks corresponding to cells that had undergone a definite number of divisions were identified in the fluorescence histograms. From a histogram with N peaks,
Constitutive hTERT expression does not immortalize T lymphocytes

The fraction of cells in this peak.

m

ml were stimulated with immobilized anti-CD3 (OKT3, 10

m

1

solution anti-CD28 (CK248, 1

m

1

After 5 days of culture, CD8

m

Ab (see below), varied from 1 to 5%. Transduced cells were selected in 1.0

g/ml puromycin for 1 wk.

To induce endogenous telomerase activity, complete PBMC (10^6 cells/

m

X

N

expression in hTERT-transduced CD8^+ T lymphocytes.

Results

Expression of telomerase activity in hTERT-transduced CD8^+ T lymphocytes

We isolated CD8^+ T cells from peripheral blood of healthy donors by magnetic sorting and stimulated them with irradiated allogeneic PBMC and PHA to trigger cell proliferation. Three days later, the cell population was infected with an amphotrophic MSCV virus (27) containing the puromycin N-acetyl transferase gene (confer-

ring resistance to puromycin) under the control of the phospho-
glycerol kinase promoter and a complete hTERT cDNA under the control of the viral long terminal repeat. As expected from the work of others (7, 8), primary human lung fibroblasts infected with the same virus expressed telomerase activity and did not undergo senescence (data not shown).

Infected lymphocytes were selected in puromycin, and telomerase activity was measured in nuclear extracts of resistant cells by TRAP. As shown in Fig. 1, A and C, transduced lymphocytes expressed high levels of telomerase activity, similar to those found in fibroblasts transduced with the same construct. Enzyme activity was ~80 times higher than that in cells derived from the same

Telomerase repeat amplification protocol (TRAP) assays and immunofluorescence

Nuclear extracts were prepared as described (30) and TRAP was performed according to (31) using the ACX-anchored return primer. Relative signal intensity of the repeat bands was measured by phosphor imaging analysis. To compare telomerase activity of the different cell populations, the intensity of the signals from individual reactions was normalized to the cell equivalents of extract added. Signals that fell into the range in which there was a linear correlation between signal intensity and cell equivalents were used for quantification (see Fig. 1C). For indirect immunofluorescence, cells were diluted in complete culture medium, dropped onto glass slides coated with polylysine, and incubated for 2 h at 37°C. After washing and fixation with 4% paraformaldehyde in PBS for 15 min at room temperature, cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min before blocking with 2% BSA/0.1% Tween 20 in PBS for 30 min at room temperature. After washing, cells were incubated with primary Abs for 1 h at room temperature. Mouse anti-nuclear pore Ab (Ab) was a gift from Dr. Susan Gasser (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). Rabbit anti-hTERT Ab (0.4 μg/ml) was affinity-purified and will be described elsewhere (M. Amacker and J. Lingner, manuscript in preparation). Incubation with secondary Abs, Alexa 488-conjugated goat anti-rabbit Ab (Molecular Probes), and Cy3-conjugated goat anti-mouse Ab (The Jackson Laboratory, Bar Harbor, ME) were conducted for 45 min at room temperature. Images were acquired on a Zeiss LSM 410 confocal microscope (Fig. 2A) and on a Coolview Photonics CCD Zeiss Axiophot microscope (Fig. 2B).

Telomere length determination by flowFISH

To measure cellular telomere length, cells were hybridized in situ with a fluorescent telomere-specific peptide nucleic acid probe, according to previously described, slightly modified protocols (20, 33, 34). To follow the evolution of a single population of lymphocytes during long-term culture, cell samples were frozen at different time points and analyzed in a single flowFISH experiment. Frozen cells (10^5–5 × 10^6) were thawed, washed in PBS, and resuspended to 10^7 cells/100 μl of a hybridization mixture (Dako, Glostrup, Denmark) containing 70% dimethylformamide and a telomere-specific FITC-conjugated (C,TA)n peptide nucleic acid probe. After 10 min at 82°C samples were incubated overnight at room temperature in the dark. Control samples were resuspended in hybridization solution without probe to obtain background fluorescence values. After hybridization, cells were spun down and washed twice with 4 ml PBS at 40°C for 10 min and finally resuspended in PBS containing 0.1% BSA, 10 μg/ml RNase A (Boheringer Mannheim, Indianapolis, IN), and 0.1 μg/ml propidium iodide (Calbiochem-Novabiochem, La Jolla, CA). After 4 h at room temperature in the dark, cells were analyzed on a FACSscan flow cytometer (Becton Dickinson, Sunnyvale, CA) or stored at 4°C before analysis. Signals from FITC-labeled beads (Quantum premixed; Flow Cytometry Standards, San Juan, PR) were measured at the beginning and at the end of every experiment. Signals were acquired in linear scale mode, and events were gated according to propidium iodide fluorescence to restrict analysis to cells with diploid DNA content, as described before (33).

