Immortalization of Human CD8+ T Cell Clones by Ectopic Expression of Telomerase Reverse Transcriptase

Erik Hooijberg, Janneke J. Ruizendaal, Peter J. F. Snijders, Esther W. M. Kueter, Jan M. M. Walboomers and Hergen Spits

*J Immunol* 2000; 165:4239-4245; doi: 10.4049/jimmunol.165.8.4239
http://www.jimmunol.org/content/165/8/4239

References  This article cites 42 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/165/8/4239.full#ref-list-1

Why The JI? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

Subscription  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2000 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Immortalization of Human CD8+ T Cell Clones by Ectopic Expression of Telomerase Reverse Transcriptase¹

Erik Hooijberg,²* Janneke J. Ruizendaal,²* Peter J. F. Snijders, † Esther W. M. Kueter, * Jan M. M. Walboomers, † and Hergen Spits³*

Repetitive senescence of T cells is correlated with erosion of telomere ends. Telomerase plays a key role in maintaining telomere length. Therefore, it is thought that telomerase regulates the life span of T cells. To test this hypothesis, we have over-expressed human telomerase reverse transcriptase in human CD8+ T cells. Ectopic expression of human telomerase reverse transcriptase led to immortalization of these T cells, without altering the phenotype and without loss of specificity or functionality. As the T cells remained dependent on cytokines and Ag stimulation for their in vitro expansion, we conclude that immortalization was achieved without malignant transformation. The Journal of Immunology, 2000, 165: 4239–4245.

The ends of linear eukaryotic chromosomes, which are called telomeres, consist of DNA-protein complexes ending in a large duplex loop (1). They serve to maintain chromosomal integrity and prevent end-to-end fusion of the chromosomes. Telomere length is not constant over time. The telomeric ends have a length of 5–15 kb in humans and shorten by 50–100 bp per cell division in normal somatic cells (2, 3). When telomeric ends get too short, cells will enter a state of replicative senescence followed by crisis and cell death. Thus, telomere shortening may prevent unlimited proliferation of human somatic tissues. Telomere shortening is counteracted by the ribonucleoprotein enzyme complex called telomerase, which has two key components, the telomerase reverse transcriptase (TERT)⁴ and telomerase RNA, which is used as a template to elongate telomeric ends (for reviews see Refs. 4–6). The crucial role of human (h)TERT in maintaining telomeric length and subsequently of the replicative life span of cells has been demonstrated recently. It has been documented that ectopic expression of hTERT, in cell types without endogenous expression of hTERT, led to elongation of the telomeres and to an increased life span of foreskin fibroblasts, retinal pigment epithelial cells, and endothelial cells (7–9), indicating that endogenous expression of hTERT, led to immortalization of these T cell clones, without altering the phenotype and without loss of specificity or functionality. As the T cells remained dependent on cytokines and Ag stimulation for their in vitro expansion, we conclude that immortalization was achieved without malignant transformation.

¹Department of Immunology, The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, and ²Department of Pathology, Section Molecular Pathology, University Hospital Free University, Amsterdam, The Netherlands

Received for publication April 14, 2000. Accepted for publication July 24, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Grant 96-1275 from the Dutch Cancer Society, Amsterdam.

2 Current address: Department of Pathology, Section Molecular Pathology, University Hospital Free University, De Boelelaan 1117, NL-1081HV, Amsterdam, The Netherlands. E-mail address: erik.hooijberg@azvu.nl

3 Address correspondence and reprint requests to Dr. Hergen Spits, Department of Immunology, The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, NL-1066CX, Amsterdam, The Netherlands. E-mail address: hergen@nki.nl

4 Abbreviations used in this paper: TERT, telomerase reverse transcriptase; hTERT, human TERT; GFP, green fluorescent protein; PD, population doubling; TRAP, telomeric repeat amplification protocol.

Copyright © 2000 by The American Association of Immunologists 0022-1767/00/$02.00
Materials and Methods

Cell lines

The virus-producing cell line Phoenix, the melanoma cell line Mel-AKR, and the EBV-transformed B cell lines JY and EBV-AKR, were grown in medium consisting of Iscove’s medium (Life Technologies B.V., Breda, The Netherlands) supplemented with 5–10% FCS (BioWhittaker, Verviers, Belgium), penicillin, and streptomycin (Boehringer Mannheim, Mannheim, Germany).

