Targeting p53 for Adoptive T-Cell Immunotherapy

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ABSTRACT

p53 gene mutations occur in most human cancers and result in an altered protein product that accumulates within the cell. Although the observed endogenous human CTL response to p53 is weak, high-affinity, human MCE-specific CTLs have been generated from HLA A2.1 transgenic mice immunized with human CTL epitope peptides. In this study, we examine the ability of HLA A2.1-restricted and human p53-specific CTLs from HLA A2.1 transgenic mice to suppress the growth of p53-overexpressing human tumors in severe combined immunodeficient (SCID) mice. In vitro, murine p53149-157-specific CTLs selectively lysed the p53-overexpressing pancreatic carcinoma cell line Panc-1 but did not recognize HLA A2.1+ tumor cells or HLA A2.1+ normal human fibroblasts. Furthermore, in vivo, the growth of established human tumor xenografts in SCID mice was significantly reduced and survival was prolonged after the administration of p53-specific CTLs but not after the administration of control CTLs or PBS alone. Following treatment with p53149-157-specific CTLs, regressing Panc-1 tumors were infiltrated by the CD8+ CTLs, as demonstrated by immunohistochemistry. These findings suggest that p53149-157-specific and HLA A2.1-restricted murine CTLs suppress the growth of established Panc-1 tumors following adoptive transfer into SCID hosts and prolong their survival.

INTRODUCTION

The human p53 tumor suppressor protein is an important regulator of cell cycle progression and is normally expressed at low levels. Mutations in the p53 gene, which are generally diverse, single-base missense mutations (1), contribute to the development of up to 50% of all cancers (2, 3). Mutations of p53, which abrogate its function as a suppressor of cell division (4, 5), are associated with high nuclear and cytoplasmic concentrations of the p53 protein (6, 7). Because these mutations result in the alteration of a single amino acid in tumors that overexpress p53 (3), the majority of the mutant protein resembles wild-type p53. A testable hypothesis is that tumor cells that overexpress mutant p53 still process and present one or more equivalent peptides to the immune system, as do normal cells. For these reasons, we have pursued the use of the wild-type epitopes of the human p53 protein as targets of a murine immune response to transgenic HLA A2.1 mice.

Intracellular proteins are continuously processed through the action of intracellular proteases generating small peptide fragments (8). Cytotoxic CD8+ CTLs recognize MHC class I molecules complexed with these 8–11 amino acid residue fragments termed MCEs.3 MCEs from several human tumor associated proteins have been identified and have been used to generate tumor-specific CTLs in vitro (9).

Although overexpressed p53 has been extensively evaluated as a target for immunotherapy, it has been difficult to generate a human CTL response to p53 (10–12).

p53-specific and HLA-restricted CTL responses have been generated to human p53 in HLA A2.1 transgenic mice (13, 14). By immunizing these mice with a number of wild-type p53-derived MCEs, CTLs were generated that recognized human HLA A2.1+, p53-overexpressing tumor cells in vitro. Moreover, these CTLs did not appear to recognize HLA A2.1+ human cells with normal p53 expression. A further demonstration of the ability of p53-specific CTLs to discriminate between cells that have normal levels of p53 and those with overexpression is the work of Vierboom et al. (15). They successfully eradicated murine p53-transfected tumors following the adoptive transfer of murine wild-type p53-specific CTLs. Here, we demonstrate the in vitro recognition of a p53 overexpressing HLA A2.1+ human pancreatic adenocarcinoma cell line by murine CTLs that recognize a wild-type epitope of human p53 in the context of HLA A2.1. In addition, we demonstrate the in vivo therapeutic efficacy of the p53-specific CTLs in an adoptive transfer model using pancreatic adenocarcinoma heterotopic and orthotopic xenografts in SCID mice.

MATERIALS AND METHODS

Cell Lines. Previously described cell lines used in these experiments included T2 (16), Saos (17), K562 (18), Panc-1 (19), and AsPC-1 (20) and were obtained from the American Type Culture Collection. HLA A2.1+ fibroblasts were generated from HLA A2.1+ human donors, under a protocol approved by the City of Hope Institutional Review Board. Saos-p53 was derived from transfection of the p53-deficient parent cell line with mutated p53 (R to H at position 175) as described previously (13). Human cell lines Panc-1, AsPC-1, T2, and K562 were maintained in RPMI, whereas Saos, Saos transfectants, and human HLA A2.1+ fibroblasts were maintained in DMEM. All media were supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The HLA status of each cell line was determined by PCR on genomic DNA, as well as by IF, as described (13).