Results

Expression of telomerase activity in hTERT-transduced CD8^+ T lymphocytes

the mean number of cell divisions (MNCD) was calculated according to the formula

\[
MNCD = \frac{X}{n_i}
\]

where \(n_i\) is the number of cell divisions corresponding to peak \(i\) and \(X_i\) is the fraction of cells in this peak.

To induce endogenous telomerase activity, complete PBMC (10^6 cells/ml) were stimulated with immobilized anti-CD3 (OKT3, 10 μg/ml) plus soluble anti-CD28 (CK248, 1 μg/ml) in the presence of 150 U/ml of IL-2. After 5 days of culture, CD8^+ cells were isolated by magnetic sorting, and telomerase activity was determined.

Retrovirus construction and infection

The hTERT retroviral construct was made by PCR amplification of full-length hTERT cDNA that was inserted into the mouse stem cell virus (MSCV) pac vector (27) provided by Dr. D. R. Littman (New York University Medical Center, New York, NY). Amphototropic viruses were generated by the transfection of this plasmid into 293T cells (28). CD8^+ bulk cultures and clones were infected 3 days after stimulation according to a spin infection protocol (29) repeated three times at daily intervals. The efficiency of transduction, estimated by staining with specific anti-hTERT Ab (see below), varied from 1 to 5%. Transduced cells were selected in 1.0 μg/ml puromycin for 1 wk.

FIGURE 2. Ectopic expression of hTERT at the single-cell level. Indirect immunofluorescence of fixed CD8^+ T lymphocytes, A, Double-staining with anti-nuclear pore Ab (left) and anti-hTERT Ab (right) of nontransduced (control) and hTERT-transduced cells from bulk cultures. Bar, 5 μm. B, Staining with anti-hTERT Ab of nontransduced cells (control; left) and hTERT-transduced cells (right) of the CTL clone LAU 203 0.3/3. Bar, 15 μm. Note that the pictures in (A) were acquired on a confocal microscope, whereas (B) shows pictures taken with a lower resolution light microscope.
lymphocyte population but transduced with a control vector. These results indicate that the introduced hTERT cDNA is expressed in T lymphocytes as efficiently as in fibroblasts, and that in both cell types hTERT is the component that limits telomerase activity. Nuclear extracts from cells transduced with the empty vector or the hTERT construct were prepared 3 wk after stimulation with PHA, when the transiently induced endogenous telomerase activity had declined to barely detectable levels. For any given extract there was a linear correlation between the signals due to telomerase products (as measured by phosphor imager) and the amount of extract added (Fig. 1B), indicating that the efficiency of the PCR step did not vary between reactions. Addition of increasing amounts of extract from vector-transduced, telomerase-negative cells to a TRAP reaction with 3000 cell equivalents of hTERT-transduced lymphocytes did not significantly affect the level of telomerase activity measured (Fig. 1D), indicating that at the concentrations used, lymphocyte extracts do not inhibit telomerase activity.

To compare maximal endogenous telomerase activity with the one due to the transgene, we also performed TRAP assays with extracts of CD8+ T cells activated with immobilized anti-CD3 Ab plus soluble anti-CD28 Ab, a method that has been reported to efficiently induce telomerase activity in cultured human T cells (14, 17). Extracts were prepared 5 days after stimulation, at the peak of induced telomerase activity. As shown in Fig. 1, the activity detected in activated normal lymphocytes was approximately four times lower than that in hTERT-transduced cells. This difference could reflect low endogenous enzyme activity in all activated normal cells, or high telomerase levels restricted to a small fraction of cells. To address this question we stained cells, by indirect immunofluorescence, with an Ab specific for hTERT (M.Amacker and J. Lingner, manuscript in preparation). Observation by confocal microscopy showed a pattern of distinct spots in the nucleus, as previously described for tumor cells (35), in >90% of the lymphocytes transduced with hTERT, whereas only background fluorescence was detected in nontransduced control cells (Fig. 2A). No strongly stained cells were detectable among lymphocytes activated with anti-CD3/anti-CD28 Abs (data not shown), indicating that the difference between the average telomerase activity of the transduced and activated control lymphocytes is mainly due to a lower level of endogenous hTERT in all normal activated cells. Transduced cells could be expanded for several weeks with no loss of hTERT expression.

