Full-Length Dominant-Negative Survivin for Cancer Immunotherapy

Vladimir Pisarev, Bin Yu, Raoul Salup, Simon Sherman, Dario C. Altieri, and Dmitry I. Gabrilovich

1University of Nebraska Medical Center Eppley Cancer Center, University of Nebraska Medical Center, Omaha, Nebraska; 2H. Lee Moffitt Cancer Center and Department of Interdisciplinary Oncology, University of South Florida, Tampa, Florida; and 3Cancer Center and Department of Cancer Biology, University of Massachusetts School of Medicine, Worcester, Massachusetts

ABSTRACT

Purpose: The goal of this study is to investigate the possibility of using dendritic cells (DCs) transduced with the full-length dominant-negative survivin for cancer immunotherapy.

Experimental Design: Mononuclear cells were collected from HLA-A2-positive healthy volunteers and patients with prostate cancer. DCs were transduced with an adenoviral vector containing a full-length, dominant-negative survivin gene. After three rounds of stimulation, the T-cell response against three different survivin-derived HLA-A2-matching peptides was tested in IFN-γ enzyme-linked immunospot and CTL assays.

Results: Seven of eight healthy volunteers and cancer patients showed a significant response to at least two different survivin-derived epitopes in the enzyme-linked immunospot assay. One patient responded to only one peptide. All four healthy volunteers and two of three patients tested demonstrated a specific CTL response against T2 target cells loaded with one survivin-derived epitope. Two donors and two patients had a significant CTL response against two different epitopes. Significant cytotoxicity activity was seen against HLA-A2-positive MCF-7 tumor cells that express survivin. That response was specific for survivin and was MHC class I restricted. Because survivin is expressed in CD34+ hematopoietic progenitor cells (HPCs), we tested whether the antisurvivin CTLs can recognize normal HPCs. The incubation of survivin-specific CTLs with CD34+ cells did not significantly decrease the colony-forming ability of HPCs.

Conclusions: DCs transduced with dominant-negative survivin induce potent survivin-specific CTL responses able to recognize and kill tumor cells. This response does not significantly affect HPCs. Thus, this study may provide rationale for immunotherapeutic clinical trials using a DC vaccine transduced with the dominant-negative survivin.

INTRODUCTION

The success of cancer immunotherapy is dependent on using suitable tumor-associated antigens (TAAs). An ideal TAA should have several important features. First, TAA should induce T cells that recognize tumors but not normal cells. Second, TAA should be expressed in tumors from a significant proportion of the patients and in a significant proportion of tumor cells. Third, it is important that TAA belongs to molecules, which are obligatory for the survival of tumor cells. Immunization against such TAA(s) would overcome tumor escape attributable to antigenic variation because losing the survival-related molecule may result in tumor cell death. At this time, there are very few molecules that fit these criteria. Survivin may be one such rare candidate.

Survivin is a Mr 16,500 antiapoptotic protein that belongs to the inhibitory apoptotic protein family. It preferentially blocks mitochondrial-dependent apoptosis by targeting caspase 9 and SMAC/DIABLO (second mitochondria-derived activator of caspases/direct inhibitory apoptotic protein-binding protein with a low isoelectric point). It also plays a critical role in mitosis, microtubule stability, and embryonic development (see Ref. 1 for review). Survivin is expressed at a high level in virtually every human cancer. The overexpression of survivin has been documented in squamous cell carcinoma (2); esophageal, colon, gastric, and pancreatic carcinomas (3–7); the majority of breast carcinomas (8); bladder tumors (9); hepatocellular carcinoma (10); melanoma (11); and other malignancies. The overexpression of survivin in cancer patients correlates with a more aggressive disease, poor survival (12, 13), and drug resistance (5, 14). Normal, terminally differentiated adult tissues do not express survivin. However, some of the following normal adult cells also express survivin: thymocytes (15); CD34+ bone marrow-derived hematopoietic progenitor cells (HPCs; Ref. 16); basal colonic epithelial cells (17); and activated endothelial cells (18).

