Inhibition of melanoma tumor growth in vivo by survivin targeting

Douglas Grossman1, Paul J. Kim3, Jeffrey S. Schechner1, and Dario C. Altieri4,5

Departments of 1Dermatology and 3Pathology and the Boyer Center for Molecular Medicine, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536

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A role of apoptosis (programmed cell death) in tumor formation and growth was investigated by targeting the apoptosis inhibitor survivin in vivo. Expression of a phosphorylation-defective survivin mutant (Thr34→Ala) triggered apoptosis in several human melanoma cell lines and enhanced cell death induced by the chemotherapeutic drug cisplatin in vitro. Conditional expression of survivin Thr34→Ala in YUSAC2 melanoma cells prevented tumor formation upon s.c. injection into C.B.17 severe combined immunodeficient-beige mice. When induced in established melanoma tumors, survivin Thr34→Ala inhibited tumor growth by 60–70% and caused increased apoptosis and reduced proliferation of melanoma cells in vivo. Manipulation of the antiapoptotic pathway maintained by survivin may be beneficial for cancer therapy.

Regulation of apoptosis (programmed cell death) is critical for normal embryonic development and for homeostasis in adult tissues (1). Dysregulation of this process with increased resistance to cell death is a common feature of malignant cells (2) and represents a significant obstacle to therapy of human cancer (3). Apoptosis resistance in melanoma (4) accounts for its poor response to chemotherapy (5) and has been correlated with increased aggressiveness and decreased patient survival (11–15).

Maximally expressed in the G2/M phase of the cell cycle, survivin physically associates with mitotic spindle microtubules and regulates progression through mitosis (16). Transformed cells are exquisitely sensitive to manipulation of this checkpoint, because interference with survivin expression/function using dominant-negative mutants affecting the baculovirus IAP repeat (BIR; ref. 17) or survivin antisense resulted in dysregulation of mitotic progression (18) and spontaneous apoptosis (8, 19, 20). This response is unique to survivin and not observed with other apoptosis inhibitors potentially contributing to neoplasia, because antisense inhibition of Bel-2 increased sensitivity to apoptosis but was insufficient in itself to induce cell death (21). Here, we targeted survivin in melanoma in vivo by using regulated expression of a phosphorylation-defective Thr34→Ala BIR mutant.

Methods

Survivin Dominant-Negative Mutant and Transient Transfections. The Thr34→Ala mutation was introduced by site-directed mutagenesis into the 1.6-kb human survivin cDNA (9) by using the oligonucleotide 5′-GGCTGCAGCCTGCACCATGACTG-3′ and the geneEDITOR system (Promega) according to the manufacturer’s instructions and cloned into the green fluorescent protein (GFP) marker plasmid pEGFPC1 (CLONTECH).

Human melanoma lines YUSAC2, YUGEN8, and LOX were kindly provided by Ruth Halaban (Yale Univ. School of Medicine, New Haven, CT) and maintained as described (8). Transient transfections were performed and apoptotic index was assessed after 48 h by nuclear morphology by using 4,6-diamidino-2-phenylindole staining as described (8).

Table 1. Induction of apoptosis in melanoma cell lines by expression of survivin Thr34→Ala

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>YUSAC2</th>
<th>LOX</th>
<th>YUGEN8</th>
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<tbody>
<tr>
<td>GFP-vector</td>
<td>2</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>GFP-survivin</td>
<td>12</td>
<td>20</td>
<td>19</td>
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<tr>
<td>GFP-survivin (Thr34→Ala)</td>
<td>40</td>
<td>55</td>
<td>42</td>
</tr>
</tbody>
</table>

The melanoma cell lines YUSAC2, LOX, and YUGEN8 were transfected separately with the indicated GFP-containing plasmids as described previously (8). At 48 h after transfection, cell nuclei were scored morphologically as normal or apoptotic by 4,6-diamidino-2-phenylindole staining and fluorescence microscopy as described previously (8). Data are expressed as percent apoptosis based on counting approximately 100 cells per condition and are the mean of two independent transfection experiments.

Generation of Inducible Transfectants in Melanoma Cells. The wild-type survivin cDNA and the survivin Thr34→Ala mutant were cloned into the EcoRI and HindIII-SpeI sites, respectively, of pTet-splice (Stratagene) downstream of the regulatory sequences of the tetracycline (tet)-resistance operon. The plasmid pTA-Neo, containing the tet-controlled transactivator sequence downstream of the tet operon and a neomycin resistance gene, was kindly provided by D. Schatz (Yale Univ. School of Medicine). In this tandem plasmid system, tet prevents transactivator binding to the tet operon and transcription of the transgene; in the absence of tet, the transactivator up-regulates its own transcription and the transgene is expressed (22).