In addition to bulk cultures of freshly isolated CD8+ T lymphocytes we infected two Ag-specific CTL clones with the same retroviral hTERT construct. Clone NH 55 recognizes influenza matrix (FluMA) peptide 58–66 (23), whereas clone LAU 203 0.3/3 is specific for the tumor-associated Ag Melan-A26–35 (24) (see Materials and Methods). Light microscopy of cells stained with anti-hTERT Abs revealed that >90% of hTERT-transduced cells expressed high levels of hTERT protein, whereas control cells did not show any staining above background (Fig. 2B). At the resolution obtained with the light microscope the punctuate pattern seen in Fig. 2A is not resolved.

**Ectopic hTERT expression does not prevent growth arrest of CD8+ T lymphocytes**

To determine whether ectopic hTERT expression extended the lifespan of T lymphocytes, we expanded cells infected with MSCV virus containing hTERT cDNA or with the empty vector (carrying only the puromycin resistance gene), as well as nontransduced cells, by periodic stimulation with PHA plus irradiated PBMC in the presence of IL-2. Living cells were counted at least once a week to determine the number of population doublings. Bulk cultures of nontransduced CD8+ T lymphocytes underwent 16–19 population doublings before proliferation ceased, in agreement with previous reports (36, 37). Cell populations expressing the hTERT transgene stopped growing after a similar number of population doublings as matched control populations (Fig. 3, A and B). After growth arrest, cells not only failed to proliferate when re-stimulated with PHA and irradiated feeder cells, but massive cell death by apoptosis was often observed instead (data not shown).
The apparent lag in the growth kinetics of the transduced population shown in Fig. 3A probably reflects the effect of inhibitory products generated by dying cells eliminated by the puromycin selection. Note that no lag is observed when growth curves of cells transduced with the hTERT construct and with the empty virus are confronted (Fig. 3B).

Similar experiments were performed with two Ag-specific CTL clones, whose rate of proliferation was continuously followed by CFSE staining (25, 26). At weekly intervals, cell aliquots were stained with CFSE and analyzed by flow cytometry 3 and 7 days later to estimate the mean number of cell divisions during these periods. Comparison of the CFSE data with viable cell counts indicated that cells underwent about twice as many divisions as population doublings. Similarly to what we had observed in the bulk cultures, hTERT transduction did not affect the proliferative capacity of either CTL clone (Fig. 4, A and B). CFSE staining of cells after growth arrest revealed that the cells had ceased to divide. This indicates that the constant number of living cells in the arrested cultures is not the consequence of equilibrium between cell division and cell death.

**Ectopically expressed hTERT maintains CD8\(^+\) T lymphocyte telomeres**

We used flowFISH to monitor telomere length in cultured CD8\(^+\) T lymphocytes. The technique quantifies, by flow cytometry, the fluorescent signals from individual cells after in situ hybridization with a fluorescent peptide nucleic acid probe specific for telomere repeats (20, 33, 34). Frozen aliquots of cells collected at different times from the same population were all processed in the same experiment. Cells were stained with propidium iodide, and telomere fluorescence was measured only in G1 phase cells. Representative histograms are shown in Fig. 5. The fluorescence of every individual cell is proportional to the total telomere length of its chromosomes. An estimate of the mean telomere length is obtained by comparing cellular signals with those from fluorescent bead standards, applying the equation derived by Rufer et al. (20).

An increase in telomere fluorescence of freshly isolated CD8\(^+\) T lymphocytes could be observed during the first 2 wk of culture after the initial stimulation. Thereafter telomere signals gradually declined until the time when the cells stopped dividing (Fig. 3, C and D). In contrast, transduction with hTERT cDNA resulted in a progressive increase in telomere length. When the cells stopped proliferating, telomeres had reached an average length slightly higher than the peak observed 2 wk after the first stimulation. The coefficient of variation of all the fluorescence histograms is similar, indicating that the decrease in telomere length during culture as well as the increase after hTERT transduction affects the entire population of cells and not only a subpopulation. This homogeneity correlates well with the uniform staining of the transduced cell population.

**FIGURE 4.** In vitro growth and telomere length evolution in CTL clones NH 55 and LAU 203 0.3/3. Infections with hTERT retroviral constructs were conducted on days 4–6. Mean numbers of cell division were determined by weekly staining of cells with CFSE and analysis of CFSE fluorescence histograms 3 and 7 days later (A and B). Telomere length was measured by flowFISH (C and D). Black, Background; no shading/dashed line, cells at the time of transduction (day 4); no shading/solid line, nontransduced control cells on day 30; gray, hTERT-transduced cells on day 30.

