Targeting p53 for Adoptive T-Cell Immunotherapy

Todd M. McCarty, Xiping Liu, Ji-Yao Sun, Elizabeth A. Peralta, Don J. Diamond, and Joshua D. I. Ellenhorn

Departments of Surgery [T. M. M., X. L, E. A. P., J. D. I. E.] and Hematology [J-Y. S., D. J. D.], City of Hope National Medical Center, Duarte, California 91010

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 p53-specific CTL lines 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_,57-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_,57-specific CTLs, regressing Panc-1 tumors were infiltrated by the CD8+ of control CTLs or PBS alone. Following treatment with pS3,49_,S7- the administration of p53-specific CTLs but not after the administration in SCID mice was significantly reduced and survival was prolonged after

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.

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 in 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. MCEs from several human tumor associated proteins have been identified and have been used to generate tumor-specific CTLs in vitro (9).

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2 To whom requests for reprints should be addressed, at City of Hope National Medical Center, Department of General and Oncologic Surgery, 1500 East Duarte Road, Duarte, CA 91010. Phone: (626) 359-8111 ext. 3095; Fax: (626) 301-8885; E-mail: jellenhorn@simplelink.coh.org
3 The abbreviations used are: MCE, minimal cytotoxic epitope; SCID, severe combined immunodeficient; IF, indirect immunofluorescence; HCMV, human cytomegalovirus; CRA, chromium release assay; IHC, immunohistochemistry; TAA, tumor-associated antigen.
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University of Virginia, Charlottesville, VA) with the immunodominant human p53 nonamer peptide spanning amino acid residues 149–157 (p53149–157; Refs. 13 and 14). p53149–157-specific CTLs were generated and cloned via limiting dilution, as described previously (13). The p53149–157-specific clonal line was maintained by weekly restimulation in the presence of irradiated p53149–157 peptide-loaded T2 cells and syngeneic murine splenocyte feeders in the presence of 10% Rat T-Stim (Collaborative Biomedical Products, Bedford, MA). Murine anti-HCMV control CTLs were generated in a similar manner by immunizing transgenic mice with a human HLA A2.1-specific HCMV nonamer peptide spanning amino acid residues 495–503 (HCMV495–503), as described (21). p53149–157-specific CTLs (5 × 10²), HCMV495–503-specific CTLs, or equivalent volumes of PBS were injected i.v. via tail vein 48 h following Panc-1 tumor xenograft implantation. Animals with heterotopic tumors were then injected with 10⁷ units of recombinant interleukin 2 (Cetus Corp., Emeryville, CA) i.p. or PBS daily for 7 days. Heterotopic tumor volume was measured every 2 days and calculated as width × length × depth. Orthotopic tumor response was assessed by disease-specific survival time following tumor cell injection.

Anti-CD8 Immunohistochemistry. Two-week established heterotopic tumors were removed 72 h following tail vein CTL injection and immediately frozen. Acetone-fixed frozen sections were treated with purified rat antimonouse CD8a IgG2a antibody (PharMingen, San Diego, CA). Bound antibodies were detected by incubation with peroxidase.

Statistical Analysis. To compare changes in tumor volume (mm³) 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 t 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 t 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 in Vitro. p53149–157-specific CTLs were maintained in vitro using p53149–157-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, p53149–157 CTLs were also tested for recognition of T2 cells pulsed with HCMV495–503. The p53149–157 CTLs demonstrated p53149–157-specific lytic activity (Fig. 1). Recognition of endogenously processed p53 by human p53149–157-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* fibroblasts. In addition, the human p53-specific murine CTLs did not lyse the human natural killer cell-sensitive erytholeukemia 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 × 10⁸ 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 × 10⁶ 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
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, p53<sub>149</sub>-<sub>157</sub>-specific CTLs, HCMV<sub>495</sub>-<sub>503</sub>-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 p53<sub>149</sub>-<sub>157</sub>-specific CTLs demonstrated a significant reduction in tumor number and size, as compared to animals receiving HCMV<sub>495</sub>-<sub>503</sub> CTLs or PBS (Fig. 4). Significant differences in tumor volume over time were found between the treatment groups, with the p53<sub>149</sub>-<sub>157</sub>-specific CTL treatment arm having statistically significantly smaller tumor volumes compared to HCMV<sub>495</sub>-<sub>503</sub> CTLs or PBS (P < 0.001). The mean tumor volume at 28 days for the p53<sub>149</sub>-<sub>157</sub> CTL treatment group was significantly lower than both the HCMV<sub>495</sub>-<sub>503</sub> CTLs or the PBS group (251 mm<sup>3</sup> versus 1400 mm<sup>3</sup>, P = 0.0002; and 251 mm<sup>3</sup> versus 1928 mm<sup>3</sup>, P = 0.0007). No statistically significant difference in mean tumor volume was found between the HCMV<sub>495</sub>-<sub>503</sub> CTLs and PBS groups (1400 mm<sup>3</sup> versus 1928 mm<sup>3</sup>, P = 0.24). Overall, more animals with palpable tumor had complete tumor regression following p53<sub>149</sub>-<sub>157</sub> CTL treatment than HCMV<sub>495</sub>-<sub>503</sub> CTLs or PBS treatment (52 versus 4 and 0%, respectively), and tumors, when present, were significantly smaller in the p53<sub>149</sub>-<sub>157</sub> 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 p53<sub>149</sub>-<sub>157</sub> 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 p53<sub>149</sub>-<sub>157</sub>-specific CTL therapy resulted in the selection of tumor cells with lower levels of p53 expression, persistent tumors following the administration of p53<sub>149</sub>-<sub>157</sub>-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 p53<sub>149</sub>-<sub>157</sub>-specific CTLs (data not shown).

