Survivin Enhances Fas Ligand Expression via Up-Regulation of Specificity Protein 1-Mediated Gene Transcription in Colon Cancer Cells

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Cancer cells are thought to possess mechanisms for evading the host's immune surveillance system. Survivin, a member of the inhibitor-of-apoptosis family overexpressed by cancer cells, inhibits Fas-mediated apoptosis induced by immune cells. In addition, cancer cells express Fas ligand (FasL) on their surfaces as a counterattack against immune cells. Mechanisms by which cancer cells express FasL, including involvement of survivin, are unclear. In the present study, we demonstrated that survivin up-regulated FasL expression and investigated how this might occur. Quantitative immunostaining showed correlation between survivin and FasL protein expression in colon cancer tissues ($r = 0.79$). FasL expression was up-regulated in LS180 colon cancer cells transfected with the survivin gene. Transfectants showed increased cytotoxicity against a Fas-sensitive human T leukemia cell line, Jurkat. In contrast, FasL expression was down-regulated in SW480 cells transfected with a small inhibitory RNA to prevent survivin expression. Survivin gene transfectants showed increased DNA binding of transcription factor specificity protein 1 (Sp1) to the FasL promoter, and up-regulation of Sp1 phosphorylation at serine and threonine residues; the total amount of Sp1 was unchanged. Thus, survivin enables cancer cells not only to suppress immune cell attack by inhibiting Fas-mediated apoptotic signaling, but to attack immune cells by induction of FasL. *The Journal of Immunology,* **2004, 172: 3922–3929.**

C ytotoxic T lymphocytes and NK cells play a pivotal role in immune surveillance, being chiefly responsible for elimination of virus-infected cells, transplants, and tumors (1). CTL and NK cells kill target cells in two distinct ways. One is the formation of pores in target cell membranes by secretion of perforin, followed by target cell penetration by granzymes cosecreted with perforin. The other is Fas ligand (FasL)[2]-mediated triggering of target cell surface receptors (Fas) that induce apoptosis (2, 3). However, the immune systems of cancer patients almost always fail to eliminate the tumor. Cancer cells therefore have been thought to have properties enabling them to evade a host's immune surveillance system.

Several proteins that inhibit Fas-mediated apoptotic signaling have been identified, including the *bcl*-2 family and the inhibitor-of-apoptosis (IAP) family (4, 5). Survivin, a member of the latter family, has been shown to block Fas-mediated apoptosis through direct inhibition of caspase-3 and -7, which act as terminal effectors in the apoptotic protease cascade (6, 7). Kawasaki et al. (8) examined apoptosis in colorectal tumor cells by in situ labeling, analyzing the relationship between apoptosis in tumor cells and survivin expression. They found that 53.2% of tumors (91 of 171) showed immunostaining for survivin, while also undergoing less frequent apoptosis. They also found survivin expression to be associated with poor patient survival. These results indicated that colon cancer cells may protect themselves from attack by CTL and NK cells by expressing survivin.

We and other investigators previously have demonstrated that colonic adenocarcinoma cells expressed FasL on the cell surface and induced apoptosis in Jurkat T cell leukemia cells, which express Fas (9, 10). Okada et al. (11) investigated apoptosis in tumor-infiltrating lymphocytes (TIL) obtained from colorectal tumors; using in situ nick translation, TIL in the 61% of colorectal tumors (25 of 41) that expressed FasL were more likely to show apoptosis than TIL in FasL-negative tumors. These investigators also found that patients with high numbers of apoptotic TIL had poorer outcomes than those with few apoptotic TIL. These results indicated that colon cancer cells may aggressively counterattack immune cells by expressing functional FasL, thus escaping the immune surveillance system.

However, the mechanisms by which cancer cells express FasL are not well defined, and whether survivin is involved in these pathways is unclear. In the present study, we hypothesized that survivin expression by cancer cells not only protects against attack from immune cells, but also counterattacks immune cells by inducing FasL expression.

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[2] Abbreviations used in this paper: FasL, Fas ligand; BIR, baculovirus IAP repeat; DAB, diaminobenzidine; IAP, inhibitor of apoptosis; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium-inner salt; pAb, polyclonal Ab; PKC, protein kinase C; siRNA, small inhibitory RNA; Sp1, specificity protein 1; TIL, tumor-infiltrating lymphocyte.

Materials and Methods

Patients and frozen tissue samples

Colorectal adenoma and cancer tissues, as well as adjacent normal colorectal mucosa counterparts, were obtained from patients undergoing surgery at Sapporo Medical University Hospital or Hokkaido Gastroenterology Hospital (Sapporo, Japan). Before the acquisition of these tissues, informed consent was obtained explaining the investigational nature of the study. Tissues were immediately frozen and stored in liquid nitrogen. Tissues were also stained with H&E, and were reviewed by well-experienced gastrointestinal pathologists.
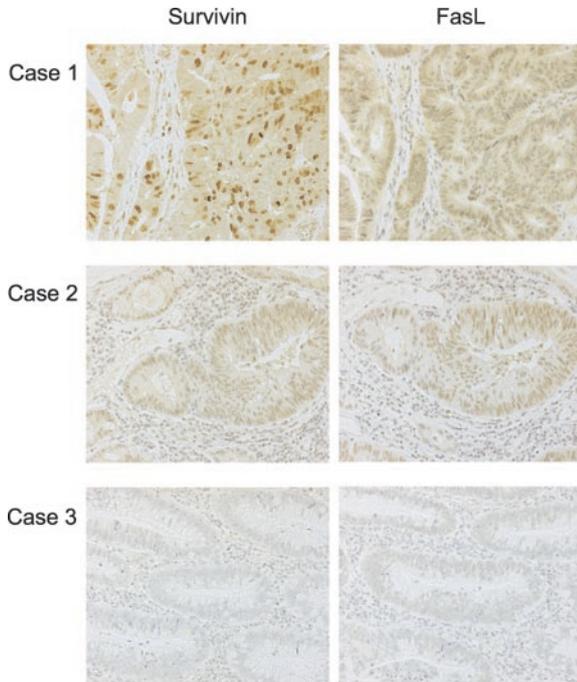
FIGURE 1. Representative specimens after immunostaining for survivin and FasL. Serial sections obtained from colon cancer tissues were immunostained for survivin (*left*) and FasL (*right*). Bright field images of immunostained tissues were captured using a digital camera (original magnification, ×200).