YUSAC2 cells (8) were transfected in six-well plates by the addition of 0.8 μg of pTet-splice containing either the wild-type survivin cDNA or the survivin Thr34→Ala mutant, 0.8 μg of pTA-Neo, 0.5 μg of tet (Sigma), and 5 μl of Lipofectamine (Life Technologies, Gaithersburg, MD) per well. After 9 h, the transfection medium was aspirated and replaced with serum-containing medium in the presence of 0.5 μg/ml tet. Forty-eight h after transfection, cells were trypsinized, washed, and replated at low density in 15 × 150-mm plates in fresh medium containing 1.5 mg/ml G418 (Life Technologies), 2 mM sodium hydroxide, and 0.5 μg/ml tet. This selection medium was changed every 6 days, and after 3 weeks colonies were transferred to U-bottom microtiter wells for expansion and screening on the basis of tet-regulated differential growth (survivin Thr34→Ala) or induction of survivin-immunoreactive material (wild-type trans-

Abbreviations: IAP, inhibitor of apoptosis; tet, tetracycline; BIR, baculovirus IAP repeat; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling.

To whom reprint requests should be addressed at: Yale University School of Medicine, BCCM3436B, 295 Congress Avenue, New Haven, CT 06536. E-mail: dario.altieri@yale.edu.

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fectants. Clones stably transfected with the wild-type survivin (YUSAC2/WT) and survivin Thr34→Ala (YUSAC2/T34A-C4 and YUSAC2/T34A-E5) were isolated and maintained in selection medium in the presence of G418 and tet.

Expression and Function of Survivin Thr34→Ala in Vitro. Western blotting was performed as described (8) by using a polyclonal antibody reactive against both native and mutant (Thr34→Ala) survivin. A novel rabbit polyclonal antibody was raised against the survivin peptide L28EGCACT*PERMAEAGFI44 containing phosphorylated Thr34 (T*). The serum was precleared over a nonphosphorylated peptide-Sepharose column, and unbound material was affinity-purified over a phosphorylated peptide-Sepharose column. This antibody to phosphorylated survivin Thr34 (α-survivinT34*) recognized wild-type survivin after in vitro phosphorylation by baculovirus-expressed p34<sup>cdc2</sup>-cyclin B1, but not unphosphorylated wild-type survivin or survivin Thr34→Ala after incubation with p34<sup>cdc2</sup>-cyclin B1 (25). Blots were stained with α-survivinT34* (10 μg/ml) overnight at 4°C.

For DNA content analysis, both nonadherent and adherent cells were recovered and pooled. Cells then were fixed, permeabilized, and stained with propidium iodide as described (8). Percentages for the sub-G<sub>1</sub> fraction (left marker), corresponding to apoptotic cells, and the G<sub>2</sub>/M fraction (right marker), corresponding to mitotic cells, were derived from histograms obtained with CELL QUEST software (Becton Dickinson). For terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) analysis of cultured cells, cells were plated on coverslips treated with 2% gelatin (Sigma).
and incubated 48 h in the presence or absence of tet. TUNEL staining was carried out by using the ApopTag kit (Intergen, Purchase, NY) according to the manufacturer’s instructions as described (8). Cisplatin (Sigma) was solubilized (30 mM) in dimethylformamide and stored at 4°C.

**Tumor Formation in CB.17 Mice.** We determined that YUSAC2 cells consistently form localized tumors in 6- to 8-week-old CB.17 SCID/beige mice (Taconic Farms, Germantown, NY) approximately 2–3 weeks after s.c. injection of 2–3 × 10⁶ cells. We have monitored animals for up to 4 months, and neither mortality nor gross metastasis is associated with increasing tumor size (up to 5,000 mm³) or ulceration. One day before injection, mice were shaved on the right flank, and the regular drinking water was replaced with 5% sucrose alone or containing 100 µg/ml tet as described (22). Cells were harvested in log-phase growth, washed twice in PBS, resuspended in PBS (12 × 10⁶ cells/ml), and injected (0.25 ml, 3 × 10⁶ cells) s.c. The drinking water was changed every 2–3 days. Tumor size was determined by the product of two perpendicular diameters and the height above the skin surface.

