Dendritic Cells Transduced with Full-Length Wild-Type p53
Generate Antitumor Cytotoxic T Lymphocytes from
Peripheral Blood of Cancer Patients

Ekaterina Yu. Nikitina, Joseph I. Clark, James van Beynen, Sunil Chada, Arvind K. Virmani, David P. Carbone, and Dmitry I. Gabrilovich

Cardinal Bernardin Cancer Center, Loyola University Medical Center, Maywood, Illinois 60153 [E. Y. N., J. I. C., J. v. B., D. I. G.]; Introgen Therapeutics, Inc., Houston, Texas 77054 [S. C.]; Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas 75235 [A. K. V.]; and The Vanderbilt Cancer Center, Vanderbilt University, Nashville, Tennessee 37232 [D. P. C.]

ABSTRACT

Accumulation of wild-type or mutant p53 protein occurs in ~50% of human malignancies. This overexpression may generate antigenic epitopes recognized by CTLs. Because normal cells have undetectable levels of p53, these CTLs are likely to be tumor specific. Here, for the first time, we test the hypothesis that full-length wild-type p53 protein can be used for generation of an immune response against tumor cells with p53 overexpression. T cells obtained from nine HLA-A2-positive cancer patients and three HLA-A2-positive healthy individuals were stimulated twice with dendritic cells (DCs) transduced with an adenovirus wild-type p53 (Ad-p53) construct. Significant cytotoxicity was detected against HLA-A2-positive tumor cells with accumulation of wild-type p53 but not against HLA-A2-positive tumor cells with normal (undetectable) levels of p53 or against HLA-A2-negative tumor cells. This response was specific and mediated by CD8+ CTLs. These CTLs recognized HLA-A2-positive tumor cells expressing normal levels of p53 protein after their transduction with Ad-p53 but not with control adenovirus. Stimulation of T cells with Ad-p53-transduced DCs resulted in generation of CTLs specific for p53-derived peptide. These data demonstrate that DCs transduced with the wild-type p53 gene were able to induce a specific antitumor immune response. This offers a new promising approach to immunotherapy of cancer.

INTRODUCTION

Many types of cancer, including breast, lung, head and neck, and others, have been found to be associated with mutations in the tumor suppressor gene p53 (1–5). The majority of solid tumors continue to express point mutant p53 proteins rather than losing expression via truncations or deletions (6). In human cancer, there is very little or no heterogeneity in these mutations within a given tumor, and they are often present, even in the earliest tumors examined (7). Mutations in the p53 gene often result in overexpression of the p53 protein in tumor cells, and accumulation of p53 may also occur in tumors without mutations in the p53 gene (reviewed in Ref. 8). This may lead to the generation of multiple epitopes that could be recognized by CTLs. We and others have demonstrated the generation of antitumor immune responses using mutant p53 peptides as antigens (9–11). However, this approach to cancer immunotherapy has serious limitations. p53 mutations occur at many different sites in the p53 molecule, making it necessary to identify the site of mutation in each patient before therapy. Most importantly, MHC-binding CTL epitopes may not represent mutant portions of p53 protein. An alternative strategy uses antigenic epitopes in wild-type sequences common to the vast majority of tumor-derived p53 proteins. Recently, wild-type p53 peptide-specific CTLs were generated from human and murine responding lymphocytes, some of which recognized p53-overexpressing tumor cells in vitro (12–17). However, because of the highly polymorphic nature of MHC class I peptide-binding sites, only certain oligopeptides can be successfully used in certain patients. Besides, a peptide that does bind to an individual’s class I MHC may not be sufficiently presented by the MHC class II, the molecules that play a critical role in induction of CD4+ T-cell immune responses. Additionally, responses directed against only a single epitope might not be as effective as polyepitope responses. One strategy to overcome these problems is to rely, not on a single peptide, but rather to use full-length p53 protein, thereby taking advantage of the relative overexpression of the whole p53 molecule in most human tumors.