Survivin is a short-lived protein with a half-life of about 30 min (2). A ubiquitin proteasome pathway regulates its degradation. It is expected that the overexpression of survivin in the cytoplasm of tumor cells and rapid degradation by proteasome-related mechanism result in an increased expression of survivin-derived epitopes on the tumor cell surface. These epitopes in association with MHC class I molecules may represent targets for CTLs. Immune responses to survivin include the generation
of antibodies (19) and type 1 T-helper cells and CTL responses in cancer patients (20, 21). Human HLA-A2-positive dendritic cells (DCs) pulsed with survivin-derived HLA-A2-matching peptide induced CD8+ effector T cells against survivin DNA-transfected or peptide-pulsed nontumor targets (20). In a recent study, colorectal carcinoma cells were recognized by survivin-specific T lymphocytes, and the survivin-specific MHC class I-restricted T lymphocytes were activated in response to HLA/survivin-peptide complexes expressed by tumor cells (22). These findings demonstrated that survivin could be considered a valuable TAA for immunotherapy.

Most of the TAAs exist in the form of MHC class I-restricted peptides. However, a peptide-based approach to cancer immunotherapy has some limitations. Defined peptide epitopes are often different from the epitopes expressed on the majority of targeted tumors, presumably because of differences in TAA-proteasomal processing. TAA may undergo posttranslational modifications (23–25) that may allow tumors to escape from recognition after immunization with a native form of antigen. MHC class I-restricted peptides require a selection of patients based on MHC class I alleles. These limitations stimulated the search for more comprehensive approaches to use TAA for vaccination against cancer.

The introduction of the genes into DCs may represent such an alternative approach. The overexpression of selected TAA genes in DCs would allow for the 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 was presented separately on the cell surface and recognized by specific CTLs (26). Adenovirus provides a high efficiency of DC transduction. Different adenovirus constructs have been used successfully for DC-based immunotherapy in preclinical settings (27–32).

In this study, for the first time, we investigated the possibility of using a full-length survivin gene in cancer immunotherapy. Using cells isolated from healthy volunteers and cancer patients, we demonstrated that DCs transduced with dominant-negative survivin induced a survivin-specific CTL response against several different epitopes and that this response did not suppress HPCs.

### MATERIALS AND METHODS

#### Healthy Volunteers and Cancer Patients.

Six HLA-A2-positive and two HLA-A2-negative healthy volunteers (age, 27–48 years) were enrolled in this study along with four cancer patients (age, 57–65 years). All patients had histologically confirmed prostate cancer with no prior chemotherapy (Table 1).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gleason score</th>
<th>PSA (ng/ml)</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>57</td>
<td>5</td>
<td>3</td>
<td>T2AN1M0</td>
</tr>
<tr>
<td>P-2</td>
<td>65</td>
<td>5</td>
<td>7.6</td>
<td>T2CN1M0</td>
</tr>
<tr>
<td>P-3</td>
<td>62</td>
<td>6</td>
<td>6.3</td>
<td>T2CN1M0</td>
</tr>
<tr>
<td>P-4</td>
<td>62</td>
<td>7</td>
<td>4.9</td>
<td>T2CN1M0</td>
</tr>
</tbody>
</table>

* PSA, prostate-specific antigen.

#### Tumor Cell Lines and Reagents.

MCF-7 and K562 cells were obtained from American Type Culture Collection (Manassas, VA). MCF-7 is a HLA-A2-positive breast tumor cell line with documented overexpression of survivin. The T2 cell line is a processing-deficient lymphoma expressing transporter associated with antigen processing-I mutation. K562 is a HLA class I negative erythroleukemia cell line that is sensitive to natural killer lysis. These cell lines were maintained in a complete culture medium [(CCM) RPMI 1640 containing 10% FCS; Life Technologies, Inc., Grand Island, NY]. Recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4, IL-7, and IL-2 were obtained from RDI (Flanders, NJ).

