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Coexistence of High Levels of Apoptotic Signaling and Inhibitor of Apoptosis Proteins in Human Tumor Cells: Implication for Cancer Specific Therapy[1]

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ABSTRACT

It is well known that dysfunction of the apoptotic pathway confers apoptosis resistance and results in a low sensitivity of human cancer cells to therapeutic agents. A novel strategy to overcome the resistance is to target the apoptotic pathway directly. To identify molecular targets in the apoptotic pathway that are differentially regulated in cancer and normal cells, we have examined the levels of apoptotic effectors and inhibitors in human tumor and normal cell lines as well as in cancer and normal tissues. These include three pancreatic cancer lines (BXPC-3, MIA PaCa-2, and Panc-1), four breast cancer cell lines (MDA-MB-231, MDA-MB-435, MDA-MB-361, and MCF-7), and colon carcinoma line (SW620). Additionally, breast carcinoma tissue specimens were examined. Compared with normal human fibroblast and mammary epithelial cell lines, we detected high basal levels of caspase-3 and caspase-8 activities and active caspase-3 fragments in the tumor cell lines and cancer tissues in the absence of apoptotic stimuli. Furthermore, the tumor cells expressed high levels of survivin and XIAP, two members of the inhibitor of apoptosis (IAP) protein family. When the activity of these IAPs was blocked by expression of dominant-negative mutant survivin (survivinT34A) and XIAP-associated factor 1, respectively, apoptosis was induced in tumor but not normal cell lines. Moreover, down-regulation of both survivin and XIAP significantly enhanced tumor-cell apoptosis as compared with inhibition of either survivin or XIAP alone. These results suggest that up-regulated IAP expression counteracts the high basal caspase-3 activity observed in these tumor cells and that apoptosis in tumor cells but not normal cells can be induced by blocking IAP activity. Therefore, IAPs are important molecular targets for the development of cancer-specific therapeutic approaches.

INTRODUCTION

Impairment of the apoptotic signaling pathway plays an important role in the initiation and progression of human cancers (1–4). Recent studies have shown that apoptotic cell death is also critical for the response of many cancer therapeutic agents (5–8). To develop innovative strategies for cancer therapy, it is necessary to identify molecular targets in the apoptotic pathway that are differentially regulated in normal and cancer cells. Many cellular factors involved in apoptosis have been identified and their roles in the apoptotic pathway are elucidated. Apoptosis is initiated through an extrinsic pathway by the interaction of cell death receptors with their ligands or an intrinsic pathway triggered by leakage of cytochrome *c* from mitochondria. These result in activation of a cascade of caspase proteolysis reaction (2, 9). Caspases can be divided into two groups based on the length of their prodomain and substrate specificity. The initiator caspase group includes caspase-2, caspase-8, caspase-9, and caspase-10, having long NH_2-terminal prodomains, which interact with adapter molecules to form a death-inducing signaling complex. Downstream caspases such as caspase-3, caspase-6, and caspase-7 are executioner caspases that remain dormant until the initiator caspases activate them by proteolysis (9). The activated executioner caspases cleave a number of structural and regulatory proteins, leading to apoptotic cell death (10, 11).

Recently, the IAP[4] family, including XIAP, c-IAP1, c-IAP2, ML-IAP, NAIP, survivin, and Apollon, has been characterized (2, 12–14). These proteins contain a novel ~80 amino acid motif that is defined as the BIR (12). XIAP is the most potent caspase inhibitor in the IAP family. It has been shown that XIAP prevents the proteolytic processing of procaspase-3, procaspase-6, and procaspase-7 by binding and blocking the activity of caspase-9 through its BIR 3 domain. XIAP additionally inhibits the activity of active caspase-3 through a short linker region that precedes BIR 2 (15, 16). Another IAP protein, survivin, counteracts a broad range of apoptosis stimuli such as Fas stimulation, overexpression of Bax and caspases, and chemotherapy drugs (17). At present, it is still controversial whether survivin directly binds and inhibits caspase-3. A comparison of the X-ray crystallographic structures of survivin with that of the XIAP:caspase-3 complex indicates lack of a structural basis for survivin-caspase-3 interaction (18). XIAP is detected at a low level in normal adult tissues. Survivin is expressed during fetal development but not in most adult tissues (19).

Increasing evidence demonstrated that IAPs, especially XIAP and survivin, are up-regulated in many human tumor types (4, 13, 20, 21). A recent study showed up-regulation of survivin in 77% of pancreatic duct cell adenocarcinoma, and survivin was expressed in early stages of neoplastic transition in pancreatic cancer cells. On the other hand, survivin was not detected in normal pancreatic tissues (4). Approximately 71% of breast cancers were positive for survivin expression while surrounding normal tissues are negative. An increase in the level of survivin contributed to a higher apoptotic threshold and survival of the breast cancer cells (21). Survivin was also detected in 64% of human colorectal cancers. Five-year survival rate in the stage II patients with positive survivin were significantly lower than that of the survivin-negative patients (20). Furthermore, a high level of XIAP was also detected in human tumors and has been shown to confer the resistance to chemotherapy drugs (22–24).

A logical strategy to overcome resistance to apoptosis is to directly target the apoptotic pathway. Gene therapy vectors have been used to deliver apoptosis inducer genes such as Fas ligand, Bax, and caspases; induction of apoptosis was observed in various human tumor cell lines after transduction of these genes (25–28). Another promising approach is to decrease the apoptotic threshold in tumor cells by counteracting IAP function. A XAF1, which is present in many normal tissues but is low or absent in tumors, abolishes the function of XIAP through direct interaction of the proteins (29, 30). In addition, expression of antisense or mutant survivin genes inhibited tumor prolifera-

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[4] The abbreviations used are: IAP, inhibitor of apoptosis; BIR, baculoviral inhibitor of apoptosis repeat; FBS, fetal bovine serum; RFU, relative fluorescence unit; GFP, green fluorescence protein; XAF1, XIAP-associated factor 1; ATCC, American Type Culture Collection; MOI, multiplicity of infection, pfu, plaque-forming unit, PARP, poly(ADP-ribose) polymerase.

tion and induced apoptosis *in vitro*, as well as in animal tumor models (31). One of the mutants contains an amino acid substitution from threonine (T) to alanine (A) at 34 position (survivinT34A). Expression of this construct blocked phosphorylation of survivin on Thr^{34}, resulting in dissociation of survivin-caspase-9 complex and induction of caspase-9-dependent apoptosis in tumor cells (32). Interestingly, expression of survivin T34A selectively induced apoptotic cell death in tumor cells but not in normal cells (31).

Although induction of apoptotic cell death by expression of either XAF1 or dominant-negative survivin has been demonstrated in human tumor cells, mechanisms for selective induction in tumor cells are largely unknown. In this study, we compared the levels and activities of apoptotic effectors and inhibitors in human cancer cell lines and tissues with normal cell lines and tissues. We demonstrated that human tumor cell lines have constitutively activated caspase activities in the absence of apoptotic stimuli and yet are not undergoing apoptosis. These cell lines also have high levels of survivin and XIAP. Therefore, we hypothesized that inhibition of IAPs could induce apoptotic cell death selectively in tumor cells that have an activated apoptotic signaling. Results of our study additionally demonstrated that expression of IAP-counteracting proteins such as survivinT34A and XAF1 could indeed lead to specific apoptosis in the tumor cell lines.

