Effects of Survivin Antagonists on Growth of Established Tumors and B7-1 Immunogene Therapy

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Background: **Survivin, a member of the inhibitor of apoptosis (IAP) protein family, is detectable in most types of cancer, and its presence is associated with a poor prognosis. We determined the effects of gene-based therapies that inhibit survivin function in a mouse tumor model.** ***Methods:*** **Using five to six mice per treatment group, we injected tumors derived from mouse EL-4 thymic lymphoma cells with plasmids encoding antisense survivin, a dominant-negative mutant survivin, and the T-cell costimulator B7-1. Expression of endogenous survivin and the proteins encoded by the injected plasmids were examined by immunohistochemical staining of tumor sections and by western blot and flow cytometry analyses of isolated tumor cells. Tumor growth, the generation of antitumor cytotoxic T-lymphocyte (CTL) activity, apoptosis, and the contribution of leukocyte subsets to antitumor activity were measured. All statistical tests were two-sided.** ***Results:*** **Large (1.0-cm diameter) tumors had approximately 10-fold more survivin than small (0.2-cm diameter) tumors. At 28 days after injection, antisense and dominant-negative mutant survivin plasmids statistically significantly inhibited the growth of both small (*P* = .006 and *P* = .0018, respectively) and large (*P*<.001 for both plasmids) EL-4 tumors compared with tumors injected with empty plasmid. The growth of large tumors was further inhibited by intratumoral injection with antisense survivin and B7-1 (*P* = .004); thus, inhibition of survivin expression renders large tumors susceptible to B7-1-mediated immunotherapy. Mice whose tumors were completely eradicated by injection of B7-1 remained tumor free for 26 days after re-injection with EL-4 cells (when the experiment ended). Compared with tumors injected with empty plasmid, tumors injected with survivin-based plasmids had increased apoptosis, and animals bearing such tumors generated more antitumor CTLs.** ***Conclusion:*** **Intratumoral injection of plasmids that block survivin expression and stimulate the generation of tumor-specific CTLs may be beneficial for the treatment of large lymphomas. [J Natl Cancer Inst 2001;93:1541–52]**

The suppression of programmed cell death (apoptosis) contributes to carcinogenesis by prolonging the life span of neoplastic cells, which facilitates the accumulation of cancer-causing gene mutations. Suppression of apoptosis also permits growth factor-independent cell survival, promotes resistance to antitumor immunity, allows cells to proceed through cell cycle checkpoints that would otherwise induce apoptosis, and enables tumors to survive in nutrient-poor ectopic environments. Suppression of apoptosis hinders many forms of cancer treatment, including cancer immunotherapy, chemotherapy, and radiation therapy *(1–3).*

Programmed cell death involves an evolutionarily conserved cascade of events that are controlled by inhibitor of apoptosis (IAP) proteins, such as survivin, apollon, MLIAP (i.e., melanoma inhibitor of apoptosis), XIAP (i.e., X-linked inhibitor of apoptosis protein), cIAP1 (i.e., cellular inhibitor of apoptosis), cIAP2, and NAIP (i.e., neuronal apoptosis inhibitory protein) *(4,5),* and by Bcl-2 family members that either promote (i.e., Bax, Bak, Bcl-xS, Bad, and Bid) or counteract (i.e., Bcl-2, Bcl-xL, and Mcl-1) apoptotic cell death signals *(6–8).* IAP proteins inhibit the executors of apoptosis, including caspases 3 and 7 *(9),* and modulate the inducible transcription factor, NF-kappa B. IAP proteins contain variable numbers of a unique 80-amino-acid residue repeat, the baculovirus inhibitor of apoptosis repeat (BIR) domain, and a single carboxy-terminal RING Zn-finger motif *(2).* Survivin, a recently identified IAP member *(10),* exists primarily as a dimer in cells. Unlike other IAPs, survivin contains a single zinc-binding BIR domain at its amino-terminus, followed by a long amphipathic α helix instead of a RING finger, which generates a "bow-tie" shape in the dimer *(11–13).* Survivin is highly expressed during human embryonic and fetal development, but it is absent from all adult tissues except for the endometrium *(14)* and basal keratinocytes *(15).* Survivin is highly overexpressed in most common cancers of the lung, colon, pancreas, prostate, and breast, and its expression in these cancers is associated with an unfavorable prognosis *(10,16–20).*

Survivin is the only apoptosis inhibitor yet described that is expressed in a cell cycle-dependent manner in G_2 and M phases. During mitosis, survivin localizes to mitotic spindle microtubules, centrosomes, pericentrosomal microtubules, and the cytokinetic remnant. Survivin is required to preserve the integrity of the mitotic apparatus *in situ,* and in transformed cell lines, it forms a novel apoptotic checkpoint *(21,22).* Overexpression of survivin initiates cell division by inducing resistance to G_1 arrest and thereby accelerating the entry into S phase. Overexpression of survivin also induces activation of the cyclin-dependent kinase 2 (Cdk2)–cyclin E complex, phosphorylation of the retinoblastoma protein, and release of p21 from Cdk4. p21 then interacts with mitochondrial procaspase 3 to suppress Fas-mediated cell death *(23–25).* Therefore, when overexpressed in the tumors of adult mammals, survivin has oncogenic potential because it can overcome the G_2/M checkpoint to ensure mitotic progression *(22).*

Caspase-3, a protease that plays a key role in a proteolytic cascade within the apoptosis signal pathway, is activated by numerous cell death-inducing signals. Like other IAP proteins,

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See "Notes" following "References."

survivin directly inhibits caspase-3, which is essential for suppression of caspase-mediated cleavage of centrosome-associated p21 *(26).* Survivin also binds to the IAP inhibitor Smac/Diablo, and that binding may free other IAP members to suppress caspases *(27).* Survivin's interaction with the mitotic spindle apparatus places it in a unique position to regulate the supramolecular assembly of cell death and cell cycle regulatory molecules present within the centrosome.

Antiapoptosis proteins are now regarded as important targets in cancer therapy. Antisense complementary DNA (cDNA) and oligonucleotides that reduce the expression of Bcl-2 inhibit the growth of certain tumor cell lines *in vitro (28–30).* Similarly, antisense oligonucleotides that reduce survivin expression in tumor cells induce apoptosis and polyploidy, decrease colony formation in soft agar, and sensitize tumor cells to chemotherapy *in vitro (31–34).* Cells that depend on survivin for their survival cannot complete cytokinesis without survivin, resulting in multinucleated cells *(32).* Survivin may be required for tumor angiogenesis, since it is highly expressed in newly formed blood vessels in response to vascular endothelial growth factor and basic fibroblast growth factor *(35,36)* and mediates angiopoietin inhibition of endothelial cell apoptosis *(37).* Hence, therapies that inhibit survivin expression and/or function may prevent tumors from acquiring an adequate blood supply.

Immunotherapy, the treatment of tumors with immunostimulators, including the lymphocyte costimulatory cell adhesion molecules B7-1, VCAM-1 (i.e., vascular cell adhesion molecule-1), and ICAM-1 (i.e., intercellular cell adhesion molecule-1), is often effective against small tumors but not against large tumors, perhaps because tumors become increasingly resistant to immune attack as they grow *(38–40).* It is possible that barriers to cancer immunotherapy can be overcome by targeting a tumor's weapons of survival (e.g., proangiogenic factors), defense (e.g., antiapoptotic and immunosuppressive factors), and attack (e.g., Fas ligand [FasL] and receptor-binding cancer antigen expressed on SiSo cells [RCAS]) *(41).*

Here, we investigate whether the inhibition of survivin expression or function can inhibit tumor growth *in situ.* Because survivin potentially protects tumors from immune attack by preventing programmed cell death, we also examined the effects of survivin antagonists on the efficacy of cancer immunotherapy using B7-1, which has been shown to stimulate potent antitumor immunity when introduced into tumors *in situ.*

MATERIALS AND METHODS

Cell Culture and Monoclonal Antibody Isolation

EL-4 thymic lymphoma cells, which were derived from C57BL/6 (H-2b) mice, were purchased from the American Type Culture Collection (ATCC), Manassas, VA, and cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) supplemented with 10% fetal calf serum, 50 U/mL penicillin–streptomycin, 2 m*M* L-glutamine, and 1 m*M* pyruvate. Rat hybridomas secreting monoclonal antibodies (MAbs) against mouse CD8 (hybridoma 53–6.72), CD4 (hybridoma Gk1.5), and natural killer (NK) cells (hybridoma PK136) were purchased from the ATCC. Hybridomas 53–6.72 and PK136 were grown in RPMI-1640 medium (Life Technologies, Inc.), whereas Gk1.5 was grown in DMEM; other conditions were the same as those used to culture EL-4 cells. Hybridomas were injected intraperitoneally into pristane-primed nude mice. We purified MAbs from ascites by ammonium sulfate precipitation and dialysis against phosphate-buffered saline (PBS). The titer of the purified MAbs was determined at a maximum dilution of 1 : 2000 by flow cytometry with the use of a FACScan (Becton Dickinson, San Jose, CA).

Animals and Experimental Tumor Model

Female C57BL/6 mice, 6–9 weeks old, were obtained from the Animal Resource Unit, Faculty of Medicine and Health Science, University of Auckland, Auckland, New Zealand. The tumors were established by the subcutaneous injection of 2×10^5 EL-4 cells into the left flank of the mice. Tumor size was determined by measuring two perpendicular diameters. The animals were killed when their tumors reached 1–1.5 cm in diameter, in accord with Animal Ethics Approval (University of Auckland). All experiments included five or six mice per treatment group, and each experiment was repeated at least once.

Construction of Expression Plasmids

Antisense survivin plasmid. Total RNA was extracted from EL-4 cells with the use of 4 *M* guanidine (Fluka Biochemika Ltd., Schweiz, Switzerland) as described previously *(42),* and 1 μg of RNA was reverse transcribed with the use of SuperScript II Reverse Transcriptase (Life Technologies, Inc.) to generate complementary DNA (cDNA). The cDNA was used as a template to amplify a 509-base-pair DNA fragment encoding mouse survivin with the use of the polymerase chain reaction (PCR) and the oligonucleotide primers 5′-GAGTCGTCTTGGCGGAGG-3′ (sense primer) and 5′-CTTAGATGTGGCATGTCACTC-3′ (antisense primer). The resulting PCR product, which contained the entire coding region of mouse survivin (nucleotides 75–583; GenBank accession No. NM_009689) *(43),* was subcloned into pGEM-T (Promega Corp., Madison, WI) by A-T ligation, liberated by digestion with *Apa*I and *Not*I, and directionally cloned into the *Apa*I and *Not*I sites of expression vector pCDNA3 (Invitrogen, Carlsbad, CA) in an antisense orientation with respect to the promoter on the plasmid.