Immunostaining

Immunostaining for survivin and FasL was performed using a standard three-stage indirect immunoperoxidase technique on 5-μm serial tissue sections. Briefly, fixed tissue sections were rehydrated in graded alcohols and then rinsed in a running water bath, with endogenous peroxidase activity quenched by preincubating slides in 3% hydrogen peroxide in a light-impermeable chamber. After washing in deionized water, slides were incubated for 10 min at 121°C in 10 mM sodium citrate buffer (pH 6). After washing slides with PBS, rabbit anti-human survivin polyclonal Ab (pAb) (Novus Biologicals, Littleton, CO) or rabbit anti-human FasL IgG mAb (Santa Cruz Biotechnology, Santa Cruz, CA) was applied at a 1/125 dilution, and tissues were incubated for 1 h in a humid chamber. After washing again in PBS, the tissues were incubated with biotinylated anti-rabbit IgG pAb (DAKO, Carpenteria, CA) for 15 min. After washing in PBS, the slides were incubated with streptavidin conjugated to HRP (DAKO) for 15 min and washed again in PBS. Slides were incubated with a liquid diaminobenzidine (DAB) substrate-chromogen system (DAKO) for the indicated time period (2 min, unless otherwise specified) to identify bound Ab. After final washes in PBS and distilled water, slides were counterstained with a 50% dilution of Gill's hematoxylin for the indicated period of time (1 min, unless otherwise specified), dehydrated in graded alcohols, and mounted with a coverslip using Permount. For all specimens, control sections were processed identically at the same time, except that primary Ab was not applied. Specifically, control sections were cut from within 5 μm of fully immunostained sections, and were treated with secondary Ab, DAB, and hematoxylin counterstained for precisely the same times as for experimental sections. Accordingly, all differences between experimental and control sections would reflect DAB identification of the relevant protein.

Quantification of immunostaining

Bright field images of immunostained tissues under 200-fold magnification were captured using a Nikon microscope (ECLIPS E600; Nikon, Tokyo, Japan) and a Nikon digital camera (DXM 1200; Nikon). Image analysis was performed using Photoshop Elements software (Adobe Systems, Mountain View, CA), as previously reported (12, 13), with some modifications. Briefly, digitized images of tissue sections were converted to grayscale images after subtraction of blue and green components. Three areas in the cytoplasm of cancer cells and nonneoplastic stromal cells were selected for analysis of mean brightness using histogram tools in the software. Immunostaining intensity of cancer cells was calculated as a bright-

ness ratio between cancer cells and nonneoplastic stromal cells. This analysis was performed by two independent observers.

Cell culture

Human colonic adenocarcinoma cell lines, LS180 and SW480, and Jurkat human T leukemia cell line were obtained from American Type Culture Collection (Manassas, VA). LS180 and SW480 cells were cultured in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FBS and grown at 37°C in a humidified atmosphere of 5% CO_2. Jurkat cells were cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS.

Quantification of survivin and FasL mRNA

The expression of survivin and FasL mRNA was determined by a quantitative RT-PCR using an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA). Approximately 2×10^6 cells of cultured cells were homogenized with 1 ml of ISOGEN reagent (Nippon Gene, Toyama, Japan), and total RNA was isolated according to the manufacturer's protocol. The quantity of total RNA was determined by UV spectrophotometry using Gene Quant DNA/RNA (Amersham Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized using TaqMan reverse-transcription reagents (Applied Biosystems). The reaction mixture (50 μl final volume) contained 1 μg of total RNA; 5 μl of 10 × reverse-transcription buffer; 5.5 mM $MgCl_2$; 500 nM dATP, dCTP, dGTP, and dTTP; 20 U of RNase inhibitor; 62.5 U of MultiScribe reverse transcriptase; and 2.5 μM of random hexamers. Reverse transcription was performed at 48°C for 10 min, 95°C for 30 min, and 95°C for 5 min. Gene-specific primers and fluorescent hybridization probes for survivin used in quantitative PCR were as follows: forward primer, 5′-AAG AAC TGG CCC TTC TTG GA-3′; reverse primer, 5′-CAA CCG GAC GAA TGC TTT T-3′; and probe, 5′-CCA GAT GAC GAC CCC ATA GAG GAA CA-3′ (14, 15). Two splice variants of survivin, survivin-2B (retaining a part of intron 2 as a cryptic exon) and survivin-ΔEx3 (lacking exon 3), were not detected by this set of primers and probe (16). Quantitative RT-PCR was performed using a TaqMan Universal PCR Master Mix (Applied Biosystems). The reaction mixture (50 μl final volume) contained 5 μl of cDNA solution, 25 μl of 2× Master Mix, 200 nM forward and reverse primers, and 100 nM probe. The amplification cycles were 95°C for 15 s and 60°C for 1 min, and repeated for 40 cycles. Quantitative PCR for FasL was performed using Predeveloped TaqMan Assay Reagents for Gene Expression (Applied Biosystems), according to manufacturer's protocol. Amounts of PCR products were determined by a TaqMan 5′ nuclease assay (17). The starting quantity of specific mRNA in an unknown sample was quantitated by preparing a standard curve using known dilutions of standard RNA obtained from SW480 cells. A standard curve was generated on the basis of a linear relationship between the first cycle number at which the fluorescence signal significantly increased the threshold cycle (C_T) value and the logarithm of the starting quantity (18). Quantity of target mRNA in unknown samples
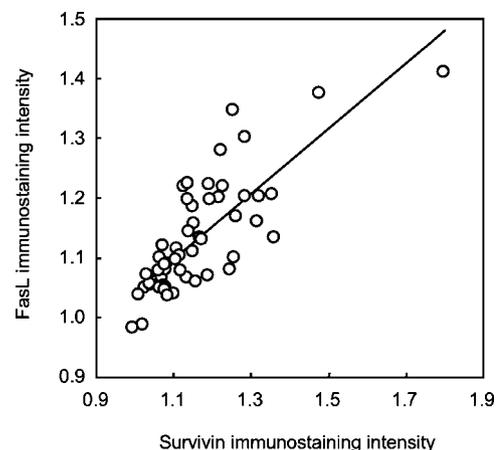