#### Adenoviral Vectors.

Replication-defective control adenovirus and the adenovirus-containing wild-type p53 gene were obtained from Intron Therapeutics (Houston, TX). Adenoviruses containing human wild-type (Ad-Surv) or Thr34-Ala mutant (Ad-mSurv) survivin were described in detail elsewhere (34).

#### Peptides.

Control peptide LLGRNSFEV derived from wild-type p53 (sequence 264–272) has demonstrated a high affinity for HLA-A2 and was used in our previous studies (32). One HLA-A2-matched survivin-derived peptide, 95ELTLGERFLKL114 (ELT), has been described previously to bind HLA-A2 and induce HLA-A2-restricted T cells in cell culture (20, 21). Two additional epitopes, 104LDRERAKNK113 (LDR) and 61FKELEGWEP69 (FKE), were selected by the PaProC algorithm (35, 36) for optimal proteasomal cleavage prediction and by SYFPEITHI algorithm for HLA-A2 binding (37). ELT and LDR were predicted as high-affinity binding epitopes, whereas FKE was expected to demonstrate poor binding to HLA-A2 (prediction scores were 19, 15, and 2, respectively). To increase affinity for HLA-A2 of the latter epitope, two amino acids in anchor positions 2 and 9 have been substituted. The resulting peptide FLELEGWEP (FLE) and other peptides were synthesized by SynPep (Dublin, CA) and tested for binding to HLA-A2.

#### MHC Stabilization Assay.

Survivin-derived peptides were dissolved in DMSO and analyzed using T2 cells expressing HLA-A2. Briefly, 5 × 10^5 T2 cells were incubated in a volume of 0.5 ml with 250 µg/ml of each peptide in AIM-V medium containing 0.1% fetal bovine serum, 2 mM l-glutamine, and 5 × 10^-5 M β-mercaptoethanol in a 48-well cell culture cluster 3548 (Costar, Cambridge, MA) at 5% CO_2 and 26°C for 12 h, followed by a 2.5-h incubation at 37°C. To exclude the possible influence of DMSO (final concentration, <1%) on the assay, control cells (no peptide added) were incubated with the same amount of DMSO as cells with the peptides. After the incubation, surface HLA-A2 molecules were stained for 30 min with 10 µl/sample mouse anti-HLA-A2 monoclonal antibodies (One Lambda, Inc., Canoga Par, CA). Cells were then washed and treated with a 1:200 dilution of donkey antimouse IgG (H+L) F(ab')_2 labeled with phycoerythrin (RDI) for 30 min. Cells were analyzed by flow cytometry using a FACSCalibur and CellQuest software (BD Immunocytometry Systems, San Francisco).

#### TABLE 1 Patients enrolled in the study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>T2AN1M0</td>
</tr>
<tr>
<td>P-2</td>
<td>T2CN1M0</td>
</tr>
<tr>
<td>P-3</td>
<td>T2CN1M0</td>
</tr>
<tr>
<td>P-4</td>
<td>T2CN1M0</td>
</tr>
</tbody>
</table>
Jose, CA). Fig. 1 demonstrates that novel survivin-derived epitope LDR binds to HLA-A2, whereas FKE possess relatively poor binding activity. As predicted, modified epitope FLE displays increased binding activity compared with all other tested peptides.