T cell blasts and T cell clones

T cell blasts were prepared by incubation of 5 × 10⁶ PBMC per ml in Yssel’s medium supplemented with 1% normal human serum and 2 µg/ml PHA. T cell blasts and Ag-specific T cell clones were cultured as previously described (27). Briefly, 3 × 10⁵ cells/well were cultured weekly with a mixture of 1 × 10⁶ irradiated (80 Gy) allogeneic PBMC/ml and 1 × 10⁵ irradiated EBV-B cells (JY), supplemented with 100 ng/ml PHA and 20 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands) in Yssel’s medium. In some experiments, T cell clones were cultured with cytokines without weekly stimulations. The following concentrations of cytokines were used: 20 IU/ml IL-2, 10 ng/ml IL-7 (PeproTech, Rocky Hill, NJ), and 10 ng/ml IL-15 (Peprotech). Cell cultures were kept in incubators at 37°C in humidified air containing 5% CO₂.

Construction of the retroviral hTERT vector

The full-length coding sequence of hTERT was isolated from pGRN145 (kindly provided by Geron, Menlo Park, CA) as a 3.5- kb EcoRI-Ncol fragment. The hTERT fragment was subsequently ligated into the polylinker of LZRS-linker-internal ribosomal entry site-GFP (28). Correct cloning of hTERT was confirmed by restriction enzyme analysis. This construct designated LZRS-hTERT-IRES-GFP was used to produce retroviral supernatant as previously described (28, 29). As control, we used LZRS-polylinker-IRES-GFP.

Transduction method

The recombinant human fibronectin fragments transduction procedure (RetroNectin; Takara, Otsu, Japan) was based on a method originally developed by Hanenberg et al. (30) with the modifications described by Heemskerk et al. (28). T cells were prestimulated with PHA (31) or with a feeder cell mixture containing PHA and IL-2 for 32–48 h before transduction. Subsequently, the target cells were plated on RetroNectin-coated dishes (maximum 5 × 10⁶ cells/petri dish with a diameter of 3 cm) in 0.5 ml of complete medium mixed with 1 ml of thawed retroviral supernatant. Cells were cultured at 37°C for 6 h or overnight, washed, and transferred to 24-well culture plates (Falcon plastics; Becton Dickinson Labware, Mountain View, CA). The capability of hTERT retrovirus to induce telomerase activity was determined in cultures of primary human keratinocytes devoid of detectable telomerase activity (data not shown).

Flow cytometric analysis

CD2, CD3, CD4, CD8, CD25, CD27, CD28, CD38, CD45, CD45RA, CD45RO, CD54, CD69, CD80, anti-TCRαβ, anti-HLA-DR, and anti-HLA-class I mAbs (all from Becton Dickinson) directly labeled with PE were used for flow cytometric analysis. HLA-A2 tetramers containing the M1/Melan-A²⁷–₃₅, the Tyrosinase₃₆₈–₃₇₆ or the influenza-A₅₈–₆₆ epitope were used for flow cytometric analysis. HLA-A2 tetramers containing the M1/Melan-A²⁷–₃₅, the Tyrosinase₃₆₈–₃₇₆ or the influenza-A₅₈–₆₆ epitope labeled with PE were prepared as previously described (28). Stained cells were analyzed using a FACScan (Becton Dickinson), and the data were processed with CellQuest computer software.

Chromium release assays

Cytotoxicity of T cell clones was determined using a standard chromium release assay as previously described (32). All assays were performed in the presence of a 50-fold excess of unlabeled K562 cells to block nonspecific lysis of the target cells. The spontaneous release varied between 10 and 25% of the maximum. SD of triplicate determinations never exceeded 10%.