**FIGURE 5.** Typical examples of telomere fluorescence histograms. Total telomere fluorescence in single cells of CD8\(^+\) T lymphocyte bulk cultures was measured by flowFISH. Black, background; no shading, control cells (transduced with empty vector); gray, hTERT-transduced cells.
populations with anti-hTERT Abs. Telomere fluorescence measurements in transduced and nontransduced CTL clones after 30 days of culture show that ectopic hTERT expression results in telomere lengthening also in these cells (Fig. 4, C and D).

Discussion

Most differentiated human cells do not express telomerase activity, and their telomeres shorten during proliferation. This can be prevented by ectopic expression of hTERT. Freshly isolated, mature T lymphocytes are peculiar in the sense that they transiently express telomerase activity upon activation; in this respect they resemble hematopoietic stem cells in expansion (13). However, the amplitude of telomerase expression declines with repeated stimulation cycles, and telomeres do shorten (16). We have found that telomere length increases in freshly isolated T cells during 2 wk after the first stimulation, before it starts to decline. This suggests that during the initial period following T lymphocyte activation, endogenous telomerase activity is sufficient to increase telomere length. However, it might also reflect selection for naive T cells with long telomeres. Telomere lengthening has also been described in normal, in vivo proliferating B lymphocytes (19). Results from a recent study (38) suggest that both hTERT and the RNA template subunit of telomerase (hTR) are up-regulated after lymphocyte activation, and that there is a correlation between the levels of hTERT expression and telomerase activity. Our experiments with hTERT-transduced T lymphocytes cultured for extended periods show that hTERT levels are not limiting.

As in fibroblasts (7), ectopic expression of hTERT induces telomere lengthening in T cells. But unlike fibroblasts or endothelial cells (39), hTERT-transduced T lymphocytes cease to proliferate after a similar number of population doublings or cell divisions as control cells. This indicates that telomere shortening is not the only process that limits the life span of T cells. However, it cannot be excluded that one or a few telomeres continue to shorten in hTERT-transduced cells and trigger growth arrest when they reach a critical size. To rule out this unlikely hypothesis it will be necessary to quantify individual telomere length by Q-FISH (40).

Our results are reminiscent of the finding that immortalization of human keratinocytes and breast epithelial cells depends on additional changes besides hTERT expression, namely, on inactivation of the Rb protein/p16 pathway (11). One explanation for the different requirements for the immortalization of fibroblasts, on the one hand, and certain epithelial cells, on the other, is that the culture conditions for the latter are suboptimal and lead to activation of the p16/Rb stress pathway. A similar explanation may apply to T lymphocytes. Progressive p16 accumulation during T cell in vitro proliferation has indeed been reported (41), but the culture system used did not allow restimulation of cells with PHA, suggesting that it was inadequate for maximal expansion of T cells by this method.

An alternative explanation for the growth arrest of CD8+ T lymphocytes under the conditions used in our experiments may be that the stimulation requirements of T lymphocytes change during clonal expansion as part of a differentiation process of naive into memory cells. Thus, the apparent senescence of these cells may not be due to the intrinsic limitation of the proliferative capacity of the cells, but rather to the loss of the ability to proliferate in response to the stimuli provided (PHA + feeder cells plus IL-2). One change that might reduce responsiveness is the decrease in the expression of the costimulatory receptor CD28 in T cells that have undergone a high number of divisions. It has been reported that CD28-mediated costimulation is necessary to prevent cell death during T cell activation (42) and that CD28 expression declines during in vitro culture and is virtually absent in senescent cells (43). We have compared CD28 expression on lymphocytes transduced with hTERT or with the control vector, at different times of culture and in senescent cells (data not shown). In both populations we observed a similar gradual loss of CD28 expression during culture. Loss of CD28 may be one of the causes why growth-arrested T cells respond to restimulation with PHA and feeder cells by apoptosis rather than proliferation. Down-regulation of other costimulatory molecules such as CD134 (OX-40) and CD154 (CD40L), or IL receptors such as IL-4R, IL-7R, and IL-15R and the IL-2R common γ chain (4) may also affect responsiveness of these cells to TCR-mediated signaling. According to this hypothesis, transduction of CD8+ T cells with components that restore their capacity to respond to the same signals as naive cells should allow the cells to proliferate further until their telomeres become critically short. We predict that, as in fibroblasts, the latter limit can indeed be overcome by ectopic expression of hTERT. Thus, hTERT may be required but is not sufficient to immortalize human T lymphocytes.

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