Human Monocyte-Derived Dendritic Cells Pulsed with Wild-Type p53 Protein Efficiently Induce CTLs against p53 Overexpressing Human Cancer Cells

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ABSTRACT

Purpose: Dendritic cells are the most potent antigen-presenting cells for initiating cellular immune responses. Dendritic cells are attractive immunoregulatory cells for cancer immunotherapy, and their efficacy has been investigated in clinical trials. The tumor suppressor gene p53 is pivotal in the regulation of apoptosis, and p53-based immunization is an attractive approach to cancer immunotherapy because of the accumulation of p53 protein in malignant but not in normal cells. It has been shown that dendritic cells transduced with an adenoviral wild-type p53 (wt-p53) construct mediate the antitumor immune responses against p53-overexpressing tumor cells. We examined whether monocyte-derived human dendritic cells pulsed with the purified full-length wt-p53 protein were also capable of inducing the specific antitumor responses against p53-overexpressing tumors in vivo.

Experimental Design: Immature dendritic cells generated in the presence of interleukin-4 and granulocyte/macrophage colony-stimulating factor from monocytes of HLA-A2- or HLA-A24-positive healthy individuals were pulsed with the purified p53 protein. Uptake of p53 protein by human dendritic cells was assessed by Western blotting and immunohistochemical staining using anti-p53 antibody. Induction of p53-specific CTL response was also evaluated by the cytotoxic assay against p53-overexpressing human tumor cells.

Results: Both Western blot and immunohistochemical analysis showed the accumulation of p53 protein in human immature dendritic cells. T cells obtained from HLA-A2- or HLA-A24-positive healthy donors were stimulated twice with p53 protein-pulsed dendritic cells and then applied to the cytotoxicity assay against p53-overexpressing target cells. The CTL activity was specific for p53-overexpressing tumor cells and MHC class I restricted. Moreover, the CTL activity generated by p53 protein-pulsed dendritic cells was nearly identical with that induced by adenoviral wt-p53-transduced dendritic cells.

Conclusions: Our results indicate that monocyte-derived human dendritic cells pulsed with the wt-p53 protein could induce the specific antitumor effect against p53-overexpressing tumors and that this in vitro model offers a new and more simple approach to the development of p53-based immunotherapy.

INTRODUCTION

Dendritic cells are highly effective antigen-presenting cells and play a central role in the induction of primary immune responses against tumor-associated antigens (1). The exceptional ability of dendritic cells to stimulate T cells is attributed to their ability to take up and present antigens, to secrete cytokines, and to express high levels of immunostimulatory molecules, such as B7.1 (CD80), B7.2 (CD86), and intercellular adhesion molecule-1 (CD54). Critical to the antigen-presenting function of dendritic cells is the fact that they can present tumor-associated antigens in the context of both MHC class I and II; thus, they can stimulate both CTL and helper T cells (2–4). Because of the advantageous character of dendritic cells compared with other antigen-presenting cells, numerous studies for vaccines based on dendritic cells have examined their efficacy in animal models (5, 6) as well as in human clinical trials (7–9).