Adenovirus provides a high-level transduction efficacy of many cell types, regardless of the mitotic status of the cell (18). Replication-defective adenoviruses with deletions in the E1 region have been directly injected into people in several human clinical trials (reviewed in Ref. 19). DCs4 are the most potent antigen-presenting cells and are very effective in stimulation of

4 The abbreviations used are: DC, dendritic cell; GM-CSF, granulocyte/macrophage-colony stimulating factor; CCM, complete culture medium; IL, interleukin; FLU, influenza virus.
primary and secondary immune responses (20). They are one of the best vehicles for delivery of tumor-specific antigens to the immune system. Successful transduction of DCs with green fluorescent protein and model antigens has been reported (21–23). Transduced DCs were able to effectively present the recombinant protein antigens. Thus, therapy with genetically modified DCs using adenovirus for delivery of antigenic epitopes has many potential advantages. In our preliminary study, we have demonstrated that DCs may be effectively transduced with an adenovirus expressing wild-type human p53 (24). However, whether this approach may generate immune response against self-protein (p53) and whether this response is sufficient to recognize and eliminate tumor cells remained unknown. In this study, we have generated in vitro anti-p53 immune response in T cells from eight cancer patients and three healthy individuals and demonstrated that this response is highly specific for tumors with accumulation of p53. This indicates that DCs transduced with wild-type p53 gene can be used in cancer immunotherapy.

PATIENTS AND METHODS

Patients. Forty-one patients were tested for the presence of HLA-A2 alleles. Nine HLA-A2-positive patients (ages, 48–78), together with three HLA-A2-positive healthy volunteers (ages, 25–38), were enrolled in this study (Table 1). Informed consent was obtained from all patients and donors, and the protocol was approved by the Institutional Review Board of Loyola University Medical Center. All patients had advanced stages of lung or head and neck cancer and no chemo- or radiation therapy for at least 2 years prior the study except one case (F. G.), where chemotherapy was finished 8 months before radiation therapy for at least 2 years prior the study.

Table 1. Patients enrolled in the study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Histological type</th>
<th>Diagnosis</th>
<th>Stage</th>
<th>p53 IC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>W. L.</td>
<td>M</td>
<td>58</td>
<td>Adenocarcinoma</td>
<td>NSCLC</td>
<td>T,N,M, stage IV</td>
<td>NA</td>
</tr>
<tr>
<td>E. C.</td>
<td>F</td>
<td>76</td>
<td>Squamous cell carcinoma</td>
<td>NSCLC</td>
<td>T,N,M, stage IV</td>
<td>–</td>
</tr>
<tr>
<td>M. M.</td>
<td>F</td>
<td>78</td>
<td>Adenocarcinoma</td>
<td>NSCLC</td>
<td>T,N,M, stage IIIA</td>
<td>NA</td>
</tr>
<tr>
<td>J. P.</td>
<td>M</td>
<td>56</td>
<td>Bronchogenic carcinoma</td>
<td>NSCLC</td>
<td>T,N,M, stage IIIA</td>
<td>NA</td>
</tr>
<tr>
<td>F. G.</td>
<td>M</td>
<td>70</td>
<td>Squamous cell carcinoma</td>
<td>HNC</td>
<td>T,N,M, stage IV</td>
<td>–</td>
</tr>
<tr>
<td>C. G.</td>
<td>F</td>
<td>60</td>
<td>Adenocarcinoma</td>
<td>NSCLC</td>
<td>T,N,M, stage IV</td>
<td>NA</td>
</tr>
<tr>
<td>J. B.</td>
<td>M</td>
<td>42</td>
<td>Nasopharyngeal carcinoma</td>
<td>HNC</td>
<td>T,N,M,cm, stage IV</td>
<td>–</td>
</tr>
<tr>
<td>J. T.</td>
<td>M</td>
<td>45</td>
<td>Adenocarcinoma</td>
<td>NSCLC</td>
<td>T,N,M, stage IIIA</td>
<td>–</td>
</tr>
<tr>
<td>J. C.</td>
<td>M</td>
<td>71</td>
<td>Adenocarcinoma</td>
<td>NSCLC</td>
<td>T,N,M, stage IIIB</td>
<td>–</td>
</tr>
</tbody>
</table>

*IC, immunohistochemistry; NSCLC, non-small cell lung carcinoma; HNC, head and neck cancer; NA, samples were not available; +, positive staining for p53; –, negative staining for p53.