**Cell Isolation.** DCs and T cells were obtained from the peripheral blood of the volunteers and patients as described previously (38), with some modifications. Briefly, mononuclear cells were incubated overnight in CCM (RPMI 1640, 10% FCS, and antibiotics). Nonadherent cells were collected, and adherent cells were cultured for 6–7 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 2 and day 4. DCs were collected, washed in serum-free medium, and used in additional studies. Collected nonadherent cells were centrifuged over 13.7% metrizamide gradient (Sigma, St. Louis, MO). Cells in interface contained enriched population of DCs. These directly isolated DCs were washed in serum-free medium and used for infection with adenoviruses. Cells from the pellet were used for isolation of T cells on a T-cell enrichment column (R&D Systems, Minneapolis, MN). For the stimulation of T cells in enzyme-linked immunospot (ELISPOT) assay, DCs (2 × 10^5 cells/well) were incubated in serum-free medium and used in flow cytometry. After a 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 2ml of CCM for an additional 24h. After incubation, 1.5ml of the medium were removed, and 1.5 × 10^6 T lymphocytes were added in 1.5ml of CCM containing 25ng/ml IL-7. Interleukin-2 (1.5ng/ml) was added 2 days later. After 7 days of culture, T cells were collected and restimulated with infected DCs generated from the first blood sample mononuclear cells using GM-CSF and IL-4 as described above. Interleukin-7 (25ng/ml) was added immediately after restimulation, and IL-2 (1.5ng/ml) was added 2 days later. T cells were restimulated again 1 week later using DCs directly isolated from the second blood sample as described above.

**CTL Assay.** Cytolysis was measured with a standard 6-h chromium release assay. Target cells were labeled with 100μCi of Na_2^1^1^C^r^O_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 51Cr release was <20% of that of maximum release. For competition assays, unlabeled K562 cells were added to the effector cells in 10-fold excess of 51^Cr^-labeled target cells. For the peptide-specific CTL assay, T2 cells (1.6 × 10^5 cells/well) were loaded with 10μg/ml β2-microglobulin (Sigma) and 100μM control or survivin-derived peptides. After overnight incubation, cells were washed, labeled with 51Cr, and used as targets for CTL assay. Control T2 cells were incubated with β2-microglobulin alone.

**IFN-γ ELISPOT Assay.** ELISPOT assay was performed as described previously (39), with some modifications. The 96-well multiscreen hemagglutinin filtration plates (Millipore, Bedford, MA) were coated with 50μl of mouse antihuman IFN-γ monoclonal antibody (MAB285; R&D Systems; final concentration, 12.5μg/ml). After overnight incubation at 4°C, wells were washed four times with PBS. The remaining proteinbinding sites were blocked by incubating plates for 2 h at 37°C with 20μl/well RPMI 1640 supplemented with 10% human serum. Cells obtained after three rounds of stimulation with survivin-transduced DCs (10^5 cells/well) were incubated in quintuplicate together with freshly generated DCs (2 × 10^4 cells/well), 10μg/ml specific or control peptides, and 2ng/ml IL-2. After 24 h of incubation at 37°C, wells were washed six times with PBS-containing 0.05% Tween 20. Plates were then incubated overnight at 4°C with 100μl (5μg/ml) of biotinylated goat antihuman IFN-γ antibody (BAF285; R&D Systems), and wells were washed six times with PBS-containing 0.05% Tween 20 and incubated for 2 h at room temperature with 50μl (1.25μg/ml) of avidin-alkaline phosphatase (Sigma). Wells were washed three times with PBS containing 0.05% Tween 20, washed three times with PBS, and then incubated with 50μl of substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Sigma) for 10–15 min. The reactions were stopped by discarding the substrate and washing the plates under tap water. The plates were then air dried, and colored spots were counted using a stereomicroscope.
Colony Formation. CD34⁺ cells were isolated from the peripheral blood of two healthy donors using a magnetic bead separation kit and column from Miltenyi Biotec (Auburn, CA). Cells were placed in 24-well plates and cultured overnight in CCM with 20 ng/ml GM-CSF. After that time, CD34⁺ cells were collected, washed, and cultured at different ratios with T cells obtained after three rounds of stimulation with either Ad-mSurv-DCs or untreated DCs as described above. Cells were incubated in triplicate for 4 days and then plated in semi-solid medium supplemented with cytokines supporting the growth of myeloid and erythroid colonies Methocult H4435 (StemCells Technology, Vancouver, Canada). Colonies were counted on days 12–14.