Much attention has been directed to the problem of how and what antigens should be pulsed to the dendritic cells. In this regard, p53 protein is one of the most attractive candidates for dendritic cell–based immunotherapy, because this protein is found abundantly in ~50% of human malignancies but not in normal tissues (10). The p53 tumor suppressor gene plays a key role in cell growth control and differentiation (11). This gene containing missense mutations is often associated with a prolonged half-life, resulting in accumulation of the inactivated protein within the nuclei and cytoplasm of cancer cells. It has been shown that overexpression of the mutant p53 protein results in the presentation of p53 peptides by MHC class I molecules on the surface of tumor cells (12, 13). This may lead to the generation of multiple epitopes that could be recognized by CTLs. Indeed, previous studies have reported the successful generation of antitumor immune responses against tumors bearing a mutant p53 protein following either vaccination in vivo or stimulation of peripheral blood mononuclear cells (PBMC) in vitro using mutant or wild-type p53 (wt-p53) peptide-pulsed dendritic cells (14–16). However, this approach to cancer immunotherapy has some serious limitations. One strategy to overcome these limitations is to use the entire p53 sequences as an antigen.
One of the best vehicles for delivery of the entire p53 sequences to dendritic cells is the use of virus vectors encoding full-length wt-p53 gene. In fact, recent studies have reported that dendritic cells transduced with a recombinant replication-deficient adenovirus vector expressing wt-p53 gene were able to effectively present the recombinant protein antigen (p53) and could induce specific immune response against p53 protein in vitro and in vivo (17, 18). However, in terms of the clinical use, this effective approach with virus vectors still leaves several critical problems concerning safety. Accordingly, in this study, we showed that monocyte-derived human dendritic cells pulsed with purified full-length wt-p53 protein could also generate p53-specific CTLs in vitro. In addition, the CTL activity generated by whole p53-protein pulsed dendritic cells was almost the same as that by adenoviral wt-p53 (Ad-p53)—transduced dendritic cells. These results offer a new effective approach to the p53-based immunotherapy and provide justification for continuing the preclinical and clinical development of full-length wt-p53-pulsed dendritic cells.

MATERIALS AND METHODS

Tumor Cell Lines and Reagents. Tumor cell lines SW620 and KATO-III were maintained in RPMI 1640 supplemented with 10% FCS, 100 units/mL penicillin, and 100 mg/mL streptomycin, referred to as complete medium. Both cell lines are HLA-A2/A24 positive. The transformed embryonic kidney cell line 293 was grown in DMEM with high glucose concentration (4.5 g/L), supplemented with 10% FCS, 100 units/mL penicillin, and 100 mg/mL streptomycin. The 293 cells were used for the production of adenovirus vectors. Recombinant human cytokines granulocyte/macrophage colony-stimulating factor, interleukin-4 (IL-4), tumor necrosis factor-α (TNF-α), and IL-7 were purchased from Genzyme Techne, (Minneapolis, MN) and IL-2 was from Roche (Mannheim, Germany). 51Cr sodium chromate was obtained from NEN Life Science Products (Boston, MA).

p53 Protein and Adenovirus Vectors. Full-length wt-p53 protein as well as mutant p53 protein (His727 mutation) were kindly provided by Kyowa Hakko (Tokyo, Japan). The recombinant, replication-deficient adenovirus vector, Ad-p53, encodes full-length human wt-p53 cDNA. This virus was obtained by lysis of infected 293 cells. Titers of viral stocks were determined with a plaque-forming assay using 293 cells.

Cell Isolation. Monocyte-derived dendritic cells and T cells were obtained from peripheral blood of HLA-A2- or HLA-A24-positive healthy volunteers. In brief, mononuclear cells were isolated by sedimentation over Ficoll-Hypaque and were subsequently allowed to adhere in culture flasks for 1 hour at 37°C. Nonadherent cells were separated from the rest of mononuclear cells by gentle washing and were cryopreserved in RPMI 1640 with 20% FCS and 10% DMSO for further use. The remaining (adherent) cells were cultured for 6 days in the presence of granulocyte/macrophage colony-stimulating factor (50 ng/mL) or IL-4 (50 ng/mL) and used as immature dendritic cells.

Generation of Anti-p53 CTLs. p53-specific CTLs were generated as follows. On day 6, immature dendritic cells were incubated with wt-p53 protein at a final concentration of 0.2 μg/mL at 37°C for 24 hours. Subsequently, the protein-pulsed dendritic cells were activated with TNF-α (50 ng/mL) for 72 hours. In another experiments, immature dendritic cells were infected with Ad-p53 at the multiplicity of infection of 100 plaque-forming units per cell for 24 hours. Nonadherent mononuclear cells were then cocultured with p53 protein-pulsed or Ad-p53-transfected dendritic cells at a ratio of 10:1 in the wells of a 24-well plate in a final volume of 2 mL/well complete medium containing IL-2 (10 units/mL) and IL-7 (5 ng/mL). After 7 days of culture, the responder cells were collected and restimulated with p53 protein-pulsed or Ad-p53-transfected dendritic cells again. Fifty percent of the medium were replaced on days 3 and 5 after each stimulation by complete medium supplemented with IL-2 (10 units/mL) and IL-7 (5 ng/mL). On 14 day of coculture, the responder cells were harvested and assessed for cytolytic activity in a standard 6-hour chromium release assay.