Measurement of telomerase activity and hTERT mRNA levels

Cell lysates for analysis of telomerase activity were prepared from ~1 × 10⁶ cells using the CHAPS (3-[3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) detergent method as described before (33, 34). Briefly, cells were suspended in 50 µl lysis buffer. After homogenization, the suspension was placed on ice for 30 min. Subsequently, the cells were spun down at maximum speed in a microcentrifuge for 30 min at 4°C. The supernatant was transferred to a new tube and snap-frozen in liquid nitrogen. The amount of protein was determined using the Bio-Rad protein detection kit (Bio-Rad Laboratories, Veendael, The Netherlands). From the resulting pellet, RNA was isolated using RNAzolB (Campro Scientific, Veendael, The Netherlands) for subsequent hTERT mRNA analysis.

Telomerase activity was determined in samples containing various amounts of protein (representative for 100–10,000 cells used) by the telomeric repeat amplification protocol (TRAP). The TRAPEze kit ( Oncor, Gaithersburg, MD) was used according to instructions of the manufacturer. To determine the specificity of the assay, the protein sample was heated for 10 min at 70°C to inactivate telomerase and tested in parallel experiments. No stepeladder patterns were observed after this preheating step. Following separation of TRAP products on polyacrylamide gels, autoradiography was performed overnight at ~80°C using intensifying screens. Relative telomerase activities were quantified by densitometric evaluation of the TRAP ladder bands relative to the corresponding internal controls. Semiquantitative RT-PCR for hTERT was performed essentially as previously described (34) except that 25 instead of 30 PCR cycles were run to ensure linearity of the amplification reaction. First-strand cDNA synthesis was performed on 100 ng of total RNA using antisense primers for both hTERT and the housekeeping gene snRNP U1A in a single reaction. Subsequent RT-PCR for hTERT and snRNP U1A were performed in a single reaction as well, using primers previously described (34). Resulting PCR products were run in duplicates on the same agarose gel and blotted to the same nylon membrane, followed by hybridization with radiolabeled hTERT and snRNP U1A-specific oligonucleotide probes, respectively. Signal intensities were measured after exposure of the hybridized filters to a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Relative levels of hTERT mRNA were calculated according to the following formula: intensity ratio hTERT/snRNP U1A.

Telomere length assay

For measurement of telomere length, genomic DNA was extracted from ~1.5 × 10⁶ cells using the PureGene DNA extraction kit (Gentra Systems, Minneapolis, MN). Five micrograms of DNA were digested with BgIII (Boehringer Mannheim) and electrophoresed through a 0.7% agarose gel before Southern blotting. The blot was subsequently hybridized to a [32P]ATP end-labeled telomeric oligoprobe (T2G3G4). Autoradiography was performed for 5 days. To assess the median telomere length, the peak signal intensity was determined by PhosphorImager analysis at a short exposure time to avoid problems of overexposure of the signal.

Results

Enhancement of expansion of human T cells upon ectopic expression of hTERT

We introduced hTERT cDNA in a human CD8⁺ T cell clone to test the effect of ectopic expression of hTERT on expansion and extension of the life span of human T cells. The T cell clone (AKR-IL7-clone no. 4) has been obtained after stimulation of patient-derived peripheral T cells with autologous melanoma cells genetically engineered to produce IL-7 (E. Hooijberg, J. J. Ruizendaal, and H. Spits, manuscript in preparation). This T cell clone is specific for the HLA-A2-restricted Mart-1/Melan-A²⁷–₃₅ epitope. After establishment of this T cell clone, the cells were cultured for 6–8 wk before transduction. In this period, the cells went through 2 PD per week, giving a total number of 12–16 PD.

To address the question whether the introduction of hTERT had an effect on T cell expansion, we followed the expression of green fluorescent protein (GFP) upon further culturing of hTERT-IRES-GFP and control-GFP-transduced T cells. The level of GFP expression in hTERT-IRES-GFP-transduced cells showed an exponential increase over time in two independent experiments with T cells from the same clone from different frozen batches (Fig. 1). In a period of 7 wk, the percentage of hTERT-IRES-GFP-positive cells increased from the initial 5% and 3%, respectively, measured 5 days after retroviral transduction, to 95% on day 53 (Fig. 1). The percentage of hTERT-IRES-GFP-positive cells remained stable (~95%) for another period of 5 wk, after which this experiment of coculturing hTERT-IRES-GFP-positive and -negative cells was terminated. Based on weekly counting of the cells and the percentages of GFP-positive and -negative cells, we calculated the...
average weekly expansion of hTERT-IRES-GFP-positive T cells and of the untransduced cells to be 10-fold (3 PD) and 5-fold (2 PD), respectively.

