Expression and Targeting of the Apoptosis Inhibitor, Survivin, in Human Melanoma

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The newly described apoptosis inhibitor survivin is expressed in many human cancers and appears to play a critical part in both apoptosis regulation and cell cycle progression. Its potential role in malignant melanoma is unknown. In a panel of 30 malignant melanomas, survivin was strongly expressed in all cases (15 of 15) of metastatic malignant melanomas and 13 of 15 cases of invasive malignant melanomas by immunohistochemistry. In invasive malignant melanomas, survivin was also expressed in the *in-situ* component of the lesion. Survivin expression was found in all cases (11 of 11) of nevi, but not in melanocytes in sections of normal skin. The apoptosis inhibitor bcl-2 was expressed in 26 of 30 cases, but generally at lower levels than that of infiltrating lymphocytes. The mitotic index, as assessed by MIB-1 staining, was consistently higher in metastatic than invasive malignant melanomas. Assessment of apoptotic index by *in situ* end-labeling revealed extremely low rates of apoptosis in most malignant melanomas. Survivin expression by western blotting was detected in four human metastatic malignant melanoma cell lines but not in cultured normal human melanocytes. Transfection of both YUSAC-2 and LOX malignant melanoma cells with green fluorescence protein-conjugated survivin anti-sense or green fluorescence protein-conjugated survivin dominant negative mutant (Cys85Ala) resulted in increased apoptosis in the absence of other genotoxic stimuli. Two-color flow cytometry confirmed that YUSAC-2 cells transfected with survivin anti-sense expressed less endogenous survivin and exhibited an increased fraction of cells with sub-G_1 DNA content. These data demonstrate that apoptosis inhibition by survivin may participate in the onset and progression of malignant melanomas, and suggest that therapeutic targeting of survivin may be beneficial in patients with recurrent or metastatic disease. ***Key words: antisense/bcl-2/GFP/IAP protein/nevus. J Invest Dermatol 113:1076–1081, 1999***

Malignant melanoma (MM) is the most serious of all cancers arising in the skin, and its incidence is increasing more rapidly than in any other cancer (Urist and Karnell, 1994). It accounts for over 7000 deaths in the U.S.A. annually and the average lifetime risk is approaching one in 80 (Johnson *et al*, 1998). In the case of metastatic MM, the response to chemotherapy is poor and 5-year survival ranges from 10 to 50% (Koh, 1991). The general mechanism by which most chemotherapeutic agents act is induction of apoptosis (Fisher, 1994), and their low therapeutic efficacy in this disease likely relates to a relative inability to induce apoptosis in melanoma compared with other malignant cell types (Li G *et al*, 1998). In addition, cells within MM lesions demonstrate an inherently low level of spontaneous apoptosis (Mooney *et al*, 1995; Stauton and Gaffney, 1995) and resistance to apoptosis has been correlated with increased metastatic potential in animal models of melanoma (Glinsky *et al*, 1997).

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Abbreviations: BIR, baculovirus IAP repeat; GFP, green fluorescence protein; IAP, inhibitor of apoptosis;. MM, malignant melanoma.

The molecular basis for apoptosis resistance in MM is not well understood. Defects at multiple levels of the cell death program have been described, ranging from ineffective extracellular signaling and caspase-8 activation (Irmler *et al*, 1997; Griffith *et al*, 1998; Thomas and Hersey, 1998) to unbalanced expression of inhibitors of apoptosis. Given its ability to block upstream initiation of the caspase cascade, the anti-apoptotic protein bcl-2 (Adams and Cory, 1998) has been extensively investigated in MM. Several groups (Cerroni *et al*, 1995; Collins and White, 1995; Selzer *et al*, 1998) found expression of bcl-2 in MM comparable with that in benign nevi, whereas another (Tang *et al*, 1998) found decreased bcl-2 expression in MM. In a large series of uveal MM, bcl-2 expression did not correlate with disease progression (Mooy *et al*, 1995).

In addition to bcl-2, another gene family of inhibitors of apoptosis (IAP) has recently been identified. Evolutionarily conserved from viruses to mammalian cells, IAP proteins share a highly conserved cysteine/histidine-rich (baculovirus IAP repeat, BIR) motif and are thought to block the downstream effector phase of programmed cell death by directly inhibiting terminal caspase-3 and -7 (LaCasse *et al*, 1998; Deveraux and Reed, 1999). Among IAP proteins, survivin (Ambrosini *et al*, 1997; Tamm *et al*, 1998) has attracted attention for its overexpression in most human cancers, and for its association with aggressive forms of neuroblastoma (Adida *et al*, 1998) and decreased survival in colorectal cancer (Kawasaki *et al*, 1998). At the molecular level, survivin is expressed in the G_2/M phase of the cell cycle in a cycle-regulated

manner and physically associates with the mitotic spindle to counteract apoptosis (Li F *et al*, 1998). In this study, we sought to investigate the expression of survivin and its potential function in MM. We found that survivin expression is a common feature of MM and that targeting of survivin in a MM cell line is sufficient to cause apoptosis.