MATERIALS AND METHODS

Human Tumor and Normal Cell Lines

Pancreatic cancer cell lines BXPC-3, MIA PaCa-2, and Panc-1, colon cancer cell line SW620, and normal immortalized human mammary epithelial cell line MCF-10A were obtained from the ATCC (Manassas, VA). Breast cancer cell lines MDA-MB-231, MDA-MB-361, and MCF-7 were from ATCC, and MDA-MB-435 was kindly provided by Dr. Zhen Fan (M. D. Anderson Cancer Center, Houston, TX). Primary normal human dermal fibroblast cell line HDF was purchased from Emory University Skin Disease Center (Atlanta, GA). BXPC-3 and SW620 cell lines were cultured in RPMI medium, and MIA PaCa-2 cells were cultured in DMEM (Mediatech, Herndon, VA). Human breast cancer cell lines were maintained in DMEM:Ham's F-12 medium (50:50; Mediatech). All above media were supplemented with 10% FBS (Hyclone, Logan, UT), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Mediatech). HDF was maintained in DMEM with 20% FBS. MCF-10A cells were cultured in DMEM:Ham's F-12 medium supplemented with 20 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, 2 mM L-glutamine, and 5% FBS.

Human Normal and Breast Cancer Tissues

Frozen human breast cancer and normal tissues were obtained from the Emory Healthcare Tissue Procurement Facility according to an approved Institutional Review Board protocol. The identification number and pathological diagnosis of each sample were provided by the Tissue Procurement Facility. Paired breast cancer and normal tissues were obtained from the patients during operation to resect the breast cancer. The normal tissues were selected from the normal breast areas by the pathologists at Emory University. Some of the breast cancer tissue samples were obtained from the breast cancer patients during surgical resection of the cancer without obtaining paired normal samples. Normal breast tissues from normal subjects undergoing breast reduction surgery were also collected for this study.

Detection of Caspase Activity in Human Tumor and Normal Cell Lines or in Breast Cancer and Normal Tissues

Caspase-3, caspase-8, and caspase-9 activities were examined either in cell lysates or in viable cells by fluorometric assays.

Determination of Caspase Activity in Solution Using Cell Lysates. Human tumor and normal cell lines were cultured in specific media as described above. To examine the basal caspase-3, caspase-8, and caspase-9 activities, the cell pellets from each cell line at 80% confluence were collected and lysed with the lysis buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $NaH_2PO4/NaHPO_4$ (pH 7.5), 130 mM NaCl, 1% Triton X-100, and 10 mM $Na_2P_2O_7$ 10 H_2O. The tissue lysates were prepared from frozen normal and cancer tissues by homogenizing in the lysis buffer. Caspase activity in the cell lysates was examined using substrates specific for caspase-3-like (Ac-DEVD-AFC), which detects activities of caspase-3, caspase-7, and caspase-10, caspase-8 (Ac-LETD-AFC), or caspase-9 activity (Ac-LEHD-AFC) according to a standard protocol (Bio-Rad Laboratories, Hercules, CA). The results were measured by a fluorescence microplate reader (Bioteck FL600 Fluorometer, Winooski, VT). RFUs were standardized to RFU/μg or RFU/10 μg protein. For each experiment, control groups with specific caspase inhibitors, including caspase-3 inhibitor (Z-DEVD-CHO; BD PharMingen, San Diego, CA), caspase-8 inhibitor (Z-LETD-CHO; BD PharMingen), and caspase-9 inhibitor (Z-LEHD-CHO; Alexis Biochemicals, San Diego, CA), were done to ensure specificity of the assay.

Examination of Intracellular Caspase Activity in Viable Human Tumor Cell Lines. Intracellular caspase-3 activity in viable tumor or normal cell lines were examined by a Caspase-3 Intracellular Activity Kit II using PhiPhiLux-G_2D_2 as a substrate (OncoImmunin, Inc., Gaithersburg, MD). Tumor cell lines were cultured on chamber slides to 80% confluence and then reacted with the caspase-3-activity detection kit for 60 min according to the methods provided by the manufacture. After washing with PBS and counterstaining with 10 μg/ml Hoechst 33342 (Molecular Probes, Inc., Eugen, OR), the slides were observed under an inverted fluorescence microscope (Nikon Eclipse E800; Nikon Instrument, Inc., Melville, NY). The fluorescence intensity in the cells represents the level of active caspase-3. Fluorescence images were taken using Optronics Magnafire digital imaging system (Meyer Instrument, Houston, TX).

Construction of Plasmids for Expression of Caspase Genes and Examination of the Effect of Expression of Caspase Genes on Human Tumor and Normal Cell Lines

The pcDNA3 plasmids containing a procaspase-3 gene or an autocatalytic Rev-caspase-3 gene, which was engineered by switching the position of the small subunit to the front of the large subunit, were kindly provided by Dr. Emad S. Alnemri at Thomas Jefferson University in Philadelphia, Pennsylvania (33). Procaspase-9 gene was obtained from Dr. Xiaodong Wang at University of Texas Southwestern Medical Center (Dallas, TX). Procaspase-8 gene was obtained from Dr. Xiaojing Li at Emory University (Atlanta, GA). The procaspase-3, procaspase-8, procaspase-9, and Rev-caspase-3 genes were cloned into an Adtrack-CMV vector, which also expressed a GFP marker gene (Ref. 34; provided by Dr. Bert Vogelstein at Johns Hopkins University, Baltimore, MD). The positive clones were selected and amplified for transfection study. To determine the effect of expression of caspase genes on tumor and normal cell lines, the plasmids were transfected into cultured tumor or normal cell lines in 24-well tissue culture plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Forty-eight h after transfection, the cells were stained with Hoechst 33342 and examined under an inverted fluorescence microscope. The percentage of apoptotic cells within transfected cell populations was determined by counting the number of GFP-positive cells with apoptotic nuclear morphology within all GFP-positive cells.

Adenoviral Vector Production

AdXAF1 vector was provided by Dr. Robert Korneluk (Solange Gauthier-Karsh Molecular Genetics Laboratory, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada). AdsurvivinT34A vector was produced in our laboratory using the AdEasy system, obtained from Dr. Bert Vogelstein at Johns Hopkins University, according to a standard protocol (34). SurvivinT34A gene was generated by PCR-directed mutagenesis from pcDNA3-survivin plasmid containing a wild-type survivin gene (Dr. Dario Altieri, Yale University, New Heaven, CT; Ref. 32). The survivin T34A gene was cloned into the Adtrack-CMV shuttle plasmid and then transferred to AdEasy 1 plasmid through homogeneous recombination as described previously (34). Adenoviral vector expressing the survivinT34A gene (AdsurvivinT34A) was produced by transfecting the AdEasy plasmid containing the survivinT34A gene into a human embryonic kidney cell line 293 (ATCC). The viral vectors were additionally amplified in 293 cell line, and high titer viral vectors were purified by centrifugation and CsCI banding.

Examination of Effects of Expression of SurvivinT34A and XAF1 Genes on Human Tumor and Normal Cell Lines

To determine the percentage of apoptotic cell death induced by expression of survivinT34A and/or XAF1 genes, 3×10^3/well of human tumor or normal cells were plated in 96-well tissue culture plates. The cells were transduced with AdsurvivinT34A or AdXAF1 vector at a MOI of 100 pfu. Some groups received both AdsurvivinT34A and AdXAF1 vectors at MOI of 100 pfu. Seven days after the viral vector transduction, the percentage of viable cells was determined by staining cells with crystal violet followed by extraction of the dye for measuring absorbance at 590 nm with a microplate reader (μQuart; Biotek Instrument, Inc.). The percentages of viable cells in experimental groups were calculated using the absorbance value from the AdGFP control group as 100%.