FIGURE 2. Correlation between immunostaining intensity of survivin and FasL in colon cancers. Bright field images of immunostained tissues were captured using a digital camera at 200-fold magnification. Brightness of immunostaining was analyzed using the Photoshop Elements software. Immunostaining intensity of survivin and FasL in cancer cells was calculated as a brightness ratio between cancer cells and nonneoplastic stromal cells.
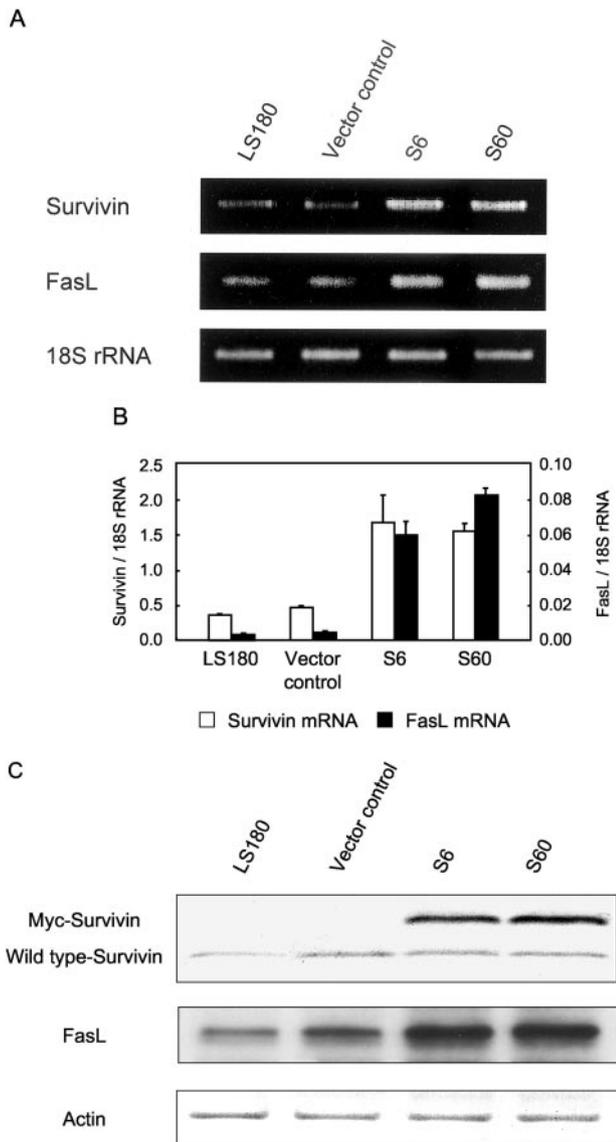
A

B

C

FIGURE 3. Up-regulation of FasL expression in survivin gene transfectants. Expression level of survivin and FasL mRNA in parent LS180 cells, control vector transfectants (vector control), and survivin gene transfectants (S6 and S60) was examined using RT-PCR. PCR products were analyzed by agarose gel electrophoresis (*A*) and by quantitative methods (*B*). Amounts of survivin mRNA (□) and FasL mRNA (■) were normalized as ratios to the amount of 18S rRNA. Expression of survivin and FasL protein was analyzed using Western blotting (*C*).

was determined from the C_T value using the standard curve. A control without template was included in each experiment. Nontemplate controls, standard dilutions, and samples were assayed in duplicate. To compare amounts of mRNA encoding survivin and FasL in different samples, the quantity of specific mRNA was normalized as a ratio to the amount of 18S rRNA (19), which was determined using TaqMan ribosomal RNA control reagents (Applied Biosystems), according to manufacturer's protocol. PCR products were also confirmed by agarose gel electrophoresis.

Transfection of wild-type survivin and specificity protein 1 (Sp1) into colonic cancer cells

A plasmid encoding human survivin, pcDNA3-*myc*-survivin, and human Sp1, pCMV4-Sp1/flu, were kindly provided by J. Reed (The Burnham Institute, La Jolla, CA) and J. Horowitz (North Carolina State University, Raleigh NC), respectively. Transfections were performed using LipofectAmine Plus reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Briefly, 10^5 LS180 cells were cultured in six-well culture plates (Costar, New Bedford, MA) in 2 ml of DMEM supplemented

with 10% FBS, and incubated for 24 h. After washing the cells with OPTI-MEM medium (Invitrogen) two times, 800 μl of OPTI-MEM medium and 200 μl of LipofectAmine reagent containing either 3 μg of pcDNA3-*myc*-survivin or pcDNA3-*myc* were added. After 4 h of incubation, medium was exchanged to 2 ml of DMEM supplemented with 10% FBS, and cells were incubated for an additional 24 h. At the end of incubation, after washing the cells with serum-free medium two times, 2 ml of DMEM supplemented with 10% FBS and 1000 μg/ml geneticin sulfate (Invitrogen) was added. Geneticin-resistant clones were obtained by limiting dilution method. For the transfection of Sp1 expression vector, pCMV4-Sp1/flu and pcDNA3-*myc* were cotransfected into LS180 cells.