MATERIALS AND METHODS

Antibodies A new polyclonal antibody was generated in rabbits by immunization with full-length recombinant human survivin (M_r 16.5 kDa), and purified from serum by protein A-Sepharose (Pharmacia, Piscataway, NJ) chromatography. It was developed and used for these studies because it demonstrated a much higher affinity in immunohistochemistry and western blotting than the monoclonal antibody 8E2 used in previous studies (Lu *et al*, 1998). This rabbit polyclonal antibody reacts with all tumors previously shown to express survivin by 8E2 staining and identifies a 16.5 kDa survivin band in all cell lines examined by western blotting. Because it has not yet been characterized for intracellular staining of permeabilized cells by flow cytometry, 8E2 was used for those experiments. A control rabbit antibody was similarly prepared from nonimmune rabbit serum. Antibodies to MIB-1 (Ki-67) and bcl-2 were purchased from Zymed Laboratories (San Francisco, CA).

Cells and cell lines Normal human melanocytes were harvested from proliferating foreskin cultures maintained in the presence of phorbol ester, as described elsewhere (Bohm *et al*, 1995), and kindly provided by Dr Ruth Halaban (Yale University School of Medicine). The melanoma cell lines YUSIT-1, YUSAC-2, and YUGEN-8 were established from cases of human metastatic MM (Halaban *et al*, 1991, 1997) and maintained in Dulbecco's minimal Eagle's medium (Gibco BRL, Grand Island, NY) containing 5% fetal calf serum (Gemini Bio-Products, Calabasas, CA), 2 mM L-glutamine (BioWhittaker, Walkersville, MD), 100 U penicillin per ml (BioWhittaker), and 100 μg streptomycin per ml (BioWhittaker). The human metastatic melanoma line LOX (Fodstad *et al*, 1988) was kindly provided by Dr Michael Bromberg (Yale University School of Medicine) and maintained in Dulbecco's minimal Eagle's medium containing 20% fetal calf serum, glutamine, and antibiotics as above.

Immunohistochemistry Cases of MM with sufficient tissue for analysis were selected from 01/95 through 05/98 using the Yale Dermatopathology Laboratory database, and all selected cases were initially reviewed by a dermatopathologist (J.M.M.). Five micrometer tissue sections were cut and slides were baked overnight in a 60°C oven. Sections were deparaffinized in xylene for 5 h, washed twice in ethanol, and endogenous peroxidase activity was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Following washing in ethanol and then water, slides were placed in a 4-quart Wear-Ever pressure cooker (Mirro, Manitowoc, WI) containing 1.5 l of 9 mM sodium citrate, pH 6.0, that had been brought to a boil. The lid was secured, and heating was continued (for approximately 6 min) until the pressure valve released. Slides were gently cooled by filling the pressure cooker with tap water, and then washed three times with water and once with phosphate-buffered saline (PBS), pH 7.0. Staining was performed using either a Histostain-Plus kit (Zymed) with 3,3′-diaminobenzidine as the chromogen, or a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) with 3-amino-9-ethylcarbazole as the chromogen, according to the manufacturer's instructions. Slides were placed over moistened paper towels in a covered tray. For survivin staining, the primary antibody was applied at a concentration of 0.5 μg per ml in PBS, pH 7.0, containing 0.5% bovine serum albumin and 5% normal goat serum (Vector Laboratories), and incubated overnight at 4°C. For MIB-1 and bcl-2, the primary antibody was diluted with dilution buffer (Zymed) 1:1 and 1:2, respectively, and staining was carried out for 1 h at room temperature. At the end of the incubation, slides were washed two times in water, counterstained for 10 s with Harris hematoxylin (Sigma, St Louis, MO), and washed again in water, prior to coverslipping.

Apoptotic cells in tumors were identified in deparaffinized sections by *in situ* end-labeling with terminal deoxynucleotidyl transferase and peroxidase-conjugated anti-digoxigenin using the ApopTag kit (Intergen, Purchase, NY) according to the manufacturer's instructions. A 1:10 dilution of terminal deoxynucleotidyl transferase was found to be optimal for staining apoptotic cells in two cases of basal cell carcinoma, giving indices comparable with that reported by others (Mooney *et al*, 1995). Several high-power (×400) fields each containing approximately 1000 cells were examined. Because the percentage of apoptotic cells was very low (generally <1%), the apoptotic index was reported as the number of positive cells per field.