Western Blot Analysis of Protein Levels

Human tumor and normal cells were lysed in the lysis buffer containing 50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 10 mM $Na_2P_2O_7$ 10 H_2O, 50 mM NaF, 1 mM $NaVO_4$, 1% Triton X-100, and protease inhibitor mixture tablets (Complete mini; Roche Molecular Biochemical, Indianapolis, IN). Tissue lysates of frozen normal and breast cancer tissues were obtained by adding the lysis buffer to the tissue powder produced through hammering liquid nitrogen treated tissues slices. Protein concentrations of the resulting lysates were determined using Bio-Rad protein assay kit. Fifty μg of protein were resolved on 12–15% polyacrylamide SDS gels and then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories). The membranes were blocked with 5% of nonfat milk in Tris-buffered saline for 1 h and then incubated from 1 h to overnight with primary antibodies, including goat antihuman survivin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anticleaved caspase-3 fragments (Cell Signaling Technology, Inc., Beverly, MA), rabbit anti-XIAP or XAF (Dr. Robert Korneluk), and mouse monoclonal antibodies to human PARP (BD PharMingen), β-tubulin, or β-actin (Sigma Chemical Co., St. Louis, MO). After washing the membranes with Tris-buffered saline three times, the membranes were incubated with horseradish peroxidase conjugated with antigoat, rabbit, or mouse secondary antibody for 1 h. The levels of specific proteins in each lysate were detected by enhanced chemiluminescence using ECL plus (Amersham International, Buckingham, United Kingdom) followed by autoradiography.

RESULTS

Many Tumor Cell Lines Had High Basal Levels of Caspase Activity but Were Still Able to Survive. At first, we examined the basal level activity of caspase-3, caspase-8, and caspase-9 in cell lysates of different tumor cell lines, as well as in normal cell lines using substrates specific for each caspase. Our results demonstrated that a considerable basal level of caspase activity could be detected in the tumor cell lines. Using caspase activity of normal primary human fibroblast (HDF) and normal mammary epithelial cell line (MCF-10A) as references, we found that most human tumor cell lines examined showed a higher basal level of caspase-3 activity relative to normal cell lines (Table 1). Up-regulation of caspase-8 activity was also detected in five of seven tumor cell lines. Colon cancer SW620 and pancreatic cancer MIA PaCa-2 cell lines showed very high levels of both caspase-8 and caspase-3 activities. On the other hand, the basal level of caspase-9 activity was only slightly higher in some tumor cell lines than that in the normal cell lines. To determine whether our observation *in vitro* reflected the situation in living cells, we examined intracellular caspase activity in viable cells using an Intracellular Caspase-3 Activity Kit II. In combination with Hoechst 33342 staining of cell nuclei, we found that intracellular caspase-3 activity was detected in many human tumor cell lines but not in HDF or MCF-10A cell line (Fig. 1). Although some tumor cells had a very high level of intracellular caspase-3 activity, most of the tumor cells displayed from low to intermediate levels of caspase-3 activity (Fig. 1). We consistently observed a subpopulation of cultured tumor cells that had a high level of caspase-3 activity but were not apoptotic cells (Fig. 1, *arrows*). We additionally demonstrated the presence of cleaved active caspase-3 fragments in cultured human tumor cells by Western blot analysis, using a rabbit polyclonal antibody specific for cleaved caspase-3 fragments (17–19 kDa). We detected from low to intermediate levels of cleaved caspase-3 fragments in BXPC-3, MIA PaCa-2, and SW620 cell lines (Fig. 2). However, active caspase-3 fragments were not detected in HDF cells. We also found that although active caspase-3 fragments were present in the tumor cell lines, we did not detect cleaved PARP fragments (85 kDa) in BXPC-3 and MIA PaCa-2 cells, and only a very low level of cleaved PARP fragments was detected in SW620 cells (Fig. 2).

High Levels of IAP Proteins Were Detected in Human Tumor Cells and Primary Tumor Tissues, but the Level Of Survivin Was Very Low or Absent from Normal Cell Lines and Tissues. It is generally believed that activation of caspase-3 triggers an irreversible process of apoptotic cell death. The results from our study clearly showed the presence of a high caspase-3 activity in many human cancer cells that were not undergoing apoptotic cell death and that continued to proliferate in culture. An interesting question is what

Table 1 *Basal caspase activity in human tumor and normal cell lines detected in cell lysates by a fluorometric assay*

A. Caspase activity in human pancreatic and colon cancer and primary fibroblast cell lines

	Caspase 8 activity		Caspase 3-like activity		Caspase 9 activity	
Cell lines	RFU[a] (Mean ± SD)[b]	Fold increase	RFU (Mean ± SD)	Fold increase	RFU (Mean ± SD)	Fold increase
SW620	1825 ± 38	5.2	3618 ± 158	6.2	1024 ± 37	1.6
MIA PaCa-2	1389 ± 113	3.9	2868 ± 118	4.9	864 ± 79	1.4
BXPC-3	761 ± 33	2.2	869 ± 14	1.5	581 ± 86	0.9
HDF	348 ± 149	1.0	586 ± 61	1.0	625 ± 23	1.0

B. Caspase activity in human breast cancer and normal mammary epithelial cell lines

	Caspase 8 activity		Caspase 3-like activity		Caspase 9 activity	
Cell lines	RFU (Mean ± SD)	Fold increase	RFU (Mean ± SD)	Fold increase	RFU (Mean ± SD)	Fold increase
MDA-MB-435	215 ± 46	0.5	891 ± 76	1.9	523 ± 49	0.8
MDA-MB-231	1004 ± 50	2.2	1493 ± 182	3.3	674 ± 126	1.0
MDA-MB-361	185 ± 75	0.4	1186 ± 47	2.6	808 ± 35	1.2
MCF-7[c]	854 ± 87	1.9	903 ± 71	2.0	389 ± 27	0.6
MCF-10 A	450 ± 70	1.0	460 ± 30	1.0	677 ± 63	1.0

[a] RFU/10 μg protein.
[b] SD from three repeat samples.
[c] MCF-7 cells do not express *caspase 3* gene. The caspase 3-like activity detected may be from caspase 7 (50).
Student's *t*-test for caspase 3 activity: pancreatic and colon cancer cells *versus* HDF, $P = 0.0019$ and $P < 0.005$; breast cancer cell lines *versus* MCF-10A, $P = 3.9 \times 10^{-6}$ and $P < 0.0000001$; all tumor cell lines *versus* all normal cell lines: $P = 5.9 \times 10^{-5}$ and $P < 0.000001$.