The higher expansion rate of hTERT-IRES-GFP transduced T cell fraction was not due to the retroviral-mediated gene transfer procedure or to the integration of the provirus into the genome of the T cell clone. Transduction with GFP-only control virus led to expression of the marker in 12% of the T cells as measured 5 days after the transduction (Fig. 1). The level of GFP expression remained stable in this T cell culture for a period of 53 days, after which the cells went into crisis and died. Thus, the maximum number of PD of the control-transduced cells was 30 –35, which is comparable to that of the untransduced cells of this clone (data not shown). These data indicate that retroviral transduction per se does not lead to enhanced expansion or extension of the life span of T cells.

**Extension of the life span of human T cells upon ectopic expression of hTERT**

The experiments shown in Fig. 1 strongly suggest that ectopic expression of hTERT results not only in an increased expansion but also in an extension of the life span of the cells. To obtain more information on the degree of life span extension of the hTERT-transduced cells, we performed subcloning experiments. The maximum number of PD of established CD8+ T cells transduced with hTERT-IRES-GFP or the control GFP only over time (given in days). The cultures were stimulated with a feeder cell mixture every week. On day 32, the cells were sorted in a GFP-positive and a GFP-negative fraction for further studies. The result of a second hTERT-IRES-GFP transduction experiment with a different batch of T cells of the same clone is also indicated (□).

### Ectopic expression of hTERT in human T cells does not alter functional and phenotypic characteristics

After having established that ectopic expression of hTERT leads to an extended life span, we examined whether it affected the cell surface phenotypes, the specificities, and the functions of the transduced T cells. No differences between the original T cell clone and the hTERT-transduced subsubclones were found in the expression of any of the cell surface markers tested (Table I). It is of note that the original clone as well as the hTERT-transduced T cells were negative for CD27 and CD28, and positive for CD45RO, which is the typical phenotype of memory T cells (35).

To examine whether ectopic expression of hTERT affected the specificity of the T cells, we tested these four clones (wild type no. 4, A11, D8, and F6) for binding of HLA-A2-Mart-1/Melan-A27–35 tetramers. Not only the original clone but also the three stained with the HLA-A2-Mart-1/Melan-A27–35 tetramers, but not with control tetramers (Fig. 2A). Consistent with this observation, the parental clone and the three hTERT transduced subsubclones lysed autologous melanoma cells, and peptide (Mart-1/Melan-A27–35) loaded EBV-B cells to the same extent (Fig. 2B). As expected, none of the clones lysed untreated EBV-B cells.

**Table I. Phenotypic analysis of wild-type AKR-IL7 no. 4 and hTERT-transduced subsubclones A11, D8, and F6**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Wild-Type AKR-IL7 no. 4</th>
<th>Sub-Sub-Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG control</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TCR αβ</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD3</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD8</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD25</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>CD27</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD28</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD38</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD45</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD45RA</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>CD45RO</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD54</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD69</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD80</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HLA-class I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

*The original clone and three isolated hTERT-transduced subsubclones, which, at the time of analyses, differed >45 PD in life span, were phenotyped. T cells were stained with PE-labeled antibodies and analyzed on a FACScan. Gates were set to contain live cells only. Indicated are the levels of expression in four categories as follows: MFI < 10, –; MFI 10–50, ±; MFI 50–500, +; MFI > 500, ++.*

**FIGURE 1.** Boosted expansion of hTERT-transduced human T cells. T cells were transduced with a retrovirus containing hTERT-IRES-GFP or the marker gene, GFP, only. The T cell clone used here is a tumor-specific, CD8+, HLA-A2-restricted clone recognizing the Mart1/Melan-A27–35 epitope. Indicated is the percentage of cells positive for the marker in T cells transduced with hTERT-IRES-GFP (□) or the control GFP only (○) over time (given in days). The cultures were stimulated with a feeder cell mixture every week. On day 32, the cells were sorted in a GFP-positive and a GFP-negative fraction for further studies. The result of a second hTERT-IRES-GFP transduction experiment with a different batch of T cells of the same clone is also indicated (□).
for the subsubclones (3 PD/wk). These findings demonstrate that T cells expressing ectopic hTERT maintained normal growth characteristics for prolonged periods of time.