Dominant-negative mutant survivin plasmid. We used PCR to construct a dominant-negative mutant of survivin by using the survivin cDNA as a template. Two overlapping complementary oligonucleotide primers, C84AF (5′-CACTCCCCGGGCGCAGCCTTCCTCAC-3′) and C84AR (5′-GGCTGCGCCCGGGGAGTGCTT-3′), which produced a T-to-G substitution at nucleotide 354 that introduced a diagnostic *Sma*I site, were used to change the cysteine residue at amino acid 84 to an alanine. The resulting PCR product was cloned into the *Xba*I and *Not*I sites of pCDNA3B, a plasmid identical to pCDNA3 except with a reversed polylinker. All constructs were verified by DNA sequencing.

B7-1 expression plasmid. A pCDM8-based expression plasmid encoding mouse B7-1 has been described previously *(38).*

Intratumoral Gene Transfer of Expression Plasmids

All plasmids were prepared by alkaline lysis, purified by cesium chloride gradient centrifugation at 270 000*g* for 22 hours at 20 °C, and diluted to 5 μg/μL in a solution of 5% glucose in 0.01% Triton X-100. The diluted plasmids were mixed at 1 : 1 (wt/wt) with a 0.1% solution of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate cationic liposomes (Boehringer Mannheim, Mannheim, Germany) to form a DNA–liposome complex. For single-vector transfer, small tumors (0.2–0.3 cm in diameter) were injected at multiple sites with 100 μg of DNA, whereas large tumors (0.4–0.6 cm in diameter) were injected at multiple sites with 180 μg of DNA, unless otherwise stated. For therapy using combinations of the B7-1- and survivin-based plasmids, 100 μg and 180 μg of the B7-1 plasmid DNA–liposome complex were injected at multiple sites into small tumors (0.2–0.3 cm in diameter) and large tumors (0.4–0.6 cm in diameter), respectively. Twenty-four hours later, 100 μg and 180 μg of the DNA–liposome complex containing either the antisense or dominant-negative mutant survivin vectors were injected into the same tumors, respectively.

Rechallenge of Mice With Parental Tumor Cells

Treated mice that rejected their tumors and subsequently remained tumor free were re-injected subcutaneously 7 weeks later with 2×10^6 EL-4 cells (in 0.1 mL) in the right flank.

Measurement of Tumor-Specific Cytotoxic T Lymphocytes

Splenocytes (effector cells) were harvested from mice 28 days after injection with expression plasmids, mixed with EL-4 target cells in graded effector cell-to-target cell ratios, and then incubated at 37 °C in 96-well, round-bottom plates. After a 4-hour incubation, 50 μL of supernatant was collected from each well, and cell lysis was measured with the use of the CytoTox 96 Assay Kit (Promega Corp.). Controls for nonspecific lysis of the target and effector cells were in-

cluded in each assay. After subtraction of the values produced by those controls, the percentage of cell lysis was calculated with the use of the formula: 100 × (experimental – spontaneous effector – spontaneous target)/(maximum target – spontaneous target).

Identification of Specific Leukocyte Subsets Responsible for Antitumor Immunity by Leukocyte Depletion

Mice bearing EL-4-derived tumors received intraperitoneal and intravenous injections every other day of 300 μg (in 0.1 mL) of the 53–6.72 (anti-CD8), Gk1.5 (anti-CD4), or PK136 (anti-NK cell) MAbs to deplete them of CD8-positive ($CD8^+$) T cells, $CD4^+$ T cells, and NK cells, respectively. Rat immunoglobulin G (IgG) (Sigma Chemical Co., St. Louis, MO) was injected as a control antibody. Seven days after the first MAb injections, the mice received an injection of the various expression plasmids. Depletion of individual leukocyte subsets was more than 90% effective, as determined by flow cytometry analysis of isolated splenocytes from leukocyte-depleted mice compared with mice whose leukocytes were not depleted.

Detection of Apoptosis

***In situ* detection of apoptotic cells by the TUNEL assay.** Large tumors were injected with plasmids, and after 2 days, the tumors were excised, immediately frozen in dry ice, and stored at −70 °C. Serial sections (6 μm thick) of the frozen tumors were prepared to detect apoptosis by TUNEL (i.e., terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling) staining with the use of the *In Situ* Apoptosis Detection Kit from Boehringer Mannheim. Briefly, frozen sections were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, incubated with 20 μL TUNEL reagent for 60 minutes at 37 °C, and then examined by fluorescence microscopy. Adjacent sections were counterstained with hematoxylin and mounted onto poly-L-lysine-coated slides to allow the total number of nucleated cells to be counted. The percentage of apoptotic cells was assessed in 10 randomly selected fields viewed at ×40 magnification. The apoptotic index (A/I) was calculated as follows: A/I = number of apoptotic (TUNEL-positive) cells × (100/total number of nucleated cells).

***In situ* detection of apoptotic cells by annexin-V-biotin staining.** Mice received an injection via the tail vein of 0.5 mg of annexin-V-biotin (Apoptest Biotin, product B500; Nexins Research, Hoeven, The Netherlands) 2 days after the injection of plasmids into large tumors *(44)*. The tumors were excised 30 minutes after administration of annexin-V-biotin, fixed in 4% formalin in HEPES buffer (i.e., 10 m*M* HEPES [pH 7.4], 137.5 m*M* NaCl, 1.25 m*M* $MgCl_2$, 1.25 m*M* $CaCl_2$, 6 m*M* KCl, 0.4 m*M* NaH_2PO_4, 5.6 m*M* glucose, and 0.1% bovine serum albumin) at 4 °C, decalcified in distilled water containing 4% EDTA and 4% formalin for 4 weeks, and embedded in paraffin. Sections (6 μm) were dewaxed in xylene and rehydrated, incubated with 3% H_2O_2 in PBS for 5 minutes to block endogenous peroxidase activity, and blocked with 30% normal goat serum (Vector Laboratories, Burlingame, CA). The sections were then incubated at 37 °C for 30 minutes with avidin–biotin–peroxidase complex (VECTASTAIN ABC Elite Kit; Vector Laboratories), developed in Sigma Fast DAB (3,3′-diaminobenzidine; Sigma Chemical Co.), and then mounted with coverslips in 50% glycerol in PBS and photographed. In some cases, the coverslips were removed in buffer, and the sections were restained with hematoxylin. The A/I was measured with the use of five sections and was calculated as follows: A/I = number of apoptotic cells × (100/total number of cells).

Detection of apoptotic cells by annexin-V-FLUOS staining. Large tumors were excised 2 days after intratumoral injection of plasmids, minced, and digested with collagenase for 60 minutes at 37 °C to isolate tumor cells, as described previously *(45)*. Cell preparations were filtered through a stainless-steel strainer to remove debris and matrix, and erythrocytes were removed by ammonium chloride lysis. The viability of the isolated tumor cells, as determined by trypan blue dye exclusion, was greater than 95%. The cells were stained for 10 minutes with annexin-V-FLUOS (green dye) (Boehringer Mannheim) to identify apoptotic cells and counterstained with methylene blue to determine the total number of tumor cells per field. The A/I was calculated as above.

Analysis of B7-1 and Survivin Protein Expression by Flow Cytometry

Tumors were excised 2 days after plasmid injection and digested with collagenase to produce single-cell suspensions, and the cells were cultured for 2 days with the use of the same conditions used to culture EL-4 cells. For B7-1 analysis, the cells were stained sequentially with 1G10 MAb (anti-B7-1; Pharmingen, San Diego, CA) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Sigma Chemical Co.) or with 2G5 MAb (anti-human HML-1, integrin αEβ7), an FITC-labeled isotype-matched control IgG2a MAb (Pharmingen). Antibody staining was then evaluated by flow cytometry with the use of a Becton Dickinson FACScan. For survivin analysis, the cells were harvested from EL-4 tumors that were injected previously with antisense and dominant-negative vectors or empty vector. They were permeabilized with 0.3% Triton X-100 and stained for survivin expression with the use of SURV11-A, a polyclonal rabbit anti-human survivin antibody that recognizes mouse survivin (Alpha Diagnostic, San Antonio, TX), or with rabbit IgG (Sigma Chemical Co.) as a negative control, followed by FITC-conjugated goat anti-rabbit IgG1. Stained cells were analyzed by flow cytometry.

Immunohistochemistry

Tumors were excised from mice 2 days after plasmid injection, frozen on dry ice, and stored at −70 °C in isopentane. Sections (10 μm thick) of the frozen tumors were prepared and mounted on poly-L-lysine-coated slides. The slides were then incubated for 30 minutes in 0.3% H_2O_2 in methanol to block endogenous peroxidases. Tumor sections were incubated with 1G10 (anti-B7-1 MAb), 53-6.72 (anti-CD8 MAb), Gk1.5 (anti-CD4 MAb), PK136 (anti-NK cell MAb), or the antisurvivin antibody. The bound antibodies were detected with the use of the VECTASTAIN ABC Elite Kit and 3,3′-diaminobenzidine tetrahydrochloride (Sigma Fast DAB) with metal enhancer ($CoCl_2$) tablets. The sections were counterstained with hematoxylin. As controls, rat IgG and rabbit IgG (Sigma Chemical Co.) were used as the primary antibodies.

Immunofluorescence Staining and Confocal Microscopy

Tumor sections (6 μm) prepared 2 days after plasmid injection from frozen tumors were stained with 4D11, a rat MAb (purchased from the ATCC) that recognizes large granular lymphocyte protein-1 (LGL-1) *(46)*, followed by FITC-conjugated anti-rat IgG (Sigma Chemical Co.); with rabbit anti-human survivin antibody followed by tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Sigma Chemical Co.); or with the anti-NK cell MAb (PK136) followed by TRITC-conjugated anti-rat IgG. As a control, rat IgG was used as the primary antibody. Sections were examined by confocal microscopy with the use of a Leica TCS 4D microscope (Leica Microsystems, GmbH, Heidelberg, Germany). Images were compiled from sets of three to five consecutive single optical sections with the use of Leica Scanware™ 4.2A software (Leica Lasertechnik, GmBH, Heidelberg).

Western Blot Analysis

The tumors were excised and homogenized in lysis buffer (i.e., 50 m*M* Tris [pH 7.4], 100 μ*M* EDTA, 0.25 *M* sucrose, 1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 100 μ*M* phenylmethylsulfonylfluoride) at 4 °C with the use of a motor-driven homogenizer (VirTis, Gardiner, NY). Tumor lysates from each group of mice were pooled and centrifuged at 10 000*g* for 10 minutes at 4 °C to remove tissue debris. Protein concentrations of the resulting supernatants were determined as described previously *(47)*, and 100 μg of each supernatant was resolved on 10% polyacrylamide gels containing SDS under reducing conditions and then electrophoretically transferred to Hybond C Extra nitrocellulose membranes (Amersham Life Science, Amersham, U.K.). The membranes were blocked with 3% bovine serum albumin in TBS-T (i.e., 20 m*M* Tris and 137 m*M* NaCl [pH 7.6] containing 0.1% Tween 20) for 2 hours at room temperature and then incubated in TBS-T containing the rabbit anti-human survivin antibody diluted 1 : 200 for 60 minutes at room temperature. The membranes were washed three times with TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Sigma Chemical Co.) diluted 1 : 5000 in TBS-T. Immunoreactivity was detected by Enhanced Chemiluminescence (Amersham International, Buckingham, U.K.) and autoradiography.