Western blotting Cells were pelleted, washed in PBS, suspended in ice-cold water (approximately 5×10^6 cells per ml) and placed on ice. Lysates were prepared by the addition of 2 × lysis buffer (final concentration of 2% sodium dodecyl sulfate, 50 mM Tris–HCl, and 10% glycerol). Tubes were inverted to mix, heated to 95°C for 3 min, and then stored at −20°C. Protein content was measured using a bicinchoninic acid reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. Prior to use, lysates were again heated to 95°C for 3 min and microfuged at room temperature for 10 min. Samples (50 μg) were electrophoresed at 150 V through a 4% stacking and 15% resolving acrylamide gel, and transferred on to Immobilon-P membrane (Millipore, Bedford, MA) at 60 V for 45 min. Blots were placed in PBS, pH 7.0, containing 5% nonfat milk and 0.1% Tween-20 (blocking solution), and incubated at 4°C overnight. After washing in PBS containing 0.1% Tween, blots were incubated with 1 μg survivin antibody per ml in blocking solution for 2 h at room temperature. Blots were washed in PBS/Tween, and reacted with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham Life Science, Arlington Heights, IL), diluted 1:5000 in PBS/Tween, for 30 min at room temperature. After washing in PBS/Tween, bands were visualized by enhanced chemiluminescence (Amersham) and autoradiography. To confirm equal protein loading per lane, membranes were subsequently reacted with a 1:5000 dilution of a mouse monoclonal antibody to β-actin (clone AC-74, Sigma).

Transient transfections and apoptotic index A green fluorescence protein (GFP) marker plasmid pEGFPc1 (Clontech Laboratories, Palo Alto, CA) was used to allow visualization and analysis of transfected cells. We have previously shown that forced expression in HeLa cells of the survivin anti-sense suppresses the endogenous survivin expression by western blotting (Ambrosini *et al*, 1998), and the dominant negative Cys85Ala (BIR mutation) survivin mutant results in complete loss of anti-apoptotic function (Li F *et al*, 1998). The cDNA encoding the Cys85Ala survivin mutant was excised from pcDNA3 with *Xba*I (blunted with Mung bean nuclease)/*Hin*dIII, and subcloned into the *Sma*I/*Hin*dIII sites of pEGFPc1, yielding pEGFPc1-surv.Cys85Ala. To generate the survivin anti-sense construct pEGFPc1-surv.AS, survivin cDNA was excised from pcDNA3 with *Eco*RV/*Pst*I and subcloned in the reverse orientation into the *Sma*I/*Pst*I sites of PEGFPc1. In each case, the cDNA was placed downstream of GFP and preservation of reading frame was confirmed by DNA sequencing. All plasmids were purified by ion-exchange chromatography (Qiagen, Valencia, CA). YUSAC-2 or LOX cells were plated into 12-well plates (2×10^5 cells per well) 18 h prior to transfection. Transfection of empty GFP vector or each construct was performed over a 6 h incubation period using a mixture of 1 μg plasmid DNA and 5 μl lipofectin (Life Technologies, Gaithersburg, MD) in 400 μl OptiMEM serum-free medium (Life Technologies) per well according to the manufacturer's instructions. Forty-eight hours after transfection, cells were washed with PBS, and fixed at room temperature with 4% paraformaldehyde containing 0.25% Triton X-100 for permeabilization. After 10 min, cells were washed with PBS and cell nuclei were stained using a mounting solution containing 6.5 μg 4,6-diamidino-2-phenylindole per ml (Sigma), 16% polyvinyl alcohol (Air Products and Chemicals, Allentown, PA), and 40% glycerol. Cells were viewed using a Zeiss fluorescent microscope, and transfected (green) cells were scored as apoptotic if nuclear condensation, fragmentation, or dissolution was observed. The apoptotic index was calculated as the ratio of transfected cells with apoptotic nuclei to the total number of transfected cells in each group.

Flow cytometry YUSAC-2 cells were grown to subconfluence in six-well plates, and transfection with either pEGFPc1 or pEGFPc1-surv.AS was performed over a 7 h incubation period using a mixture of 1.6 μg plasmid DNA and 5 μl lipofectamine (Life Technologies) in 1 ml OptiMEM per well. For the detection of endogenous survivin in GFP-transfected cultures, both nonadherent and adherent cells were recovered after 48 h, washed in PBS, pH 7.0, and then fixed in PBS containing 4% paraformaldehyde for 30 min at room temperature. After washing twice with PBS, cells were suspended at a concentration of 20×10^6 per ml in wash buffer consisting of PBS with 1% bovine serum albumin (Sigma), 0.1% saponin (Sigma), and 0.1% sodium azide (Fisher Scientific, Pittsburgh, PA). Normal goat serum was added at a final concentration of 10% to block nonspecific binding. After 15 min at room temperature, cells were diluted 1:1 with survivin antibody 8E2 in wash buffer to a final concentration of 15 μg per ml. Staining was carried out at room temperature for 1 h, with occasional agitations as cells settled. After washing twice in wash buffer, cells were reacted with a 1/100 dilution of phycoerythrin-conjugated $F(ab')_2$ fragment goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) in wash buffer for 30 min at room

temperature. After washing twice in wash buffers, cells were diluted in PBS containing 1% bovine serum albumin and analyzed using a FACSort (Becton Dickinson, San Jose, CA) and Cell Quest software (Becton Dickinson). Analysis was restricted to green GFP-expressing cells.