**Increased hTERT mRNA levels, telomerase activity, and stabilization of telomeres in T cells ectopically expressing hTERT**

We next examined the hTERT mRNA levels, telomerase activity, and telomere lengths in untransduced and hTERT-transduced T cells. Ten days after sorting GFP-negative and -positive cells, the levels of hTERT mRNA and of telomerase activity were assayed in the sorted cells. Semi-quantitative RT-PCR for measurement of hTERT mRNA levels, relative to a household gene, was performed on day 42 after retroviral transduction of the wild-type T cell clone no. 4, 1 wk after the last stimulation with a feeder cell mixture. At that time point, the feeder cells were completely cleared. The hTERT mRNA levels were about 5-fold increased in GFP-positive cells compared with the GFP-negative counterparts (Fig. 3A). Telomerase activity was high in both cell fractions and could be detected in as little as 5 ng of protein lysate, which is equivalent to ~100 cells (Fig. 3B). Densitometric scanning of TRAP ladder bands compared with the internal control yielded a two to three times higher OD in the lanes corresponding to the GFP-positive cells, indicating a 2- to 3-fold increased telomerase activity. Moreover, a slight decrease in mean telomere length (median length of 6–6.5 kb vs 7 kb) and a smear indicating loss of telomere integrity was observed in the GFP-negative cells compared with the GFP-positive T cell fraction (Fig. 3C). At the time of analysis, 12 PD had occurred in the GFP-negative and 18 in the GFP-positive cell populations since the transduction. A larger difference in telomere length between wild-type and the hTERT-transduced cells may be expected near the point of senescence of the wild-type clone no. 4. Unfortunately, this could not be verified because it is difficult to obtain enough material from the GFP-negative fraction or of wild-type T cells close to the point of senescence. Accurate measurement of hTERT mRNA levels, telomerase activity, and telomere lengths is also severely hampered by the presence of contaminating feeder cells, in such near-senescent cultures. In cultures with well-growing T cell clones, these irradiated feeder cells are rapidly cleared. To obtain independent proof for the biological activity of the transduced hTERT in the T cell clones, we compared the telomere lengths of untransduced wild-type T cells of the same clone with the isolated subsubclones A11, D8, and F6. Fig. 3D shows that stabilization of telomere length had occurred in the hTERT-IREs-GFP-positive T cell clones, which were subcloned twice (A11, D8, and F6). After being subcloned twice, these clones were maintained in vitro in culture for another 11 wk, which involved on average ~3 PD per week. At the time of telomere length analysis, these cells differed 60–70 PD from the cells analyzed 10 days after sorting and of the wild-type cells used in this analysis. It is apparent that the telomeres in these clones (A11, D8, F6) had not eroded and were maintained at an average length of 7 kb (Fig. 3D).

**Rescue of a tyrosinase-specific T cell clone by ectopic expression of telomerase**

To demonstrate that immortalization by ectopic expression of hTERT is not restricted to clone no. 4, we transduced another T cell clone with hTERT-IREs-GFP. This HLA-A2-restricted, CD8+, T cell clone (AKR-IL7-clone no. 108) is specific for the Tyrosinase368-376 epitope, and has been obtained from the same stimulation experiment as AKR-IL7-clone no. 4. Clone no. 108 was selected from our collection of tumor-specific T cell clones because it has been very difficult to grow and expand, with a weekly PD of less than one. Two days after the transduction, the

---

**FIGURE 2. Functional analyses of wild-type and hTERT-transduced T cells.**

A. Cells from the wild-type AKR-IL7-no. 4 T cell clone (wt #4) and of three subsubclones (A11, D8, and F6) were stained with tetramers containing the HLA-A2-restricted Mart1/MelanA27–35 epitope (thick lines). As a negative control, tetramers containing the HLA-A2-restricted influenza epitope were used (thin lines). B. These T cells were also used in a cytotoxicity assay on autologous EBV-B cells (●), peptide-loaded EBV-B cells (□), and on the autologous melanoma cells (■). Indicated is the percentage of specific chromium release of triplicate determinations. The E:T ratio was 60. The data shown are representative for three independent experiments.