Cytotoxicity Assay. Standard 6-hour chromium release assay was done to measure cytolyis. Target cells were incubated with 100 mCi Na2CrO4 for 1 hour, washed, and added to the wells of U-bottomed 96-well plates (5 × 10^3 cells per well). Effector T cells were then added in triplicates to give various E:T ratios ranging from 80:1 to 10:1. After a 6-hour incubation, the supernatants were collected, and the radioactivity was measured in a scintillation counter. The percentage of specific lysis was calculated according to the formula: % Specific lysis = [(experimental cpn - spontaneous cpn) / (maximal cpn - spontaneous cpn) × 100].

Flow Cytometric Analysis. The cell surface expression of dendritic cell markers was assessed by flow cytometric analysis. Antibodies used to evaluate the phenotype of dendritic cell were anti-HLA-DR-FITC, anti-CD80-FITC, anti-CD86-PE, and anti-CD83-PE (ImmuNoTech, Marseilles, France). Dendritic cells were stained for 20 minutes at 4°C and analyzed by the FACScan using Cell Quest software. Ten thousand cells were examined for each determination. Autologous T cells cultured with p53 protein-pulsed dendritic cells were also analyzed for the phenotype by flow cytometric analysis using anti-CD4 and anti-CD8 antibodies that were purchased from Becton Dickinson (San Jose, CA).

Immunostaining. Cytosin preparations of p53 protein-pulsed dendritic cells and tumor cell lines were fixed in 4% paraformaldehyde and stained with the primary antibody against human p53 (Ab-2, Oncogene Science, Manhasset, NY) overnight at 4°C. This staining was done according to the protocol provided by the manufacturer of Envision/HRP System (DAKO, Carpinteria, CA).

Measurement of Cytokine. Supernatants of the culture were recovered and assayed in duplicate by ELISA using commercially available reagents (PharMingen, San Diego, CA). The concentration of cytokine in the supernatant was determined by regression analysis.

Statistical Analysis. Student’s t test was used to compare differences in CTL reactivities against different tumor cell lines. Statistical significance was defined when P < 0.05.

RESULTS

Preparation of Dendritic Cells and p53 Protein. Representative phenotypic characteristics of immature and mature dendritic cells that were used in these studies are shown in Fig. 1A. Flow cytometric analysis showed that additional
maturation of immature dendritic cells in TNF-α (25 ng/mL) and prostaglandin E2 (1 μg/mL) led to the expression of HLA-DR, CD80, CD86, and CD83 markers on the cell surface. We next did Western blot analysis using antibody against human p53 to confirm the stability of full-length wt-p53 protein. Both full-length wild-type and mutated p53 proteins could be equally detected at the same level of expression, suggesting that wt-p53 protein is quite stable in vitro (data not shown).

**Uptake of Full-length p53 Protein by Human Dendritic Cells.** To confirm that human immature dendritic cell ingests the full-length wt-p53 protein spontaneously, we carried out immunohistologic analysis. Immunohistochemistry showed that immature dendritic cells pulsed with wt-p53 protein at a final concentration of 0.2 μg/mL for 24 hours exhibited intense cytoplasmic and nuclear staining of p53 protein (Fig. 2A). We also coincubated wt-p53 protein with immature dendritic cells for various periods at various concentrations to determine the optimal condition. The uptake of p53 protein was quantitatively equivalent in immature dendritic cells pulsed with 0.2 μg/mL of p53 for 24 hours and immature dendritic cells pulsed with 1.0 μg/mL of p53 for 3 hours (Fig. 2B). However, the viability of dendritic cells pulsed with p53 at 1.0 μg/mL for 24 hours was reduced (data not shown). These results suggest that immature dendritic cells can effectively ingest the full-length wt-p53 protein when pulsed at 0.2 μg/mL for 24 hours.