For DNA staining of GFP-transfected cultures, both nonadherent and adherent cells were recovered 48 h after transfection, washed in PBS, pH 7.0, and then fixed in cold 70% ethanol for 30 min on ice. After washing in PBS, cells were suspended at a concentration of 10×10^6 per ml in PBS containing 25 μg propidium iodide per ml (Sigma), 0.05% Triton X-100, and 100 μg RNase A per ml (Boehringer Mannheim, Indianapolis, IN). Staining was carried out for 45 min at room temperature, and cells were then diluted in PBS containing 1% bovine serum albumin and immediately analyzed by flow cytometry as above.

RESULTS

Expression of cell death and proliferation markers in MM These studies were carried out in 30 cases of MM, and are summarized in **Table I**. Of 15 cases of metastatic MM, all demonstrated cytoplasmic staining of tumor cells with the anti-survivin antibody (**Fig 1*A***). By contrast, no reactivity was seen with the antibody prepared from preimmune serum (**Fig 1*B***). Diffuse smooth cytoplasmic staining for survivin was easily distinguished from endogenous melanin, which displayed a distinct granular appearance (data not shown). In addition, four pigmented melanomas were stained using 3-amino-9-ethylcarbazole as the chromogen to rule out a potential role of endogenous melanin in the observed reactivity. In these cases, there was strong staining with the survivin antibody (**Fig 1*C***) and no staining with the control preimmune antibody (data not shown). Mitotic figures within the tumors demonstrated enhanced staining with the antibody to survivin (**Fig 1*D***), consistent with the association of survivin with microtubules and peak expression in G_2/M (Li F *et al*, 1998). Occasional cells also exhibited nuclear staining (**Fig 1*D***), most likely representing cells preparing to divide. Of 15 cases of invasive MM, 13 were positive for survivin expression (**Fig 1*E***). In most lesions, positive staining was also observed in the epidermal component, representing MM-*in-situ* (**Fig 1*F***). Survivin was not detected in two MM cases (7% of total). Survivin was not detected in normal epidermal melanocytes adjacent to MM lesions or in normal skin (data not shown), but was seen in all cases (11 of 11) of melanocytic nevi examined (**Fig 1*G***). Bcl-2 expression was detected in 26 of 30 (87%) MM lesions, but generally at reduced intensity compared with that of adjacent reactive lymphocytes (**Fig 1*H***). Four MM lesions were negative for bcl-2 expression (**Table I**). The mitotic index was highly variable among these tumors, consistently greater in metastatic than invasive MM (**Table I**). Rare apoptotic nuclei could be clearly identified (**Fig 1*I***). No staining was observed in the absence of terminal deoxynucleotidyl transferase (data not shown). The apoptotic index among these tumors varied within an order of magnitude, but did not appear to correlate directly with the expression of survivin or bcl-2 (**Table I**).

Survivin expression in melanoma cell lines By immunoblotting, the antibody to survivin recognized a ≈ 16.5 kDa survivin band in protein-normalized lysates of four cell lines derived from human metastatic MM (**Fig 2**). No specific bands were seen in blots