Statistical Analysis

All *in vivo* and *in vitro* experiments were performed at least twice. To compare the change in size of the small and the large tumors over time in response to treatment with plasmids encoding B7-1, antisense survivin, or a dominant-negative mutant of survivin, we used a mixed linear model fitted with number of

days and treatment as a repeated measure and treatment groups and initial (day 0) tumor size group (small or large) as explanatory variables. Tumor size at day 0 (the day of injection of plasmid encoding B7-1, antisense survivin, or a dominant-negative mutant of survivin) was included as a covariate. Because the change in tumor size over time was not monotonic, day was treated as a categorical variable. The interaction of day with treatment group and initial tumor size group was examined to determine whether the effect of treatment was independent of tumor size group, with the difference in treatment effects due to antisense survivin or dominant-negative mutant survivin, with and without B7-1 being of interest.

To compare changes in tumor size during the 21 days after mice were depleted of leukocyte subsets (T cells, B cells, or NK cells), we fitted a mixed linear model, where day 0 and day 21 were repeated measures and the interaction of day and depletion group was the outcome of interest. The particular contrasts that we investigated were the change in tumor size over time for mice that had not undergone leukocyte depletion contrasted with that for mice that had been depleted, for each type of leukocyte. This analysis was performed separately for the four treatment groups (i.e., antisense survivin, dominant-negative survivin, B7-1 plus antisense survivin, and B7-1 plus dominant-negative survivin).

All other statistical comparisons were made with the use of Student's *t* tests. All statistical tests were two-sided.

RESULTS

Relationship Between Survivin Expression and Tumor Size

Tumors become increasingly immune resistant as they grow. Here, we sought to determine whether survivin expression increases as tumors grow and to determine whether increased immune resistance was associated with increased survivin expression. Increased survivin expression might also reflect a tumor's dependence on survivin to overcome increasingly poor nutrient conditions at an ectopic site. Syngeneic EL-4 tumors were established by injection of EL-4 cells into the left flank of C57BL/6 mice, and the expression of survivin within the tumors at this ectopic site was examined. Survivin was poorly expressed in small tumors (i.e., tumors ≤0.2 cm in diameter) (Fig. 1, A). However, survivin expression increased dramatically with increasing tumor size. Tumors 1.0 cm in diameter had approximately 10-fold more survivin than 0.2-cm tumors, as measured by immunohistochemical staining of tumor sections (Fig. 1, A–C), western blot analysis of tumor homogenates (Fig. 1, J), and flow cytometry analysis of cells isolated from 0.5-cm and 1.0-cm tumors (Fig. 1, K). Survivin was expressed uniformly throughout all tumors (Fig. 1, A–C). LGL-1, the antigen recognized by 4D11, is normally expressed on EL-4 cells, on a subset (50%) of C57BL/6 NK cells, and on fewer than 2% of T cells *(46)*. We used 4D11 to detect EL-4 cells within tissue sections. Large tumors (1 cm in diameter) were composed almost entirely of 4D11-staining EL-4 cells, which coexpressed survivin (Fig. 1, D–F). Only small numbers of NK cells (Fig. 1, H) infiltrated tumors, and some of those appeared to express LGL-1 (Fig. 1, G–I). Thus, survivin expression increases uniformly throughout tumors as they enlarge, suggesting that it may be a critical factor for the survival of large tumors. We have found that survivin is not expressed in EL-4 cells in culture, presumably because the culture conditions are optimal for cell growth and survival.

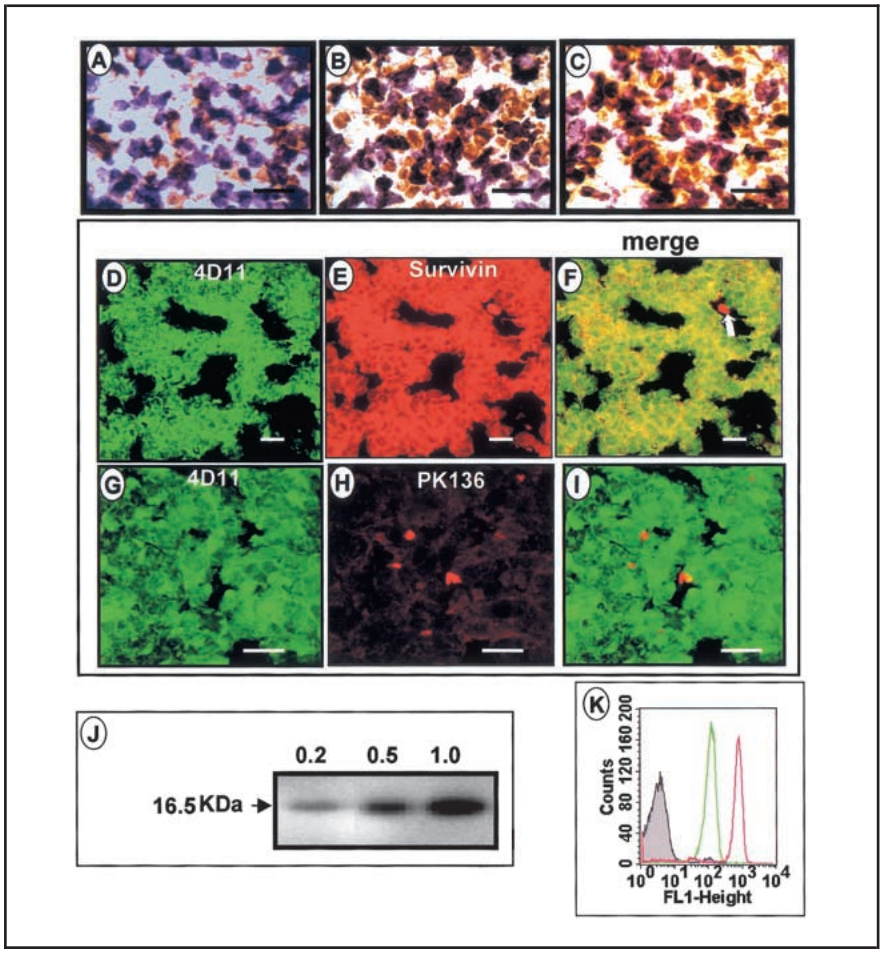

Fig. 1. Change in survivin expression with change in tumor size. **Panels A–C:** Sections prepared from established tumors, 0.2 cm (**A**), 0.5 cm (**B**), and 1.0 cm (**C**) in diameter, were immunohistochemically stained for survivin (**brown**) with a rabbit antisurvivin antibody and counterstained with hematoxylin (**blue**). **Panels D–I:** Sections prepared from established tumors of 1.0 cm in diameter were co-stained with 4D11, a monoclonal antibody specific for the EL-4 and natural killer (NK) cell marker, large granular lymphocyte protein-1 (LGL-1) (**D and G, green**), and with either the PK136 monoclonal antibody for NK cells (**H, red**) or the antisurvivin antibody (**E, red**) and were analyzed by confocal immunofluorescence microscopy. The images in **D** and **E** were merged (**F**) to determine the extent of survivin expression in EL-4-derived tumor cells. The images in **G** and **H** were merged (**I**) to determine whether NK cells expressed the LGL-1 marker. **Yellow coloration** in **F** and **I** denotes colocalization of LGL-1 and survivin and of LGL-1 and NK cells, respectively. **Arrow** in **F** denotes a survivin-positive cell that failed to stain for 4D11 and is probably an infiltrating leukocyte. **Scale bar** = 20 μm. **Panel J:** western blot analysis of tumor cell homogenates extracted from tumors 0.2, 0.5, and 1.0 cm in diameter. **Each lane** contains 100 μg of protein. **Panel K:** Survivin expression was further confirmed by flow cytometry analysis of cells extracted from 0.5-cm (**green**) and 1.0-cm (**red**) tumors. Survivin was detected with a rabbit anti-survivin antibody. The ***x*-axis** denotes mean fluorescence intensity, and the ***y*-axis** denotes relative cell number. The **gray peak** represents cells stained with isotype-matched control antibody. KDa = kilodaltons.

Effects of Antisense Survivin and Dominant-Negative Mutant Survivin Expression on Tumor Growth

We next tested the effects of two molecular reagents designed to inhibit survivin expression or function on the growth of established EL-4 tumors. One reagent, an antisense survivin expression plasmid, was expected to block the translation of endogenous survivin messenger RNA transcripts and, consequently, survivin expression. The other reagent, an expression plasmid encoding a dominant-negative mutant form of survivin, was constructed on the basis of the finding that mutation of Cys84 to Ala in the extreme C-terminal region of the BIR domain completely abrogates survivin's ability to inhibit apoptosis *(22)*. The dominant-negative mutant form of survivin was expected to block endogenous survivin function by competitively binding to survivin effectors. We examined the effects of these two reagents on 0.2-cm EL-4 tumors, which express little endogenous survivin, and on 0.4- to 0.6-cm tumors, which express high levels of survivin. Small (0.2-cm) EL-4-derived tumors injected with 100 μg of plasmid encoding either antisense survivin or dominant-negative mutant survivin showed a 40%–50% reduction in tumor diameter 28 days after plasmid injection compared with small tumors injected with empty plasmid. For example, tumors injected with empty plasmid had a mean size of 1.21 cm (95% CI = 0.92 cm to 1.50 cm) 28 days after injection, whereas tumors injected with antisense survivin or dominant-negative mutant survivin had mean sizes of 0.62 cm (95% CI = 0.34 cm to 0.90 cm; $P = .006$) and 0.50 cm (95% CI = 0.34 cm to 0.66 cm; $P = .0018$), respectively, at 28 days after injection (Fig. 2, A). In contrast, large (0.4- to 0.6-cm) EL-4-derived tumors injected with 180 μg of plasmid encoding either antisense survivin or dominant-negative mutant survivin showed an 80% reduction in size 14 days after plasmid injection compared with large tumors injected with empty plasmid (Fig. 2, B). For example, large tumors injected with empty plasmid had a mean size of 0.86 cm (95% CI = 0.66 cm to 1.06 cm) 14 days after injection, whereas tumors injected with antisense survivin or dominant-negative mutant survivin had mean sizes of 0.18 cm (95% CI = 0.12 cm to 0.24 cm; $P<.001$) and 0.18 cm (95% CI = 0.11 cm to 0.25 cm; $P<.001$), respectively, at 14 days after injection (Fig. 2, B). These effects were specific for plasmids that expressed either antisense survivin or dominant-negative mutant survivin; empty plasmids (i.e., those containing no survivin-related sequences) had, as shown previously *(38)*, no effect on tumor growth.

