Equivalent Induction of Telomerase-specific Cytotoxic T Lymphocytes from Tumor-bearing Patients and Healthy Individuals


Abstract

Although high frequencies of T lymphocytes specific for certain tumor-associated antigens have been detected in some cancer patients, increasing evidence suggests that these T cells may be functionally defective in vivo and fail to induce meaningful clinical responses. One strategy to overcome this limitation is to target novel antigens that are ignored during the natural antitumor immune response but are nevertheless capable of triggering effector T-cell responses against tumors after optimal presentation by antigen-presenting cells. Here, we show that the telomerase catalytic subunit, a largely universal tumor antigen identified by epitope deduction rather than from patient immune responses— is immunologically ignored by patients despite progressive tumor burden. Nevertheless, HLA-A2-restricted CTLs against hTERT are equivalently induced ex vivo from patients and healthy individuals and efficiently kill human tumor cell lines and primary tumors. Thus, telomerase-specific T cells from cancer patients are spared functional inactivation because of immunological ignorance. These findings support clinical efforts to target the hTERT as a tumor antigen with broad therapeutic potential.

Introduction

The characterization of TAA4 based on patient T-cell reactivity against tumors, such as melanoma, has raised the question of why such T-cell responses, detectable mostly in metastatic disease, are clinically ineffective. Recent evidence based on peptide/MHC tetramer analysis suggests that despite a potentially extensive clonal expansion of tumor-specific CD8+ cells in vivo, functional inactivation of these cells is an important mechanism for immune evasion (1). Antigen-specific cytolytic function is impaired, and cytokine production, even in response to mitogens, is absent (1). Restimulation ex vivo with cytokines may or may not be able to reverse this defect (1–3). Consequently, clinical efforts to target TAA defined by patient immune reactivity may require strategies to immunologically rescue antigen-specific T cells from functional inactivation.

In considering this issue, we turned to an alternative strategy for tumor antigen discovery that is not based on the natural antitumor T-cell responses of patients. In this method, peptide epitopes matching MHC class I binding motifs are deduced from genes known to be selectively expressed or overexpressed in tumor versus normal tissues. Using this approach, we have evaluated the hTERT as a nearly universal tumor antigen and identified the epitope I540 that binds to the most common MHC class I allele, HLA-A2 (4). I540 peptide triggers specific CTLs that kill hTERT+ tumors from a wide range of histologies (4). Immunization of mice with DCs transfected with murine TERT leads to protective immunity (5), further supporting the hypothesis that telomerase may be a clinically important tumor rejection antigen. Unlike other TAA, telomerase is expressed by >85% of all human cancers but is absent in most normal cells (6–8). Moreover, the expression of telomerase in human cancer is directly linked to tumor growth and development (9, 10). Inhibition of telomerase in telomerase-positive human tumors by genetic methods (11) or through the use of peptide nucleic acid molecules (12) leads to growth arrest. Thus, tumor down-regulation of hTERT, as a means of immune escape, may itself be incompatible with sustained tumor growth. For therapeutic strategies targeting antigens that have no known role in cancer growth, the selection of antigen-deficient mutant tumors is a well-recognized limitation (13, 14).

Because hTERT was identified by deduction, it is not known whether hTERT triggers a natural in vivo T-cell response during tumor progression and, if so, whether there is evidence for functional inactivation. It does appear that the T-cell repertoire against hTERT is intact in normal donors (4) and probably in cancer patients (15), given the ability to expand specific CTLs after multiple restimulations ex vivo. Here, we directly compared T cells from cancer patients and healthy volunteers using peptide/MHC tetramers and functional assays. The following questions were addressed: (a) are hTERT-specific CTL responses measurable at baseline in cancer patients; and (b) can hTERT-specific CTLs be amplified equivalently ex vivo from tumor-bearing patients and healthy individuals? We demonstrate that neither healthy volunteers nor cancer patients exhibit an expanded pool of specific CTLs for the HLA-A2-restricted hTERT epitope and suggest that hTERT is ignored at baseline by the immune system even in the setting of active neoplasia. However, such CTLs can be equivalently amplified ex vivo from cancer patients and healthy donors without evidence of tolerance or functional inactivation.