Mice. C.B-17 SCID mice (Taconic Farms, Germantown, NY) were maintained in a specific pathogen-free environment in the mouse breeding colony at the City of Hope Animal Care Facility. Experiments were performed under a protocol approved by the Research Animal Care Committee of the City of Hope.

Cytotoxic Assay. To assess peptide specific lysis, T2 cells were labeled with 150 μCi of 51Cr (ICN, Costa Mesa, CA), and peptide-pulsed (1.0 μM) for 90 min. For cytotoxic assays involving cell lines without peptide sensitization, cells were pretreated for 18 h with 20 ng/ml IFN-γ (Collaborative Biomedical Products, Bedford, MA) and labeled with 51Cr for 90 min. Labeled target cells and diluted effector cells were cocultivated for 4 h. Supernatants were harvested (Skatron Instruments, Norway) and counted using a gamma counter (Packard Instrument Company, Meriden, CT). Specific lysis was determined as follows: % specific release = ([experimental release - spontaneous release]/(maximum release - spontaneous release)) × 100.

Human Tumor Xenografts. Heterotopic tumor xenografts were generated by injecting 5 × 106 Panc-1 cells in 200 μl of PBS into the s.c. space of adult SCID mice. Orthotopic tumors were generated by injecting 5 × 106 Panc-1 cells in 500 μl of PBS into the peritoneum of adult SCID mice. To evaluate the viability of established tumor xenografts, H&E staining was performed on tumor explants in a standard fashion.

Adoptive CTL Transfer. p53-specific CTL cell lines were generated by immunizing HLA A2.1 transgenic mice (a kind gift from Dr. Victor Englehardt, Department of Surgery, City of Hope National Medical Center, Duarte, California 91010).
Fig. 2. Cytotoxic activity of anti-p53<sub>149-157</sub> CTL effector cells using human cell line targets in a 4-h CRA. E:T ratios were 20:1, 6:1, and 2:1. The following cell lines were used as targets: Saos (HLA A2.1<sup>+</sup>, p53 deficient), p53/Saos (HLA A2.1<sup>+</sup>, p53 transfectant), Panc-1 (HLA A2.1<sup>+</sup>, p53<sup>+</sup>), AsPC-1 (HLA A2.1<sup>+</sup>, p53<sup>+</sup>), HLA A2.1 fibroblasts, and K562 (natural killer cell sensitive). SE of triplicate cultures was always <5% of the mean.

Statistical Analysis. To compare changes in tumor volume (mm<sup>3</sup>) over time among treatment groups, two-way analysis of variance was performed. Time (in days), treatment group, and the interaction between these two factors were included in the statistical model. If a significant interaction was detected, differences in mean tumor volume between treatment methods at each time point were compared using the independent <i>t</i> test. To test for an association between complete tumor regression and treatment method, Fisher’s exact test was used. Mean tumor volumes at 21 days were compared using the independent <i>t</i> test. All significance testing was done at the 0.05 level. The SAS/STAT software (SAS, Inc., Cary, NC) was used.

RESULTS

Recognition of p53<sub>149-157</sub> in Vitro. p53<sub>149-157</sub>-specific CTLs were maintained in vitro using p53<sub>149-157</sub>-pulsed T2 cells as antigen-presenting cells. Peptide-specific CTL responses were measured 6 days following stimulation in a 4-hour CRA with peptide-pulsed T2 cells as targets. As a control for peptide specificity, p53<sub>149-157</sub>-CTLs were also tested for recognition of T2 cells pulsed with HCMV<sub>495-503</sub>. The p53<sub>149-157</sub>-CTLs demonstrated p53<sub>149-157</sub>-specific lytic activity (Fig. 1). Recognition of endogenously processed p53 by human p53<sub>149-157</sub>-specific murine CTLs was assessed using the p53-deficient Saos cell line, which had been transfected with p53, as well as the p53 overexpressing human pancreatic carcinoma cell lines Panc-1 and AsPC-1 (Fig. 2). The p53-specific CTLs did not lyse the Saos cells unless they were first transfected with p53. The p53-specific CTLs also lysed the p53 overexpressing and HLA A2.1-restricted Panc-1 cells but did not lyse the AsPC-1 cells, which overexpress p53 but do not express HLA A2.1, nor did they lyse normal human HLA A2.1<sup>+</sup> fibroblasts. In addition, the human p53-specific murine CTLs did not lyse the human natural killer cell-sensitive erythroleukemia K562 cell line (Fig. 2).