**hTERT-transduced T cells are still dependent on cytokines and Ag stimulation for survival and expansion**

An extension of the life span of somatic cells may lead to abnormal growth characteristics. We examined the growth characteristics of the three isolated subsubclones (A11, D8, and F6) in the absence and presence of cytokines. We cultured the hTERT-transduced T cells in medium without cytokines, or with added IL-2, IL-7, IL-15, or mixtures of these cytokines. IL-7 alone was not capable of supporting survival of either of the subsubclones, nor of the original clone. The hTERT-transduced cells could be maintained in medium containing IL-2 or IL-15 for a period up to 3 mo (longer periods were not tested) without significant expansion. Like the original, untransduced clone, the subsubclones remained highly dependent on periodic activation for their growth. The expansion of T cell cultures in a feeder cell mixture containing IL-2 and PHA was about 5 for the original clone (2 PD per week) and about 10
Fractions 10 days after cell sorting, which was 7 wk after transduction. RT 2 snRNP U1A were compared between GFP-positive and GFP-negative cell fractions mentioned above. IC indicates the internal control. Densitometric scanning of TRAP ladder bands compared with the internal control yielded a two to three times higher OD in the lanes corresponding to the GFP-positive cells, indicating a 2- to 3-fold increased telomerase activity. C. Telomere lengths were determined on GFP-positive and GFP-negative cells 10 days after sorting, which is 7 wk after transduction. The transduced cells went through 2 PD a week.

FIGURE 3. Determination of mRNA levels, telomerase activity, and telomere length in wild-type and hTERT-transduced T cell clones. T cell cultures were stimulated every week with a feeder cell mixture. One week after stimulation, hTERT mRNA levels, telomerase activity, and telomere lengths were determined. Experimental details are given in Materials and Methods. A, The levels of mRNA of hTERT and the household gene snRNP U1A were compared between GFP-positive and GFP-negative cell fractions 10 days after cell sorting, which was 7 wk after transduction. RT - and RT + indicate cDNA synthesis in the absence or presence of added reverse transcriptase respectively. The relative hTERT mRNA levels were about 5-fold higher in GFP-positive cells compared with the GFP-negative counterparts. B, Telomerase activity was measured down to 5 ng of protein, which is equivalent to 100 cells, derived from cell lysates of the same cell fractions mentioned above. IC indicates the internal control. Densitometric scanning of TRAP ladder bands compared with the internal control yielded a two to three times higher OD in the lanes corresponding to the GFP-positive cells, indicating a 2- to 3-fold increased telomerase activity. C, Telomere lengths were determined on GFP-positive and GFP-negative cells 10 days after sorting, which is 7 wk after retroviral transduction of the cells. At the time of analysis, 12 PD had occurred in the GFP-negative fraction and 18 in the GFP-positive fraction since retroviral transduction. D, Telomere lengths were also determined on early passage wild-type AKR-IL7 no. 4 T cells (lane 1), and on three subsubclones, A11, D8, and F6, in lanes 2, 3, and 4 respectively. These clones differed 60–70 PD from the hTERT-transduced no. 4 clone (35 PD) and of other T cell clones reported previously (17, 37). These data indicate that constitutive expression of hTERT dramatically extends the life span of this CD8 + CTL clone to a point that one can speak of immortalization. Additional deliberate interference with the cell cycle was not necessary to confer life-span extension to these T cells. The immortalization should be caused by constitutive hTERT expression because transduction of T cells with the GFP control virus did not lead to an increased expansion, nor to an extension of their life span. The constitutive hTERT expression in hTERT-transduced no. 4 cells correlated with a 5-fold higher hTERT mRNA expression and 3-fold higher enzymatic activity compared with untransduced T cells as measured 7 days after the last stimulation. Expansion of no. 4 T cells requires restimulation of these cells every 7–10 days. The cells do not become “resting” in this period of time. To determine whether hTERT transduction into CD8 + T cells results in sustained mRNA expression and enzymatic activity independent of the activation stage of the cells, we used freshly isolated CD8 + T cells. These bulk T cells can be maintained alive without repeated restimulation for a more prolonged period of time than the cloned T cells. T cells transduced with hTERT-IRE5-GFP