**Influence of wt-p53 Protein on Differentiation and Activation of Human Monocyte-Derived Dendritic Cells.** To evaluate the effect of wt-p53 protein on the differentiation and activation of human immature dendritic cells, we analyzed the cell surface phenotype of dendritic cells pulsed with wt-p53 protein at 0.2 μg/mL for 24 hours or control immature dendritic cells by flow cytometry. The intensity of expression of surface markers, such as costimulatory and adhesion molecules, remained stable on wt-p53 protein-pulsed dendritic cells compared with that on control immature dendritic cells (Fig. 3). We next examined whether exposure to TNF-α (50 ng/mL) could induce maturation on dendritic cells pulsed with wt-p53 protein. TNF-α increased in the expression levels of surface markers, including HLA-DR, CD80, CD86, and CD83, in a time-dependent manner (Fig. 3). These results suggest that the wt-p53 protein had no apparent influence on dendritic cell maturation.

We also examined whether p53 protein could alter the cytokine production profiles of dendritic cells. After a culture period of 3 days, the supernatants of control immature dendritic cells and wt-p53 protein-pulsed immature dendritic cells were harvested, and the levels of IFN-γ in the supernatants were measured by ELISA. Both control and p53 protein-pulsed immature dendritic cells produced low to undetectable amounts of IFN-γ (0.101 ± 0.048 and 0.303 ± 0.247 pg/mL, respectively).
respectively), indicating that p53 protein had no apparent effect on cytokine production of dendritic cells.

**Induction of p53-Specific CTL Response against Tumor Cells.** We selected two HLA-A2/A24 human tumor cell lines, SW620 and KATO-III, as targets for p53-specific CTLs. Human colorectal cancer cell line SW620 exhibits overexpression of p53 caused by the specific mutation at codon 273, whereas human gastric cancer cell line KATO-III is p53 null. Immunohistochemistry confirmed that SW620 cells are p53 positive and KATO-III cells are p53 negative (Fig. 4). SW620 and KATO-III cells were positive for MHC class I expression (19, 20).

p53-specific CTLs were generated with p53 protein-pulsed dendritic cells from autologous T cells of three healthy HLA-A2+ donors, and the CTL response against two target cell lines was assessed by a standard 6-hour 51Cr release assay. As shown in Fig. 5A, effector T cells stimulated with wt-p53 protein-pulsed dendritic cells could effectively lyse p53-overexpressing SW620 cells. On the other hand, p53-null KATO-III cells were only minimally lysed. Although mononuclear cells primed by Ad-p53-transduced dendritic cells also specifically lysed p53-overexpressing SW620 cells, the lytic activity was almost the same as that stimulated with wt-p53 protein-pulsed dendritic cells. Phenotypic analysis of effector T cells indicated that the percentages of CD4+ and CD8+ cells were 68.13% and 24.41%, respectively. To further verify the specificity of the CTL response, the cytotoxicities of p53-specific CTLs, human lymphokine-activated killer (LAK) cells, and untreated T cells were examined against SW620 and KATO-III cell lines. LAK cells were generated from nonadherent mononuclear cells in the presence of IL-2 (100 units/mL) for 3 days. The lytic activity of CTLs induced by p53 protein-pulsed dendritic cells against SW620 cells was comparable with that of LAK cells. In contrast, LAK cells effectively killed KATO-III cells, whereas cells were only minimally lysed by CTLs (Fig. 5B). These results were consistent with CTLs primed by Ad-p53-infected dendritic cells.

To show the broad applicability of p53 protein-pulsed dendritic cells, we examined whether p53-specific CTLs could be generated from HLA-A24+ healthy volunteers. Besides the HLA-A2 allele, HLA-A24 is one of the most frequently expressed HLA-A alleles. CTL responses against mutant p53-expressing SW620 cells, but not against p53-null KATO-III cells, were induced with wt-p53-pulsed dendritic cells obtained from two HLA-A24+ healthy volunteers (Fig. 6). These results suggest that wt-p53 protein-pulsed dendritic cells are likely to present multiple p53 epitopes on different class I HLA molecules, such as HLA-A24 and HLA-A2.