We performed immunohistochemical staining of the 0.4- to 0.6-cm tumors 2 days after they were injected with the survivin-containing or empty plasmids to detect survivin expression. Tissue sections from tumors that had been injected with the dominant-negative mutant survivin plasmid had high levels of survivin, as detected by an antibody that recognizes both human and mouse survivin (Fig. 3, B). This survivin presumably reflects the combined levels of endogenous wild-type mouse survivin (Fig. 3, A) and overexpressed dominant-negative mutant survivin (Fig. 3, B). In contrast, endogenous survivin expression was undetectable in tissue sections from 0.4- to 0.6-cm tumors that had been injected with the antisense survivin plasmid (Fig. 3, C). We confirmed these immunohistochemical results by performing western blot analysis of tumor homogenates using survivin antibodies (Fig. 3, E). Survivin-specific antibodies de-

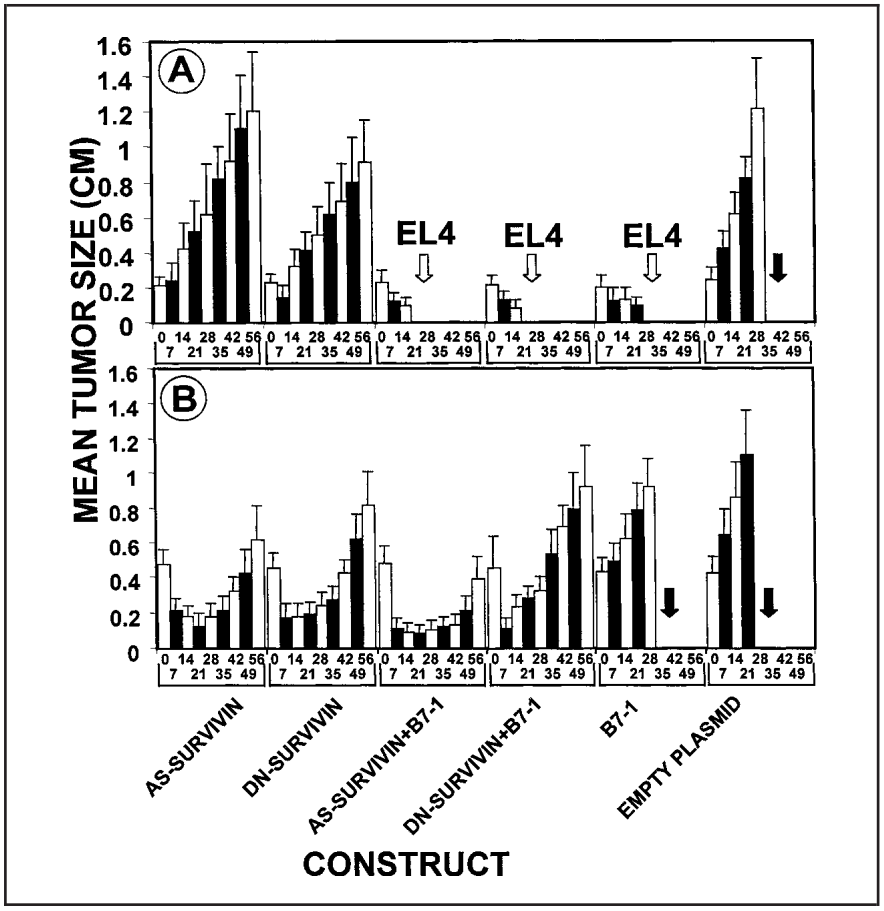

Fig. 2. Effects of overexpression of B7-1 and inhibition of survivin expression on tumor growth. Established EL-4-derived tumors approximately 0.2 cm (**A**) and 0.4–0.6 cm (**B**) in diameter were injected with DNA–liposome complexes containing either 100 μg (**A**) or 180 μg (**B**) each of the indicated expression plasmids. For combination therapy, antisense survivin plasmid was administered 24 hours after the B7-1 plasmid. Day 0 refers to the day of the first plasmid injection. Tumor size (as measured by two perpendicular diameters, in centimeters) was monitored for 56 days (day of experiment is indicated by the **numbers beneath each group of bars**); animals were killed when their tumors became larger than 1–1.5 cm in diameter (**filled arrows**). Cured mice (i.e., those whose tumors had completely regressed) were re-injected with 2×10^6 parental EL-4 cells in the opposing flank 35 days after gene transfer (**open arrows**) and then monitored for tumor regrowth for an additional 26 days. **Each bar** represents the mean tumor size with 95% confidence intervals for five mice. AS = antisense; DN = dominant negative.

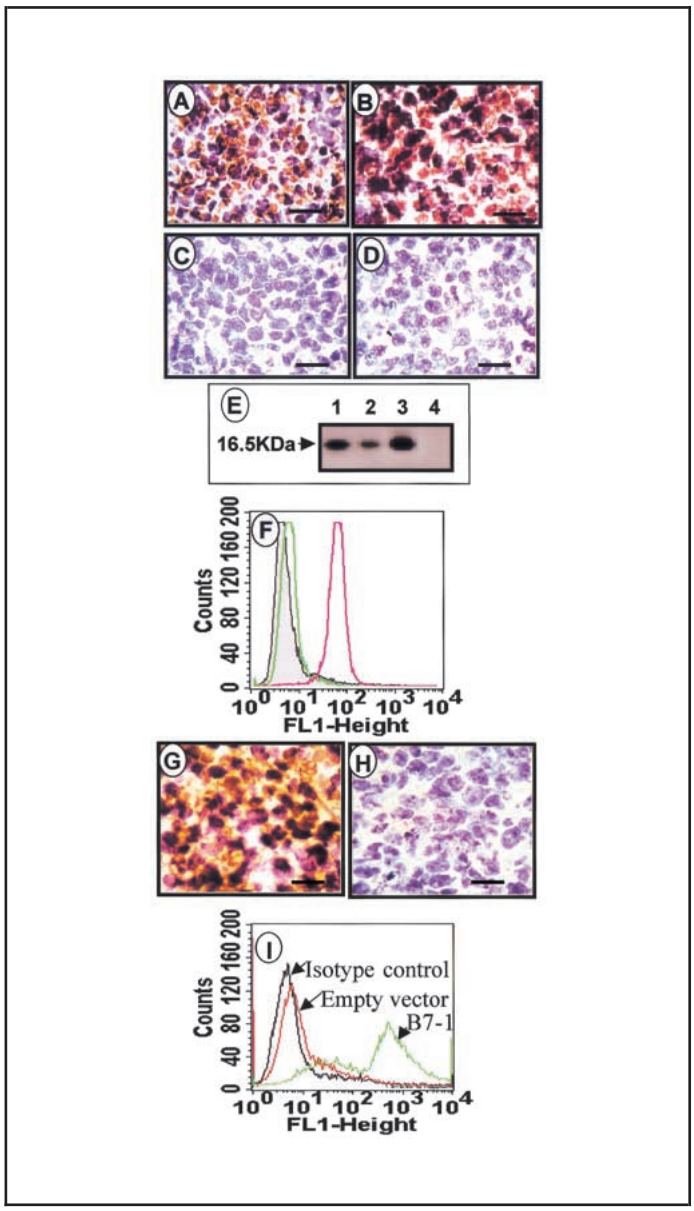

Fig. 3. Expression of survivin and B7-1 in plasmid-injected tumors. Tumors (0.4–0.6 cm diameter) were injected with empty pCDNA3 vector (**A** and **D**) or with expression plasmids encoding dominant-negative mutant survivin (**B**) or antisense survivin (**C**). Tumor sections were prepared 2 days after plasmid injection and stained for survivin (**brown**) with a rabbit anti-human/mouse survivin antibody (**A–C**) or with rabbit immunoglobulin G (IgG) (**D**). Sections were counterstained with hematoxylin (**blue**). **Scale bar** = 20 μm. **Panel E:** western blot analysis of tumor cell homogenates prepared 2 days after 0.4- to 0.6-cm-diameter (**lane 1**) and 0.2-cm-diameter (**lane 2**) tumors were injected with empty vector or after 0.4-cm to 0.6-cm tumors were injected with expression plasmids encoding dominant-negative mutant survivin (**lane 3**) and antisense survivin (**lane 4**). **Each lane** contains 100 μg of protein. **Panel F:** flow cytometry analysis of survivin expression in tumor cells extracted from 0.2-cm tumors before (**red**) and 2 days after (**green**) the tumors were injected with antisense survivin expression plasmid. The ***x*-axis** denotes mean fluorescence intensity, and the ***y*-axis** denotes relative cell number. The **gray peak** represents cells stained with isotype-matched control antibody. **Panels G and H:** Sections prepared from 0.4-cm to 0.6-cm tumors 2 days after they were injected with B7-1 expression plasmid were stained with either the anti-B7-1 monoclonal antibody (MAb), 1G10 (**G, brown/purple**), or control rat IgG antibody (**H**). Sections were counterstained with hematoxylin (**blue**). **Scale bar** = 20 μm. **Panel I:** flow cytometry analysis of cells extracted from the latter tumors injected with B7-1 (**black and green peaks**) or empty plasmid (**red peak**), stained for B7-1 with the anti-B7-1 MAb 1G10 (**green**) or an isotype-matched control MAb 2G5 to control for nonspecific staining (**black**).

tected a 16.5-kilodalton protein in cell homogenates prepared from tumors injected with the dominant-negative mutant survivin plasmid but not in homogenates from tumors injected with the antisense survivin plasmid. In addition, flow cytometry analysis of tumor cells isolated from 0.2-cm tumors showed a population of survivin-positive cells, which was absent from tumors injected with antisense survivin plasmid (Fig. 3, F). Overall, these results suggest that reagents that block survivin expression or function have the ability to slow tumor growth, especially the growth of large tumors. The results are in accord with the view that the rate of growth of EL-4 tumors is dependent on the level of survivin expression.

