The Immunogenicity of the hTERT540-548 Peptide in Cancer
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Abstract
Human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, is an attractive target antigen for cancer immunotherapy due to its expression in the vast majority of human tumors. The first immunogenic peptide described from hTERT was the HLA-A2–restricted peptide hTERT540 (ILAKFLHWL). However, much discrepancy exists about the processing and presentation of this epitope on the surface of neoplastic cells. Originally, it was described that specific CTL can be generated in vitro and that such cells are able to kill a range of hTERT+ tumor cell lines and primary tumors in a peptide-specific, HLA-A2–restricted fashion. Furthermore, it was described that vaccination of cancer patients with hTERT540 introduced functional antitumor CD8+ T cells in patients. More recently, it was described that most patients with cancer have circulating hTERT540-specific CD8+ T lymphocytes. In contrast, several other studies have concluded that hTERT540 is not presented on the surface of tumor cells and that immunization of cancer patients with hTERT540 leads to the introduction of specific T cells that do not recognize tumor cells in vivo. In the present commentary, we summarize these highly contradictory results about this potentially very important T-cell epitope. Furthermore, we describe novel data showing that naturally occurring immune responses against hTERT540 are, although rare, present in cancer patients and that such hTERT540-specific T cells are able to recognize and kill cancer cells. Hence, our data support the findings that hTERT540 peptide is presented by human tumors and can be a target for CTL-mediated tumor lysis.

During the past years, T-cell antigens have been characterized in most neoplasms, leading to the notion that host immune system recognizes cancer cells and, thus, that T cells in general have the capacity to recognize cancer cells (1). Taking advantage of the above-mentioned findings, therapeutic vaccinations against cancer have been ongoing for more than a decade. Although the effect on the course of disease in many trials has been minimal, some trials have shown clinical significance of vaccination, and there is consensus that therapeutic vaccinations are likely to represent one of the treatment options for treatment of cancer in the future (2). In this regard, human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, is an attractive potential target antigen for cancer immunotherapy. It is overexpressed in the vast majority of human tumors, whereas normal adult tissues with few exceptions do not express hTERT (3). Another important characteristic is the critical role hTERT plays in tumor development and survival. Hence, down-regulation or loss of hTERT would severely inflect the growth potential of the tumor cell. It is well established that therapeutic strategies targeting antigens not involved in tumor growth can result in the selection of antigen-loss tumor mutants that are clinically progressive (4, 5).

In recent years it has been shown that peptides derived from hTERT are naturally processed by tumors, presented on MHC molecules, and are able to trigger effector functions of specific CTL (6–9). Hence, hTERT-specific T cells generated by stimulation with specific peptides or by dendritic cells transfected with either hTERT or whole tumor mRNA are able to lyse tumor cells (10, 11). Importantly, no signs of autoimmunity have been described in connection with the introduction of immune responses against hTERT. In this regard, hTERT-specific CTL are not lysing either telomerase-positive CD34+ hematopoietic progenitor cells or activated T lymphocytes in vitro (6, 8, 9, 12). Since these successful preliminary studies, rapid progress has been made to translate these findings into new therapies in the clinic (reviewed in ref. 13). The first hTERT-based vaccination trial showed the immunologic feasibility of vaccinating patients against telomerase. hTERT-specific T lymphocytes were induced in four of seven patients with advanced breast or prostate carcinoma after vaccination with dendritic cells pulsed with hTERT peptides, resulting in partial tumor regression in one patient (14). Another clinical trial used hTERT mRNA-transfected dendritic cells in patients with metastatic prostate cancer (15). A trial investigating vaccination with hTERT peptides in patients with non–small-cell lung cancer showed immune responses in 12 of 24 evaluable patients during the primary regimen, with a complete tumor response observed in 1 patient (16). These studies justify and warrant further clinical testing to evaluate the efficacy of...
hTERT-based vaccinations, and several hTERT based clinical vaccination trials are currently ongoing.