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3 R. H. V. and J. L. S. contributed equally to this study.

4 The abbreviations used are: TAA, tumor-associated antigen; hTERT, human telomerase catalytic subunit; PBMC, peripheral blood mononuclear cell; NHL, non-Hodgkin’s lymphoma; DC, dendritic cell; MP, influenza A matrix protein; HTLV, human T-cell lymphotropic virus; mAb, monoclonal antibody.

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Materials and Methods

Donor and Patient Samples. PBMCs were obtained by leukapheresis or phlebotomy from cancer patients and normal donors after informed consent. Primary NHL cells were obtained from discarded clinical material after biopsy of involved lymphoid tissue. Tumor-infiltrating lymphocytes were obtained from a biopsy of a thigh mass in a patient with melanoma (kindly made available by Dr. G. Dranoff, Dana-Farber Cancer Institute, Boston, MA). Our Internal Review Board approved the protocols and informed consent procedures used to obtain these samples. Cell lines were from American Type Culture Collection (Manassas, VA) or as described (4).

Peptides. Peptides used were IS40 (ILAKFLHWL) from hTERT, 1476 (ILKEPVHGV) from RT-pol of HIV, F271 (FLWGPRALV) from MAGE-3, G58 (GILGFVFTL) from the matrix protein of influenza A, and L11 (LLF-GYPVVY) from the tax protein of HTLV-1. Peptides were purchased from Sigma Chemical Co. Genosys Biotechnologies (The Woodlands, TX).

MHC Class I Tetramers. Soluble HLA-A2 tetramers were prepared with IS40 peptide and β2-microglobulin as described (16) and conjugated to phycoerythrin or Alexa 488. Control tetramers were made with two HLA-A2-binding peptides (HTLV-1 tax L11 and influenza MP G58) and validated using L11-specific CD8 clones or G58-specific CTLs. Tetramers were added for 15 min at room temperature followed by mAb for 20 min. PBMCs were evaluated after enrichment for CD8 T cells using magnetic bead depletion (4).

ELISPOT Analysis. PBMCs at 2 × 10^5 cells/well were added to ImmunoSpot plates (Cellular Technology, Cleveland, OH) precoated with 10 μg/ml anti-IFN-γ mAb (Mabtech, Nacka, Sweden) in the presence or absence of peptide overnight at 37°C. After washing, wells were then incubated with 1 μg/ml biotin-conjugated anti-IFN-γ mAb (Mabtech) followed by streptavidin-alkaline phosphatase (Mabtech). Spots were developed with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium color development substrates (Promega, Madison, WI). Specific spots were calculated as the number of spots with peptide − the number of spots without peptide.

Generation and Evaluation of CTLs and T Cells. CD8-enriched PBMCs, DCs, and CD40-B cells were prepared as described (4). Peptide-pulsed DCs were added to autologous CD8-enriched T cells, and cultures were restimulated with peptide-pulsed autologous CD40-B cells as described (4). Interleukin 2 (50 units/ml; Chiron, Emeryville, CA) was introduced on day 8 and replenished as needed every 3–4 days. CTLs as effector cells were used after the third or fourth restimulation in chromium release assays, for which specific spots were added to autologous CD8-enriched T cells, and cultures were restimulated as described (4). Peptide-pulsed DCs and CD40-B cells were prepared as described (4). Labeling with negative control A2/L11 tetramer is shown for comparison. In B, photomicroscopy of a single representative cell isolated by cell sorting showed specific T-cell receptor staining (red) with diffuse CD8 staining (green). In C, A2/IS40 tetramer identified a 2.83% population of IS40-specific CD8 T cells in CTLs from patient 1 (top panels), but no A2/IS40 CD8 T cells from the same patient were identified (bottom panels). Similar results were obtained for IS40 CTLs from 2 other normal donors and 5 other cancer patients and for CD8-enriched PBMCs from 9 other normal volunteers and 7 other HLA-A2 cancer patients (Table 1).