Table I. Melanomas examined in this study

Tumor characteristics							
No./age/sex	Type[a]	Site	Depth[b]	Survivin	bcl-2[c]	Mitotic index[d]	Apoptotic index[e]
1/52/M	Metastatic MM			+	+	3.2	2
2/61/M	Metastatic MM			+	–	1.9	9
3/51/M	Metastatic MM			+	+	6.2	1
4/61/F	Metastatic MM			+	+	22.9	1
5/63/M	Metastatic MM			+	++	9.2	ND[f]
6/61/F	Metastatic MM			+	+	38.2	4
7/55/M	Metastatic MM			+	+	27.3	2
8/45/M	Metastatic MM			+	+	14.8	1
9/59/M	Metastatic MM			+	+	22.7	5
10/75/F	Metastatic MM			+	++	12.0	1
11/81/M	Metastatic MM			+	++	19.4	1
12/82/M	Metastatic MM			+	++	17.1	2
13/41/M	Metastatic MM			+	–	33.4	4
14/34/M	Metastatic MM			+	–	24.1	6
15/63/M	Metastatic MM			+	++	12.2	2
16/49/F	ss-MM	Back	0.45	+	+	2.4	2
17/72/F	ss-MM	Upper arm	0.63	+	++	2.8	2
18/35/M	ss-MM	Back	0.65	–	+	7.8	3
19/57/M	ss-MM	Chest	0.80	+	–	5.8	2
20/26/F	ss-MM	Scalp	0.85	–	++	2.5	1
21/56/M	ss-MM	Back	1.00	+	+	6.6	2
22/78/M	ss-MM	Chest	1.10	+	+	1.9	2
23/32/F	ss-MM	Thigh	1.60	+	++	<0.3	2
24/82/M	ss-MM	Back	1.60	+	+	8.4	1
25/50/M	n-MM	Shoulder	1.65	+	+	13.0	1
26/42/M	ss-MM	Shoulder	1.90	+	+	1.8	1
27/68/F	ss-MM	Shoulder	2.64	+	+	11.5	2
28/72/M	n-MM	Back	3.00	+	++	12.0	5
29/77/M	ss-MM	Upper arm	4.00	+	++	16.6	2
30/78/F	n-MM	Forearm	4.50	+	+	2.4	3

[a]Tumor types include metastatic MM, superficial spreading MM (ss-MM), and nodular MM (n-MM).
[b]Breslow depth in mm.
[c]bcl-2 expression graded as negative (–), positive (+), or strongly positive comparable with that of infiltrating lymphocytes (++).
[d]Mitotic index reflects the average percentage of MIB-1 positive cells in two representative high-power (×400) fields.
[e]Apoptotic index refers to number of apoptotic cells labeled for DNA fragmentation per high-power (×400) field.
[f]Not determined.

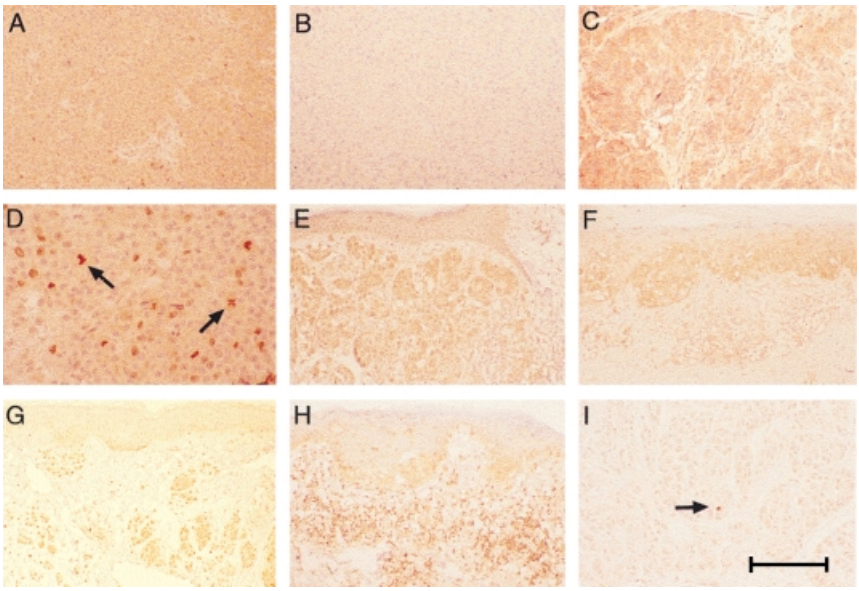

Figure 1. Immunohistochemistry for survivin, bcl-2, and apoptosis. (*A*) Metastatic MM stained with survivin antibody. (*B*) Metastatic MM stained with control preimmune antibody. (*C*) Metastatic MM stained with survivin antibody. (*D*) Metastatic MM stained with survivin antibody, demonstrating enhanced staining of mitotic figures (*arrows*). (*E*) Invasive MM stained with survivin antibody. (*F*) MM *in-situ* stained with survivin antibody. (*G*) Melanocytic nevus stained with survivin antibody. (*H*) Invasive MM stained with bcl-2 antibody, demonstrating staining of tumor but with less intensity than underlying reactive lymphocytes. (*I*) Isolated apoptotic cell positive for DNA fragmentation in invasive MM (*arrow*). The chromophore was 3,3′-diaminobenzidine in parts (*A*), (*B*), (*D–I*) and 3-amino-9-ethylcarbazole in part (*C*). *Scale bar:* (*A–C, E–H*) 200 μm; (*D, I*) 100 μm.