Effects of Antisense Survivin and B7-1 Expression on Growth of Tumors

B7-1 is a molecule expressed on dedicated antigen-presenting cells that interacts with CD28 on responding T cells and costimulates T-cell activation and proliferation *(48).* Gene transfer of B7-1 into established tumors induces the generation of tumor-specific cytotoxic T lymphocytes (CTLs) and systemic immunity that results in the rejection of small tumors *(38,39).* While the mechanism involved in this antitumor immunity is not fully understood, B7-1 may induce tumor rejection by stimulating the proliferation of antitumor T cells or by serving as an NK cell receptor. To determine whether inhibiting survivin expression would render tumors more susceptible to lysis by CTLs following B7-1-mediated immunotherapy, we injected established small and large tumors with two plasmids, one that encodes either antisense survivin or the dominant-negative mutant survivin and one that encodes B7-1, and measured tumor growth. Small (0.2- to 0.3-cm) tumors were injected with 100 μg of each plasmid (Fig. 2, A), and large (0.4- to 0.6-cm) tumors were injected with 180 μg of each plasmid (Fig. 2, B). As reported previously *(38),* small tumors regressed rapidly and completely after injection with a plasmid encoding B7-1. We observed the same result for small tumors that were injected with plasmids encoding B7-1 and either antisense survivin or the dominant-negative mutant survivin (Fig. 2, A). Because the effects of the different plasmids were dependent on initial tumor size, we analyzed the effects of plasmid injections on the sizes of the small and large tumors separately. At 14 days after plasmid injection, the mean change in size for small tumors was −0.08 cm (95% CI = −0.06 cm to −0.10 cm) for those treated with B7-1, −0.14 cm (95% CI = −0.11 cm to −0.17 cm) for those treated with B7-1 plus antisense survivin, −0.13 cm (95% CI = −0.10 cm to −0.16 cm) for those treated with B7-1 plus dominant-negative mutant survivin, 0.21 cm (95% CI = 0.14 cm to 0.28 cm) for those treated with antisense survivin, 0.09 cm (95% CI = 0.02 cm to 0.16 cm) for those treated with dominant-negative mutant survivin, and 0.38 cm (95% CI = 0.24 cm to 0.52 cm) for those treated with empty plasmid. The mean change in size for tumors treated with empty plasmid was statistically significantly different from that for tumors treated with B7-1 in the absence or presence of either survivin plasmid (P<.001 for each comparison), as well as for tumors treated with the dominant-negative mutant survivin plasmid (P = .03) but not for those treated with the antisense survivin plasmid (P = .188). In contrast, there was no statistically significant difference in the mean change in size for tumors treated with B7-1 compared with that for tumors treated with B7-1 plus either survivin plasmid

($P = .15$) or between tumors treated with B7-1 plus antisense survivin and those treated with B7-1 plus dominant-negative survivin ($P = .63$). In summary, while each of the survivin-based plasmids inhibited the growth of small tumors, neither plasmid enhanced or impaired B7-1-mediated rejection of small tumors.

As reported previously *(38)*, large tumors continued to grow after injection with a plasmid encoding B7-1. At 14 days after plasmid injection, the mean change in size for large tumors was 0.18 cm (95% CI = 0.04 cm to 0.32 cm) for those treated with B7-1, −0.43 cm (95% CI = −0.36 cm to −0.50 cm) for those treated with B7-1 plus antisense survivin, −0.23 cm (95% CI = −0.20 cm to −0.26 cm) for those treated with B7-1 plus dominant-negative mutant survivin, −0.29 cm (95% CI = −0.18 cm to −0.40 cm) for those treated with antisense survivin, −0.25 cm (95% CI = −0.21 cm to −0.29 cm) for those treated with dominant-negative mutant survivin, and 0.43 cm (95% CI = 0.34 cm to 0.52 cm) for those treated with empty plasmid. As we observed for small tumors, the mean change in size over time for large tumors treated with empty plasmid was statistically significantly different from that for tumors treated with B7-1 in the presence of either survivin plasmid and from that for tumors treated with either of the survivin-based plasmids alone ($P<.001$ for each comparison). The mean change in size over time for large tumors treated with empty plasmid was also statistically significantly different from that for tumors treated with plasmid encoding B7-1 alone ($P = .02$). However, in contrast to our observations for small tumors, the mean change in size over time for large tumors treated with B7-1 was statistically significantly different from that for tumors treated with B7-1 plus either survivin plasmid ($P = .004$ for each comparison), as well as for tumors treated with B7-1 plus antisense survivin compared with those treated with B7-1 plus dominant-negative survivin ($P = .02$). These results suggest that the combined actions of the B7-1 and antisense survivin plasmids contributed more to the reduction in the size of large tumors than did the action of either plasmid alone. For example, at day 14 after plasmid injection, large tumors injected with a combination of the B7-1 and antisense survivin plasmids had a statistically significantly ($P<.001$) smaller mean size (0.09 cm; 95% CI = 0.04 cm to 0.14 cm) than large tumors injected with empty plasmid (0.44 cm; 95% CI = 0.37 cm to 0.51 cm), with B7-1 plasmid alone (0.62 cm; 95% CI = 0.48 cm to 0.76 cm), or with antisense survivin plasmid alone (0.18 cm; 95% CI = 0.12 cm to 0.24 cm). On day 14, the mean size of the tumors injected with B7-1 plasmid alone was not statistically significantly ($P = .06$) smaller than the mean size of large tumors injected with empty plasmid, whereas the mean size of the tumors injected with antisense survivin plasmid was ($P<.001$). In contrast, the combined actions of the B7-1 and dominant-negative mutant survivin plasmids did not contribute more to the reduction in the size of large tumors than did the action of the dominant-negative mutant survivin plasmid alone. For example, when compared with the mean size of large tumors at day 14 after injection with empty plasmid, large tumors injected with B7-1 and dominant-negative mutant survivin had a mean size of 0.23 cm (95% CI = 0.16 cm to 0.30 cm; $P<.001$) at day 14 after injection, whereas large tumors injected with the dominant-negative mutant survivin plasmid alone had a mean size of 0.18 cm (95% CI = 0.11 cm to 0.25 cm; $P<.001$). These results suggest that, although each of the survivin-based plasmids appears to disrupt survivin function *in vivo*, these plasmids vary in their ability to reduce tumor growth when used in combination therapy with B7-1.

To determine whether B7-1 immunotherapy in combination with antisurvivin therapy would generate heightened systemic antitumor immunity observed previously with B7-1 alone, we challenged mice that had rejected their tumors with parental EL-4 cells. Animals previously cured of small tumors after injection with the B7-1 plasmid alone or in combination with antisense survivin or dominant-negative mutant survivin constructs remained tumor free for 26 days after they were reinjected with 2×10^6 parental EL-4 cells (Fig. 2, A). Intratumoral injection of the B7-1 plasmid led to *de novo* expression of B7-1 in approximately 90% of the tumor cells, as assessed by immunohistochemical staining of tumor sections prepared 2 days after gene transfer (Fig. 3; *compare* panels G and H). This result was confirmed by flow cytometry analysis of tumor cells extracted from B7-1-treated tumors by collagenase digestion (Fig. 3, I). These results suggest that combining plasmids that reduce endogenous survivin expression or activity with a plasmid that expresses B7-1 does not impair the generation of systemic antitumor immunity that results from B7-1-mediated immunotherapy.

Effect of Gene Dose on Therapeutic Efficacy

We reported previously *(38)* that injection of tumors with a dose of 60 μg of plasmid encoding either B7-1 or other costimulatory cell adhesion molecules was the most effective dose for inducing antitumor immunity. Gene dose dependency may reflect transfection efficiency or the degree of T-cell costimulation that results from plasmid expression, since excessive antigen/costimulation induces T-cell anergy instead of proliferation. To investigate the optimal dose requirements for tumors treated with the combination of B7-1 and antisense survivin plasmids, we first injected established tumors with different amounts of B7-1 plasmid (60–140 μg for small tumors [Fig. 4, A]; 100–220 μg for large tumors [Fig. 4, B]). Twenty-four hours later, the tumors were injected with an amount of antisense survivin plasmid equivalent to the amount of B7-1 plasmid that was injected. We found that injection of either 100 or 140 μg each of the antisense survivin and B7-1 expression plasmids caused the rapid and complete regression of small tumors, whereas injection of 60 μg of each reagent did not (Fig. 4, A). In contrast, injection of either 180 or 220 μg of both plasmids inhibited the growth of large tumors but did not cause complete tumor regression (Fig. 4, B).

Effects of Antisense Survivin Expression on Antitumor CTL Activity

To determine whether injection with the antisense survivin plasmids might stimulate antitumor immunity, we measured the antitumor CTL activity (as determined by the ability to lyse EL-4 cells) of splenocytes collected from mice 28 days after their large EL-4 cell-derived tumors were injected with the B7-1 or antisense survivin expression plasmids, either alone or in combination. We found that the anti-EL-4 CTL activity, as measured by the ratio of release of lactate dehydrogenase (LDH) from splenocyte-lysed EL-4 cells compared with the total LDH content of EL-4 target cells (expressed as percent cytotoxicity), was statistically significantly greater in animals whose tumors were injected with either the B7-1 plasmid alone ($P<.001$ at an effector-to-target cell ratio of 50 : 1) or B7-1 in combination with

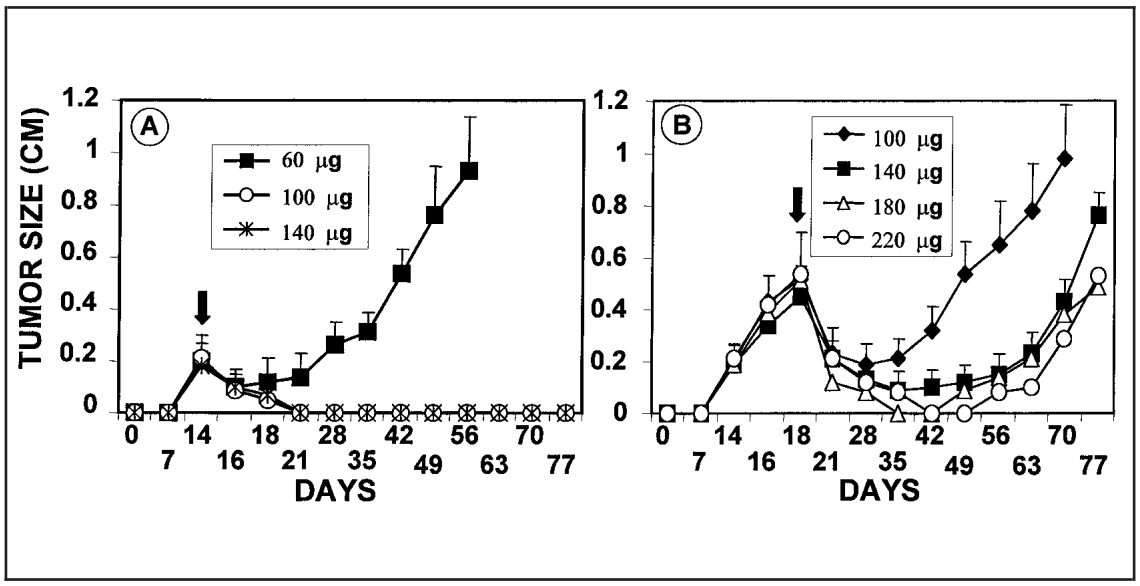

Fig. 4. Dependence of combination therapy with B7-1 and antisense survivin plasmids on plasmid dose. Tumors approximately 0.2 cm (**A**) and 0.4–0.6 cm (**B**) in diameter were injected with 60–220 μg of the B7-1 plasmid; 24 hours later, they were injected with an equal amount of antisense survivin expression plasmid. **Each point** represents the mean tumor size (as measured by two perpendicular diameters, in centimeters) of results obtained from five or six mice. **Arrows** denote the time point when plasmids were injected into tumors. **Bars** represent 95% confidence intervals.

the antisense survivin plasmid ($P<.001$ at an effector-to-target cell ratio of 50 : 1) compared with the CTL activity from animals whose tumors were injected with empty plasmid (Fig. 5). For example, splenocytes from animals whose tumors were injected with either B7-1, antisense survivin, or the combination of B7-1 and antisense survivin plasmids showed 18.8% (95% CI = 12.6% to 25%; $P<.001$), 33.6% (95% CI = 28.3% to 38.9%; $P<.001$), and 36.8% (95% CI = 28.2% to 45.4%; $P<.001$) cytotoxicity against EL-4 cells at an effector-to-target cell ratio of 50 : 1, respectively compared with splenocytes from animals whose tumors were injected with empty plasmid and showed only 7.2% (95% CI = 4.5% to 9.9%) cytotoxicity.