Tumor Cell Lines and Reagents. Tumor cell lines HTB-22 and HTB-26 were obtained from American Type Culture Collection (Rockville, MD). Tumor cell lines HCC 1419 and SCC 78 were established in the laboratory of Dr. A. Gazdar at University of Texas Southwestern Medical Center (Dallas, TX). Cells were grown in ACL-4 medium described elsewhere (26). The T2 cell line is a processing-deficient lymphoma (TAP-1 mutation). K562 is a HLA class I-negative erythroleukemia cell line that is sensitive to natural killer lysis. These cell lines were maintained in RPMI 1640 containing 10% FCS (Life Technologies, Inc., Grand Island, NY). Recombinant human GM-CSF, IL-4, IL-7, and IL-2 were obtained from RDI (Flanders, NJ). Replication-defective control adenovirus as well as adenovirus containing wild-type p53 gene were obtained from Introgen Therapeutics (Houston, TX). Peptide LLGRNS- FEV derived from wild-type p53 sequence 264–272 and peptide GILGFVFTL derived from the matrix of the influenza virus were synthesized by SynPep (Dublin, CA). Both these peptides have high affinity to HLA-A2. Purified anti-CD4 and anti-CD8 antibodies were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-HLA-A2 antibody BB7.2 was provided by Dr. M. Kast (Loyola University of Chicago).

Cell Isolation. DCs and T cells were obtained from peripheral blood of the volunteers and patients as described (27) with some modifications. Briefly, mononuclear cells were incubated with sheep red cells for 1 h at 4°C. Cells forming rosettes and demonstrated that this response is highly specific for T lymphocytes. These cells were separated from the rest of the mononuclear cells by centrifugation over gradient lymphoprep (Sigma). After removal of sheep red cells by osmotic lysis, T cells were used in further studies. Remaining mononuclear cells were cultured overnight in CCM (RPMI 1640, 10% FCS, and antibiotics). After that time, nonadherent cells were removed, and adherent cells were cultured for five to six days in 3 ml of CCM supplemented with 30 ng/ml GM-CSF and 10 ng/ml IL-4. The same amount of cytokines in 0.5 ml of CCM was added on day 3. DCs were collected, washed in serum-free medium, and used in additional studies.

CTL Generation. Two or three samples of peripheral blood were collected from cancer patients at weekly intervals. The first sample (week 1) was used for generation of the DCs, the second sample (week 2) for generation of DCs and isolation of T cells, and the third sample (when available; week 3) was used for generation of DCs. DCs were infected with Ad-p53 or Ad-c in 0.5 ml of the serum-free medium supplemented with GM-CSF and IL-4. The multiplicity of infection of 100 plaque-forming units/cell was used. After 2 h incubation at 37°C, fresh CCM supplemented with GM-CSF and IL-4 was added. DCs (2 × 10^5 cells/well) were seeded to 24-well plates and incubated in 2 ml of CCM for an additional 24 h. After that time, 1.5 ml of the medium were removed, and 1.5–2 × 10^6 T lymphocytes were added in 1.5 ml of CCM containing 25 ng/ml IL-7. IL-2 (1.5 ng/ml) was added 2 days later. After 7 days of culture, T cells were collected and restimulated with infected DCs generated from the second blood sample as described above. IL-7 (25
ng/ml) was added immediately after restimulation and IL-2 (1.5 ng/ml) 2 days later. In two cases when the third sample was available, T cells were restimulated again 1 week later exactly as described above. In some experiments, DCs were infected with FLU as described (27) and then used for stimulation of CTLs.