Transfection of small inhibitory RNA (siRNA) for survivin into colonic cancer cells

siRNA was designed to target the coding region of the survivin gene (nt 366–385, relative to the start codon), and prepared by Japan Bio Service (Saitama, Japan). ssRNAs were annealed by incubating a 20 μM concentration of each strand in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH buffer at pH 7.4, and 2 mM magnesium acetate) for 1 min at 90°C, followed by 1 h of incubation at 37°C. As a transfection control, scramble RNA was prepared to contain numbers of each nucleotide equal to those in the siRNA targeted to the survivin gene. The siRNA duplexes used in this study were as follows: survivin, 5′-GAA UUU GAG GAA ACU GCG A TT-3′ and 3′-TT CUU AAA CUC CUU UGA CGC U-5′; scramble RNA, 5′-GCA UUG GAU AAG ACG UAG A TT-3′ and 3′-TT CGU AAC CUA UUC UGC AUC U-5′.

Transfections were performed using OligofectAmine reagent (Invitrogen), according to the manufacturer's protocol. Briefly, 5×10^4 of SW480 cells were cultured in six-well culture plates (Costar) in 2 ml of DMEM supplemented with 10% FBS, and incubated for 24 h. After the cells were washed twice with OPTI-MEM medium (Invitrogen), cells were incubated in 800 μl of OPTI-MEM medium and 200 μl of OligofectAmine with the addition of 1 μM either RNA. After 4 h of incubation, 300 μl of FBS was added and cells were incubated for an additional 120 h.

Western blot analysis

Cytoplasmic proteins were extracted from colonic cancer cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) with a protease inhibitor cocktail (Sigma-Aldrich). Equal amounts of cytoplasmic proteins (10 μg/lane) were separated on a 4–20% gradient Tris-glycine gel (Invitrogen) under denaturing conditions using Tris-glycine
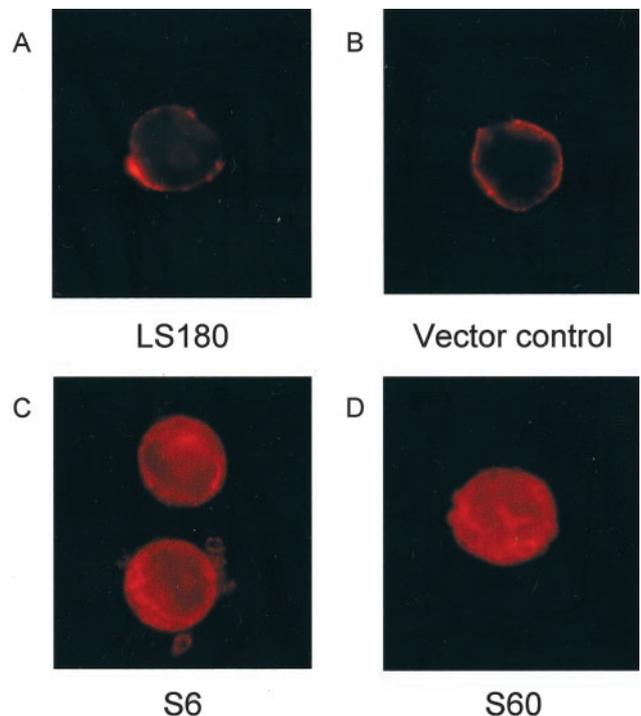

FIGURE 4. Increased cell surface expression of FasL in survivin gene transfectants. Cell surface expression of FasL by parent LS180 cells (*A*), control vector transfectants (vector control (*B*)), and survivin gene transfectants S6 (*C*) and S60 (*D*) was examined using immunofluorescence staining.

FIGURE 5. Increased FasL-mediated cytotoxicity against Jurkat cells in survivin gene transfectants. Jurkat cells alone (□) or Jurkat cells with paraformaldehyde-fixed parent LS180 cells (■), control vector transfectants (vector control, gray bar), or survivin gene transfectants (S6, ▨; S60, dotted bar) were cultured for 24 h with or without anti-human FasL IgG-neutralizing mAb or isotype-matched irrelevant hamster IgG. Viability of Jurkat cells was determined by an MTS assay.
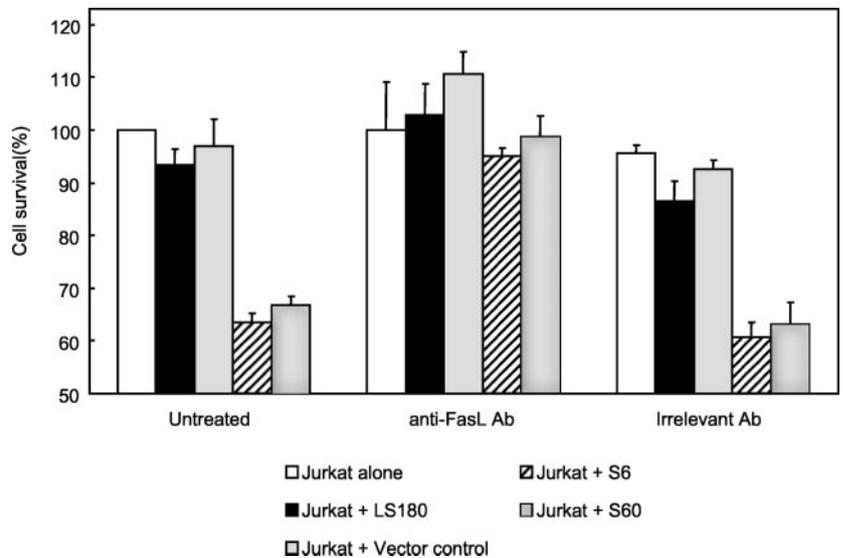
SDS running buffer (Invitrogen). Proteins were electroblotted to a nitrocellulose membrane (Invitrogen). Proteins were detected using a Western-Breeze chromogenic Western blot immunodetection kit (Invitrogen) or WesternBreeze chemiluminescent Western blot immunodetection kit (Invitrogen), according to the manufacturer's protocol. Briefly, after incubation in blocking solution for 30 min at room temperature, the membrane was incubated with mouse anti-human survivin IgG mAb (Santa Cruz Biotechnology), mouse anti-human FasL IgG mAb (BD Biosciences, Franklin Lakes, NJ), or mouse anti-human actin IgG mAb (Santa Cruz Biotechnology) at room temperature for 1 h. The membrane was washed and incubated with secondary Ab conjugated to alkaline phosphatase at room temperature for 30 min. Alkaline phosphatase labeling was detected using chromogenic substrate containing 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium or chemiluminescent substrate.