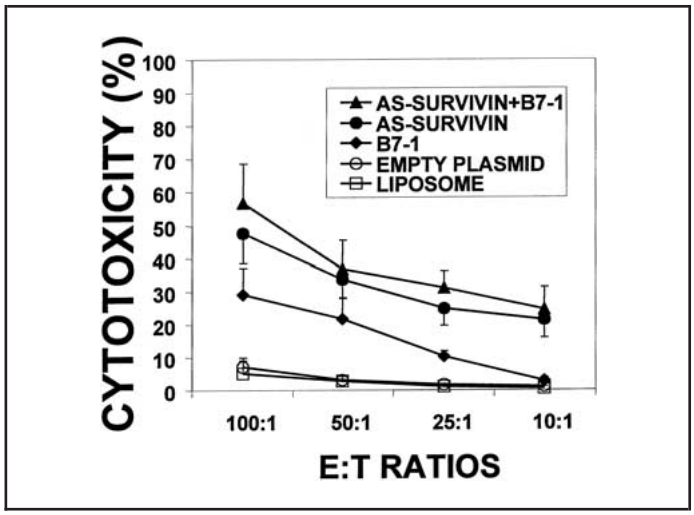

Fig. 5. Cytotoxicity in response to intratumoral expression of B7-1 and antisense (AS) survivin alone and in combination. Splenocytes, the effector cells (E), were harvested from mice 28 days after their EL-4-derived tumors were injected with DNA–liposome complexes or liposomes only and tested for their cytolytic activity against EL-4 target cells (T). The percent cytotoxicity is plotted against various effector-to-target cell ratios (E : T ratios). Control animals received empty pCDNA3 vector or the liposome transfection vehicle. **Each point** represents the mean percent cytotoxicity obtained from five or six mice. **Bars** represent 95% confidence intervals.

Identification of Immune Cells Responsible for Antitumor Activity in Response to Intratumoral Expression of B7-1, Antisense Survivin, and Dominant-Negative Mutant Survivin

We sought to determine the particular leukocyte subsets that infiltrated tumors as well as those responsible for tumor regression in response to the various treatment regimens, since this information might provide insights into the mechanisms responsible for antitumor immunity. Only small numbers of leukocytes infiltrated uninjected tumors (data not shown) or tumors injected with empty expression plasmid (Fig. 6). Large tumors injected with antisense survivin had statistically significantly ($P = .003$) more infiltrating $CD8^+$ leukocytes than large tumors injected with empty plasmid, whereas large tumors injected with dominant-negative mutant survivin were poorly infiltrated with all three types of leukocytes but had statistically significantly

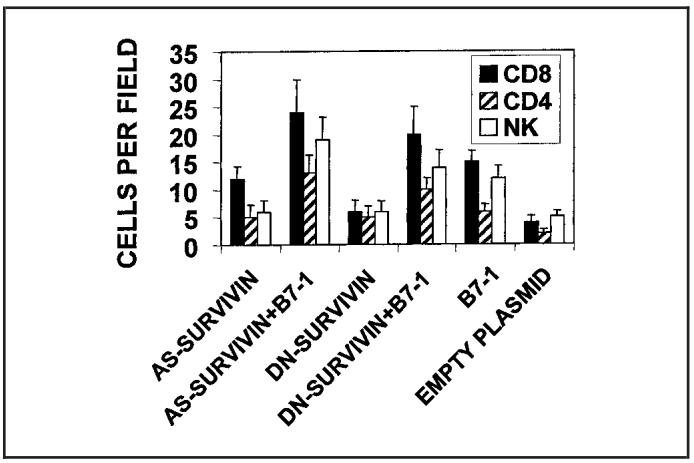

Fig. 6. Enumeration of immune cell subsets that infiltrate plasmid-injected tumors. Sections prepared 7 days after 0.4- to 0.6-cm-diameter EL-4-derived tumors were injected with the indicated plasmids were immunohistochemically stained for $CD4^+$ T cell, $CD8^+$ T cell, and natural killer (NK) cells. The mean number of each leukocyte subset was determined by counting eight to 10 fields. **Bars** indicate 95% confidence intervals. AS = antisense; DN = dominant negative.

($P = .03$) more $CD4^+$ T cells than tumors injected with empty plasmid (Fig. 6). Tumors injected with the B7-1 plasmid had statistically significantly more $CD8^+$ T cells (15.2 cells per field; 95% CI = 13.3 to 17.1 cells per field; $P<.0001$), $CD4^+$ T cells (6.4 cells per field; 95% CI = 4.8 to 8.0 cells per field; $P = .003$), and NK cells (11.7 cells per field; 95% CI = 9.6 to 13.8 cells per field; $P = .002$) than tumors injected with empty plasmid. Tumors injected with the B7-1 and antisense survivin plasmids had statistically significantly more $CD8^+$ T cells (23.8 cells per field; 95% CI = 17.1 to 30.5 cells per field; $P = .0006$), $CD4^+$ T cells (12.8 cells per field; 95% CI = 9.0 to 16.6 cells per field; $P = .0009$), and NK cells (18.8 cells per field; 95% CI = 15.8 to 22.0 cells per field; $P<.0001$) than tumors injected with empty plasmid. Tumors injected with the B7-1 and the dominant-negative mutant survivin plasmids had statistically significantly more $CD8^+$ T cells (19.8 cells per field; 95% CI = 14.6 to 25.0 cells per field; $P = .0006$), $CD4^+$ T cells (9.6 cells per field; 95% CI = 6.0 to 13.2 cells per field; $P = .005$), and NK cells (13.8 cells per field; 95% CI = 10.6 to 16.9 cells per field; $P = .002$) than tumors injected with empty plasmid. In general, more $CD8^+$ T cells and NK cells than $CD4^+$ T cells were present in all plasmid-injected tumors.

Antitumor immunity is largely mediated by $CD8^+$ T, $CD4^+$ T, and NK cells, and the contribution of each leukocyte subset to tumor regression varies according to the type of tumor and the specific approach to immunotherapy. Because the presence of a particular leukocyte subset in a tumor does not necessarily imply the direct involvement of that subset in antitumor immunity, we performed leukocyte depletion experiments to identify the infiltrating leukocyte subsets that contributed to the regression of the plasmid-injected EL-4 tumors. For tumors expressing B7-1 and either antisense survivin or dominant-negative mutant survivin, we found that there was a difference in tumor growth, depending on the particular leukocyte subset depleted; i.e., tumors depleted of each of the individual leukocyte subsets grew more than tumors not depleted of any leukocyte subset. Tumors injected with either the antisense survivin plasmid or the dominant-negative mutant survivin plasmid alone grew more when they were injected with MAbs to deplete them of $CD8^+$ T cells, NK cells, or $CD4^+$ T, $CD8^+$ T, and NK cells combined than when they were injected with MAbs to deplete them of $CD4^+$ T cells or in the absence of any leukocyte depletion (Table 1). Thus, whereas B7-1 in combination with either antisense survivin or the dominant-negative mutant survivin caused the complete regression of 0.2-cm tumors, resulting in mean decreases in tumor size at day 21 compared with day 0 of −0.23 cm (95% CI = −0.17 cm to −0.30 cm) and −0.22 cm (95% CI = −0.16 cm to −0.28 cm), respectively, antibody depletion of all three leukocyte subsets destroyed antitumor immunity, resulting in the growth of all tumors and leading to mean increases in tumor size of 0.58 cm (95% CI = 0.49 cm to 0.66 cm) and 0.65 cm (95% CI = 0.56 cm to 0.73 cm), respectively. Injection of 0.2-cm tumors with antisense survivin or the dominant-negative mutant survivin led to mean increases in tumor size at day 21 compared with day 0 of 0.32 cm (95% CI = 0.20 cm to 0.44 cm) and 0.19 cm (95% CI = 0.12 cm to 0.26 cm), respectively, whereas antibody depletion of all three leukocyte subsets resulted in the enhanced growth of all tumors resulting in mean increases in tumor size of 0.59 cm (95% CI = 0.49 cm to 0.68 cm) and 0.64 cm (95% CI = 0.52 cm to 0.76 cm), respectively. In contrast, depletion of $CD4^+$ T cells had little effect on the growth of tumors injected with antisense survivin or dominant-negative mutant survivin, as reflected by mean increases in tumor size at day 21 compared with day 0 of 0.25 cm (95% CI = 0.16 cm to 0.33 cm) and 0.19 cm (95% CI = 0.17 cm to 0.21 cm), respectively, which were similar or identical to the mean increases in sizes of 0.32 cm and 0.19 cm, respectively, for the tumors of non-leukocyte-depleted control mice.

These results suggest that $CD8^+$ T and NK cells cause the regression of EL-4 tumors in response to therapy with either antisense survivin or dominant-negative mutant survivin, alone or in combination with B7-1, whereas $CD4^+$ T cells contribute less to tumor rejection.

Effects of Antisense and Dominant-Negative Mutant Forms of Survivin on Tumor Cell Apoptosis

We reported previously *(38)* that B7-1-mediated antitumor immunity is accompanied by augmented CTL activity involving both the perforin and FasL pathways, suggesting that programmed cell death is responsible for tumor rejection. We there-

Table 1. Difference in tumor size at day 21 after treatment of plasmid-injected tumors with monoclonal antibodies (MAbs) to deplete leukocyte subsets*

	Expression plasmids injected							
	AS-survivin		AS-survivin + B7-1		DN-survivin		DN-survivin + B7-1	
MAb	Mean (95% CI)	P†	Mean (95% CI)	P†	Mean (95% CI)	P†	Mean (95% CI)	P†
Anti-CD4	0.25 (0.16 to 0.33)	.25	0.27 (0.22 to 0.33)	<.0001	0.19 (0.17 to 0.21)	.95	0.46 (0.40 to 0.52)	<.0001
Anti-CD8	0.52 (0.48 to 0.56)	.003	0.49 (0.42 to 0.57)	<.0001	0.58 (0.48 to 0.68)	<.0001	0.56 (0.49 to 0.63)	<.0001
Anti-NK	0.51 (0.46 to 0.56)	.004	0.49 (0.42 to 0.57)	<.0001	0.57 (0.48 to 0.66)	<.0001	0.56 (0.52 to 0.60)	<.0001
Anti-CD4/8/NK	0.59 (0.49 to 0.68)	.0002	0.58 (0.49 to 0.66)	<.0001	0.64 (0.52 to 0.76)	<.0001	0.65 (0.56 to 0.73)	<.0001
No MAb	0.32 (0.20 to 0.44)		−0.23 (−0.17 to −0.30)		0.19 (0.12 to 0.26)		−0.22 (−0.16 to −0.28)	
Rat IgG‡	Not determined		−0.23 (−0.16 to −0.29)		Not determined		Not determined	

*Mice were injected 7 days before plasmid injection and every alternate day for the duration of the experiment with anti-CD4 (Gk1.5), anti-CD8 (53–6.72), and anti-natural killer (NK) cell (PK136) MAbs or simultaneously with all three MAbs. Tumor sizes were monitored for 21 days following plasmid injection. Each value represents the mean difference in tumor size, in centimeters, from day 0 to day 21 for leukocyte-type depletion versus no depletion (no MAb) from five mice. AS = antisense; DN = dominant-negative; CI = confidence interval.