**CTL Assay.** Cytolysis was measured with a standard 6-h chromium release assay. Target cells were labeled with 100 μCi of Na$_2^{51}$CrO$_4$ for 1 h, washed, and dispensed into the wells of U-bottomed 96-well plates. Different numbers of the effector cells were added in duplicates to generate different E:T ratios. The radioactivity released into supernatants was measured in a scintillation counter. In all experiments, the level of spontaneous $^{51}$Cr release was <20% of that of maximum release. For competition assays, unlabeled K562 cells were added to the effector cells in 50-fold excess of $^{51}$Cr-labeled target cells. In the experiments with neutralization of CD4 or CD8 cells, effector and target cells were incubated in the presence of 1 μg of either purified anti-CD4 or anti-CD8 antibodies. For the peptide-specific CTL assay, T2 cells (1.6 × 10$^7$/well) were loaded with 10 μg/ml of β$_2$-microglobulin (Sigma) and 100 μM p53 or FLU peptides. After overnight incubation, cells were washed, labeled with $^{51}$Cr, and used as targets for CTL assay. In control, T2 cells were incubated with β$_2$-microglobulin alone.

**Surface Expression of HLA-A2 on Tumor Cells.** Tumor cells were labeled with mouse anti-HLA-A2 monoclonal antibody BB7.2 used as a hybridoma supernatant. After 20 min incubation on ice, cells were washed and treated for 20 min with FITC-conjugated goat antimouse antibody (Sigma). In control samples, cells were incubated with secondary antibody alone. Analysis was performed on FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA).

**p53 Immunohistochemistry.** Tumor cell lines were grown on glass coverslips, fixed in 4% paraformaldehyde, and stained using anti-p53 antibody (DO-7 clone) and LSAB2 System (Dako, Carpinteria, CA). Staining was performed according to the manufacturer’s protocol. Immunostaining of formalin-fixed, paraffin-embedded tumor tissues from patients was performed using the same antibody by QualTec Molecular Laboratories (Santa Barbara, CA).

**Statistical Analysis.** Statistical analysis was performed using parametric methods and JMP statistical software (SAS Institute, Inc., Cary, NC). Paired t test was used for group analysis. For each individual case, differences were considered as significant if analyzed values were more than 2 SD higher than the control values.

**RESULTS**

**Generation of CTL Response against Tumor Cells.** Four tumor cell lines selected as targets for p53-specific CTLs were described elsewhere (15, 28, 29): (a) HLA-A2-positive breast adenocarcinoma cell line HTB-26 has a mutation in codon 280 of the p53 gene that results in nuclear accumulation of p53; (b) HLA-A2-positive breast adenocarcinoma cell line HTB-22 has an accumulation of wild-type p53 in the cytoplasm; (c) HLA-A2-negative breast adenocarcinoma cell line HCC-1419; and (d) HLA-A2-positive non-small cell lung cancer line SCC-78. Both, HCC-1419 and SCC-78 did not have p53 overexpression. We have confirmed those data by immunostaining of these four tumor cell lines with anti-p53 antibody (Dako; data not shown). Expression of HLA-A2 on these cells was analyzed by flow cytometry using anti-HLA-A2-specific antibody.
(BB7.2). Similar levels of HLA-A2 expression were detected in HTB-22 and SCC-78 cell lines, and a slightly higher level was found in HTB-26 cells (Fig. 1). HCC-1419 tumor cells were HLA-A2 negative. Thus, in this study, we used two HLA-A2-positive tumor cell lines with accumulation of mutant or wild-type p53 (HTB-26, HTB-22) and two control cell lines with normal (undetectable) level of p53 expression, one HLA-A2 negative (HCC-1419) and the other HLA-A2 positive (SCC-78).

T cells isolated from 9 HLA-A2-positive patients and 3 HLA-A2-positive healthy volunteers were stimulated twice with Ad-p53 transduced DCs, and CTL response against the four tumor cell lines was measured. Two types of the statistical analysis have been performed. For analysis of each individual case, the level of cytotoxicity against tumor cells with p53 overexpression (HTB-22 and HTB-26) was considered as significant if it was more than 2 SDs higher than the level of cytotoxicity against control cell lines (HCC-1419 and SCC-78) at the same E:T ratios. As expected, the levels of cytotoxicity varied from patient to patient. However, in 3 donors and 7 patients, the level of cytotoxicity against tumor cells with p53 overexpression (HTB-22 or HTB-26) was more than 3 SDs higher than the highest values of cytotoxicity against control tumor cells. In one patient (M. M.), it was 2 SDs higher, and only in one patient (J. T.) was it at the 2 SD threshold (Fig. 2).