Detection of FasL by immunofluorescence cell staining

Cells were grown for 48 h on two-well culture slides (BD Biosciences). Cells were incubated with biotin-conjugated mouse IgG mAb against human FasL (BD Biosciences) for 1 h at 4°C after fixation with 2% paraformaldehyde for 30 min at 4°C. The cells were then incubated with a streptavidin-PE conjugate (BD Biosciences) for 30 min at 4°C, and visualized with a fluorescence microscope. Staining with PE-conjugated mouse IgG mAb (BD Biosciences) was performed as a negative control.

Cell proliferation assay

Viability of Jurkat cells cocultured with survivin gene transfectants was determined with a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium-inner salt (MTS) assay, CellTiter 96 AQueous One Solution Cell proliferation assay (Promega, Madison, WI). Survivin gene transfectants (10^5 cells) were cultured in 96-well culture plates (Costar) in 100 μl of DMEM supplemented with 10% FBS, and incubated for 24 h. Cells then were fixed with 2% paraformaldehyde for 1 h at 4°C. After washing the cells, 5×10^4 Jurkat cells in 100 μl of RPMI 1640 supplemented with 10% FBS were added to be incubated for an additional 24 h. At the end of incubation, 20 μl of MTS solution was added to be incubated for 2 h at 37°C. The amount of soluble formazan from reduction of MTS by viable Jurkat cells was assessed by measurement of absorbance at 490 nm using a microtiter plate reader (Spectla Fluor; TECAN, Maennedorf, Switzerland). To block FasL-mediated cytotoxicity, Jurkat cells were cocultured with fixed survivin gene transfectants in the presence of hamster anti-human FasL IgG-neutralizing mAb (MBL, Nagoya, Japan), or isotype-matched irrelevant hamster IgG (Immunotech, Marseille, France).

EMSA

Nuclear proteins were extracted from colonic cancer cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) with a protease inhibitor cocktail (Sigma-Aldrich).

An oligonucleotide probe containing the Sp1-binding motif of the FasL promoter (5′-AAA TTG TGG GCG GAA ACT TCC AGG G-3′) was prepared and end labeled with biotin by Sigma Genosys Japan (Ishikari,

Japan). Detection of Sp1-oligonucleotide complex was performed using a LightShift chemiluminescent EMSA kit (Pierce). Briefly, nuclear protein (5 μg) was incubated with 20 fmol of biotin-labeled oligonucleotide for 20 min at room temperature in binding buffer consisting of 10 mM Tris at pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 50 ng of poly(dA·dT), and 0.05% Nonidet P-40. The specificity of the Sp1 DNA binding was determined in competition reactions in which a 200-fold molar excess (4 pmol) of unlabeled oligonucleotide was added to the binding reaction. Products of binding reactions were resolved by electrophoresis on a 6% polyacrylamide gel (Invitrogen) using 1/2× Tris-borate EDTA (TBE) buffer (Invitrogen). Sp1-oligonucleotide complex was electroblotted to a nylon membrane (Invitrogen). After incubation in blocking buffer for 15 min at room temperature, the membrane was incubated with streptavidin-HRP conjugate for 30 min at room temperature. The membrane was incubated with chemiluminescent substrate for 5 min, and allowed to expose radiographic film. Band intensity of Sp1-oligonucleotide complex was semiquantified using Photoshop Elements software after conversion to digitalizing image using an image scanner (GT9700 F; EPSON, Tokyo, Japan).

Immunoprecipitation

Nuclear proteins (350 μg) from survivin gene transfectants were immunoprecipitated with 4 μg of rabbit anti-human Sp1 pAb and 20 μl of protein G PLUS-agarose (Santa Cruz Biotechnology) in PBS overnight at 4°C. The Sp1-Ab-protein G complexes were pelleted at 1000 × g, and then washed four times with PBS.

The Sp1 protein immunoprecipitate was resuspended in Western blot sample buffer and boiled for 3 min. Immunoprecipitate then was electrophoresed, followed by transfer to a nitrocellulose membrane. The blots were exposed to rabbit pAbs against phosphoserine and phosphothreonine (Chemicon International, Temecula, CA) for visualization.

Results

Correlation between survivin and FasL expression was observed in colon cancer tissue

To clarify the relationship between survivin and FasL expression in colon cancer cells, we determined expression of survivin and FasL protein in colorectal cancer tissues using immunostaining of serial sections. Fig. 1 presents representative examples of immunostaining for survivin and FasL. Strong immunostaining for FasL was seen more frequently in cancer cells with strong immunostaining for survivin.

To confirm this tendency, we quantified the intensity of survivin and FasL immunostaining in cancer cells by determining brightness of the cytoplasm in cancer cells and nonneoplastic stromal cells in captured digital images. The immunostaining intensity in cancer cells was expressed as a brightness ratio calculated between cancer cells and nonneoplastic stromal cells. As shown in Fig. 2,
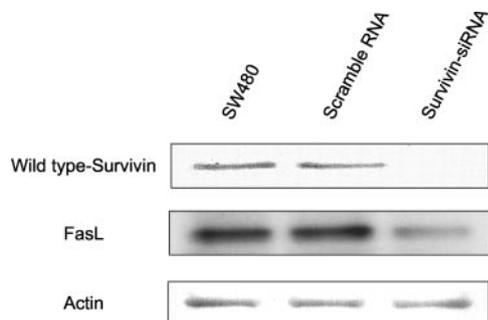
FIGURE 6. Down-regulation of the FasL expression by inhibition of survivin expression. Expression of survivin and FasL protein in SW480 cells was determined using Western blotting 120 h after transfection of an siRNA against survivin (survivin-siRNA) or scramble RNA.

a strong correlation between survivin and FasL immunostaining intensity in cancer cells was observed ($r = 0.79$), indicating a likely interrelationship in expression of these two molecules.