Statistical Analysis. Statistical analysis was performed using parametric methods and JMP statistical software (SAS Institute Inc., Cary, NC). A paired t test was used for group analysis. For each individual case, differences were considered significant if analyzed values were >2 SDs higher than in control.

RESULTS

In the initial “proof-of-concept” experiments, we tested the adenoviral construct containing full-length wild-type survivin (Ad-Surv). After three rounds of stimulation with Ad-Surv-DCs, T-cell response against one survivin-derived epitope (ELT) was evaluated in ELISPOT and CTL assays. All four tested HLA-A2 donors demonstrated a significant specific response against this peptide (Fig. 2, A and B). The immune response to three other survivin-derived epitopes was measured using cells from two volunteers by ELISPOT assay. As shown in Figs. 2A and 3A, a significant response against peptides LDR and FKE, but not to modified peptide FLE, was detected in both tested individuals. The results of these experiments suggest that DCs trans-
duced with Ad-Surv were able to induce a specific immune response. Clinical use of a full-length gene encoding potent antiapoptotic protein (survivin) is very problematic. Therefore, we tested the adenoviral construct containing mutant survivin. It has been made by substituting Thr with Ala in position 34 and designated T34A. This mutation abolishes the p34cdc2-phosphorylation site and makes this mutant protein dominant negative (34). Because this is the only single amino acid substitution, there was a very high probability that DCs transduced with mutant survivin could generate CTLs against multiple epitopes derived from the wild-type protein. For instance, this substitution does not affect the sequence of any of HLA-A2-specific epitopes used in preliminary experiments.

Because of the dominant-negative nature of the T34A construct, we asked whether the transduction of DCs with Ad-mSurv might induce apoptosis in these cells and thus prevent the induction of T-cell responses. DCs were transduced with either Ad-Surv or Ad-mSurv and incubated in complete medium without cytokines for 24, 48, and 72 h. Apoptosis in the green fluorescent protein-positive cell population was measured by flow cytometry using staining with phycoerythrin-conjugated Annexin V and 7-amino actinomycin D. A 3-day culture of untreated DCs in CCM resulted in a gradual increase in the apoptosis of these cells (from 3.7 ± 2.5% before the start of the culture to 58.1 ± 7.4% after 3 days of culture; \( P < 0.01 \)). DCs transduced with Ad-Surv demonstrated a similar level of apoptosis after a 3-day culture (53.0 ± 5.5%; \( P > 0.05 \)). The same proportion of apoptotic DCs was found after transduction with Ad-mSurv (51.9 ± 6.8%; \( P > 0.05 \)). No differences from the control were seen at any other time point (24 and 48 h; data not shown). Thus, the transduction of DCs with T34A mutant survivin did not result in enhanced apoptosis of these cells.

We measured immune response after three rounds of in vitro stimulation with Ad-mSurv-DCs in four HLA-A2* healthy individuals and four HLA-A2* patients with early-stage prostate cancer. After these three rounds of stimulation, almost all (>98%) viable cells were CD3+ T cells. CD4+ cells represented 65–75% of these cells, and CD8+ cells represented 25–35% of these cells. A specific immune response against the ELT peptide was determined in all eight individuals (Figs. 3B and 4) who were tested. In addition, all four healthy individuals and two of four patients had a significant response against another survivin-derived peptide, LDR. One healthy individual and two patients showed a significant response against the FKE peptide, whereas no response was detected against the modified FLE peptide (Fig. 4). Thus, with one exception (P-2), all of the tested healthy individuals and patients showed a significant response to at least two different survivin-derived peptides (Fig. 4). The two most immunogenic epitopes (ELT and LDR) were used in a CTL assay. For the analysis of each individual case, the level of cytotoxicity against target T2 cells loaded with specific peptides (ELT or LDR) was considered as significant if it was >2 SDs higher than the level of cytotoxicity against target T2 cells loaded with the control p53-derived peptide. As expected, the levels of cytotoxicity varied from individual to individual. In all four healthy individuals and two of three tested patients, the level of cytotoxicity against ELT-pulsed T2 cells was significantly higher than that against T2 cells pulsed with the control peptide (Fig. 5A). Two donors and two patients also had a significant response to the LDR peptide. To investigate the antitumor effect of stimulation with Ad-mSurv breast tumor cells, MCF-7 cells were used as targets. These cells are HLA-A2 positive and express a high level of survivin. Significant cytotoxic activity was determined in all four tested donors (Fig. 5B). Because T cells were cultured for a long period of time with IL-2, it was possible that part of this activity could be caused by the accumulation of lymphokine-activated killer cells. To test this possibility, the CTL assay was performed in 10-fold excess of unlabeled K562 cells, the target for natural killer and lymphokine-activated killer cells. The presence of K562 cells decreased the level of cytotoxicity. It still remained higher than that against T2 cells pulsed with the survivalin targeting constructs.