†Data comparing differences in tumor growth resulting from each leukocyte-type depletion versus no depletion were analyzed with the use of contrasts in a mixed linear model analysis to calculate the statistical significance of values between the antibody-treated group and the group not treated with MAb. *P* values refer to the comparison of change in tumor size from day 0 to day 21 for leukocyte-type depletion versus no depletion. All statistical tests were two-sided (Student's *t* test).

‡Rat immunoglobulin G (IgG) was used as the antibody control for the combination treatment of B7-1 with antisense survivin.

fore performed *in situ* TUNEL staining of tumor sections to detect the DNA strand breaks that characterize apoptotic cells. We measured the A/I 2 days after tumors were injected with B7-1 alone, with B7-1 and either antisense survivin or dominant-negative mutant survivin, with antisense survivin and dominant-negative survivin mutant alone, or with empty plasmid (Fig. 7, K). TUNEL staining revealed that tumors expressing either antisense survivin or dominant-negative mutant survivin plasmids had greater percentages of apoptotic cells (15% [95% CI = 10% to 20%; $P = .001$] and 25% [95% CI = 17% to 33%; $P<.001$], respectively) than tumors injected with empty plasmid (Fig. 7, K; *compare* Fig. 7, panels A and B with panel D). Tumors injected with B7-1 and either antisense survivin or dominant-negative mutant survivin plasmids had even greater percentages of apoptotic cells (28% [95% CI = 19.2% to 36.8%; $P<.001$] and 35% [95% CI = 26.4% to 43.6%; $P<.001$], respectively) than tumors injected with empty plasmid (Fig. 7, C and K). Similar results were obtained with the use of annexin-V-FLUOS to stain apoptotic cells extracted from tumors (Fig. 7, G–K) and annexin-V-biotin to stain apoptotic cells in tumor sections (Fig. 7, K). The higher A/I values obtained by annexin-V staining compared with TUNEL staining presumably reflect the increased sensitivity of annexin-V, which detects a very early hallmark of apoptosis.

DISCUSSION

Here, we report that survivin expression in tumors increases dramatically with increasing tumor size. Factors that induce survivin expression in tumors have yet to be identified. Angiogenic agents, such as vascular endothelial growth factor and basic fibroblast growth factor, which are induced in tumors in response to hypoxia, induce survivin expression in endothelial cells *(35,36).* Such angiogenic factors may indirectly aid tumor survival because they may increase the survival of endothelial cells that form blood vessels required to feed the growing tumor. In this context, survivin appears to mediate, at least in part, the ability of angiopoietin to stabilize vascular structures during angiogenesis *(37).* Thus, inhibiting survivin expression within tumors might be expected to impair the ability of angiopoietin to stabilize vascular structures during angiogenesis, thereby acting as a type of antiangiogenic therapy by inducing nascent tumor blood vessels to undergo apoptosis and preventing tumors from developing a blood supply.

We have shown that inhibition of survivin expression or function cripples large tumors such that they either regress or have severely impaired growth. The tumors that regressed failed to grow back to their original size at the initiation of gene therapy for at least 6 weeks and only began to grow again after 28 days, possibly because, by that time point, the therapeutic vectors had been degraded. It is possible that repeated administration of antisurvivin plasmids may cause sustained inhibition of tumor growth, as has been achieved with antiangiogenic therapy *(49).* The inhibition of survivin expression with either antisense or dominant-negative mutant forms of survivin clearly illustrates the dependence of large tumors on high levels of survivin expression. In contrast, inhibition of survivin expression in small tumors, where it is weakly expressed, had little effect on tumor growth, although the small tumors continued to grow at slightly reduced rates compared with tumors injected with empty plasmid. It is possible that the antisense and dominant-negative mutant survivin expression plasmids were degraded by the time the small tumors had become dependent on the protective properties of survivin.

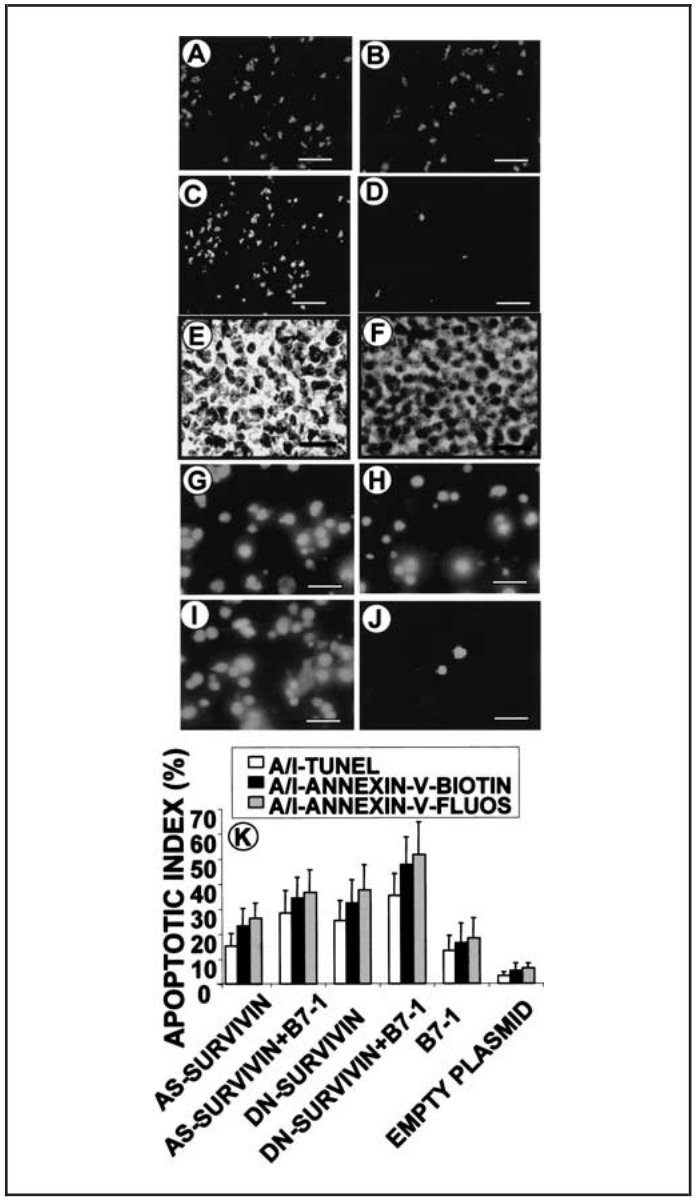

Fig. 7. Effect of inhibition of survivin expression on tumor cell apoptosis. Two days after plasmid injection, tumor sections were stained by the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling (TUNEL) method (**A**–**D**), or cells isolated from tumors were stained with annexin-V (**G**–**J**). Tumors were injected with plasmids expressing antisense survivin (**A and G**), dominant-negative mutant survivin (**B and H**), antisense (AS) survivin and B7-1 (**C and I**), or empty plasmid (**D and J**). For the determination of the total number of cells, tumor sections adjacent to TUNEL-stained sections were counterstained with hematoxylin (**E,** TUNEL assay), and annexin-V-stained, isolated tumor cells were co-stained with methylene blue (**F,** annexin-V assay). **Scale bar** = 20 μm. The number of apoptotic (**gray**) cells detected by TUNEL staining of tumor sections or by annexin-V staining of isolated tumor cells was determined for 10 randomly selected fields viewed at ×40 magnification. **Panel K:** The apoptotic index (A/I) is the number of apoptotic (TUNEL-positive or annexin-V-positive) cells × (100/total number of cells). **Bars** indicate 95% confidence intervals. AS = antisense; DN = dominant negative.

The increased expression of survivin in growing tumors parallels the acquisition of immune resistance of tumors as they enlarge. Thus, as reported here and previously *(38),* small tu-

mors can be eradicated completely by B7-1-mediated immunotherapy, but large tumors are refractory to such treatment. A pivotal finding in the present work was that inhibiting survivin expression in large tumors rendered them susceptible to B7-1-mediated immunotherapy. This result would be expected if survivin was protecting tumor cells from immune attack. We found that the efficacy of combination treatment with B7-1 and antisense survivin plasmids was dependent on plasmid dose, with administration of the highest dose of therapeutic plasmids always resulting in the greatest reduction in tumor size. However, whereas high plasmid doses caused the complete eradication of small tumors, large tumors were not similarly affected by doses of each plasmid of 100 μg or greater.

Tumors become resistant to immunotherapy by a variety of mechanisms *(50)*. They may secrete immunosuppressive factors *(51)*, develop mutant or variant cells that can avoid the immune response *(52)*, or develop the ability to counterattack the immune system with their own immune-based weapons. Alternatively, tumors may become resistant to CTL-mediated cell lysis *(3)* or infiltration by CTLs because of endothelial anergy *(53)*. CTLs kill target cells through the interaction of FasL on T cells with Fas, a mediator of apoptosis, on target cells. There are now several examples of how tumor cells can act like CTLs and kill antitumor T cells. For example, tumor cells expressing FasL can potentially counterattack and kill Fas-expressing CTLs *(54)*. In addition, some tumors and tumor cell lines express the membrane-associated molecule RCAS1, which is thought to bind a putative receptor on leukocytes, causing the inhibition of leukocyte cell growth and the induction of apoptosis *(41)*.

We suggest that survivin renders tumor cells more resistant to antitumor CTLs, thus acting as an important immunosuppressive factor. Accordingly, we found that inhibiting survivin expression in combination with B7-1 immunotherapy leads to increased apoptosis of tumor cells, which could be due, at least in part, to an increased susceptibility to attack by pre-existing antitumor T cells.

The inhibition of survivin expression led to a slight increase in the antitumor CTL response, as measured by an increase in the number of splenocytes in treated animals able to lyse EL-4 cell targets. However, we think that it is unlikely that survivin has direct immunostimulatory properties *per se*. Rather, the increase in CTLs in response to survivin inhibition may arise if cell fragments from apoptotic or necrotic tumor cells were taken up by dendritic cells, and the tumor antigens on such fragments were presented to the immune system indirectly. Cell fragments may be generated from dying tumor cells deprived of survivin, a protein that would otherwise protect them against an increasingly unfavorable, nutrient-poor, ectopic environment. Survivin plays a similar role in protecting interleukin 3 (IL-3)-dependent pre-B cells against IL-3 withdrawal *(10)*. This protective role could explain, in part, the increased tumor cell apoptosis after inhibition of survivin.