**DISCUSSION**

The p53 mutations or functional inactivation in human cancers lead to accumulation of p53 protein, whereas normal cells have very low levels of p53 expression. This makes p53 protein a potent target for immunotherapy of human cancer. Here, we show...
that CTLs induced by in vitro activation with dendritic cells pulsed with full-length wt-p53 protein elicited effective killing of p53-overexpressing human tumor cell lines. Several groups have reported previously that MHC class I–bound p53 peptides induced p53-specific CTLs, and several clinical trials are currently in progress (14–16); these CTLs are however mostly much less effective against p53-overexpressing tumor cells in spite of very effective cell lysis against peptide-pulsed targets. These observations suggest the limitation of peptide-based vaccine therapy and led us to examine whether monocyte-derived dendritic cells pulsed with the entire recombinant sequence of p53 protein efficiently induce p53-specific CTLs in vitro.

Recently, Nikitina et al. (17, 18) have reported that dendritic cells transduced with full-length wt-p53 gene using an adenovirus vector could generate a specific antitumor immune response. Overexpression of the entire sequence of wt-p53 in dendritic cells might present several different epitopes not only on the MHC class I but also on the MHC class II that induces CD4+ T-cell immune response. This strategy may be quite attractive; the application of adenovirus vector however made its clinical use difficult concerning safety. In addition, immunomodulatory proteins coded by an adenoviral vector might be expressed in dendritic cells, recognized as antigens compounded into cells, and scarcely presented on the MHC class II molecules. Thus, we suggest an alternative method of p53 protein-based immunotherapy using dendritic cells pulsed with full-length wt-p53 protein. After incubation with whole p53 protein, dendritic cells may process the endocytosed p53 proteins into peptides that are bound to MHC class II molecules and be presented on the cell surface for stimulation of CD4+ T helper lymphocytes. In addition to this classic mechanism of antigen presentation, dendritic cells turned out to be capable of processing exogenous proteins by an alternative pathway leading to peptide presentation on MHC class I molecules, which is called cross-presentation.

Because immature dendritic cells are highly effective in taking up and processing antigens compared with mature dendritic cells, we used monocyte-derived immature dendritic cells to be pulsed with wt-p53 protein. Immunohistochemical analysis showed a sufficient ingestion of pulsed p53 protein (Fig. 2). Generation of antitumor immunity by dendritic cells is intimately linked to dendritic cell maturation stage. Recent reports have shown that recombinant adenovirus infection induces partial maturation of dendritic cells via a nuclear factor-κB–dependent pathway (21). We also found that infection of adenovirus slightly up-regulated the expression of costimulatory and MHC class II surface molecule 3 days after infection (data not shown). On the other hand, p53 protein-pulsed dendritic cells showed no distinct alteration of the expression of surface markers (Fig. 3). Accordingly, we concluded that the maturation of p53 protein-pulsed dendritic cells would be indispensable. We next asked which activators would be suitable for maturation of p53 protein-pulsed dendritic cells. Many dendritic cell activators, including CD40 ligand, various cytokines, bacterial or viral products, calcium ionophores, and others (22–27), had been already reported to up-regulate the costimulatory molecule expression, secrete IL-12, and effectively present antigens. Among these dendritic cell activators, TNF-α is one of the most commonly used cytokines for this purpose. Although TNF-α alone is not sufficiently potent to cause full maturation of dendritic cells, it was selected as a dendritic cell stimulator in this study based on clinical applicability.

Fig. 5 Cytolytic reactivity of PBMC-derived CTLs against SW620 and KATO-III human cancer cells. Mean ± SD from three wells. A, cell lysis activity of T cells from three HLA-A2+ healthy volunteers stimulated with Ad-p53-transfected dendritic cells (left) or wt-p53 protein-pulsed dendritic cells (right) against target cells was assessed by 6-hour standard 

\[ ^{31} \text{Cr} \] release assay. Each experiment was done in triplicates. Points, mean at four different E:T ratios; bars, SD. B, CTLs were compared with LAK cells and untreated T cells, which served as positive and negative controls, respectively. Top, Ad-p53-transfected dendritic cells; bottom, wt-p53 protein-pulsed dendritic cells.