T cells from 4 patients and donors had significantly higher cytotoxicity against HTB-26 than HTB-22 tumor cells; 5 patients and donors had a higher response to HTB-22 than to HTB-26 cells, and in 3 cases, the differences were not significant (Fig. 2). Thus, 8 of 9 patients and all 3 tested donors demonstrated significantly higher cytotoxicity against HLA-A2-positive tumor cell lines with accumulation of p53 protein than against control tumor cell lines. The higher level of HLA-A2 expression on HTB-26 cells did not necessarily render a higher level of cytotoxicity. To analyze statistical differences in all studied patients and donors, we used a paired t test. The highest values of cytotoxicity against tumor cells with p53 overexpression (HTB-22 or HTB-26) were compared with the highest values of cytotoxicity against control tumor cells (HCC-1419 or SCC-78) at the same E:T ratios. The level of cytotoxicity against p53-overexpressing tumor cells was significantly higher than that against control tumor cells ($P = 0.0003$).

Overexpression of p53 was detected in tumor samples from two patients, and normal (undetectable) levels were found in the samples from three patients (Table 1). T cells from both patients (J. B. and F. G.) with p53 accumulation in their tumors and two of three patients with normal levels of p53 (E. C. and J. C.) demonstrated a significant level of cytotoxicity against HTB-22 and HTB-26 tumor cells. One patient with a normal level of p53 in the tumor showed no antitumor response after stimulation with Ad-p53 DCs.

We also investigated the ability of T cells stimulated with Ad-p53 DCs to recognize one p53-specific epitope. Because of the limited number of cells available from several patients, this analysis was performed using T cells from 3 donors and 7 patients. T2 cells were loaded with HLA-A2-matched peptides derived from either wild-type p53 or FLU. In all 3 tested healthy individuals and 4 of 7 tested cancer patients, significantly higher cytotoxicity was detected against p53-derived peptide than FLU-derived, peptide-loaded targets (Fig. 3).

Cytotoxicity against Tumor Cells Is Specific and Mediated by CD8+ CTLs. The surface phenotype of T cells was analyzed in two donors and two patients. In all four cases, the majority of cells were CD3+CD4- (40–60%). CD3+CD8+ cells represented 20–30% of the cell population, and CD56+ represented 20–30%. To investigate the nature of the effector cells, the cytotoxic assay was performed in the presence of 50-fold excess of unlabeled K562 cells (target for natural killer

![Fig. 2 Specific cytotoxicity of T cells against tumor cells with p53 accumulation. T cells from 9 HLA-A2-positive patients (shown with initials) and three healthy volunteers (D-1, D-2, and D-3) were stimulated twice with wild-type p53-transduced DCs as described in “Patients and Methods.” CTL response was measured in duplicates against four different tumor cell lines. Averages are shown; bars, SD. ●, HTB-26; ○, HTB-22; △, SCC-78; □, HCC-1419.](image)
cells). It resulted in some decrease in the cytotoxicity against specific target cells (HTB-22 and HTB-26). However, in all four experiments performed, the cytotoxicity remained significantly higher than against control targets (SCC-78 and HCC-1419; Fig. 4A). The presence of 50-fold excess of unlabeled K562 cells did not abrogate p53 peptide-specific CTL activity in two tested healthy volunteers (Fig. 4B). Preincubation of the effector cells with anti-CD4 antibody did not change the level of response against specific target cells, whereas anti-CD8 antibody significantly reduced the cytotoxicity (Fig. 4C). Thus, these data indicate that CD8$^+$ CTLs mediate the observed cytotoxicity against tumor cells.

To confirm the specificity of the CTL response, T cells isolated from two healthy volunteers were stimulated twice with DCs infected with FLU. This resulted in 25% of the specific cytotoxicity against FLU-derived peptide (Fig. 5A). This was comparable with the level of the specific cytotoxicity against p53-derived peptide after two rounds of stimulation with Ad-p53 DCs. Additional stimulation of T cells resulted in increased specific response (data not shown). However, because all of our experiments were performed using two rounds of stimulations, we did not escalate the level of cytotoxicity and directly compared the ability of anti-FLU and anti-p53 CTLs to recognize tumor cells. In contrast to T cells stimulated with Ad-p53 DCs, the T cells stimulated with DC-FLU did not specifically recognize and kill tumor cells with p53 accumulation (Fig. 5B).