Our results suggest that survivin is an important therapeutic target. However, other antiapoptotic factors besides survivin, such as Bcl-2, and Bcl-xL, are also widely expressed in many tumors. It was reported previously that Bcl-2 is expressed in the EL-4 lymphoma *(55)*. We presume that an arsenal of therapeutic agents that simultaneously target survivin, Bcl-2, and other immunosuppressive factors would hold even greater promise than the antisurvivin therapy described in our study. Because survivin, unlike other potential therapeutic targets, is expressed in few adult tissues *(14,15)*, we predict that survivin-specific therapies are likely to produce few, if any, side effects. Our study indicates that inhibiting survivin expression to restore susceptibility to programmed cell death could break down this barrier to cancer immunotherapy and should be investigated as an adjunctive therapy for the treatment of large immune-resistant tumors.

REFERENCES

(1) Reed JC. Dysregulation of apoptosis in cancer. J Clin Oncol 1999;17: 2941–53.

(2) Reed JC, Bischoff JR. BIRinging chromosomes through cell division—and survivin' the experience. Cell 2000;102:545–8.

(3) Jaattela M. Escaping cell death: survival proteins in cancer. Exp Cell Res 1999;248:30–43.

(4) LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene 1998;17: 3247–59.

(5) Duckett CS, Nava VE, Gedrich RW, Clem RJ, Van Dongen JL, Gilfillan MC, et al. A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. EMBO J 1996;15:2685–94.

(6) Reed JC. Bcl-2 and the regulation of programmed cell death. J Cell Biol 1994;124:1–6.

(7) Nagata S. Apoptosis by death factor. Cell 1997;88:355–65.

(8) Yang E, Korsmeyer SJ. Molecular thanatopsis: a discourse on the BCL2 family and cell death. Blood 1996;88:386–401.

(9) Wright ME, Han DK, Hockenbery DM. Caspase-3 and inhibitor of apoptosis protein(s) interactions in *Saccharomyces cerevisiae* and mammalian cells. FEBS Lett 2000;481:13–8.

(10) Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917–21.

(11) Verdecia MA, Huang H, Dutil E, Kaiser DA, Hunter T, Noel JP. Structure of the human anti-apoptotic protein survivin reveals a dimeric arrangement. Nat Struct Biol 2000;7:602–8.

(12) Chantalat L, Skoufias DA, Kleman JP, Jung B, Dideberg O, Margolis RL. Crystal structure of human survivin reveals a bow tie-shaped dimer with two unusual alpha-helical extensions. Mol Cell 2000;6:183–9.

(13) Muchmore SW, Chen J, Jakob C, Zakula D, Matayoshi ED, Wu W, et al. Crystal structure and mutagenic analysis of the inhibitor-of-apoptosis protein survivin. Mol Cell 2000;6:173–82.

(14) Konno R, Yamakawa H, Utsunomiya H, Ito K, Sato S, Yajima A. Expression of survivin and Bcl-2 in the normal human endometrium. Mol Hum Reprod 2000;6:529–34.

(15) Chiodino C, Cesinaro AM, Ottani D, Fantini F, Giannetti A, Trentini GP, et al. Communication: expression of the novel inhibitor of apoptosis survivin in normal and neoplastic skin. J Invest Dermatol 1999;113:415–8.

(16) Adida C, Haioun C, Gaulard P, Lepage E, Morel P, Briere J, et al. Prognostic significance of survivin expression in diffuse large B-cell lymphomas. Blood 2000;96:1921–5.

(17) Sarela AI, Macadam RC, Farmery SM, Markham AF, Guillou PJ. Expression of the antiapoptosis gene, survivin, predicts death from recurrent colorectal carcinoma. Gut 2000;46:645–50.

(18) Islam A, Kageyama H, Takada N, Kawamato T, Takayasu H, Isogai E, et al. High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene 2000;19:617–23.

(19) Tanaka K, Iwamoto S, Gon G, Nohara T, Iwamoto M, Tanigawa N. Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. Clin Cancer Res 2000;6:127–34.

(20) Monzo M, Rosell R, Felip E, Astudillo J, Sanchez JJ, Maestre J, et al. A novel anti-apoptosis gene: re-expression of survivin messenger RNA as a prognosis marker in non-small-cell lung cancers. J Clin Oncol 1999;17: 2100–4.

(21) Reed JC, Reed SI. Survivin' cell-separation anxiety. Nat Cell Biol 1999; 1:E199–200.

(22) Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998;396:580–4.

(23) Suzuki A, Hayashida M, Ito T, Kawano H, Nakano T, Miura M, et al. Survivin initiates cell cycle entry by the competitive interaction with Cdk4/p16(INK4a) and Cdk2/cyclin E complex activation. Oncogene 2000;19:3225–34.
(24) Ito T, Shiraki K, Sugimoto K, Yamanaka T, Fujikawa K, Ito M, et al. Survivin promotes cell proliferation in human hepatocellular carcinoma. Hepatology 2000;31:1080–5.
(25) Suzuki A, Ito T, Kawano H, Hayashida M, Hayasaki Y, Tsutomi Y, et al. Survivin initiates procaspase 3/p21 complex formation as a result of interaction with Cdk4 to resist Fas-mediated cell death. Oncogene 2000;19:1346–53.
(26) Shin S, Sung BJ, Cho YS, Kim HJ, Na NC, Hwang JI, et al. An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. Biochemistry 2001;40:1117–23.
(27) Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000;102:33–42.
(28) Webb A, Cunningham D, Cotter F, Clarke PA, di Stefano F, Ross P, et al. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. Lancet 1997; 349:1137–41.
(29) Miayake H, Tolcher A, Gleave ME. Chemosensitization and delayed androgen-independent recurrence of prostate cancer with the use of antisense Bcl-2 oligodeoxynucleotides. J Natl Cancer Inst 2000;92:34–41.
(30) Baba M, Iishi H, Tatsuta M. *In vivo* electrophoretic transfer of bcl-2 antisense oligonucleotide inhibits the development of hepatocellular carcinoma in rats. Int J Cancer 2000;85:260–6.
(31) Li F, Ackermann EJ, Bennett CF, Rothermel AL, Plescia J, Tognin S, et al. Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. Nat Cell Biol 1999;1:461–6.
(32) Chen J, Wu W, Tahir SK, Kroeger PE, Rosenberg SH, Cowsert LM, et al. Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-independent growth. Neoplasia 2000;2:235–41.
(33) Grossman D, McNiff JM, Li F, Altieri DC. Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. J Invest Dermatol 1999; 113:1076–81.
(34) Olie RA, Simoes-Wust AP, Baumann B, Leech SH, Fabbro D, Stahel RA, et al. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res 2000;60:2805–9.
(35) O'Connor DS, Schechner JS, Adida C, Mesri M, Rothermel AL, Li F, et al. Control of apoptosis during angiogenesis by survivin expression in endothelial cells. Am J Pathol 2000;156:393–8.
(36) Tran J, Rak J, Sheehan C, Saibil SD, LaCasse E, Korneluk RG, et al. Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. Biochem Biophys Res Commun 1999;264:781–8.
(37) Papapetropoulos A, Fulton D, Mahboubi K, Kalb RG, O'Connor DS, Li F, et al. Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. J Biol Chem 2000;275:9102–5.
(38) Kanwar J, Berg R, Lehnert K, Krissansen GW. Taking lessons from dendritic cells: multiple xenogeneic ligands for leukocyte integrins have the potential to stimulate anti-tumor immunity. Gene Ther 1999;6:1835–44.
(39) Kanwar JR, Kanwar RK, Pandey S, Ching LM, Krissansen GW. Vascular attack by 5,6-dimethylxanthenone-4-acetic acid combined with B7.1 (CD80)-mediated immunotherapy overcomes immune resistance and leads to the eradication of large tumors and multiple tumor foci. Cancer Res 2001;61:1948–56.
(40) Sun X, Kanwar JR, Leung E, Lehnert K, Wang D, Krissansen GW. Gene transfer of antisense hypoxia inducible factor-1α enhances the therapeutic efficacy of cancer immunotherapy. Gene Ther 2001;8:638–45.
(41) Nakashima M, Sonoda K, Watanabe T. Inhibition of cell growth and induction of apoptotic cell death by the human tumor-associated antigen RCAS1. Nat Med 1999;5:938–42.
(42) Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
(43) Li F, Altieri DC. The cancer antiapoptosis mouse survivin gene: characterization of locus and transcriptional requirements of basal and cell cycle-dependent expression. Cancer Res 1999;59:3143–51.
(44) Bronckers AL, Goei SW, Dumont E, Lyaruu DM, Woltgens JH, van Heerde WL, et al. *In situ* detection of apoptosis in dental and periodontal tissues of the adult mouse using annexin-V-biotin. Histochem Cell Biol 2000;113:293–301.
(45) Gibson-D'Ambrosio RE, Samuel M, D'Ambrosio SM. A method for isolating large numbers of viable disaggregated cells from various human tissues for cell culture establishment. In Vitro Cell Dev Biol 1986;22:529–34.
(46) Mason LH, Ortaldo JR, Young HA, Kumar V, Bennett M, Anderson SK. Cloning and functional characteristics of murine large granular lymphocyte-1: a member of the Ly-49 gene family (Ly-49G2). J Exp Med 1995; 182:293–303.
(47) Peterson GL. Determination of total protein. Methods Enzymol 1983;91:95–119.
(48) Chen L, Ashe S, Brady WA, Hellstrom I, Hellstrom KE, Ledbetter JA, et al. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. Cell 1992;71:1093–102.
(49) Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature 1997; 390:404–7.
(50) Hersey P. Impediments to successful immunotherapy. Pharmacol Ther 1999;81:111–9.
(51) Wojtowicz-Praga S. Reversal of tumor-induced immunosuppression: a new approach to cancer therapy. J Immunother 1997;20:165–77.
(52) Villunger A, Strasser A. The great escape: is immune evasion required for tumor progression? [news]. Nature Med 1999;5:874–5.
(53) Griffioen AW, Damen CA, Mayo KH, Barendsz-Janson AF, Martinotti S, Blijham GH, et al. Angiogenesis inhibitors overcome tumor induced endothelial cell anergy. Int J Cancer 1999; 80:315–9.
(54) O'Connell J, Bennett MW, O'Sullivan GC, Collins JK, Shanahan F. Fas counter-attack—the best form of tumor defense? [news]. Nat Med 1999;5:267–8.
(55) Grimaitre M, Werner-Favre C, Kindler V, Zubler RH. Human naive B cells cultured with EL-4 T cells mimic a germinal center-related B cell stage before generating plasma cells. Concordant changes in Bcl-2 protein and messenger RNA levels. Eur J Immunol 1997;27:199–205.

NOTES

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