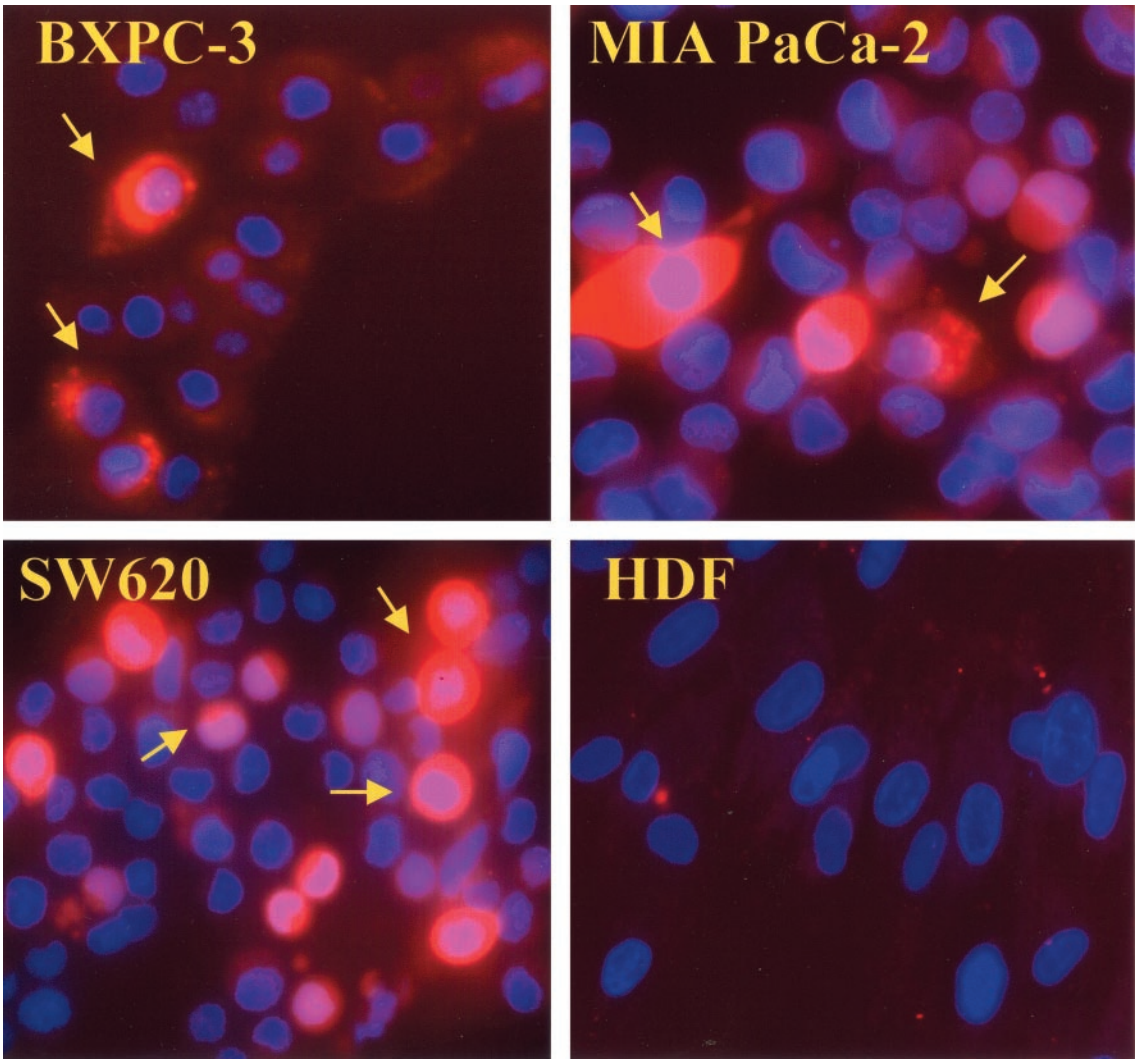

Fig. 1. Detection of intracellular caspase-3 activity in viable cells by a fluorometric assay using Caspase-3 Intracellular Activity Kit II. The cells were cultured on chamber slides for 24 h and then the substrate for caspase 3 was added into the culture medium for 60 min. After staining with Hoechst 33342 (*blue*), the cells were then observed under an inverted fluorescence microscope. *Arrows* indicate tumor cells with a high level of intracellular caspase 3 activity (*red*, cytoplasmic staining) but are not apoptotic (*blue*, nuclei).

cellular factors are responsible for blocking the progress of downstream apoptotic cascades. It has been shown that IAP family proteins are able to inhibit activated caspase-3 and caspase-9 activities (15, 17, 18, 35, 36). To understand the relationship between the presence of high levels of basal caspase-3 activity and levels of IAP expression in human tumor cell lines, we examined the protein levels of XIAP and survivin in several human tumor and normal cell lines. We found that survivin and XIAP proteins were highly expressed in most tumor cell lines, including breast cancer MDA-MB-231, MDA-MB-435, and MCF-7, pancreatic cancer MIA PaCa-2, BXPC-3, and Panc-1, and colon cancer SW620 cell lines (Fig. 3). On the other hand, a low level of XIAP was detected in HDF and MCF-10A cell lines (Fig. 3). Although survivin was not detected in most normal adult tissues, we detected a very low level of survivin in HDF and MCF-10A cells (Fig. 3). We additionally found that the level of survivin increased slightly as the normal cell lines were passed *in vitro* for many passages (data not shown). A low level of survivin expression in normal cell lines may be necessary for the cells to survive in culture. The presence of high levels of IAPs in tumor cells may prevent the progress of apoptotic response through direct or indirect inhibition of active caspases by IAPs and block the apoptotic pathway in the cells.

To confirm that our observation on the tumor cell lines was not an artifact of cells in culture, we examined the expression of survivin and the presence of active caspase-3 fragments in the tissue lysates from 34 paired and nonpaired breast cancer and normal tissues by Western blot analysis. We detected a high level of survivin in 72% of breast cancer tissues but not in any normal breast tissues. Using an antibody specific for cleaved active caspase-3 fragments (17–19 kDa), we also found that breast cancer tissues that were positive for survivin had a high level of active caspase-3 as well. However, active caspase-3 fragments were not detected in any normal tissues (Fig. 4, *A* and *B*). Four breast cancer tissues that did not have a detectable level of active caspase-3 fragments also lacked expression of survivin (Fig. 4*A*, patient no. 1, data not shown for other three cases). We additionally examined caspase-3 activity in the tissue lysates using a fluorometric assay with a caspase-3 substrate (Ac-DEVD-AFC). Consistent with the results of Western blot analysis, we observed a higher caspase-3 activity in the cancer tissues than that of the paired or nonpaired normal breast tissues (Fig. 4, *A* and *B*). We also analyzed caspase-3 activity from 33 breast cancer and normal tissue lysates *in vitro*. As compared with 15 normal breast tissue samples, a significant high level of caspase-3 activity was detected in eighteen breast cancer

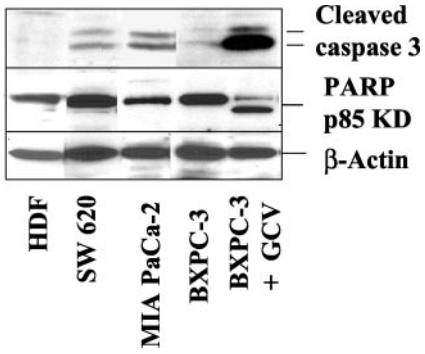

Fig. 2. Examination of the levels of active caspase-3 and cleaved PARP fragments in tumor cell lines by Western blot analysis. The tumor cell lines cultured at 70% confluence were collected for Western blot analysis. The level of cleaved caspase-3 fragments (17 and 19 kDa) and PARP (85 kDa) in the cell lysates was examined using antibodies specific for active caspase-3 or PARP. Normal cell line HDF was used as a control. A positive control sample was obtained from treating the herpes simplex virus thymidine kinase gene-modified BXPC-3 cells with 5 μg/ml ganciclovir (GCV) for 2 days, which induced apoptotic cell death in the cells (49). Although detectable basal level of active caspase-3 was seen in BXPC-3 cells, a marked increase in the level of cleaved caspase-3 and PARP p85 fragments was detected in BXPC-3 cells undergoing GCV-induced apoptosis.

tissues (Fig. 4*C*; Student's *t* test, $P = 0.0011$). These results suggested the presence of both activated apoptotic and antiapoptotic factors in primary breast cancer tissues.