**Analysis of Apoptosis and Proliferation in vivo.** Apoptotic cells in vivo were identified by TUNEL staining as described above. An apoptotic index was obtained by counting the average number of TUNEL-positive cells in 12 low-power (×100) fields, each containing approximately 4,000 cells. For determination of proliferating cells in vivo, mice were injected i.p. with 50 mg/kg BrdUrd (Sigma) in PBS, and tumors were excised 2 h later. Tissue sections were stained for BrdUrd by using a kit (Zymed) according to the manufacturer’s instructions. A proliferative index was obtained by counting the average number of BrdUrd-positive cells in 12 high-power (×400) fields, each containing approximately 1,000 cells.

**Reestablishment of Tumor Cell Lines in Vitro.** Tumors were excised surgically and skin and s.c. tissues were dissected away. Tumors were cut into small pieces with a sterile razor blade and dissociated into a single-cell suspension by vortexing in PBS. After removal of insoluble debris, cells were washed twice in PBS, resuspended in selection medium, and cultured for two to three passages.

**Results and Discussion**

A survivin Thr³⁴→Ala mutation, which abolishes a phosphorylation site for the main mitotic kinase p34cdc2-cyclin B1, caused spontaneous apoptosis (i.e., a dominant-negative effect) when overexpressed in HeLa carcinoma cells (25). Transfection of three different human melanoma cell lines (8) with GFP conjugates of survivin Thr³⁴→Ala also resulted in morphologic signs of apoptosis including chromatin condensation and DNA fragmentation (Table 1). By contrast, GFP (wild-type)-survivin did not affect melanoma cell viability (Table 1).

One of these melanoma lines, YUSAC2, was stably transfected with survivin Thr³⁴→Ala or the wild-type survivin cDNA under the control of a tet-regulated (“tet-off”) promoter system (22). Upon withdrawal of tet from the culture medium, subclone YUSAC2/T34A-C4 strongly expressed a 16.5-kDa induced survivin band by Western blotting (Fig. Ll). By contrast, no modulation of survivin expression was observed when tet was present in the culture medium (Fig. 1A). Similar results of tet-regulated induction of survivin Thr³⁴→Ala were obtained with another subclone, YUSAC2/T34A-E5 (not shown). Reactivity by Western blotting with phosphosurvivin-specific antibody (α-survivinT34*) was considerably reduced in tet− cultures of YUSAC2/T34A-C4 as compared with tet+ cultures of YUSAC2/WT cells (Fig. 1B).

Tet-regulated expression of the Thr³⁴→Ala mutant resulted in a progressive, time-dependent accumulation of apoptotic cells with hypodiploid (sub-G₁) DNA content and coincident loss of mitotic (G₂/M) cells, as assessed by propidium iodide staining and flow cytometry (Fig. 1C). Tet-deprived YUSAC2/T34A-C4 cells exhibited apoptotic nuclear morphology and stained intensely for internucleosomal DNA fragmentation by TUNEL (Fig. 1D). In addition to spontaneous apoptosis, induction of survivin Thr³⁴→Ala increased the sensitivity of YUSAC2 cells to the chemotherapeutic drug cisplatin. A 3-day culture of YUSAC2/T34A-C4 cells in the presence of tet and cisplatin resulted in a population of both apoptotic cells and cells arrested in G₂/M (Fig. 2A), consistent with previous reports (23) of cisplatin-treated melanoma cells. Cultures treated with cisplatin and deprived of tet revealed a 2.2-fold increase in apoptotic cells, as compared with either treatment alone (Fig. 2A).

Next, we asked whether interference with survivin function by regulated expression of survivin Thr³⁴→Ala mutant could block melanoma tumor formation in CB.17 severe combined immunodeficient-beige mice. Untransfected YUSAC2 cells readily formed localized nodular amelanotic tumors in mice composed
of sheets of large, epithelioid malignant cells that stained positively for survivin and HMB-45, a marker of human melanoma cells (24; Fig. 2B). Tumor formation by YUSAC2 cells was not affected by the presence or absence of tet in the drinking water (Fig. 3A; Table 2). By contrast, YUSAC2/T34A-C4 cells did not form tumors in 13 of 15 (87%) animals when tet was withheld from the drinking water (Fig. 3A; Table 2). All of these animals remained tumor-free for an additional 3-month observation period. The two tumors that formed in tet-deprived animals were considerably smaller in size and appeared with a markedly delayed onset compared with those in animals given tet (Table 2). The slightly smaller size of tumors formed in the presence of tet by survivin Thr34→Ala subclones compared with untransfected YUSAC2 cells (Table 2) may be due to minimal leakiness of the promoter system in vivo. Prevention of melanoma tumor formation in vivo by survivin Thr34→Ala also was obtained with subclone YUSAC2/T34A-CE5 (Table 2).