**Fig. 3** Typical example of enzyme-linked immunospot (ELISPOT) assay. A, donor 4 from experiments described in Fig. 2. B, donor 6 from experiments described in Fig. 4. Cells were stimulated with indicated peptides, and the number of spots in each well is shown.
Fig. 4  Antisurvivin response after stimulation with adenoviruses containing Thr\(^{34}\)-Ala mutant-survivin dendritic cells (Ad-mSurv-DCs). Dendritic cells (DCs) isolated from four HLA-A2-positive donors (D-1, D-2, D-5, and D-6) and four prostate cancer patients (P-1 to P-4) were transduced with Ad-mSurv and used in three rounds of stimulation of autologous T cells. Cells were then stimulated with p53-derived (control) or survivin-derived peptides, and the number of IFN-γ-producing cells was evaluated in enzyme-linked immunospot (ELISPOT) assay. Each experiment was performed in quintuplicate. *, statistically significant differences from control ($P < 0.05$).
control peptide (Fig. 5B); however, these levels indicate that a significant part of the observed antitumor toxicity was independent of natural killer cells.

To establish the specificity of the antisurvivin immune response, two types of experiments were performed. First, two HLA-A2-negative donors were stimulated with Ad-mSurv-DCs exactly as described above, and CTL activity against T2 cells loaded with p53-derived (control) or survivin-specific (ELT and LDR) peptides was measured as targets in standard 6-h ⁵¹Cr release assay. All experiments were performed in duplicate. B, MCF-7 cells were used as a target. In parallel, CTL assay was performed in the presence of 10-fold excess of unlabeled “cold” K562 cells. Cytotoxicity was measured as described in “Materials and Methods.”

The data described above demonstrated that Ad-mSurv could be used to induce a survivin-specific CTL response. This response could recognize and kill tumor cells; however, survivin is also expressed in some normal cells, predominantly CD34⁺ HPCs. We asked whether antisurvivin CTLs could recognize and affect normal CD34⁺ cells. Antisurvivin CTL response was induced and verified in healthy individuals 1 and 2 as described above. CD34⁺ cells were isolated from the peripheral blood of these two individuals using the magnetic beads separation technique. CD34⁺ cells were then cultured overnight with GM-CSF, washed, and incubated with autologous T cells stimulated three times with Ad-mSurv-DCs (CTLs) or autologous T cells cultured for the same period only with IL-2. After 4 h of coincubation, cells were seeded in triplicate into a semi-solid medium supporting the growth of myeloid and erythroid colonies. The number of colonies was counted 2 weeks later. Only at the T cells/CD34⁺ cells ratio 20:1 survivin-specific CTLs slightly (P < 0.05) decreased the number of colony-forming unit, granulocyte-macrophage colonies. This effect was not observed at other ratios (Fig. 7, A and B). These data suggest that survivin-specific CTLs do not significantly inhibit HPCs.
not incubated with T cells. Incubation of ELT-pulsed CD34\(^+\) cells with the survivin-specific CTLs significantly decreased the number of myeloid and erythroid colonies (Fig. 7C). In contrast, when CD34\(^+\) cells were pulsed with control peptide, no such decrease was found.