Establishment of Xenograft Tumor. To determine whether a tumor could be eliminated by murine CTL targeting p53, a human pancreatic carcinoma xenograft was established in SCID mice. Adult SCID mice were injected s.c. or i.p. with 5 × 10<sup>6</sup> human Panc-1 tumor cells. To assess the presence of viable tumor in the s.c. model, animals were sacrificed and palpable tumors were explanted 48 h after tumor cell injection and subjected to H&E staining. The H&E stain demonstrated a well-organized, poorly differentiated carcinoma with established capillary formation (Fig. 3A). To assess the presence of viable tumor in the orthotopic model, animals were sacrificed 48 h following i.p. injection of 5 × 10<sup>6</sup> human Panc-1 tumor cells. H&E stain showed organized tumor localized to the immediate peripancreatic region adjacent to the pancreatic capsule (Fig. 3B). If left untreated tumors in the orthotopic model progressed to invade the pancreatic capsule (Fig. 3C).}

Fig. 1. Peptide-specific cytotoxic activity of CTL effector cells using peptide-pulsed T2 cells as targets in a 4-h CRA. E:T ratios were 30:1, 10:1, and 3:1. A, cytotoxicity of the anti-p53<sub>149-157</sub> CTL cell line against T2 cells pulsed with either p53<sub>149-157</sub> (<big>Φ</big>) or HCMV<sub>495-503</sub> (<big>Φ</big>) peptides. B, cytotoxicity by the anti-HCMV<sub>495-503</sub> CTL cell line against T2 cells pulsed with either HCMV<sub>495-503</sub> (<big>Φ</big>) or p53<sub>149-157</sub> (<big>Φ</big>) peptides. SE of triplicate cultures was always <5% of the mean.
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pancreas and surrounding tissues (data not shown). Thus, viable, organized, and progressive tumor was present by 48 h.

Heterotopic Tumor Response following Adoptive CTL Transfer. To assess the impact of adoptive CTL transfer on established tumor xenografts, p53149_l57-specific CTLs, HCMV495_503-specific CTLs or PBS was administered i.v. to SCID mice 2 days following tumor cell injection. In a representative experiment, heterotopic tumors that were challenged with p53149-l57-specific CTLs demonstrated a significant reduction in tumor number and size, as compared to animals receiving HCMV495_503 CTLs or PBS (Fig. 3). Significant differences in tumor volume over time were found between the treatment groups, with the p53149-l57-specific CTL treatment arm having statistically significantly smaller tumor volumes compared to HCMV495_503 CTLs or PBS (P < 0.001). The mean tumor volume at 28 days for the p53149_l57 CTL treatment group was significantly lower than both the HCMV495_503 CTLs or the PBS group (251 mm3 versus 1400 mm3, P = 0.0007; and 251 mm3 versus 1928 mm3, P = 0.0007). No statistically significant difference in mean tumor volume was found between the HCMV495_503 CTLs and PBS groups (1400 mm3 versus 1928 mm3, P = 0.24). Overall, more animals with palpable tumor had complete tumor regression following p53149_l57 CTL treatment than HCMV495_503 CTLs or PBS treatment (52 versus 4 and 0%, respectively), and tumors, when present, were significantly smaller in the p53149-l57 CTL group than control groups (P < 0.0001; Table 1). The addition of i.p. recombinant interleukin 2 in animals with heterotopic Panc-1 tumors challenged with p53149-l57 CTLs resulted in no demonstrable difference in tumor growth as compared to those who received i.p. PBS (data not shown).

p53 Expression in Persistent Heterotopic Tumors. To determine if p53149-l57-specific CTL therapy resulted in the selection of tumor cells with lower levels of p53 expression, persistent tumors following the administration of p53149_l57-specific CTLs were removed and tested for p53 expression by IHC. No difference in the level of p53 expression was noted between persistent tumors and those that had not been treated with p53149-l57-specific CTLs (data not shown).