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**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 x 10<sup>6</sup> Panc-1 cells. Arrow, capillary magnification, X400. B, H&E stain of orthotopic peripancreatic tumor removed 48 hours following intraperitoneal injection of 5 x 10<sup>6</sup> Panc-1 cells magnification, X400. C, CD8<sup>+</sup> IHC staining of 2-week established s.c. heterotopic Panc-1 tumor 72 h following tail vein infusion of 5 x 10<sup>7</sup> p53<sub>149</sub>-<sub>157</sub>-specific CTLs. Magnification, X400. D, CD8<sup>+</sup> IHC staining of 2-week established s.c. heterotopic Panc-1 tumor 72 h following tail vein infusion of 5 x 10<sup>7</sup> HCMV<sub>495</sub>-<sub>503</sub>-specific CTLs. Magnification, X400.
These responses were generated in mice following immunization with tumor derived from cells that had been transfected with demonstrated the ability of murine wild-type p53-specific CTLs to approach. (22), it is an attractive target for an adoptive T-cell immunotherapy protein p53 is the most frequently identified TAA in human cancer getting more generally expressed TAAs. Because the tumor suppressor exclusively expressed by tumor tissue (9). Most of the identified responses are progressing at an accelerated pace. Currently, most of these findings illustrate, in mice, that an enhanced cellular immune response to murine wild-type p53 can mediate the selective recognition and destruction of tumor cells that overexpress mutant murine p53. Furthermore, there was no evidence of autoreactivity toward cells with normal levels of p53 expression (15, 23, 24).

p53-specific cellular immune responses in humans have been more difficult to demonstrate. CTLs from human peripheral blood have been derived against a number of HLA A2.1-binding peptides of p53 (10, 11, 25). With the exception of one report, which describes the derivation of a single CTL clone from an individual that recognizes p53 overexpressing human tumors (12), p53 peptide-specific CTLs generated from human peripheral blood are incapable of killing p53 overexpressing tumor cells (10, 11). A likely interpretation of this data is that tolerance to p53 through either thymic elimination of high-affinity p53-specific CTLs or peripheral anergy to human p53 prevents the generation of human CTL with high enough affinity to kill tumor cells that overexpress p53.

Our laboratory and others have previously generated high-affinity, human p53-specific CTLs using transgenic mice with a peptide immunization approach (13, 14). Although the murine CTLs recognized p53 overexpressing human tumors in vitro, the ability to achieve a response to a solid tumor mass in vivo has yet to be evaluated. This study was performed in an attempt to elucidate the in vivo tumor response of adaptively transferred human p53, 149-157-specific murine CTLs. Pancreatic carcinoma was selected as a xenograft tumor model to study because of its frequent p53 overexpression, lack of effective clinical therapy, and largely fatal outcome. To develop a reliable in vivo tumor model, 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 tumors 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 on 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 individual cellular characteristics, technique, and fluorescence intensity of the conjugate molecule. Although this can range from a few hundred to many thousand receptors per cell, sensitivity in the current model is limited to a resolution down to 1000 molecules per cell (26).

**DISCUSSION**

Attempts to target human TAAs by enhancing tumor-specific CTL responses are progressing at an accelerated pace. Currently, most of the TAAs recognized by CTL were derived from cell lineage-specific proteins or oncogene or tumor suppressor gene products that are not exclusively expressed by tumor tissue (9). Most of the identified TAAs are lineage specific and are only expressed on a limited number of different tumor types. To date, this phenomenon has severely limited the widespread applicability of the specific immunotherapy approach. Recent emphasis has been placed on identifying and targeting more generally expressed TAAs. Because the tumor suppressor protein p53 is the most frequently identified TAA in human cancer (22), it is an attractive target for an adoptive T-cell immunotherapy approach.

Previous murine studies have demonstrated that it is possible to generate a cytotoxic cellular immune response to murine p53, resulting in the eradication of mutant p53 overexpressing murine tumors. These responses were generated in mice following immunization with wild-type p53-derived peptide pulsed on dendritic cells (23), as well as following immunization with a vaccinia viral vector expressing wild-type p53 (24). In addition, studies by Vierboom et al. (15) have demonstrated the ability of murine wild-type p53-specific CTLs to eradicate tumor derived from cells that had been transfected with p53. These findings illustrate, in mice, that an enhanced cellular immune response to murine wild-type p53 can mediate the selective recognition and destruction of tumor cells that overexpress mutant murine p53. Furthermore, there was no evidence of autoreactivity toward cells with normal levels of p53 expression (15, 23, 24).

![Fig. 5. Disease-specific survival of animals with orthotopic Panc-1 tumors following adoptive CTL transfer. Animals were treated 2 days following i.p. tumor cell injection with 5 × 10^7 p53, 149-157 CTL (B), HCMV49-503 control CTL (B), or PBS (A). Data points, one of three representative experiments, with four animals per treatment group.](image-url)
contrast, recent evidence suggests that small 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 this proof of 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 CTX 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 fails 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, resulting in prolonged survival.


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