Overexpression of Procaspase and Active Caspase Genes Induced Apoptotic Cell Death in Both Tumor and Normal Cell Lines. Next, we evaluated the feasibility of induction of apoptosis by overexpression of procaspase-8, procaspase-9, and procaspase-3 genes and an autocatalytic Rev-caspase-3 gene in tumor and normal cell lines. The plasmids containing procaspase-8, procaspase-9, and procaspase-3 genes or an autocatalytic Rev-caspase-3 gene were transfected into tumor and normal cell lines, and the number of GFP positive cells that were undergoing apoptotic cell death was determined under a fluorescence microscope after counterstaining cell nuclei with Hoechst 33342. We found that expression of procaspase-8 and procaspase-9 induced apoptotic cell death in both tumor and normal cell lines. Expression of procaspase-3 gene only induced a low level of apoptotic cells in the cell lines. However, expression of Rev-caspase-3 gene induced a high level of apoptotic cells in both tumor and normal cell lines (Fig. 5). Of all cell lines detected, we found the highest percentages of apoptotic cells (>80%) in several normal cell lines such as HDF, MCF-10A, and primary human dermal endothelial cells after expression of Rev-caspase-3 gene (Fig. 5 and data not shown). On the other hand, tumor cell lines MIA PaCa-2 and SW620, which expressed high levels of IAPs, showed a relatively low sensitivity to apoptosis induction by overexpression of caspase genes as compared with HDF cell lines (Fig. 5).

Expression of SurvivinT34A and XAF1 by Adenoviral Vectors Induced Apoptotic Cell Death Preferentially in Tumor Cell Lines. Because high levels of proapoptotic factors were present in most tumor cells but not in normal cells, we hypothesized that inhibition of antiapoptotic factors would lower the resistance to apoptosis and induce apoptotic cell death specifically in the tumor cells. To test this hypothesis, we examined the feasibility of induction of apoptosis in human tumor cell lines by overexpression of IAP-counteracting proteins such as survivinT34A and XAF1. Human tumor and normal cell lines were transduced with adenoviral vectors containing survivinT34A or XAF1 gene. An adenoviral vector expressing only a GFP gene was used as a control vector. Consistent with the hypothesis, our results showed that expression of survivinT34A or XAF1 gene by adenoviral vectors induced apoptosis preferentially in tumor cell lines but not in normal cell lines (Fig. 6, *A* and *B*). As shown in the Fig. 6, *A* and *B*, expression of survivinT34A or XAF1 induced different amounts of apoptotic cells in the tumor cells, resulting in various levels of remaining viable cells. At 7 days after viral vector transduction, ~40–88% of the tumor cells were killed by expression of either survivinT34A or XAF1 gene (Fig. 6, *A* and *B*). Moreover, a combination of AdsurvivinT34A and AdXAF1 transduction, which inhibited both survivin and XIAP function, significantly enhanced the apoptosis induction as compared with expression of either survivinT34A or XAF1 alone (Fig. 6, *A* and *B*; Student's *t* test, $P < 0.05$ for MIA PaCa-2, SW620, and MDA-MB-231 cell lines). This increase was greater in SW620 cells with a very high level of caspase-3 activity (Fig. 6*A*; Student's *t* test, $P = 0.001$). On the other hand, few apoptotic cells were observed in normal cell lines such as HDF and MCF-10A after overexpression of survivinT34A or XAF1 gene alone or a combination of both genes (Fig. 6, *A* and *B*). In addition, we also demonstrated that differential apoptosis induction in human tumor and normal cell lines was not attributable to differences in the gene expression because high levels of survivinT34A and XAF1 were detected in the normal cell lines transduced with either AdsurvivinT34A or AdXAF1 vector as shown by Western blots (Fig. 6*C*). We also found a high basal level of XAF1 in MCF-10A cells but not MDA-MB-231 cells, which further suggested that the level of XAF1 was down-regulated in human breast cancer cells (Fig. 6*C*).

It has been shown that XIAP inhibits the caspase-3 activation and blocks activity of active caspase-3 and caspase-9 (15, 16). We have examined caspase-3 and caspase-9 activities in tumor and normal cell lines after transduction of the cells with the adenoviral vectors for 3 days. We observed increases in caspase-3 and caspase-9 activity after blocking XIAP function by XAF1 in several tumor cell lines (Fig. 7, *A* and *B*). However, inhibition of survivin function by survivinT34A increased caspase-9 activity but did not significantly increase the caspase-3 activity in the tumor cell lines (Fig. 7, *A* and *B*). Expression of survivinT34A or XAF1 in normal cell lines also slightly increased caspase-3 or caspase-9 activity in those cells (Fig. 7, *A* and *B*). However, the levels of capase-3 and capase-9 activity in normal cells after the adenoviral vector transduction were still very low as compared with the tumor cells.

DISCUSSION

Increasing evidence indicates that development of resistant mechanisms for apoptosis confers high survival ability and low drug sensitivity to many cancer cell types (1, 6, 8). A promising approach to induce apoptotic cell death or to enhance the effectiveness of chemotherapy drugs is to target the apoptotic pathway directly. Sev-

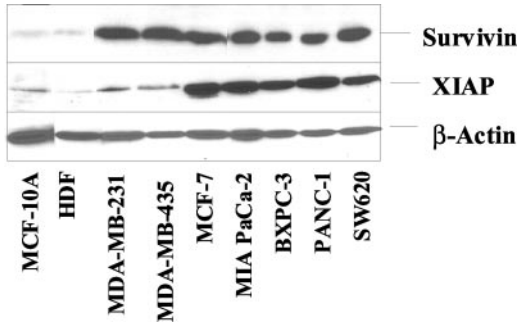

Fig. 3. Examination of the levels of survivin and XIAP expression in human tumor and normal cell lines by Western blot analysis. A high level of survivin (16.5 kDa) was detected in all breast, pancreatic, and colon cancer cell lines examined. Expression of survivin was very low in normal cell lines such as MCF-10A and HDF. Expression of XIAP (53 kDa) was found in both normal and tumor cell lines. However, the level of XIAP was up-regulated in all tumor cell lines.