Fig. 3. Evaluation of established human Panc-1 tumor xenografts in SCID mice. A, H&E stain of heterotopic s.c. tumor removed 48 h following subcutaneous injection of 5 × 10⁶ Panc-1 cells. Arrow, capillary magnification. ×400. B, H&E stain of orthotopic peripancreatic tumor removed 48 hours following intraperitoneal injection of 5 × 10⁶ Panc-1 cells magnification. ×400. C, CD8⁺ IHC staining of 2-week established s.c. heterotopic Panc-1 tumor 72 h following tail vein infusion of 5 × 10⁷ p53149_l57-specific CTLs. Magnification, ×400. D, CD8⁺ IHC staining of 2-week established s.c. heterotopic Panc-1 tumor 72 h following tail vein infusion of 5 × 10⁷ HCMV495_503-specific CTLs. Magnification, ×400.

Fig. 4. In vivo heterotopic tumor volume response following adoptive CTL transfer. Animals with 2-day established tumors were injected i.v. with 5 × 10⁷ p53149_l57 CTLs (■), HCMV495_503 Control CTLs (●), or PBS (▲), and tumor response was measured over 28 days. Data points, one of five representative experiments, with six animals per treatment group.
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Table 1  Heterotopic tumor response 21 days following PBS or CTL challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>n (total)</th>
<th>n (tumor)</th>
<th>Tumor volume (mm³)</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>27</td>
<td>27</td>
<td>1243</td>
<td>900-2100</td>
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<td>1082</td>
<td>860-1946</td>
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<td>p53 CTL</td>
<td>25</td>
<td>12*</td>
<td>365*</td>
<td>125-1850</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.0001.

Survival Advantage in Orthotopic Xenograft Animals. To better assess the potential therapeutic impact of adoptive CTL transfer, animals were challenged with CTLs 48 h after i.p. tumor cell injection. Untreated animals with orthotopic tumors progress with a disease pattern similar to that seen in primary pancreatic carcinoma. Specifically, these animals develop an infiltrating tumor of the pancreas with subsequent peripancreatic lymph node metastasis, obstructive jaundice, peritoneal carcinomatosis, and eventual liver metastasis. If left untreated, animals will progress to a state of advanced cancer cachexia and succumb to disease in 6–9 weeks. Animals which received p5349-157-specific CTLs at 48 h demonstrated prolonged disease-specific survival compared to those who received control HCMV495-503 CTLs or PBS (Fig. 5). In fact, half of the animals developed no clinical signs or symptoms of malignancy and had no evidence of carcinoma at necropsy 20 weeks following CTL challenge.

p53 CTLs Infiltrate Panc-1 Xenografts in SCID Mice. To evaluate whether tumor elimination and growth inhibition of Panc-1 xenografts was associated with infiltration of the tumors by CTL, IHC studies were performed. IHC was performed on established heterotopic Panc-1 tumors 72 h following CTL injection into SCID mice. Heterotopic tumor xenografts from mice challenged with p5349-157-specific CTLs appear to have significantly more CTL infiltration than those receiving control HCMV495-503 CTLs or PBS (Fig. 5). In fact, half of the animals developed no clinical signs or symptoms of malignancy and had no evidence of carcinoma at necropsy 20 weeks following CTL challenge.

In the current study, we screened a number of human cancer cell lines by IHC and IF to determine the expression of p53 and the MHC class I molecule, HLA A2.1. The tumor lines tested included the human pancreatic carcinoma cell lines Panc-1, AsPC-1, BxPC, MIA PaCa-2, Capan-1, Capan-2, HS766T, and CFPAC-1 (data not shown). Of these, Panc-1 displayed the strongest dual expression of both p53 and HLA A2.1. Interestingly, only 40–50% of the Panc-1 cell population overexpressed p53 by IHC or IF, whereas nearly 95% of the cells strongly expressed HLA A2.1 (following IFN–γ pretreatment; data not shown). Similar results were obtained when IHC for p53 expression was performed on established Panc-1 xenografts in SCID mice (data not shown).