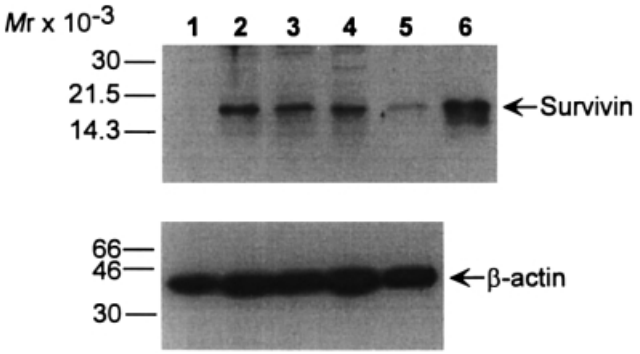

Figure 2. Expression of survivin in human melanoma cell lines. Protein-normalized lysates (50 μg) from normal melanocytes (*lane 1*), YUSAC-2 (*lane 2*), YUSIT-1 (*lane 3*), LOX (*lane 4*), and YUGEN-8 (*lane 5*) were electrophoresed, blotted and reacted with antibodies to survivin (≈16.5 kDa, *top panel*) or β-actin (≈ 42 kDa, *bottom panel*). *Lane 6* contained 50 ng recombinant survivin (16.5 kDa).

stained only with preimmune serum antibody (data not shown). Levels of survivin protein were highest in the YUSAC-2, YUSIT-1, and LOX cell lines, whereas only faint expression was seen in YUGEN-8 (**Fig 2**). Survivin expression was not detected in normal melanocytes (**Fig 2**). Staining for β-actin confirmed equal protein loading per lane (**Fig 2**).

Targeting endogenous survivin in melanoma cells Survivin-expressing YUSAC-2 cells transfected with GFP constructs exhibited bright green fluorescence (**Fig 3**, *middle panel*). The nuclei of these cells were visualized by 4,6-diamidino-2-phenylindole staining, and scored as either normal or apoptotic based on nuclear morphology. Under these experimental conditions, forced expression of anti-sense or of a dominant negative Cys84Ala BIR mutant induced morphologic changes characteristic of apoptosis in GFP-expressing cells (**Fig 3**, *bottom panel*). As shown in **Fig 4**, this resulted in a 3-fold increase in the apoptotic index of cells transfected with either survivin anti-sense or the BIR mutant compared with control cells transfected with empty GFP vector. Similar results were obtained in LOX cells (**Fig 4**). By flow cytometry, there was decreased endogenous survivin expression in YUSAC-2 cells transfected with survivin anti-sense compared with empty GFP vector (**Fig 5*A***). In addition, cells transfected with survivin anti-sense demonstrated a 10-fold increase in the sub-G_1 fraction, corresponding to apoptotic cells (**Fig 5*B***).

DISCUSSION

This study has shown that survivin, a newly identified inhibitor of apoptosis, is broadly expressed in human MM. Second, direct targeting of survivin in MM cell lines was sufficient to trigger spontaneous apoptosis in the absence of other genotoxic or chemotherapeutic agents. These observations are particularly relevant because resistance to apoptosis is a common feature of MM and is perceived as a critical obstacle to achieving long-lasting therapeutic remissions.

Using a novel high-affinity antibody, survivin expression was detected in 93% of the tumors and in all of the melanoma cell lines examined. All of the metastatic lesions were positive and the two cases of MM that were negative for survivin were both minimally invasive lesions, consistent with previous studies (Ambrosini *et al*, 1997; Adida *et al*, 1998; Kawasaki *et al*, 1998) in which survivin expression correlated with tumor aggressiveness. In earlier series of colorectal (Kawasaki *et al*, 1998) and gastric (Lu *et al*, 1998) cancer, survivin was found in various fractions of malignant cells whereas it was homogeneously expressed in 100% of MM cells, perhaps a reflection of the higher affinity antibody used in these studies. In addition, survivin was expressed in the MM-*in-situ* component of many lesions. In contrast to bcl-2, survivin was not detected in normal skin. Although survivin was not expressed in epidermal melanocytes or in normal cultured melanocytes, it was consistently expressed in melanocytic nevi that are often precursors to MM (Crucioli and Stilwell, 1982; Clark, 1991). We have recently shown that survivin is broadly expressed in nonmelanoma skin cancer and in premalignant keratinocytic lesions of Bowen's disease and actinic keratosis (Grossman *et al*, 1999). Taken together, these studies suggest that survivin expression may be an important early step in the development of most malignant skin tumors.