We next investigated whether expression of survivin Thr34→Ala could affect the growth of already established melanoma tumors. For these experiments, 30 mice were injected with YUSAC2/T34A-C4 cells and tet was provided to permit tumor formation. After 3 weeks, tumors became palpable (20–50 mm³) and tet was withheld from the drinking water of 20 animals. Tumors of animals then were monitored for an additional 3 weeks. Tumors of animals maintained on tet exhibited exponential growth during the 3-week observation period (Fig. 3B). Tumors of animals maintained on tet exhibited exponential growth during the 3-week observation period (Fig. 3B).
period (Fig. 3B). By contrast, withdrawal of tet in established tumors was associated with a significant \( P < 0.0001 \) reduction (60–70%) in growth rate (Fig. 3B).

The long-term effects of survivin targeting in established melanoma tumors were investigated in 10 additional animals in which tet had been withdrawn. At 8 weeks, animals were killed and histologic examination of these tumors was performed. Tumors in animals deprived of tet contained massive areas of necrosis, compared with minimal loss of cell viability in tumors of animals maintained on tet (Fig. 3C).

Next, we examined whether the decreased growth rate in survivin-targeted tumors was associated with an increased rate of spontaneous apoptosis in melanoma cells. Whereas tumors from animals maintained on tet contained low numbers of apoptotic cells, tumors from animals deprived of tet revealed consistently increased numbers of TUNEL-positive cells throughout the 21-day observation period of predicted expression of survivin Thr34→Ala (Fig. 3D). In control experiments, no cells were stained for TUNEL in the absence of terminal deoxynucleotidyl transferase (not shown).

Given the role of survivin in controlling apoptosis (16) and ploidy at cell division (18), we next examined the rate of melanoma cell proliferation by BrdUrd incorporation in the absence or presence of survivin targeting. Whereas tumors from animals maintained on tet demonstrated an initial burst of proliferative activity, tumors from animals deprived of tet revealed no proliferative burst and consistently lower levels of BrdUrd-labeled cells throughout the observation period (Fig. 3D). No cells were stained for BrdUrd in the absence of BrdUrd injection (not shown).

Finally, we wish to confirm that the suppressive effect of survivin targeting on tumor growth and viability in vivo could be attributed to spontaneous apoptosis induced by tet-regulated expression of survivin Thr34→Ala. For these experiments, cell lines were reestablished in vitro from several melanoma tumors excised from animals deprived of tet and analyzed for tet-regulated induction of apoptosis in vitro. In all cases, these cells retained tet responsiveness as removal of tet from the culture medium was associated with generation of hypodiploid (apoptotic) cells by DNA content analysis (Fig. 3E) and in agreement with the data presented above (Fig. 1). The persistence of some viable cells in tet-deprived tumors may reflect an inability to remove tet completely from the animal and achieve maximal transgene expression in vivo. In addition, inhibition of survivin may not eliminate nondividing cells given its selective action during the G2/M phase of the cell cycle (16).

In summary, we have used a tet-regulated molecular antagonist of survivin, i.e., a dominant-negative mutant, to interfere with the apoptotic balance in melanoma tumors in vivo. The Thr34 residue in survivin corresponds to a unique p34cdc2 phosphorylation site, and mutagenesis of Thr34→Ala completely suppressed survivin phosphorylation by p34cdc2-cyclin B1 in vitro and in vivo (25). Based on the survivin crystal structure, Thr34 appears ideally positioned to modulate protein recognition potentially mediated by the survivin BIR in a phosphorylation-dependent manner (17). The ability of survivin Thr34→Ala mutant to localize to mitotic spindle microtubules and associate with p34cdc2-cyclin B1 (25) suggests that its dominant-negative effect may reflect interference with phosphorylation of endogenous survivin. Consistent with this prediction, tet-regulated expression of survivin Thr34→Ala resulted in considerable inhibition of survivin phosphorylation on Thr34, as determined by Western blotting with a T34* phosphorylation-specific antibody. Functionally, this resulted in spontaneous apoptosis of melanoma cells in vitro and in vivo, enhanced sensitivity to a chemotherapeutic drug (cisplatin), suppression of de novo melanoma tumor formation, and growth inhibition of already established melanoma tumors. Consistent with a potential effect on the apoptosis balance at mitosis, expression of survivin Thr34→Ala also resulted in loss of cells with G2/M DNA content in vitro and decreased proliferation of melanoma cells in vivo. Combined with the selective overexpression of survivin in cancer but not in normal tissues (9, 10), these data suggest that manipulation of the survivin pathway alone or with chemotherapy may be therapeutically useful in the treatment of melanoma and other malignancies.

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