An intriguing question raised by this study is how a tumor mass could be completely eradicated by p53-specific murine CTL transfer when overexpression of p53 occurs in only a percentage of its component cells. The answer may derive from the limitations in sensitivity of IHC and IF compared to antigen recognition by CTL. IHC and IF sensitivity is dependent on numerous variables including sensitivity of IHC and IF compared to antigen recognition by CTL. An intriguing question raised by this study is how a tumor mass could be completely eradicated by p53-specific murine CTL transfer when overexpression of p53 occurs in only a percentage of its component cells. The answer may derive from the limitations in sensitivity of IHC and IF compared to antigen recognition by CTL. IHC and IF sensitivity is dependent on numerous variables including sensitivity of IHC and IF compared to antigen recognition by CTL.
contrast, recent evidence suggests that as little as a single antigen: MHC complex per cell can trigger a lytic CTL response (27), a level far below the sensitivity of IHC or IF. Because the vast majority of Panc-1 cells strongly express HLA class I, a small number of p53 peptide complexes may be sufficient to generate the in vivo tumor response observed in this study. Furthermore, IHC and IF measure cellular intact p53, and we cannot estimate from those techniques the amount of appropriately processed p53 peptides that potentially could be presented in the context of HLA class I on the tumor cell surface. In proof of this principle, other HLA A2.1+ human tumor cell lines with lower levels of p53 overexpression were recognized by the p53(149-157) CTLs in vitro in an antigen-specific manner, as measured by tumor cell lysis or CTL cytokine secretion (tumor necrosis factor-α). Interestingly, this in vitro response correlated with in vivo tumor response as well (data not shown).

This study is the first report of an in vivo human tumor response to p53-specific CTLs. We used two separate human pancreatic adenocarcinoma xenograft models as a proof of this principle. The s.c. xenograft model allows for an assessment of tumor volume in response to treatment. Unfortunately, it falls short as an accurate model of clinical pancreatic cancer. Previous attempts to establish an orthotopic pancreatic cancer model in immunocompromised mice have been limited by the necessity for direct intrapancreatic injection of tumor cells via laparotomy (28). Although i.p. injection of pancreatic carcinoma cell lines has been performed in the past, it resulted in diffuse intraperitoneal carcinomatosis without specific targeting to the pancreas (29). In contrast, the intraperitoneal Panc-1 model used in this study mimics the natural course of human pancreatic cancer. Panc-1 cells are unique because, when injected i.p., they track directly to the pancreas and quickly organize into an invasive tumor, which may be due to the favorable microenvironment provided by the host pancreas. Tumor progression in these animals proceeds with regional lymph node metastasis, causing biliary obstruction and subsequent jaundice, and is soon followed by i.p. carcinomatosis, retroperitoneal tumor involvement and hepatic metastasis. As expected, death is due to an overwhelming tumor burden. This fortuitous model is helpful because the disease course is very similar to that seen in primary human pancreatic cancer, allows a survival analysis following treatment, and provides insight into the impact of therapy on the clinical course of disease. In this study, it appears that some orthotopic tumors were eradicated and/or growth inhibited by the p53(149-157)-specific CTLs, resulting in prolonged survival.

The hypothesis that the tumor response observed in this study was the result of tumor infiltration, recognition and elimination by the p53(149-157)-specific CTL is supported by the immunohistochemical detection of murine CD8+ CTL infiltration of established Panc-1 tumors in SCID mice. Large numbers of CD8+ cells were seen to infiltrate tumors in animals challenged with p53(149-157)-specific CTLs. The infiltrating CD8+ cells appear to track to the tumor site and likely orchestrate its eradication, either through direct tumor cell lysis and/or the production of cytokines that facilitate tumor cell death. A potential strategy to selectively target p53-overexpressing human tumors, without relying on an adaptive p53-specific CTL response in the host, is to genetically transfer the high-affinity, p53(149-157)-specific T-cell receptor from murine to human T cells. This approach would bypass the need for the host to amplify its own immune response, a situation that presumably is not effective for most human malignancies. Use of autologous T cells genetically modified with murine p53(149-157)-T-cell receptor may substitute therapeutically for an effective autologous response to tumor and provide a generalized approach to immunotherapy of p53 overexpressing human tumors.

REFERENCES

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