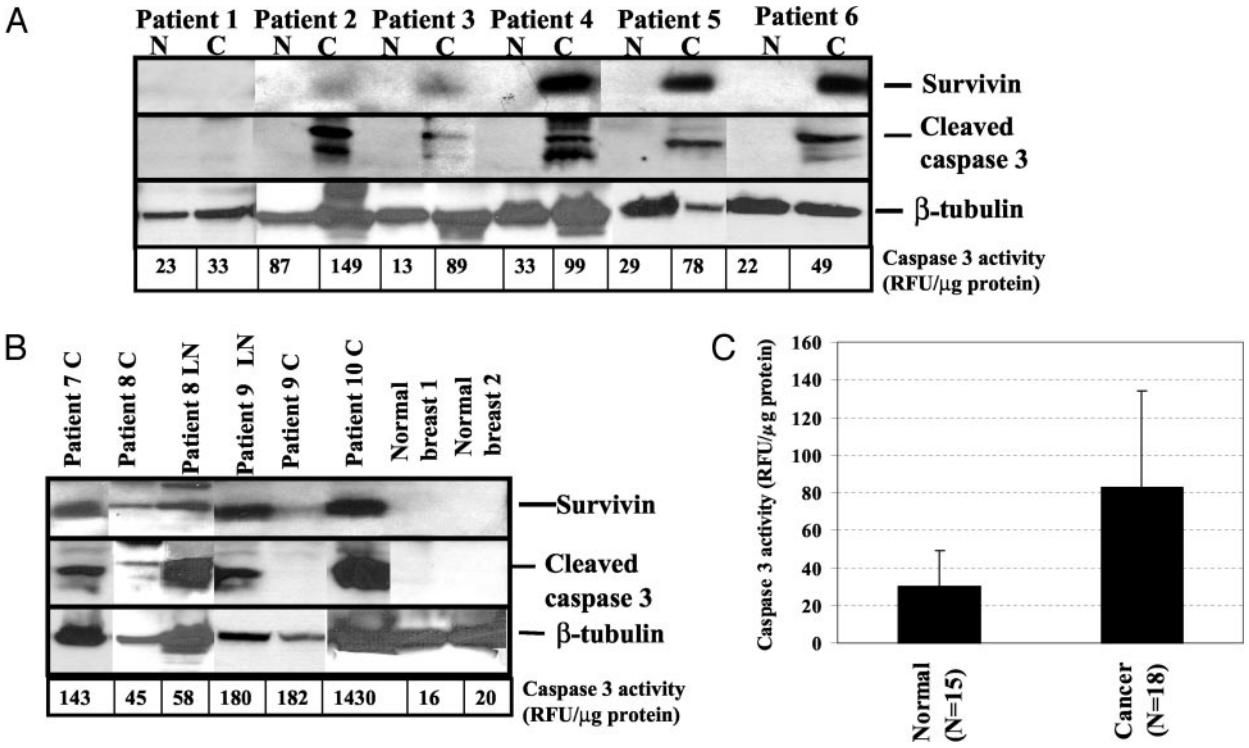

Fig. 4. Detection of the levels of survivin and active caspase-3 in breast normal and cancer tissues by Western blot analysis and a fluorometric assay. *A*, levels of survivin and active caspase-3 fragments in paired normal and cancer tissue samples from the breast cancer patients. *B*, levels of survivin and active caspase-3 fragments in breast cancer tissues and normal breast tissues obtained from breast reduction surgery. *C*, comparison of average caspase-3 activity detected by a fluorometric assay in cell lysates obtained from normal and breast cancer tissues. *A* and *B*, 50 μg of total protein from tissue lysates were examined for the levels of survivin and active caspase-3 fragments by Western blot analysis. N, normal tissue; C, cancer tissue; LN, draining lymph node. The same lysate samples were also examined for the caspase-3 activity using a fluorometric assay. The RFU/μg of protein for each sample is also shown in *A* and *B*. *C*, average caspase-3 activity from 15 normal and 18 cancer tissue samples were compared. The caspase-3 activity was >2-fold higher in the tumor tissues than that of the normal tissues. There was a significant difference in the caspase-3 activity between breast cancer and normal tissues (Student's *t* test, $P < 0.005$).

eral laboratories reported that apoptotic cell death could be induced in tumor cells by activation of upstream apoptosis signaling such as expression of Fas ligand, caspases, and Bax or inhibition of downstream antiapoptotic factors such as expression of dominant-negative survivinT34A, XAF1, and smac (25–29, 31, 37). However, the question regarding which molecular targets in the apoptotic pathway have the greatest potential for tumor specific therapy has yet to be answered.

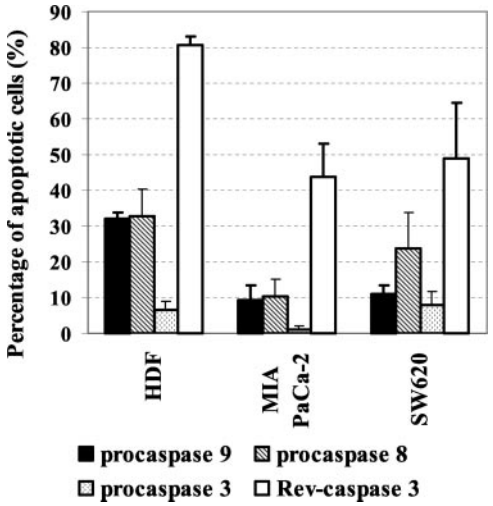

Fig. 5. Induction of apoptotic cell death by overexpression of procaspase-8, procaspase-9, and procaspase-3 genes or an autocatalytic Rev-caspase-3 gene. Human tumor and normal cell lines were cultured in 24-well plates for 24 h and then transfected with 1 μg of AdtrackCMV plasmids containing procaspase-8, procaspase-9, procaspase-3, or Rev-caspase-3 gene. At 48 h after transfection, the cells were stained with Hoechst 33342, and the percentage of apoptotic cells in transfected cell population, which were cells expressing GFP marker gene (*green*) and showing apoptotic nuclei as detected by Hoechst 33342 staining (*blue*), was examined in each transfected group. The numbers in the figure represent mean values from the examination of three to five fields under a ×40 lens with total cell numbers from 80 to 300 depending on transfection efficiency for tumor cell lines.

To address this question, we have examined the regulatory factors in the apoptotic pathways in human tumor and normal cells. We found that in comparison with normal cell lines and tissues, many tumor cell lines and tissues had activated apoptotic signaling such as high levels of caspase-8 and caspase-3 activities in the absence of apoptotic stimuli. Experimental results from our study additionally revealed that these human tumor cell lines and tissues also had high levels of survivin and XIAP. On the basis of our observations, we hypothesized that the genetic and molecular changes associated with malignant transformation lead to activation of the apoptotic cascade, yet the cancer cells are able to avoid death by up-regulating IAPs, which inhibits activity of the caspases and blocks apoptotic cascades. We additionally hypothesized that we could restore the final apoptotic events specifically in tumor cells by inhibiting IAP function through expression of IAP-counteracting proteins such as survivinT34A and XAF1.

Cellular apoptosis or survival results from a balance between apoptotic and survival factors. One of the logical therapeutic approaches for induction of apoptosis in tumor cells is to activate caspases. Several studies have shown that overexpression of initiator caspase-8 and caspase-9 or executioner caspase-3 and caspase-7 genes induced apoptotic cell death in human tumor cell lines (28, 38). Expression of a modified, constitutively active caspase-3 gene (Rev-caspase 3) induced apoptosis in the absence of upstream stimuli in 293 and MCF-7 cells (33).