Fig. 6 Cytolytic reactivity of T cells obtained from two HLA-A24+ healthy donors exposed to wt-p53 protein-pulsed dendritic cells was also assessed by CTL assay. Points, mean from three wells; bars, SD.
Another important area of our investigation was to define the appropriate maturation state of dendritic cells. It has been reported that Ad-p53-transduced dendritic cells activated with CD40 ligand were able to break tolerance to self-p53 protein and induce potent antitumor response in mice, whereas nonactivated p53-expressing dendritic cells were unable to overcome the tolerance (17). Moreover, recent studies have shown that dendritic cells that ingest apoptotic tumor cells or pulsed with tumor cell lysate could also effectively generate tumor-specific T-cell response in vitro when mature (28, 29). In our study, flow cytometric analysis showed that treatment with TNF-α induced maturation of p53 protein-pulsed dendritic cells in a time-dependent manner (Fig. 3). Accordingly, we used p53-expressing dendritic cells stimulated with TNF-α for 3 days in subsequent experiments. However, it has been reported recently that dendritic cells exhibited a direct cytotoxic effect on tumor cells and that immature dendritic cells undergoing maturation induced a much stronger tumor-specific growth inhibitory effect than immature dendritic cells or mature dendritic cells through TNF-α-dependent and TNF-α-independent mechanisms (30).

We showed that intratumoral injection of nonactivated bone marrow–derived dendritic cells transduced with adenovirus expressing murine wt-p53 gene could mediate a greater systemic immune response against tumor cells than those of dendritic cell alone or control vector-transduced dendritic cells (31). Immature dendritic cells delivered locally at the site of tumors can be expected to ingest adjacent tumor cells and present additional tumor antigens in vivo, which may be facilitated by maturing dendritic cell–mediated direct cell lysis and tumor-specific CTL responses. Therefore, dendritic cells undergoing maturation might be more appropriate than fully mature dendritic cells, when a clinical trial of intratumoral administration of p53 protein-pulsed dendritic cells is tested.

To confirm the successful generation of CTLs from PBMC of a healthy HLA-A2-positive donor in vitro with p53 protein-pulsed dendritic cells that recognize the wt-p53 epitopes, we examined the lytic activity against target the human tumor cell lines SW620 and KATO-III. These HLA-A2- and HLA-A24-positive tumors showed either accumulation of mutant p53 molecules with a point mutation at codon 273 or expression of a p53-null genotype with no p53 accumulation (Fig. 4). We detected high CTL responses against mutant p53-expressing SW620 cells after 7-day in vitro stimulation of PBMC with p53 protein-pulsed dendritic cells; however, p53-null KATO-III cells were not effectively killed by CTLs (Fig. 5a). The observation that LAK cells lysed KATO-III target cells (Fig. 5b) suggest that the low killing activity of CTLs against KATO-III cells is due to the lack of p53 antigen presentation. In other words, the effector cells are likely to be p53-specific CTLs. We also showed that p53-specific CTLs could be generated from PBMC of a HLA-A24-positive donor in vitro by using p53 protein-pulsed dendritic cells in the same in vitro stimulation method (Fig. 6). Based on these findings, we speculate that multiple p53 peptides are presented on HLA class I molecules and recognized by autologous CTLs. Our findings indicate that dendritic cells pulsed with p53 protein can overcome the limitations of peptide-based vaccines, such as a priori knowledge of the patient’s HLA haplotype to select appropriate peptides compatible with that particular haplotype. Therefore, it may permit a more potent immune response involving the presentation of epitopes from all possible restriction elements. To answer this question, we are currently investigating the lytic activity of p53-specific CTLs against target tumor cells pulsed with different p53-derived peptides.

Taken together, our study indicate that monocyte-derived human dendritic cells pulsed with purified full-length wt-p53 protein can generate p53-specific CTLs whose lytic activity against p53-overexpressing target cells is similar to that induced by stimulation with Ad-p53-transduced dendritic cells in vitro. To the best of our knowledge, this is the first report describing the CTL induction with full-length wt-p53 protein-pulsed dendritic cells. Our data offer a new and potentially valuable option of p53-based immunotherapy for human cancer.

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