**hTERT540: A T-Cell Epitope**

The first immunogenic peptide described from hTERT was the HLA-A2–restricted peptide hTERT540 (ILAKFLHWL). HLA-A2 is found among nearly 50% of Caucasians, Asians, and Hispanics and 33% of African Americans and is consequently a very important HLA type for use in immunotherapy. Vonderheide et al. (8) showed that hTERT540 peptide–specific CTL can be generated in vitro and that these are able to kill a range of hTERT tumor cell lines and primary tumors in a peptide-specific, HLA-A2–restricted fashion. Other studies have confirmed that hTERT540-specific CTL of cancer patients specifically lysed a variety of HLA-A2* cancer cell lines, showing immunologic recognition of endogenously processed hTERT540 peptide (6, 17). In addition, the direct isolation of the hTERT540 peptide from the groove of HLA-A2 on primary tumor cells has been shown by mass spectroscopy (18). As mentioned above in the first hTERT vaccination trial, seven HLA-A2* breast or prostate cancer patients were vaccinated with dendritic cells pulsed with hTERT540 (14). The treatment introduced functional antitumor hTERT540-specific T cells in the patients. Recently, 19 breast cancer patients were vaccinated with hTERT540 in Montanide adjuvant with granulocyte macrophage colony-stimulating factor. Peripheral blood hTERT-specific CD8+ T cells were introduced by the treatment, and an association between hTERT-specific CD8+ T-cell immune response and overall survival in the patients was reported (19). Different reports have been given with regard to the frequency of spontaneously induced hTERT540-specific CTL in peripheral blood of cancer patients. Maeccker et al. (20) reported that CTL specific for hTERT540 could be detected in patients with multiple myeloma, but only in a very limited number of patients. In contrast, Filaci et al. (21) recently described that most patients with cancer have circulating hTERT540-specific CD8+ T lymphocytes. The authors could detect hTERT540-specific CD8+ T cells directly ex vivo in 20 of 22 cancer patients (18 prostate cancer patients) with frequencies ranging from 0.1% to as high as 1.4%.

**Fig. 1.** A, ex vivo FACS analyses of hTERT540-specific CD8+ T cells from leukapheresis samples from 4 renal cell carcinoma (RCC), 10 melanoma (MM), and freshly isolated peripheral blood mononuclear cells from 9 prostate cancer (PC) patients were done on a FACS Aria (BD Biosciences) using phycoerythrin-conjugated pentameric complexes consisting of HLA-A*0201-hTERT540 (ILAKFLHWL), HLA-A*0201-HIV-1 pol476-484 (ILKEPVHGV), and HLA-A*0201-CMV pp65495-503 (NLVPMVATV; Proimmune). T cells were costained with CD3-FITC and CD8–allophycocyanin monoclonal antibodies and dead cells were gated out using 7-amino-actinomycin D (BD Immunocytometry Systems). hTERT540 cells detected in one melanoma patient (solid triangle) were used for further analyses. B, HLA-A2–restricted T-cell responses against hTERT540 as measured by IFN-γ ELISPOT ex vivo. T lymphocytes were thawed and plated 24 h later at 5 × 10⁵ per well in duplicates either without or with the hTERT540 peptide. Peripheral blood lymphocytes from 4 renal cell carcinoma, 9 prostate cancer, and 10 melanoma were examined. Points, average number of peptide-specific spots (after subtraction of spots without added peptide) calculated for each patient using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers, LLC). The hTERT540–specific ELISPOT response from the melanoma patient in A (solid triangle) is shown by a solid lozenge. C, FACS plot and ELISPOT wells depict the hTERT540–specific T-cell response from the melanoma patient marked by a solid triangle and solid lozenge in A and B, respectively.
hTERT540: Not a T-Cell Epitope

In striking contrast to the above described data, Ayyoub et al. (22) reported that the hTERT540 peptide is not presented on the surface of tumor cells in the context of HLA-A2. Thus, hTERT540-specific CTL were generated from peripheral blood lymphocytes from melanoma patients that showed strong cytotoxicity against HLA-A2+ target cells in the presence of exogenously loaded peptide. However, no cytotoxicity was observed against melanoma and colon cancer cells despite their expression of hTERT and HLA-A2. Transfection of melanoma cells with a plasmid containing an hTERT minigene resulted in recognition by specific CTL, but only for the minimal minigene coding for the exact 9-amino-acid peptide. The investigators incubated a long hTERT peptide (534-554) with proteasomes and analyzed the product by mass spectrometry and cytotoxic assays. These results suggested inefficient antigen processing of hTERT, resulting in lack of peptide presentation on the cell surface, which explains why tumor cells were not recognized by the CTL.

Parkhurst et al. (23) initiated a clinical protocol to vaccinate patients with metastatic cancers with hTERT540. In this study, 14 patients with metastatic cancer were vaccinated with hTERT540 in Montanide. However, although they generated peptide-specific T cells, these did not recognized either telomerase+ tumors or cells transduced with a recombinant adenovirus encoding the full-length hTERT gene, and no clinical benefit was observed. Recently, these data were confirmed by Purbhoo et al. (24), who generated both a hTERT540-specific CTL clone as well as a specific high-affinity recombinant soluble T-cell receptor. The investigators could not detect the hTERT540 peptide on the surface of tumor cells using either the clone or the T-cell receptor and concluded that the peptide is not a naturally presented epitope.