Overexpression of survivin enhanced FasL expression

To determine whether survivin affected expression of FasL, we transfected a control vector (pcDNA3-*myc*) or a wild-type survivin gene expression vector (pcDNA3-*myc*-survivin) into LS180 colon cancer cells that express survivin and FasL transcripts at only low levels (9). As shown in Fig. 3, two survivin gene transfectants (S6 and S60) showed higher expression of survivin gene and *myc*-tagged survivin protein than parent LS180 cells or control vector transfectants (vector control). Because these transfectants showed increased resistance against anti-human Fas IgM Ab-mediated apoptosis, the *myc*-tagged survivin protein was functional (data not shown). In these survivin gene transfectants, expression of FasL mRNA and protein also was dramatically up-regulated. To determine whether cell surface expression of FasL also was enhanced, immunofluorescence staining of cells for FasL was performed. Enhanced staining in the outer membrane of survivin gene transfectants indicated increased cell surface expression in these transfectants (Fig. 4).

To confirm that FasL expressed on surfaces of survivin gene transfectants was functional, coculture was performed between survivin gene transfectants and Fas-expressing T cell leukemia cells (Jurkat cells). Cytotoxicity of survivin gene transfectants against Jurkat cells was quantified by an MTS assay. As shown in Fig. 5, significantly fewer Jurkat cells cocultured with survivin gene transfectants remained viable than in cocultures with parent LS180 cells or vector control. Because this reduction was inhibited by a neutralizing anti-human FasL mAb, but not by an irrelevant mAb, the increased cytotoxicity of survivin gene transfectants was mediated by FasL.

Interestingly, when we transfected LS180 cells with an expression vector for livin, an IAP family member (20), livin did not enhance expression of FasL transcripts (data not shown).

FasL expression was diminished by down-regulation of survivin

For further evaluation of the survivin effect on expression of FasL, we examined changes in FasL expression after transfecting an siRNA targeted to survivin (survivin-siRNA) into SW480 cells, which express more survivin and FasL transcript than LS180 cells (9). As shown in Fig. 6, introduction of siRNA completely abrogated expression of survivin protein at 5 days after transfection; no reduction of survivin protein was observed in cells transfected with scramble RNA, which contained equal numbers of individual nucleotides. Along with down-regulation of survivin protein, expres-

sion of FasL protein was less in survivin-siRNA-transfected cells than in parent SW480 cells or scramble RNA transfectants. In addition, FasL expression on the cell surface was decreased in the survivin-siRNA transfectants (Fig. 7). These results indicated that survivin regulates expression of FasL.

Survivin enhanced the binding of Sp1 to the FasL promoter

Previous reports demonstrated that transcription factor Sp1 was required for basal transcription from the FasL promoter (21). We therefore transfected Sp1 expression vector into LS180 cells, confirming that Sp1 up-regulated the expression of FasL. We next investigated DNA-binding activity of Sp1 to the FasL promoter in Sp1 gene transfectants using EMSA. In this assay, we used a biotinated oligonucleotide containing the Sp1-binding motif and encompassing nucleotide positions -288 to -263 relative to the FasL translation initiation site. As shown in Fig. 8, Sp1-oligonucleotide complex was up-regulated in Sp1 gene transfectants compared with parent LS180 cells and the vector control. FasL protein expression on the cell surface also was up-regulated in LS180 cells transfected with Sp1 expression vector (Fig. 9). These results confirmed the importance for FasL expression of increased Sp1 DNA binding to the FasL promoter.

We then determined DNA-binding activity of Sp1 to the FasL promoter in survivin gene transfectants and survivin-siRNA transfectants. Sp1-oligonucleotide complex was increased in survivin gene transfectants similarly to Sp1 gene transfectants (Fig. 10). In contrast, this complex was decreased in survivin-siRNA transfectants compared with parent SW480 cells and scramble RNA transfectants. These results indicate that survivin enhanced the binding activity of Sp1 to the FasL promoter.
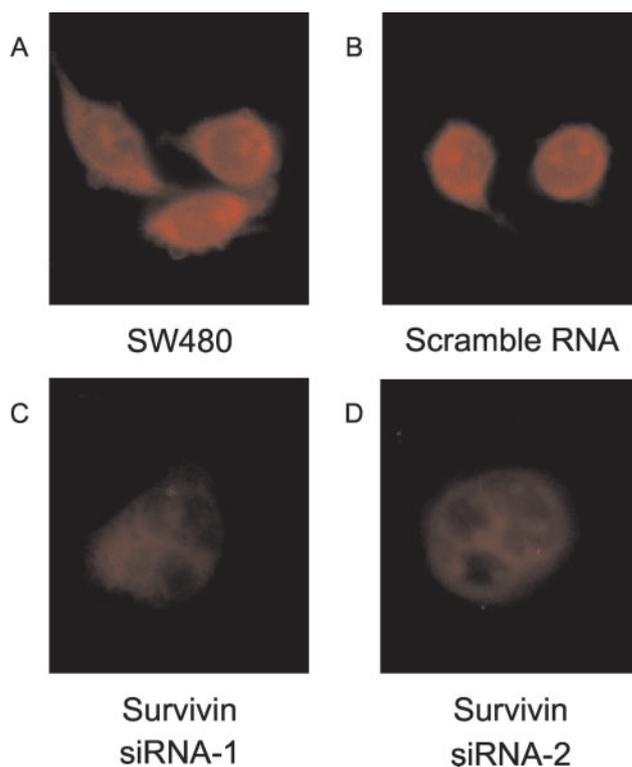