Results

Evaluation of Baseline CTL Responses to the hTERT Peptide in HLA-A2-positive Cancer Patients and Healthy Donors. To evaluate the extent to which cancer patients respond in vivo to the IS40 hTERT epitope, we used tetramer analysis on PBMCs from cancer patients and healthy donors. Tetrameric complexes of HLA-A2 and the IS40 peptide (A2/IS40) were generated according to published methods (16) and validated using three IS40-specific CTL lines generated from normal donors. For each normal donor CTL line, a clear population of A2/IS40 CD8 T cells (ranging from 1 to 3% with fluorescence intensity two to three orders of magnitude above background) was identified after four ex vivo stimulations (Fig. 1A). Specific staining was confirmed by immunofluorescence microscopy (Fig. 1B).

We then used the A2/IS40 tetramer to probe peripheral blood from HLA-A2 healthy individuals (n = 10) and cancer patients (n = 8). For each individual tested, the percentage of A2/IS40 CD8 T cells in peripheral blood was ≤0.03% of CD8 T cells, the limit of tetramer detection (Table 1 and Fig. 1). The percentage of CD8 T cells that labeled with a negative control tetramer (A2/L11 constructed using the LLGYPVYV peptide derived from the tax gene of HTLV-1) was also less than the limit of detection for each individual. In contrast, 8 of 10 normal donors and 4 of 8 cancer patients were found to have >0.03% influenza-specific CD8 T cells that labeled with the positive control A2/G58 tetramer constructed using the GILGFVFTL HLA-A2-restricted peptide epitope from MP.

We corroborated these results using an IFN-γ ELISPOT assay to evaluate the frequency of IS40-specific CTLs based on single cell cytokine secretion in response to peptide. In every normal donor (n = 9) and cancer patient (n = 5) tested, ELISPOT analysis paralleled the tetramer data: there were no hTERT IS40-specific or tax L11-specific spots from 2 × 10^5 PBMCs/well (Table 1). In contrast,
specific spots were seen in response to influenza MP G58 in the same normal donors or patients for whom the A2/G58 tetramer identified influenza-specific CD8+ cells.

Similarly, tetramer analysis of tumor-infiltrating lymphocytes indicated that IS40-specific CD8+ cells were either very rare or absent. Biopsy samples from six HLA-A2 patients were evaluated: four low baseline frequency could be expanded ex vivo with A2/I540, A2/L11, or A2/G58 tetramers above background stimulations were required before

Similar to CTL cultures from healthy individuals, at least three were monitored by tetramer analysis to identify I540-specific CTLs. Patient CTL cultures identified among CTLs. After four ex vivo stimulations, IS40/HLA-A2 tetramers identified a 1–3% population of IS40-specific CD8+ T cells (Fig. 1C) for each patient.

Functionality of hTERT-specific CTLs from Cancer Patients. Specific cytotoxic effector function of CTL lines from cancer patients was evaluated by using peptide-pulsed target cells (to test peptide specificity) and telomerase-positive tumor cells (to test tumor specificity). For each donor tested, CTLs demonstrated peptide-specific cytotoxicity of targets pulsed with the IS40 peptide compared with targets pulsed with irrelevant peptides (F271 MAGE-3 or I476 RT-pol) or β2-microglobulin alone (Fig. 2, A–C). As a comparison, patient-derived CTLs specific for the neo-HIV antigen I476 RT-pol achieved the same degree of peptide-pulsed target lysis as IS40-specific CTLs (Fig. 2F).

Patient-derived CTLs were evaluated for tumor specificity using a panel of telomerase-positive tumor cell lines. For 5 of 6 patients, cytotoxicity was observed against HLA-A2+ (but not HLA-A2 negative) tumor cell lines (Fig. 2, D–F). Specificity was further investigated by using the hTERT-negative/HLA-A2+ sarcoma cell line U2OS that had been infected either with a retroviral vector encoding the full sequence of hTERT (U2OS-TERT) or with a virus specifying only a drug-resistance marker (U2OS-pB; Ref. 4). As shown in Fig. 2G, hTERT-specific CTLs lysed U2OS-TERT but not U2OS-pB cells. Consistent with MHC restriction, anti-HLA-A2 mAb but not control anti-CD20 mAb inhibited lysis of both wild-type tumor targets and U2OS-TERT cells (Fig. 2H).