functionality of these cells as autologous melanoma cells, and peptide-loaded EBV-B cells were lysed, whereas EBV-B cells without exogenous peptide were not (data not shown).

Discussion

T cells express hTERT upon activation (23). Despite the presence of telomerase, the telomere ends of T cells cultured in vitro erode, giving rise to replicative senescence (20, 36). This raises the question whether the replicative life span of T cells is regulated solely by hTERT. This is a relevant question because some cell types like keratinocytes require interference with cell cycle control mechanisms as well as ectopic hTERT expression for significant extension of their replicative life span (10). Here, we investigated the life span of two CD8 + T cell clones transduced with hTERT. The subsubclones of the hTERT-transduced no. 4 T cells have undergone >100 PD at the time of writing, which is much more than the maximum number of PD of the untransduced cells or of control-transduced no. 4 clone (35 PD) and of other T cell clones reported previously (17, 37). These data indicate that constitutive expression of hTERT dramatically extends the life span of this CD8 + CTL clone to a point that one can speak of immortalization. Additional deliberate interference with the cell cycle was not necessary to confer life-span extension to these T cells. The immortalization should be caused by constitutive hTERT expression because transduction of T cells with the GFP control virus did not lead to an increased expansion, nor to an extension of their life span. The constitutive hTERT expression in hTERT-transduced no. 4 cells correlated with a 5-fold higher hTERT mRNA expression and 3-fold higher enzymatic activity compared with untransduced T cells as measured 7 days after the last stimulation. Expansion of no. 4 T cells requires restimulation of these cells every 7–10 days. The cells do not become “resting” in this period of time. To determine whether hTERT transduction into CD8 + T cells results in sustained mRNA expression and enzymatic activity independent of the activation stage of the cells, we used freshly isolated CD8 + T cells. These bulk T cells can be maintained alive without repeated restimulation for a more prolonged period of time than the cloned T cells. T cells transduced with hTERT-IRE5-GFP
or with GFP-control virus were isolated and restimulated with PHA and feeder cells. The hTERT-transduced CD8⁺ T cells showed high levels of hTERT mRNA expression and enzymatic activity on days 7, 10, 14, and 22 after stimulation. The levels of expression and activity were comparable at all time points. In contrast, both mRNA expression and enzymatic activity had dropped to baseline levels already 7 days after activation of GFP-control transduced CD8⁺ T cells and of untransduced control T cells (results not shown). Because after 2–3 wk these three bulk CD8⁺ T cell fractions have become in a resting state, we conclude that hTERT transduction leads to sustained mRNA expression and telomerase activity independent of the activation stage of the T cells.