FIGURE 7. Decreased cell surface expression of FasL in survivin-siRNA transfectants. Cell surface expression of FasL in parent SW480 cells (*A*), scramble RNA transfectants (*B*), and transfectants with siRNA against survivin (*C* and *D*) was examined using immunofluorescence staining.
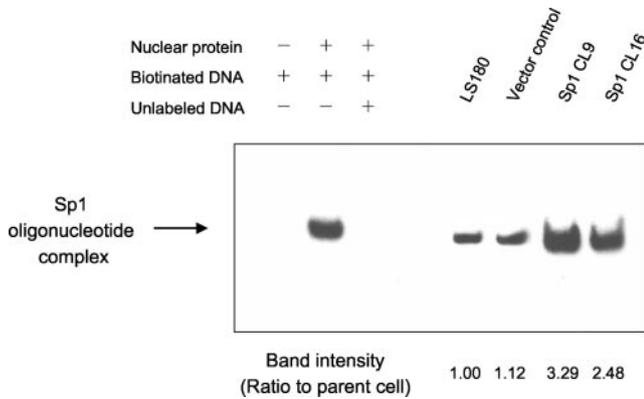
FIGURE 8. Enhanced DNA binding of Sp1 to the FasL promoter in Sp1 gene transfectants. DNA-binding activity of Sp1 to the FasL promoter in parent LS180 cells, control vector transfectants (vector control), and Sp1 gene transfectants (Sp1 CL9 and Sp1 CL16) was examined using EMSA. Specificity of Sp1 DNA binding was determined in competition reactions in which a 200-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. Band intensity of digitalized image was semiquantified using Photoshop Elements software. Band intensity was shown as a ratio to parent cell.

Survivin increased phosphorylation of Sp1

Increased Sp1 protein expression as seen with Sp1 gene transfectants appears to be one mechanism by which survivin enhances DNA-binding activity of Sp1 to the FasL promoter. Increased Sp1 phosphorylation may be another; phosphorylation of Sp1 protein at serine and threonine residues has been reported to enhance its DNA-binding activity (22, 23). To clarify these possibilities, we assessed expression of Sp1 and phosphorylated Sp1 in both survivin gene transfectants and survivin-siRNA transfectants. Sp1 protein from nuclear extracts of transfectants was immunoprecipitated using anti-human Sp1 pAb and immunoblotted with anti-Sp1, anti-phosphoserine, or anti-phosphothreonine pAb. As illustrated in the *upper panel* of Fig. 11*A*, band intensity of total Sp1 in survivin gene transfectants did not differ significantly from that in parent LS180 cells or vector control. However, phosphoserine and phosphothreonine band intensities were increased in survivin gene transfectants. In addition, phosphoserine and phosphothreonine band intensities in survivin-siRNA transfectants were less than in parent SW480 cells or scramble RNA transfectants (Fig. 11*B*). These results indicated that survivin enhanced DNA-binding activity of Sp1 not by up-regulation of the Sp1 protein expression, but by enhancing Sp1 phosphorylation at serine and threonine residues.

Discussion

We previously reported that colonic cancer cells expressed functional FasL protein on their surfaces, concluding that FasL expression acts importantly evading immune surveillance by counterattacking immune cells (9). However, the mechanism by which cancer cells expressed FasL has remained elusive.

In the present study, we found that survivin up-regulated FasL expression by augmenting gene transcription. In addition, DNA-binding activity of Sp1 to the FasL promoter was enhanced in the survivin gene transfectant, while activity was down-regulated by inhibition of survivin expression by survivin-siRNA.

Interestingly, although the total amount of Sp1 protein in cells overexpressing survivin did not differ from amounts in parent cells or cells transfected with control vector, the amount of phosphorylated Sp1 was increased in survivin-overexpressing cells. These
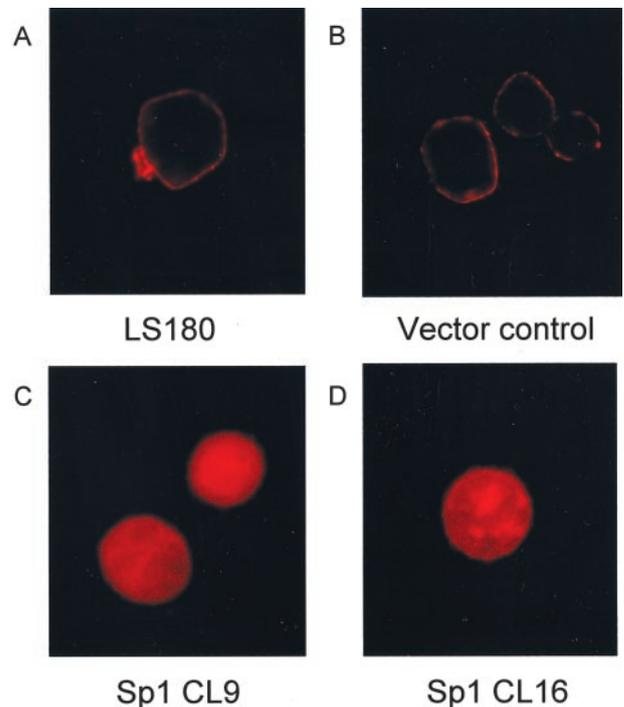

FIGURE 9. Increased cell surface expression of FasL in Sp1 gene transfectants. Cell surface expression of FasL in parent LS180 cells (*A*), control vector transfectants (vector control (*B*)), and Sp1 gene transfectants (Sp1 CL9 (*C*) and CL16 (*D*)) was examined using immunofluorescence staining.

results indicated that survivin enhanced the DNA-binding activity of Sp1 not by up-regulation of Sp1 protein expression, but by intensifying Sp1 phosphorylation. The mechanism by which the survivin enhances phosphorylation of Sp1 has been unclear; Bolton et al. and Chen et al. (24, 25) showed that survivin interacted directly with the catalytic domain of aurora-B kinase, a serine/threonine kinase involved in regulation of mitosis, and enhanced its activity. This suggests that survivin may induce phosphorylation of Sp1 by activation of aurora-B kinase.