In this study, we examined apoptosis induction by expression of

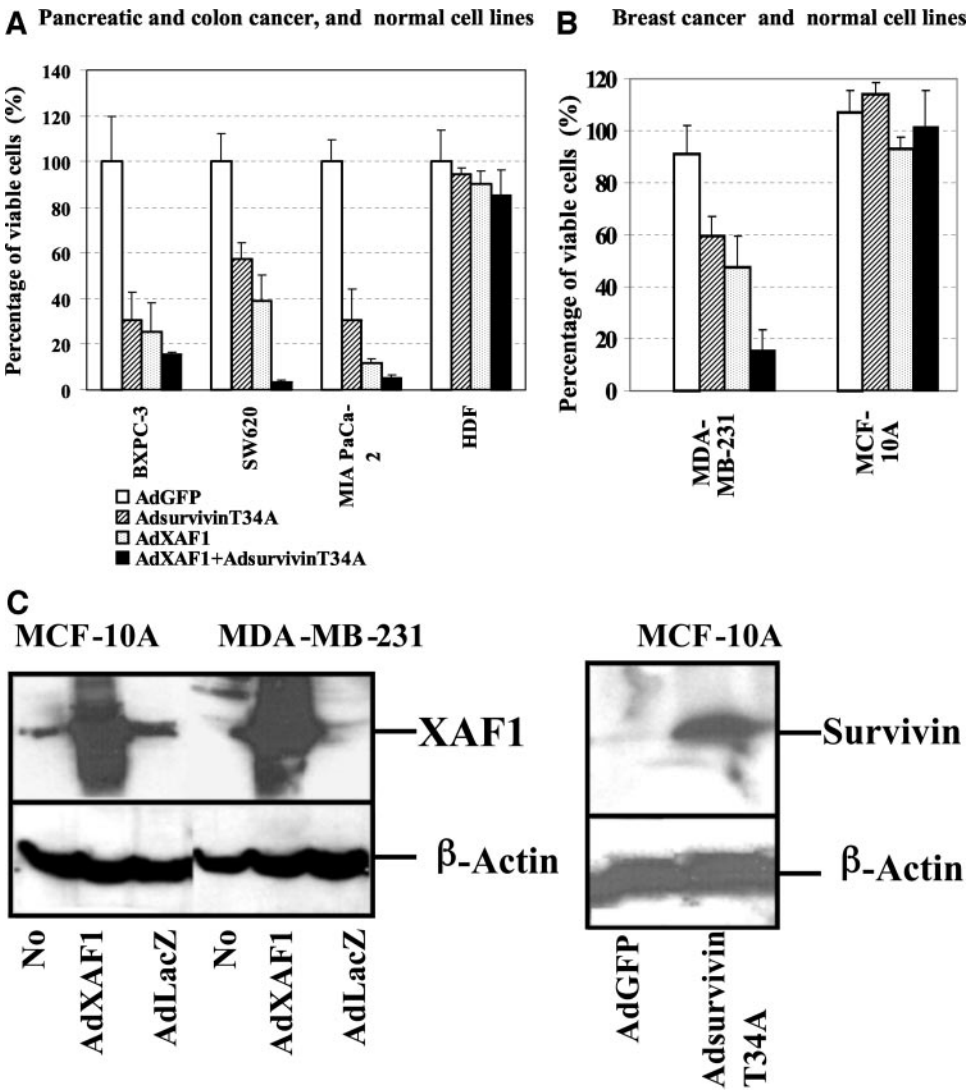

Fig. 6. Effects of expression of survivinT34A and/or XAF1 genes on human tumor and normal cell lines. *A* and *B*, induction of apoptotic cell death in human pancreatic, colon, and breast tumor cell lines by overexpression of survivinT34A and/or XAF1 genes. The tumor and normal cell lines were cultured in 96-well plates for 24 h and then transduced with the adenoviral vector at a MOI of 100 pfu. Over 80% of the cells were transduced with the adenoviral vectors as determined from AdGFP or AdsurvivinT34A vector-transduced groups. The percentage of viable cells at 7 days after transduction was determined by staining the cells with crystal violet followed by extraction of the dye for measuring absorbance at 590 nm. The absorbance value of the cells transduced with control vector AdGFP serves as a relative cell number of 100%. Each value in the bar figure represents a mean value of three to four repeat samples. Similar results were found in three repeat experiments. Student's *t* test was used to determine the significant difference between the percentage of viable cells in the groups treated with AdsurvivinT34A alone or ADXAF1 alone with the groups received both AdsurvivinT34A and AdXAF1. A significant difference in the percentage of viable cells was detected in the groups transduced with both AdsurvivinT34A and AdXAF1 as compared with the groups transduced with AdsurvivinT34A or AdXAF1 vector alone (Student's *t* test: MIA PaCa-2, $P < 0.05$; SW620, $P < 0.005$; MDA-MB-231, $P < 0.01$). *C*, detection of expression of survivinT34A or XAF1 in normal cell lines after transduction with either AdsurvivinT34A or AdXAF1 vectors by Western blot analysis. MDA-MB-231 and MCF-10A cells were transduced with AdsurvivinT34A, AdXAF1, AdGFP, or AdLacZ vectors at MOI of 100 pfu for 2 days. The level of survivin or XAF1 expression was examined by Western blot analysis using antibodies specific for XAF1 and survivin. Because MCF-10A cells expressed a very low level of survivin, an increase in the level of survivin represents the amount of survivinT34A expressed from the AdsurvivinT34A vector.

procaspase-8, procaspase-9, and procaspase-3 and Rev-caspase-3 genes in human tumor and normal cell lines. We found that although expression of procaspase-8, procaspase-9, and Rev-caspase-3 genes induced apoptotic cell death in both normal and tumor cell lines, cells with low levels of survivin and XIAP were more susceptible to apoptosis induction after expression of caspase genes. For example, we found that normal cell lines were more sensitive to apoptosis induction than that in tumor cells after overexpression of Rev-caspase-3 gene. On the other hand, tumor cells with high levels of survivin and XIAP such as SW620 and MIA PaCa-2 displayed a low sensitivity to apoptosis induction by overexpression of caspase genes. Thus, our results suggest that activation or expression of caspases alone is not a powerful strategy for induction of apoptosis in cancer cells and does not have specificity for tumor cells.

Recently, studies have revealed that tumor cells have acquired various resistant mechanisms to apoptosis. The upstream defects found in tumor cells include loss or mutation of cell death receptors and decrease in the level of Bax protein (39, 40). Deletion or silencing of the caspase-8 gene was discovered in childhood neuroblastoma (41). Although different upstream deficiencies were found in selective types of tumor cell lines and tissues, it seemed that up-regulation of IAPs was a common feature for the majority tumor types. Therefore, targeting IAPs should be a feasible approach for developing novel therapeutic strategies for many cancer types. At present, the role and mechanism of increases in IAP proteins during the process of tumorigenesis are still unknown. In particular, it is not known how survivin is up-regulated in tumors because this protein is not expressed in most normal adult tissues.

Our results that human tumor cells have both activated apoptotic signaling and elevated antiapoptotic factors are in agreement with those of several recent studies that detected a high level of caspase expression or active caspase-3 in various human tumor tissues. Studies from several laboratories showed that caspase-3 expression was up-regulated in breast cancer cell lines, breast ductal carcinoma *in situ*, and invasive breast carcinomas by Western blot analysis and immunohistochemical staining (42–44). Moreover, high levels of proapoptotic factors such as Bax and active caspase-3 and caspase-6 were detected in >90% of human breast cancer tissues (45). A strong positive correlation was also found between the levels of XIAP and cleaved caspase-3 in breast cancer tissues (45). It has also been shown that caspase-3 was overexpressed in pancreatic cancer tissues as compared with that of normal pancreas tissues (4).