Tumor cells SCC-78 express HLA-A2 and a normal (undetectable) level of p53. As was shown in Fig. 2, these cells were very poor targets for p53-specific T cells. To investigate whether SCC-78 cells would become a better target after infection with Ad-p53, these cells were infected with a multiplicity of infection of 100 plaque-forming units/cell of either control adenovirus or Ad-p53. Our previous experiments showed that this dose provides the highest level of transduction efficacy. Twenty-four h later, these cells were used as targets in the CTL assay. At that time, the viability of Ad-p53- and Ad-c-infected tumor cells was similar to that of nontreated cells (>85%). The level of spontaneous $^{51}$Cr release was also the same. T cells stimulated with Ad-p53 DCs were used as effectors. In all four experiments, infection of SCC-78 cells with Ad-p53 significantly increased their recognition by T cells (Fig. 6). In all of these cases, the level of cytotoxicity against wild-type p53-transduced targets was more than 2-fold higher than against target cells infected with the control adenovirus.

DISCUSSION

p53 protein is an attractive target for immunotherapy of cancer. Normal cells have very low levels of p53, whereas accumulation of this protein because of mutations or functional inactivation is observed in ~50% of human malignancies. This provides, in theory, potential targets for CTLs that recognize 8–9 amino acid, class I MHC-bound epitopes. Currently, several HLA-A2, HLA-A24, and HLA-B peptide epitopes have been identified (12, 13, 15, 16, 30–33). In many cases, those CTLs effectively lysed peptide-pulsed targets but were much less effective against tumor cells with p53 overexpression. Peptide-based immunization, although having the obvious advantage of using well-defined antigens, bears serious limitations: (a) patients need to be selected based on HLA class I type; (b) from a variety of possible epitopes, this method of immunization uses only one or two; (c) a single peptide epitope may not necessarily be expressed, even in MHC class I-matched tumor.

Wild-type, p53-derived, self-MHC-self-peptide complexes expressed by bone marrow-derived cells in the thymus cause negative selection of immature thymic T cells with high avidity for such complexes. This results in deletion of T cells with sufficient avidity to recognize natural wild-type p53 epitopes.
Transduced DCs and then used as effectors (3). Similar results were obtained in two other experiments. M in a CTL assay against 51 Cr-labeled target HTB-26 tumor cells (5). T2 cells loaded with either FLU-derived peptide (T2-FLU) or p53-derived peptide (T2-p53) or only with β2-microglobulin were used as targets. A standard 6-h 51Cr release assay was performed in duplicates. Averages at an E:T ratio of 60:1 are shown; bars, SD. Two experiments with similar results were performed.

**A**

Cytotoxicity is mediated by CD8+ CTLs. A, T cells from one healthy volunteer (D-1) and one patient (C.G.) were stimulated twice with p53-transduced DCs and then used as effectors (3 × 10^5 per well) in a CTL assay against 51Cr-labeled target tumor cells (5 × 10^3 cells/well) in presence of 50-fold excess (2.5 × 10^5 cells/well) of unlabeled K562 cells. The experiment was performed in duplicates, and averages are shown; bars, SD. Similar results were obtained in two other experiments. □, no K562; □, K562. B, T cells from two healthy volunteer (D-1 and D-3) were stimulated twice with p53-transduced DCs and then used as effectors (3 × 10^5 per well) in a CTL assay against 51Cr-labeled target T-2 cells (5 × 10^3 cells/well) in presence of 50-fold excess (2.5 × 10^5 cells/well) of unlabeled K562 cells. Experiments were performed in duplicates, and averages are shown; bars, SD. □, no K562; □, K562. C, T cells from one patient were stimulated twice with p53-transduced DCs and then used as effectors (3 × 10^5 per well) in a CTL assay against 51Cr-labeled target HTB-26 tumor cells (5 × 10^3 cells/well) in the presence of anti-CD4 or anti-CD8 antibodies. The experiment was performed in duplicates, and averages are shown; bars, SD. Similar results were obtained in two other experiments. ●, nontreated; ○, anti-CD4; ▲, anti-CD8.