Survivin expression in nevi may contribute to the increased resistance to apoptosis seen in nevus cells compared with normal melanocytes (Alanko *et al*, 1999), and allow for their slow continual proliferation and persistence in the skin over many decades. In MM, tissue invasion and ultimately metastasis depends on a predominance of cell growth over cell death. The low levels of apoptosis in MM lesions shown here and by others (Mooney *et al*, 1995; Stauton and Gaffney, 1995) likely involves altered expression or dysfunction of proteins that regulate both the cell cycle and the apoptotic program. Survivin may be a prime candidate for mediating both functions in MM. Its maximal expression in G_2/Mand localization to the mitotic spindle (Li F *et al*, 1998) suggest that it may be directly involved in promoting escape from cell cycle control in malignant cells. Although we found bcl-2 to be widely expressed in our panel of MM, the level of expression was

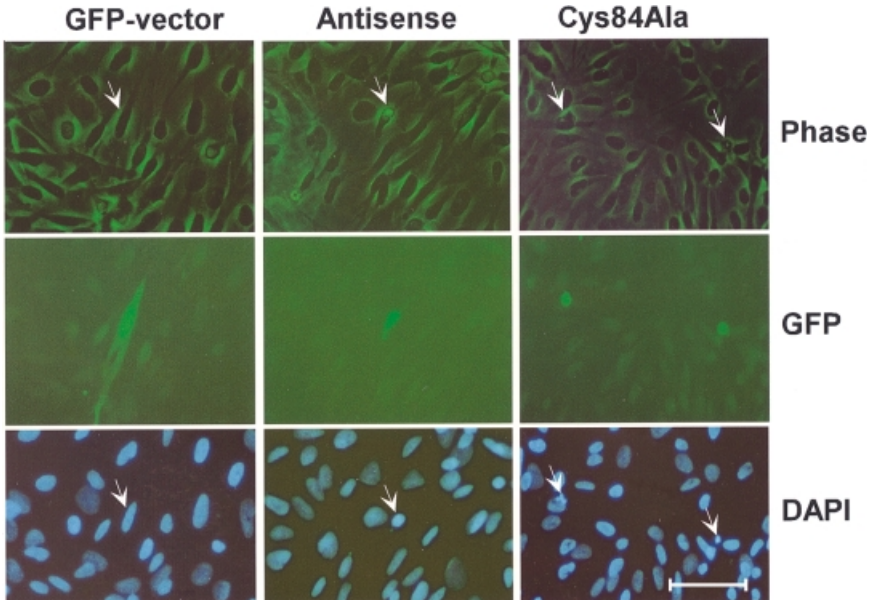

Figure 3. Analysis of transfected melanoma cells for nuclear morphology. YUSAC-2 cells were transfected with empty GFP vector, or GFP fusion products containing survivin anti-sense or the Cys84Ala BIR mutant as indicated. The cell monolayer is shown by phase-contrast microscopy (*top panel*). Transfected cells are green under fluorescent microscopy (*middle panel*) and identified by *arrows* in the other panels. Normal and apoptotic nuclear morphology is revealed by 4,6-diamidino-2-phenylindole staining (*bottom panel*). *Scale bar:* 35 μm.

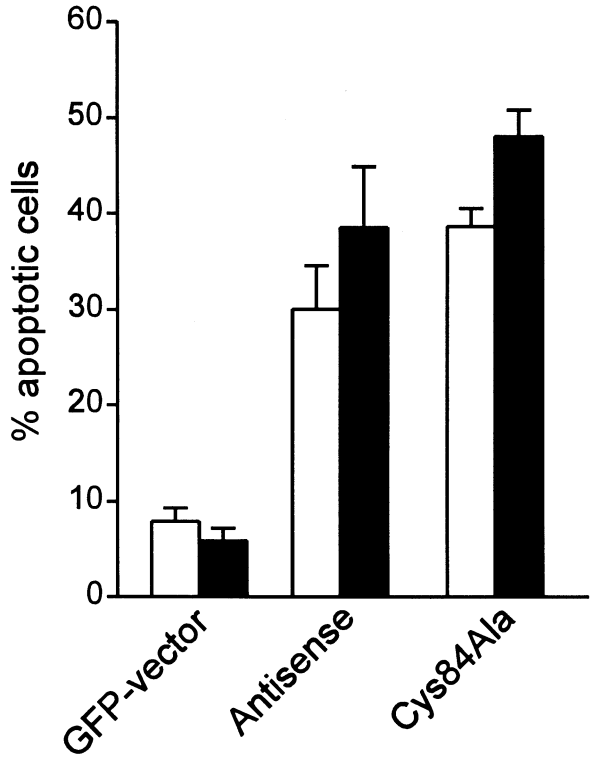

Figure 4. Effect of survivin targeting on apoptotic index. YUSAC-2 (*open bars*) and LOX (*filled bars*) were transfected with empty GFP vector, or GFP fusion products containing survivin anti-sense or the Cys84Ala BIR mutant as indicated, and nuclear morphology of green fluorescence cells was scored as normal or apoptotic at 48 h. The apoptotic index was calculated as the ratio of apoptotic to normal nuclei based on counting approximately 100 GFP-transfected cells. Error bars indicate SD of three separate experiments.