Pal et al. (26) demonstrated that protein kinase C ζ (PKCζ) directly phosphorylated Sp1 and enhanced Sp1-mediated gene
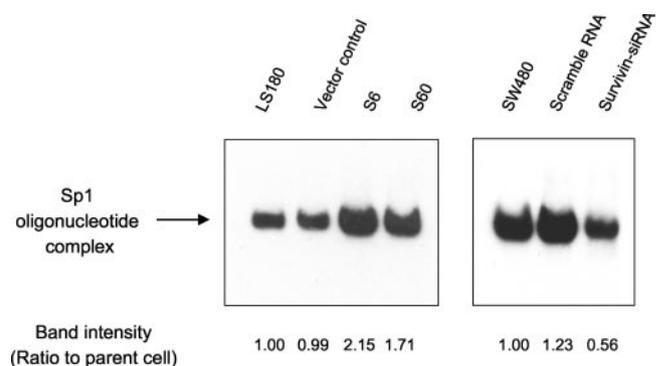

FIGURE 10. Regulation of DNA-binding activity of Sp1 to the FasL promoter by survivin. DNA-binding activity of Sp1 to the FasL promoter in parent LS180 cells, control vector transfectants (vector control), survivin gene transfectants (S6 and S60), parent SW480 cells, scramble RNA transfectants, and transfectants with siRNA against survivin (survivin-siRNA) was examined by EMSA. Band intensity of digitalized image was semiquantified using Photoshop Elements software. Band intensity was shown as a ratio to parent cell.
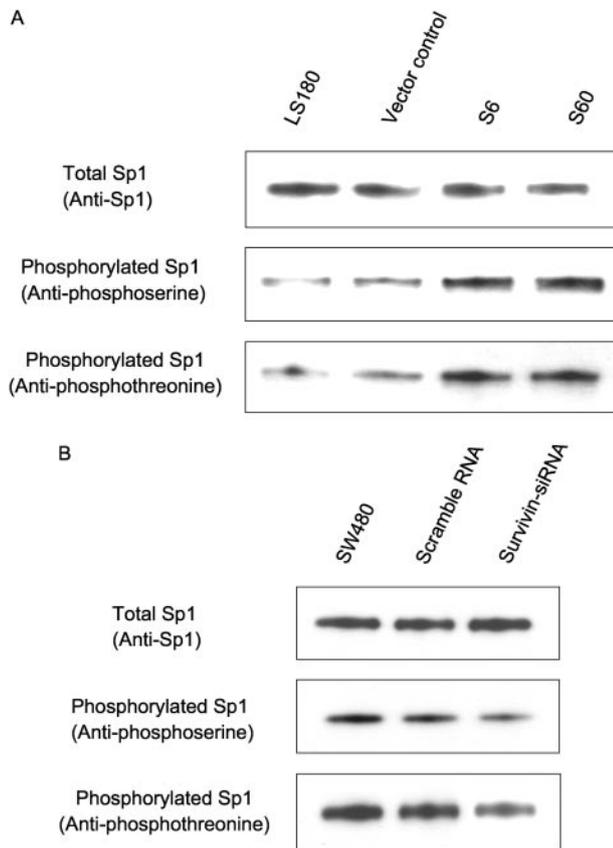
FIGURE 11. Regulation of phosphorylation of Sp1 by survivin. Nuclear extract was obtained from: *A*, parent LS180 cells, control vector transfectants (vector control), and survivin gene transfectants (S6 and S60), or *B*, parent SW480 cells, scramble RNA transfectants, and transfectants with siRNA against survivin (survivin-siRNA). Sp1 protein from nuclear extracts of transfectants was immunoprecipitated using anti-human Sp1 pAb and immunoblotted with anti-Sp1, anti-phosphoserine, or anti-phosphothreonine pAb.

transcription. Therefore, another possibility is survivin may enhance the kinase activity of PKCζ upon aurora-B kinase. We now are conducting additional experiments to clarify these possibilities.

Members of the IAP family usually contain several repeats of the baculovirus IAP repeats (BIR) domain (27). The BIR domain is essential for inhibition of caspase activity (28), and survivin includes only a single BIR domain (29). We constructed a dominant-negative mutant survivin expression vector encoding a mutant form of survivin with replacement of a cysteine residue in the BIR domain at amino acid position 84 by an alanine residue. This mutant cannot inhibit activation of caspase (30). To examine whether this caspase binding domain is required for up-regulation of FasL expression, we transfected this dominant-negative mutant survivin expression vector into SW480 cells and examined expression of FasL mRNA. Dominant-negative mutant survivin-expressing cells showed the up-regulation of FasL transcription similarly to survivin gene transfectants (data not shown). Caspase binding site in BIR domain, then, may not be required for up-regulation of FasL expression.

CTL and NK cells express FasL after activation (31–33). Because activated CTL and NK cells also express Fas on the cell surface, these cells undergo apoptosis by their own up-regulated FasL, a process termed activation-induced cell death. O'Connell et al. (34) showed coexpression of Fas and FasL was seen in colonic cancer cell lines and large areas of most colon cancer tissues. How-

ever, these cancer cells did not undergo apoptosis like activation-induced cell death. One possible explanation of these findings is cancer cells express antiapoptosis molecules including survivin and block Fas-mediated apoptotic signaling.

In conclusion, we suggest that survivin enables cancer cells to suppress attack by immune cells via inhibition of Fas-mediated apoptotic signaling, and also fight back via induction of FasL on the cancer cell surface. Thus, for cancer cells, survivin is a powerful weapon in escaping the host's immune surveillance system. Controlling survivin expression may enhance elimination of cancer cells by the immune system, opening new avenues for cancer treatment.

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