**DISCUSSION**

Survivin is a very attractive candidate for cancer immunotherapy. It is expressed in a vast majority of human tumors but not in differentiated normal cells. It is critically important for tumor cell survival because dominant-negative survivin induces apoptosis of tumor cells (34). It is conceivable that tumors may not be able to escape a strong antisurvivin CTL response. Survivin-derived peptides were successfully used in several previous studies for induction of CD8\(^+\) T-cell-specific CTL response (20, 21, 40). The use of a full-length protein for cancer immunotherapy has a number of advantages over the peptide-based approach. It allows for the induction of a multiepitope-specific CTL response and engagement of both CD4\(^+\) and CD8\(^+\) T cells, and it avoids patient selection based on MHC class I. Because DCs represent the most effective approach to the induction of antitumor immunity, and adenovirus is the most effective method for gene delivery into DCs, we used DCs transduced with adenoviral vector containing mutant dominant-negative survivin for the stimulation of an antisurvivin immune response. Adenoviral vector has another advantage. It induces DC maturation, which is very important for the stimulation of a potent immune response (41–43). Mutant (T34A) survivin does not have the p34\(^{cdk2}\)-cyclin B1 phosphorylation site, which eliminates the potentially serious problem of delivering a potent antiapoptotic activity associated with wild-type survivin to patients. Survivin is differentially expressed in cancer; however, some of the following normal adult cells also express survivin: thymocytes (15); CD34\(^+\) bone marrow-derived HPCs (16); and basal colonic epithelial cells (17). In this study, we sought to address the following two main questions: whether DCs transduced with Ad-mSurv are able to induce a survivin-specific immune response; and whether this response affects the function of HPCs to act as a sign of potential serious side effects of immunotherapy.

**Fig. 6** Immune response induced by adenoviruses containing Thr\(^{34}\)-Ala mutant survivin-dendritic cells (Ad-mSurv-DCs) is MHC class I restricted and survivin specific. A, dendritic cells (DCs) isolated from HLA-A2-negative donors were transduced with Ad-mSurv and used for stimulation of autologous T cells exactly as in experiments described in the legend to Fig. 3. T2 cells loaded with control and survivin-specific peptides were used as targets. B, DCs from HLA-A2-positive donors were transduced with Ad-p53 and then used in three rounds of the stimulation of autologous T cells. After that, cells were stimulated with p53-derived or survivin-derived peptides, and IFN-γ-producing cells were evaluated in enzyme-linked immunospot (ELISPOT) assay as described in "Materials and Methods." Each experiment was performed in quintuplicate. *, statistically significant differences from the values after cell incubation in medium alone (P < 0.05).
In our study, the expression of T34A mutant, dominant-negative survivin did not affect the viability of DCs. DCs express little of wild-type survivin, and apparently, their viability does not depend on this protein. CD8 T cells recognize TAA in a form of MHC class I-bound peptides (epitopes) on the surface of adenomatous polyposis coli. These peptides are the result of protein processing by adenomatous polyposis coli. To be successful, DCs transduced with full-length survivin should induce an immune response against several different epitopes. Therefore, using PaProC algorithm for proteasomal cleavage...
prediction and SYFPEITHI algorithm for HLA-A0201 binding, we have selected several epitopes from the human survivin sequence predicted to be optimally cleaved by proteasome and bound to HLA-A*0201 with a different affinity. ELT and LDR epitopes both have binding scores that are predicted to be high, whereas FKE has a score that is predicted to be low. We have also designed a modified epitope FLE with binding predicted to be increased by including nonpolar hydrophobic leucine and isoleucine in anchor positions 2 and 9, respectively. These replacements form the canonical HLA-A0201-binding motif. The epitope ELT has been used previously to induce strong responses in HLA-A2-positive T cells (20, 21). The determination of peptide binding to HLA-A2 cells by MHC stabilization assay confirmed the prediction (Fig. 1). In our study, antigen-specific CD8+ T-cell-mediated response was evaluated by IFN-γ ELISPOT assay in four HLA-A2-positive volunteers and four HLA-A2-positive patients. Two healthy individuals had significant responses to three survivin-derived epitopes, two individuals and three cancer patients had responses to two epitopes, and one patient had a response to only one epitope. These data indicate that Ad-mSurv-DCs were able to induce responses against multiple survivin-derived epitopes not only in healthy volunteers but also in cancer patients. No response was observed against the modified FLE peptide, although this peptide had a high binding score to HLA-A*0201 and high affinity in binding assay (Fig. 1). These data are not surprising because this modified peptide is not present in the native form of survivin, and T cells induced by the full-length survivin may discriminate the possible conformational differences between native and modified peptides. These results, however, may serve as a negative control in our experiments and confirm the specificity of the T-cell response against survivin. The specificity of CD8+ T-cell responses to survivin epitopes was further confirmed in experiments with the stimulation of T cells with Ad-p53-transduced DCs. This stimulation resulted in the accumulation of CTL precursors specific for p53-derived peptides but not survivin-derived peptides. T cells responding to survivin-derived epitopes were able to kill both targets loaded with peptide and tumor cells expressing survivin (MCF-7 cell line). This killing was MHC class I restricted and was not mediated by lymphokine-activated killer and natural killer cells. Taken together, these data demonstrate that Ad-mSurv-DCs induce in vitro survivin-specific CTLs that recognize survivin-derived epitopes in the context of MHC class I molecules.