**B**

The specificity of antitumor response. A, T cells stimulated with FLU-infected DCs are able to recognize FLU-derived epitope. T cells isolated from the HLA-A2-positive healthy donor (D-1) were stimulated twice with FLU-infected DCs or with Ad-p53 DCs. T2 cells loaded with either FLU-derived peptide (T2-FLU) or p53-derived peptide (T2-p53) or only with β2-microglobulin were used as targets. A standard 6-h 51Cr release assay was performed in duplicates. Averages at an E:T ratio of 60:1 are shown; bars, SD. Two experiments with similar results were performed. □, T2; □, T2-Flu; □, T2-p53. B, FLU-specific T cells are not able to recognize tumor cells with p53 accumulation. T cells described above were used as effectors in a CTL assay against tumor cell targets. Averages at an E:T ratio of 60:1 are shown; bars, SD. Two experiments with similar results were performed. □, DC-Ad-p53; □, DC-Flu.

Fig. 5 The specificity of antitumor response. A, T cells stimulated with FLU-infected DCs are able to recognize FLU-derived epitope. T cells isolated from the HLA-A2-positive healthy donor (D-1) were stimulated twice with FLU-infected DCs or with Ad-p53 DCs. T2 cells loaded with either FLU-derived peptide (T2-FLU) or p53-derived peptide (T2-p53) or only with β2-microglobulin were used as targets. A standard 6-h 51Cr release assay was performed in duplicates. Averages at an E:T ratio of 60:1 are shown; bars, SD. Two experiments with similar results were performed. □, T2; □, T2-Flu; □, T2-p53. B, FLU-specific T cells are not able to recognize tumor cells with p53 accumulation. T cells described above were used as effectors in a CTL assay against tumor cell targets. Averages at an E:T ratio of 60:1 are shown; bars, SD. Two experiments with similar results were performed. □, DC-Ad-p53; □, DC-Flu.

We suggest here another method of immunotherapy based on the use of full-length, wild-type p53. This approach may be devoid of many limitations of peptide-based immunization and would provide a valuable option for clinical trials. Overexpression of wild-type p53 in antigen-presenting cells would allow for presentation of several different epitopes. The feasibility of such an approach was shown previously in model experiments where each of the different minimal epitopes combined to a single fusion protein can be presented separately on the cell surface and be recognized by specific CTLs (35). DCs are the most potent antigen-presenting cells and are actively being used in cancer immunotherapy. Adenovirus provides a high efficiency of DC transduction. Different adenovirus constructs have been successfully used for DC-based immunotherapy in preclinical settings (21, 23, 36–38). Interestingly, very low or no anti-adenovirus response has been detected in this type of vaccination. We have demonstrated previously that transduction of mouse DCs with human wild-type p53 resulted in induction of p53-specific immune response (24). Although that study demonstrated that this method may be useful in principle, it did not provide answers to the main questions: whether this method can overcome tolerance to self-protein; and whether this response is effective in the killing of human tumor cells.