The notion that hTERT transduction results in a sustained functional expression is strongly supported by the observation that the subclones A11, D8, and F6 had the same telomere length of 7 kb as the wild-type clone at the time of transduction, despite the fact that the subsubclones underwent 60–70 PD after the transduction. Based on the average loss of telomere length of 50–100 bp per PD in T cells (2, 3, 20), one would expect to observe a minimum telomere end erosion of 3–3.5 kb in cell samples differing 60–70 PD. The maintenance of the telomere lengths in the hTERT⁺ sub-sub clones proves the biological activity of the transduced hTERT and indicates a correlation between the observed increase in life span of the T cells and constitutive activity of the transduced hTERT. However, whether hTERT expression is the sole cause of immortalization is not yet sure. One may argue that immortalization of the T cells is the result of hTERT expression combined with another event induced by the retroviral transduction itself either in all hTERT-transduced cells or in a very small proportion of the transduced cells. We cannot exclude these possibilities, although we consider it unlikely that a rare event that occurred in a minority of the cells which together with telomerase is responsible for the observed immortalization. We observed that, on the average, the transduced T cells underwent 3 PD and the untransduced cells 2 PD per week. Assuming that all transduced cells had a growth advantage compared with untransduced cells, one can calculate the percentage of hTERT-GFP⁺ cells expected after 6 wk of culture to increase from 5% to 76%. We observed an increase from 5% to 60% in one and from 3% to 60% in the second experiment (Fig. 1). These observations indicate that the majority of the hTERT-transduced cells have a growth advantage compared with wild-type cells. Recently, we have transduced a panel of CD4⁺ T cell clones and observed also with these clones an expansion advantage of all hTERT-transduced cells in comparison with untransduced cells (H. Yssel and H. Spits, unpublished observations). Another question is whether all hTERT-transduced cells enter a state of immortalization. The low cloning efficiency (15%) of hTERT-GFP⁺ no. 4 cells indicates that not all transduced cells are immortalized and may suggest that the site of integration of the hTERT DNA is a determining factor for immortalization. However, the cloning efficiencies of the no. 4 subclones, which should have hTERT integrated at a single site in the genome, were even lower (1.5% and 3% in two experiments with different subclones). A likely explanation for the low cloning efficiency of these subclones is that a proportion of the transduced cells lose their hTERT over time either by inactivation of the inserted DNA or by posttranscriptional inactivation.

Recently, it was reported by Liu et al. (23) that low levels of hTERT mRNA are constitutively present in CD4⁺ T lymphocytes, regardless of the telomerase activity in the cells that was activation-induced and transient, suggesting that telomerase is mainly regulated at a posttranscriptional level. However, it is possible that only a minority of the CD4⁺ T cells in the study of Liu et al. expressed constitutive telomerase mRNA levels. Moreover, while our data do not exclude posttranscriptional regulation of hTERT activity, they strongly suggest that the level of hTERT transcripts is the main regulator of the life span of T cells. Presently, we analyze the effect of constitutive hTERT expression on a large panel of CD8⁺ and CD4⁺ T cell clones to obtain insight into the regulation of telomerase in T cells in general.

It is of note that the stabilization of the telomere length as observed in the subsubclones of clone no. 4 indicates that constitutive hTERT expression does not result in telomere lengthening as has been observed in hTERT-transfected fibroblasts (7, 8) and in germinal center B cells (18, 38). The reasons for this have yet to be determined. The finding that ectopic expression of telomerase extends the replicative life span of certain cell types without net telomere lengthening is not unprecedented. In human fibroblasts transfected with hTERT, the telomeres continue to shorten to average lengths below those of untransfected cells that enter replicative senescence and crisis (39, 40). This suggests that hTERT has a role in life-span extension beyond the prevention of telomere length erosion.

Importantly, ectopic expression of hTERT did not affect the phenotype, Ag specificity, or functionality of the T cells. Of equal importance is the observation that the hTERT-transduced T cells are still dependent on cytokines and Ag stimulation for proliferation, suggesting that these T cells were not transformed. These findings are well in agreement with recent publications on the lack of induction of a transformed phenotype in hTERT-transfected normal human fibroblasts and retinal pigment epithelial cells (41, 42). The advantages of immortalized T cell clones are obvious. The availability of immortalized functional T cells with defined specificities will facilitate functional, genetic, and biochemical experiments. The limited in vitro life span of human Ag-specific T cell clones has severely hampered application of these cells in cellular therapies involving adoptive transfer of Ag-specific T cells. Our finding that tumor-specific CTL clones can be immortalized by ectopic expression of hTERT and thus can be expanded to very large numbers may lead to potential application of these cells in the treatment of cancer patients.

Acknowledgments

We thank the Geron Corporation for plasmid pGRN145 containing hTERT cDNA, Drs. L. Ma and R. D. Steenbergen for excellent technical assistance and helpful discussions, and E. Noteboom and A. Pfau for cell sorting.

References