Because survivin has been shown to be expressed in HPCs (16), the possible hematological toxicity could be a serious limiting factor in any attempt to use survivin in cancer immunotherapy. A direct comparison demonstrated that HPCs express a substantially lower level of this protein than leukemic cells (16). It suggests that HPCs may be much less sensitive to survivin-specific CTLs than tumor cells. To test this hypothesis, we isolated CD34+ HPCs from donors and, in parallel, generated survivin-specific CTLs. CD34+ cells were cultured overnight with GM-CSF to stimulate cell proliferation and the accumulation of survivin, and cells were then incubated with CTLs. We evaluated the ability of HPCs to form colonies in the presence of the appropriate growth factors and cytokines. Only a slight decrease in colony-forming activity in HPCs incubated with survivin-specific CTLs was observed as compared with T cells stimulated with untreated DCs. These data support our hypothesis and suggest that an antisurvivin CTL response may not be effective against HPCs. Recent data have demonstrated that survivin-specific CTLs lyse malignant chronic leukemia cells, although they spare activated autologous B cells or activated DCs that express survivin (40). This also confirms the absence of immunotoxicity of survivin-specific CTLs for normal cells that express less survivin than malignant cells. More animal studies in vivo are needed to further verify the safety of survivin-based vaccination.

In conclusion, we have demonstrated that DCs transduced with dominant-negative survivin were effective in the induction of the survivin-specific, multiepitope CTL response. These CTLs recognized tumor cells expressing survivin but did not significantly affect the function of HPCs. These data provide a rationale for using dominant-negative survivin in immunotherapy of cancer.

ACKNOWLEDGMENTS

We thank Dr. Sunil Chada from Introgen Therapeutics for providing Ad-p53, Dr. Charles A. Kusznyski and Linda M. Wilkie (University of Nebraska Medical Center) for help with the fluorescence-activated cell sorter analysis, and Kristi L. W. Berger (University of Nebraska Medical Center) for editorial assistance.

REFERENCES


Full-Length Dominant-Negative Survivin for Cancer Immunotherapy

Vladimir Pisarev, Bin Yu, Raoul Salup, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/9/17/6523

Cited articles
This article cites 39 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/9/17/6523.full.html#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
/content/9/17/6523.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.