To answer these questions, we selected HLA-A2-positive healthy individuals and cancer patients. We and others have demonstrated previously that DCs generated from peripheral blood progenitors of patients with advanced cancer are functionally competent (27, 39, 40). These cells transduced with wild-type p53 were used in two rounds of stimulation of autologous T cells. As targets, we used two HLA-A2-positive tumor cell lines with accumulation of wild-type or mutant p53 proteins and as control one HLA-A2-positive and one HLA-A2-negative tumor cell line with normal levels of p53. Significantly, higher cytotoxicity against tumors with p53 overexpression than against control tumor cell lines was detected in all 3 healthy individuals and 8 of 9 cancer patients. Most of the previous studies have used multiple rounds of stimulations with peptides to achieve immunological effect. Here, we used only two stimulations to test the hypothesis that even a limited number of CTLs generated against a variety of p53 epitopes is sufficient to provide antitumor response. It may explain why CTLs generated from some of the patients did not recognize HLA-A2-bound presented by MHC class I molecules on tumor cells and thus prevents immune response. Only low avidity CTLs survive the induction of self-tolerance (34). The extent of such avoidance varied between the particular class I MHC-p53 peptide combinations (34). Therefore, the use of only one or two peptide epitopes for immunotherapy may limit the efficacy of the treatment.
peptide but at the same time recognized and killed p53-overexpressing tumor. Evidently, T cells can recognize p53 epitopes bound to other than HLA-A2 MHC class I molecules. This also may explain why in some patients HTB-22 tumor cells were better targets than HTB-26, and in some patients, the reverse was seen. Apparently, the level of HLA-A2 expression on tumor cells with p53 accumulation also did not significantly affect the CTL lysis, because HTB-26 cells had substantially higher HLA-A2 expression than HTB-22. Similar levels of cytotoxicity were seen against both control tumor cell lines (HLA-A2" and HLA-A2"). Our data indicate that presentation of antigens not associated with p53 (for instance, FCS-derived peptides) was not a significant factor in this assay. Overexpression of p53 in patients’ tumors also did not affect the ability of T cells to respond to stimulation with Ad-p53-transduced DCs. Experiments with inhibition of cytotoxicity with K562 cells and anti-CD4 or anti-CD8 antibodies demonstrated that tumor cell lysis was mediated predominantly by CD8\(^+\) CTLs.

Because of a polyclonal nature of T cells generated after two rounds of stimulations with Ad-p53 DCs, it is possible that some level of cytotoxicity against tumor cells could be mediated by alloreactivity. We used four different tumor cell lines and T cells from all 3 donors, and 8 of 9 patients recognized p53-overexpressing tumor cells significantly better than control tumor cell lines. Therefore, it is highly unlikely that possible alloreactivity may affect the interpretation of the data.

Four major facts support the conclusion that lysis was specific for p53: (a) CTL activity against tumor cells with p53 accumulation was significantly higher than against cells with normal levels of p53 (Fig. 2); (b) CTLs specific for FLU did not lyse tumor cells with p53 overexpression (Fig. 5); (c) in all three donors and 4 of 7 tested cancer patients, CTLs generated after stimulation with Ad-p53 DCs had significantly higher cytotoxic activity against targets loaded with p53-derived peptide epitope than with FLU-derived peptide epitope (Fig. 3); and (d) in experiments with SCC-78 cell line (HLA-A2 positive, p53 normal), infection with Ad-p53 resulted in significantly higher cytotoxicity than after infection of these cells with control adenovirus (Fig. 6).

Stimulation of T cells with Ad-p53 DCs did not lead to generation of substantial anti-adenovirus CTL response. These findings are consistent with data reported previously that demonstrated very low or undetectable anti-adenovirus CTL response after immunization of experimental animals with adenovirus-infected DCs (21, 23, 24). One of the possible explanations of this fact could be that replication-defective adenovirus does not provide sufficient level of antigens to induce strong CTL response.

In this study, we did not address the issue of possible autoimmune reaction after induction of potent anti-p53 immune response. Although in previous experiments using immunization with p53-derived peptides such autoimmune abnormalities were not reported, this important issue remains to be resolved.

In conclusion, these data for the first time indicate that DCs transduced with full-length wild-type p53 are able to generate a CTL response specific for tumors with p53 overexpression. This response was induced not only in 3 healthy volunteers but also in patients with advanced cancer. These findings demonstrate that this approach may overcome tolerance to self-protein and may serve as a valuable option in cancer immunotherapy.

REFERENCES

2. Chiba, I., Takahashi, T., Nau, M. M., D’Amico, D., Curiel, D., Mitsudomi, T., Buchhagen, D., Carbone, D., Koga, H., Reissmann,


Dendritic Cells Transduced with Full-Length Wild-Type p53 Generate Antitumor Cytotoxic T Lymphocytes from Peripheral Blood of Cancer Patients

Ekaterina Yu. Nikitina, Joseph I. Clark, James van Beynen, et al.