Importantly, the results of our study demonstrated, for the first time, the presence of activated apoptotic signaling such as activated caspase-3 in human tumor cells that are not apoptotic. At present, the mechanism activating the apoptotic pathway remains unclear. Be-

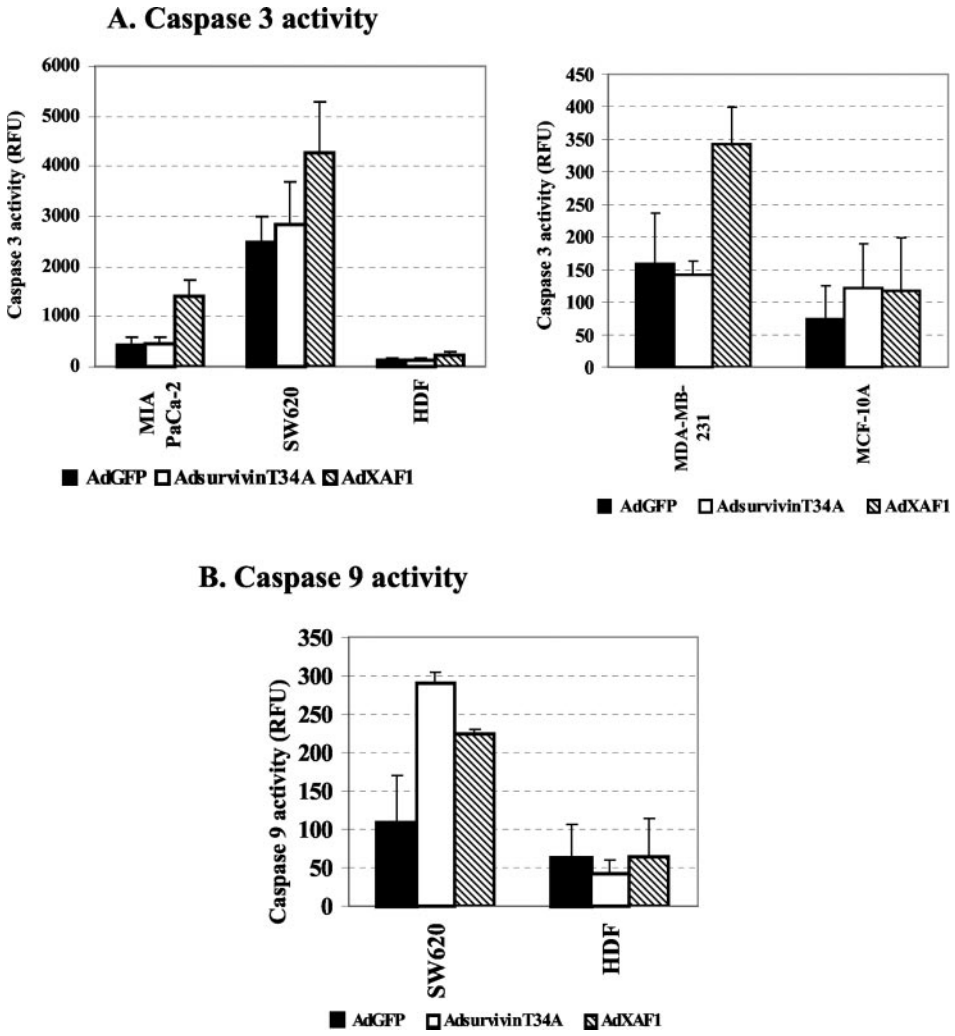

Fig. 7. Examination of the levels of caspase-3 and caspase-9 activity in human tumor and normal cell lines transduced with adenoviral vectors for 3 days. *A*, caspase-3 activity detected in cell lysates of AdsurvivinT34A or XAF1-transduced tumor and normal cell lines. *B*, detection of caspase-9 activity in cell lysates of AdsurvivinT34A or XAF1-transduced tumor and normal cell lines. The cell lysates from each treatment group were examined for caspase-3 or caspase-9 activity with caspase-specific substrates. The numbers in the figure represent mean values of three repeat groups (RFU/10 μg of protein). Similar results were obtained from repeat experiments.

cause apoptotic cell death is a common pathway to eliminate the abnormal cells, it is possible that during the process of tumorigenesis, abnormalities in the tumor cells cause activation of the apoptotic pathway and induce cell death in the majority of transformed cells. In this view, only a small fraction of the transformed cells that have up-regulated their antiapoptotic mechanisms such as IAPs are able to survive and develop into a tumor mass.

Our finding that both apoptotic and antiapoptotic factors are up-regulated in human tumor cells but not in normal cells can potentially lead to the development of novel tumor-specific therapies. Because tumor cells already have activated upstream apoptotic signaling and a relatively high level of active caspases, it is possible that potentiating caspase activity by down-regulation or counteracting IAP function could induce apoptosis specifically in tumor cells. A recent study showed that expression of the survivinT34A gene by adenoviral vector induced apoptotic cell death specifically in human tumor cell lines but not in several normal cell lines (31). In our experimental system, we observed similar results after expression of counteracting IAP proteins such as survivinT34A and XAF1 in human tumor and normal cell lines.

At present, the mechanisms of induction of apoptotic cell death by expression of survivinT34A or XAF1 have yet to be further determined. It seemed that a marked increase in caspase-3 and caspase-9 activity played a role in the apoptotic cell death of AdXAF1-transduced tumor cells because expression of XAF1 increased activity of both caspase-3 and caspase-9 in the tumor cell lines. Our result that expression of survivinT34A induced activation of caspase-9 in the tumor cell lines is also consistent with a recent study showing that association of survivin with a cellular protein, hepatitis B X-interacting protein, is required for binding survivin to procaspase-9. However, expression of survivinT34A abolishes the association between hepatitis B X-interacting protein and survivin and therefore activates caspase-9 (46). Although caspase-9 activity was increased by expression of survivinT34A, the caspase-3 activity was not significantly increased in the tumor cell lines. It is possible that the presence of a high level of XIAP in the tumor cells prevents additional activation of caspase-3. However, expression of survivinT34A inhibits the survivin function, which allows the proceeding of the apoptosis cascade in the tumor cells with a high basal level of caspase activity. Consistent with this assumption, recent studies showed that survivin level in tumor cells was regulated by phosphorylation of survivin Thr^{34}. Expression of survivinT34A or treatment with a cyclin-dependent kinase inhibitor, flavopiridol, suppressed the phosphorylation of endogenous survivin at the Thr^{34} position and resulted in loss of survivin expression and induction of apoptosis in the tumor cells (47, 48).

Furthermore, because survivin is not detected in most normal adult tissues, down-regulation of survivin function by expression of the survivinT34A gene should have a minimum effect on normal cells. Accordingly, expression of the survivin T34A gene did not induce apoptotic cell death in immortalized normal human mammary epithe-

lial cell line MCF-10A and normal fibroblast cell line HDF, although we have detected a weak expression of survivin in those cells. XAF1 counteracts the function of XIAP through competing for binding to caspases (30). It has been shown that XAF1 is expressed in normal cells and tissues but is down-regulated in tumor cell lines and tissue (29). Thus, expression of XAF1 may induce apoptosis in tumor cells with a low level or lack of XAF1 without affecting the normal cells.

In summary, we have demonstrated that IAPs are potentially novel molecular targets for the development of tumor-specific therapies. Significantly, we have detected the coexistence of both activated apoptotic signaling and up-regulated antiapoptotic factors in many human tumor cell lines and tissues but not in normal cells. Down-regulation of IAP function using agents such as small molecules, small peptides, RNA interference, and gene therapy vectors may change the balance of the apoptotic pathway and induce cell death specifically in tumor cells.

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