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Cytotoxic 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 co-secreted 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.

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.

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Department of Clinical Laboratory Medicine, Sapporo Medical University, School of Medicine, Sapporo, Japan

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1 Address correspondence and reprint requests to Dr. Naoki Watanabe, Department of Clinical Laboratory Medicine, Sapporo Medical University, School of Medicine, South-1, West-16, Chuo-ku, Sapporo 060-8543, Japan. E-mail address: watanahn@sapmed.ac.jp

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)-2H-tetrazolium-inner salt; pAb, polyclonal Ab; PKC, protein kinase C; siRNA, small inhibitory RNA; Sp1, specificity protein 1; TIL, tumor-infiltrating lymphocyte.
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 (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, Carpentry, 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 brightness 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% CO₂. 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 × 10⁶ 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 1 × reverse-transcription buffer; 5.5 mM MgCl₂; 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 (Ct) value and the logarithm of the starting quantity (18). Quantity of target mRNA in unknown samples

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).

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.
with 10% FBS, and incubated for 24 h. After washing the cells with OPTI-MEM (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 1% FBS, and cells were incubated for another 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 colon 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 GCC GCA 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 CUU UUC UGC AUC U-5'.

Transfections were performed using OligofectAmine reagent (Invitrogen), according to the manufacturer’s protocol. Briefly, 5 × 10⁵ of SW480 cells were cultured in six-well culture plates (Costar) in 2 ml of DMEM supplemented with 1% 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 colon 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 buffer (pH 8.8). Protein was analyzed using Western blotting (C).

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, 10⁷ 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 1% FBS, and cells were incubated for another 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 wild-type survivin and specificity protein 1 (Sp1) into colon cancer cells

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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 incubations with blocking solution for 30 min at room temperature, the membrane was incubated with mouse anti-human survivin IgG (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-indoly1-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 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)-2H-tetrazolium-inner salt (MTS) assay, CellTiter 96 AQueous One Solution Cell proliferation assay (Promega, Madison, WI). Survivin gene transfectants (105 cells) were cultured in 96-well culture plates (Costar) in 100 μl of DMEM supplemented with 10% FBS, and incubated for 24 h. Cells were fixed with 2% paraformaldehyde for 1 h at 4°C. After washing the cells, 5 × 104 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 (Spectra 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, IgG-neutralizing mAb (MBL, Nagoya, Japan), or isotype-matched irrelevant hamster IgG (Immuno-tech, Marseille, France).

EMSA

Nuclear proteins were extracted from colon 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 (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 EDTA, 2.5% glycerol, 5 mM MgCl2, 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 semi-quantified 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 for 20 min, 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,
Fluorescence staining of cells for FasL was performed. Determine whether cell surface expression of FasL also was enhanced, mRNA and protein also was dramatically up-regulated. 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, expression 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 biotinylated 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.

**Survivin enhanced the expression of FasL**

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. 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).

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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 biotinylated 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.
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. 11A, 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. 11B). These results indicated that survivin enhanced DNA-binding activity of Sp1 not by up-regulation of 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 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 Cζ (PKCζ) directly phosphorylated Sp1 and enhanced Sp1-mediated gene
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 pow-
erful weapon in escaping the host’s immune surveillance system.
Controlling survivin expression may enhance elimination of can-
cer cells by the immune system, opening new avenues for cancer
treatment.

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FIGURE 11. Regulation of phosphorylation of Sp1 by survivin. Nuclear
extract was obtained from: A, parent LS180 cells, control vector transfec-
tants (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 ex-
tracts of transfectants was immunoprecipitated using anti-human Sp1 pAb
and immunoblotted with anti-Sp1, anti-phosphoserine, or anti-
phosphothreonine pAb.