Immunogenicity of hTERT540

As a consequence of these highly conflicting data, we scrutinized the peripheral blood lymphocytes of renal cell carcinoma patients, prostate cancer patients, and melanoma patients for the presence of hTERT540-specific T cells directly ex vivo by HLA-A2/hTERT540 pentamer stainings by flow cytometry as well as ELISPOT (Fig. 1A and B). These data confirm the study by Vonderheide et al. (20) that hTERT540-specific T cells are present in cancer patients but only in a limited number of patients. Interestingly, we were not able to detect hTERT540-specific T cells by ELISPOT ex vivo in any of the nine analyzed prostate cancer patients. HLA-A2/hTERT540 pentamer stainings, however, revealed weak responses in a few patients. This is in contrast to the study by Filaci et al., suggesting that >90% of HLA-A2+ patients with prostate cancer harbor strong spontaneous T-cell responses against hTERT540 (up to 1.4%; ref. 21). In one of the melanoma patients, we were, however, able to detect a strong response against hTERT540 by both fluorescence-activated cell sorting (FACS; 0.2%) and ELISPOT (Fig. 1C). To further characterize the functional capacity of such hTERT540-specific T cells, HLA-A2/ hTERT540 pentamer-positive CD8 cells were sorted by FACS. The cells were sorted as single cells into 96-well plates containing a mixture of irradiated peripheral blood lymphocytes from three healthy donors, phytohemagglutinin, and interleukin-2. After expansion, the specificity of the growing clones was analyzed in standard $^{51}$Cr release assays. To this end, T2 cells either without peptide or loaded with hTERT540 (10 μmol/L) served as targets. A number of clones exclusively killed T2 cells pulsed with hTERT540 (one of these is depicted in Fig. 2). The hTERT-specific T cells were further tested for the capacity to kill cancer cell lines of different origin. As shown in Fig. 2A, the hTERT-specific T-cell clone efficiently lysed the hTERT+, HLA-A2+ colon cancer cell line SW480. To examine the antigen specificity and HLA restriction of the hTERT-specific T-cell clone, we examined the effect of blocking HLA class I by addition of the specific monoclonal antibody W6/32. Lysis could be blocked by preincubation of target cells with W6/32 (P = 0.05; Fig. 2A). Likewise, the addition of cold (unlabeled) T2 cells pulsed with the hTERT540 peptide (10 μmol/L) blocked the lysis of SW480 tumor cells, whereas the addition of T2 cells without peptide only showed a very limited dilution effect. As an additional control, no cytotoxicity was observed against the HLA-A2+ ovary cancer cell line SKOV-3 (Fig. 2A). Next, we included some of the cancer cell lines.
lines, which were not lysed by hTERT540-specific T cells in the study by Parkhurst et al. (ref. 23; i.e., the HLA-A2+ EBV transformed B lymphoblast IM-9 as well as the HLA-A2+ myeloma cell line U266). In contrast to the former study, the hTERT540-specific T-cell clone generated in our lab was able to lyse both cell lines (Fig. 2B) in a HLA-A2/peptide–restricted manner because inhibition with cold T2 cells pulsed with hTERT540 (10 μmol/L) completely blocked the killing of the target cells (P = 0.05). Consequently, our data support the findings that hTERT540 peptide is presented by human tumors, including some of the neoplastic cells, which have not been recognized in former studies.

Identification of new MHC-restricted peptide epitopes from tumor-associated antigens, including hTERT540, has most commonly been achieved through reverse immunology with the prediction of potential MHC-binding peptides according to the location of MHC peptide anchor residues. A critical issue in the strategy for CD8 T-cell target antigen identification is clearly to show that the peptide is presented and naturally processed on the surface of tumor cells. This is generally achieved by either of two methods: recognition by T-cell clones or elution of peptides from the cell surface. Hence, presentation of antigens can be confirmed by isolation and expansion of T-cell clones recognizing a specific peptide from the blood of cancer patients or normal donors, which are able to lyse tumor cells. The major puzzle about hTERT540, however, is that different groups have achieved highly convincing but different results when generating hTERT540-specific T cells: on one hand, hTERT540-specific T cells kill cancer cells, and on the other hand they do not. Although differences in T-cell avidity, culture conditions, or proteasome/immunoproteasome expression within the target cells might account for some of these highly conflicting data, we believe that the main explanation still needs to be resolved.

Alternatively, mass spectrometry can be used to confirm the presence of peptides eluted from the cell surface or from purified MHC antigens. In this regard, the direct isolation of hTERT540 peptide from the HLA-A2 binding groove of tumor cells expressing telomerase strongly supports that hTERT540 is indeed a T-cell epitope (18).

Overall, our study confirms the first results published on the hTERT540 peptide by Vonderheide and colleagues. Naturally occurring immune responses against hTERT540 are, although rare, present in cancer patients, and hTERT540-specific T cells are able to recognize and kill cancer cells. Hence, our data support the findings that hTERT540 peptide is presented by human tumors and can be a target for CTL-mediated tumor lysis.

References
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