For 1 of the 6 patients studied (patient 3, with stage IIIA multiple myeloma), IS40 CTLs lysed only peptide-pulsed targets but not tumor cell lines or U2OS-TERT cells. We were also unable to generate I476 RT-pol-specific CTLs from this donor. In contrast to the other 5 patients, patient 3 was treated with high-dose steroids before leukapheresis. The findings of peptide specificity without tumor specificity likely reflect low-avidity CTLs arising in this case from steroid immunosuppression or suboptimal culture conditions.

Patient-derived CTLs were also tested against freshly isolated, primary tumor cells. NHL cells were obtained from 2 patients (one sample was HLA-A2+, and the second was HLA-A2 negative but MHC class I+) and tested as targets for two patient-derived IS40-specific CTL lines. Both primary tumors were telomerase positive (Fig. 3A), but for each CTL, only the HLA-A2+ NHL cells were lysed (Fig. 3, B and C).

We have shown previously that IS40-specific CTLs fail to lyse normal CD34+ peripheral blood cells that express telomerase activity (4). To test further the potential of IS40-specific CTLs to lyse nonmalignant cells expressing hTERT, peripheral blood CD34+ lymphocytes were treated with phytohemagglutinin and interleukin 2 for 5 days and then used as targets in cytotoxicity assays. Although negative at baseline, CD3+ lymphocytes became strongly telomerase positive after mitogen/cytokine exposure and maintained expression

### Table 1 Frequency of antigen-specific CD8+ cells in PBMCs from HLA-A2 cancer patients and healthy donors determined by tetramer and ELISPOT analyses

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex</th>
<th>Disease</th>
<th>Stage</th>
<th>Peptide used to construct tetramer</th>
<th>Peptide used for stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41 F</td>
<td>MM</td>
<td>IA</td>
<td>HTLV-1 tax</td>
<td>I540</td>
</tr>
<tr>
<td>2</td>
<td>47 F</td>
<td>MM</td>
<td>IA</td>
<td>I540</td>
<td>HIV RT-pol</td>
</tr>
<tr>
<td>3</td>
<td>40 M</td>
<td>MM</td>
<td>IIIA</td>
<td>I476</td>
<td>hTERT</td>
</tr>
<tr>
<td>4</td>
<td>29 M</td>
<td>NHL</td>
<td>IIIA</td>
<td>I476</td>
<td>hTERT</td>
</tr>
<tr>
<td>5</td>
<td>69 M</td>
<td>Prostate Ca</td>
<td>IV</td>
<td>I476</td>
<td>hTERT</td>
</tr>
<tr>
<td>6</td>
<td>54 M</td>
<td>Prostate Ca</td>
<td>IV</td>
<td>I476</td>
<td>hTERT</td>
</tr>
<tr>
<td>7</td>
<td>40 M</td>
<td>Bladder Ca</td>
<td>II</td>
<td>I476</td>
<td>hTERT</td>
</tr>
<tr>
<td>8</td>
<td>59 M</td>
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<td>I476</td>
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</tr>
<tr>
<td>9</td>
<td>69 M</td>
<td>Lung Ca</td>
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<td>I476</td>
<td>hTERT</td>
</tr>
<tr>
<td>10</td>
<td>70 M*</td>
<td>Melanoma</td>
<td>IV</td>
<td>I476</td>
<td>hTERT</td>
</tr>
</tbody>
</table>

* HLA-A3+, HLA-A2– patient (negative control); ND, not done.

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antigen-specific CD8^+ T cells (1, 3), no hTERT-specific CD8^+ T cells were detected among uncultured peripheral blood or tumor-infiltrating lymphocytes from cancer patients. Nevertheless, the ex vivo expansion of functional hTERT-specific CTLs suggests that a population of hTERT-specific T cells escapes irreversible central tolerance mechanisms. Whether this might be attributable to insufficient antigen presentation in the thymus or elsewhere is currently unknown. Furthermore, because the induction of hTERT-specific CTLs from patients versus healthy donors was equivalent, peripherally induced tolerance to hTERT in the cancer patient is either absent or can be overcome. In support of this hypothesis, we found that for the same patient, the expansion and cytolytic function of hTERT-specific CTLs was identical to CTLs specific for the neoantigen RT-pol476 from HIV. In contrast, CTL generation against the recall viral peptide MP G58 from influenza was more robust, consistent with a secondary immune response. Unfortunately, in vivo mouse models of TERT-directed immunotherapy are limited by significant differences between mouse and human telomerase biology. In particular, telomerase is expressed widely in normal mouse tissues (in contrast to its restricted expression in humans). Therefore, tolerance may limit mouse responses to mTERT in vivo more than human responses to hTERT (5). Whether a low-frequency (in contrast to an expanded but functionally inactivated) precursor population would facilitate clinical attempts to induce immunity remains an important question for future immunotherapeutic trials in cancer.