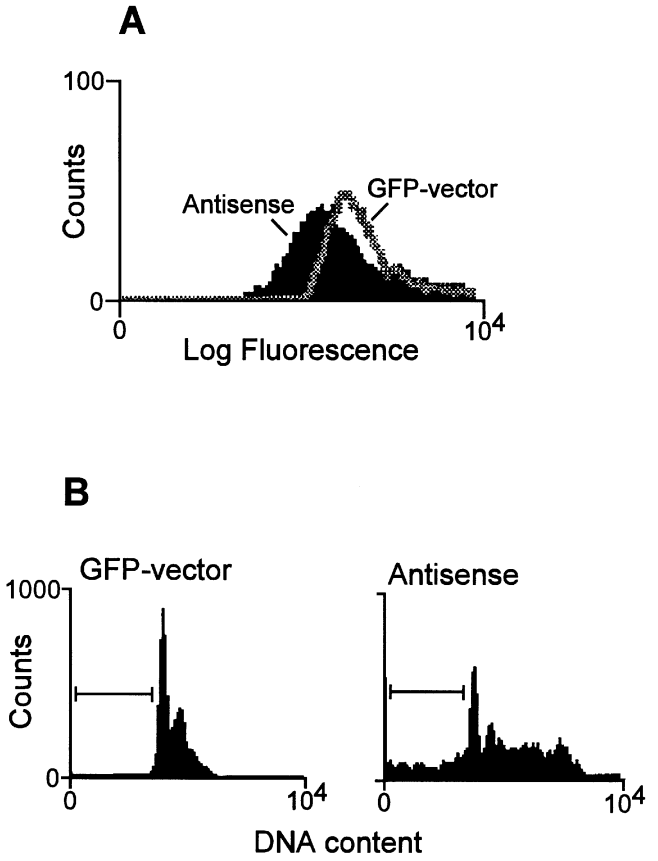

Figure 5. Effect of survivin targeting by anti-sense on endogenous expression and DNA content. Transfected YUSAC-2 cells were harvested at 48 h, fixed, and analyzed using two-color flow cytometry. (*A*) Survivin staining of GFP-expressing cells transfected with empty GFP vector (*open histogram*) or survivin anti-sense (*shaded histogram*). (*B*) Propidium iodide staining of GFP-expressing cells transfected with empty GFP vector or survivin anti-sense as indicated. The marker denotes the sub-G_1 fraction, corresponding to apoptotic cells.

reduced compared with that of normal cells, as reported by others (Cerroni *et al*, 1995; Collins and White, 1995; Selzer *et al*, 1998). Moreover, the lack of correlation between bcl-2 expression and prognosis in MM (Mooy *et al*, 1995) further suggests that bcl-2 may not be the only regulator of apoptosis resistance in MM. On the other hand, the simultaneous co-expression of survivin and bcl-2 in MM may provide two independent and non-overlapping anti-apoptotic mechanisms that together may reduce the efficacy of combination chemotherapy *in vivo*. Whereas bcl-2 counteracts the upstream initiation of apoptosis by preserving mitochondrial

integrity and blocking cytochrome *c* release (Adams and Cory, 1995), survivin and other IAP proteins interact with caspase-3 and -7 and thus inhibit the downstream effectors of the caspase cascade (LaCasse *et al*, 1998; Tamm *et al*, 1998; Deveraux and Reed, 1999).

Targeting survivin in YUSAC-2 or LOX MM cells either by anti-sense or a dominant negative survivin mutant was associated with a striking increase in the level of spontaneous apoptosis. In previous studies with HeLa cells, we have shown that forced expression of survivin anti-sense decreased proliferation (Ambrosini *et al*, 1998) and transfection of the Cys85Ala BIR mutant effectively displaced endogenous survivin from mitotic microtubules and increased caspase-3 activity (Li F *et al*, 1998). Here we show by flow cytometry that anti-sense targeting in YUSAC-2 cells suppresses the level of endogenous survivin and is associated with an increase in the sub-G_1 fraction representing apoptotic cells. This observation that targeting a single gene is sufficient to cause apoptosis in the absence of additional genotoxic or chemotherapeutic agents emphasizes the dominant part that survivin must play in maintaining the apoptosis-resistant state in these cells. In this context, perturbation of survivin expression and function in MM may alter the balance between pro-apoptotic and anti-apoptotic factors and facilitate elimination of targeted cells by apoptosis. Consistent with this exquisite vulnerability of melanoma cells to this type of genetic manipulation, anti-sense-based downregulation of bcl-2 in MM increased sensitivity to chemotherapy in immunodeficient mice (Jansen *et al*, 1998).

In conclusion, these studies support a role of apoptosis inhibition by survivin in the establishment and progression of MM. They further suggest potential therapeutic applications aimed at disrupting survivin expression and function in MM.

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