At the E:T ratios used in this study, our polyclonal populations of I540-specific CTLs never achieved 100% lysis of tumor targets, an observation typical of chromium release assays evaluating tumor antigen-specific CTLs. This may represent an artifact of the geometry and/or static nature of the in vitro experimental system, or alternatively, reflect subpopulations of resistant tumor cells. In the case of I540 CTLs, it will be important to evaluate the nature of tumor cell resistance, including potential down-regulation of hTERT and the development of alternative mechanisms for telomere lengthening.

Two main biological features of hTERT further support its clinical evaluation as a TAA: (a) >85% of all tumors express telomerase activity such that it may be possible to extend novel strategies for antigen-specific immunotherapy to more patients with common cancers; and (b) because hTERT activity has been demonstrated to be critical for tumor growth and development (9–12), the use of hTERT as an immune target may minimize the well-described risk of immune

Discussion

In this study, we demonstrate that CTLs against the telomerase reverse transcriptase hTERT are equivalently induced ex vivo from cancer patients and normal donors despite evidence of baseline immunological ignorance. hTERT-specific CD8^+ T cells could not be detected in freshly isolated peripheral blood, but ex vivo expansion of specific CTLs was identical for cancer patients and healthy individuals. For both donor types, hTERT-specific CTLs labeled brightly with peptide/MHC tetramers and killed telomerase-positive tumor cells in an MHC-restricted fashion. Thus, hTERT-reactive CTLs specific for tumor cells are neither deleted from the T cell repertoire nor specifically inactivated despite the expression of hTERT on active neoplasia and rare normal cells.

hTERT differs from classical TAA, such as melan-A/MART-1 or tyrosinase, in terms of immune surveillance. In contrast to melanoma of HLA-A2. However, no lysis of these cells was seen with I540-specific CTLs (0.0% specific lysis at E:T ratios of 30:1, 10:1, or 3:1).
escape attributable to antigen loss (13, 14). Inhibition of hTERT activity in human tumors that express telomerase activity leads to growth arrest without the appearance of telomerase-negative clones that maintain telomere length by alternative methods (11). We hypothesize that tumor antigens linked to oncogenesis may offer an important clinical advantage for immune therapies. Tumor deletion, mutation, or down-regulation of such antigens—as a consequence of therapeutically driven immune selection (13, 14)—may itself inhibit sustained tumor growth.

Despite these apparent advantages of hTERT as a TAA, we are mindful that certain normal cells express telomerase activity and may present a risk for autoimmunity in patients treated with hTERT-specific therapies. Our in vitro findings suggest that hTERT is a poor autoantigen in hematopoietic stem cells (4, 17), antigen-activated CD8+ lymphocytes (17), and as noted in this report, mitogen-activated T lymphocytes. In mice immunized with TERT mRNA-transduced DCs, antitumor immunity can be generated without the development of autoimmunity against TERT-expressing cells (5). A Phase I trial of hTERT vaccination in cancer patients underway at our institution is aimed at directly evaluating the issue of therapy-induced autoreactivity.

Finally, the demonstration of hTERT-specific CTLs from cancer patients has important implications for ongoing efforts to characterize additional tumor antigens. Unlike TAA defined from patient immunoreactivity, the hTERT epitope described in this report was deduced from primary sequence data and characterized by methods of “reverse immunology” (18). Although the method of epitope deduction carries its own difficulties—several of which have been addressed in this report—advances in genomics and proteomics suggest a growing number of candidate antigens highly expressed in most cancers but rare in normal cells. As additional work in cancer biology characterizes the function of these candidate genes, studies to define clinically relevant epitopes from these important candidate antigens can be guided by methods described for telomerase.

References


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