Suppression of survivin expression in glioblastoma cells by the Ras inhibitor farnesylthiosalicylic acid promotes caspase-dependent apoptosis

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Abstract

The Ras inhibitor farnesylthiosalicylic acid (FTS) has been shown to induce apoptosis in glioblastoma multiforme, but its mechanism of action was unknown. We show that FTS or dominant-negative Ras, by deregulating extracellular signal-regulated kinase and Akt signaling, decreases *survivin* gene transcripts in U87 glioblastoma multiforme, leading to disappearance of survivin protein and cell death. FTS affected both Ras-controlled regulators of *survivin* transcription and Ras-regulated survival signals. Thus, Ras inhibition by FTS resulted in release of the survivin ''brake'' on apoptosis and in activation of the mitochondrial apoptotic pathway: dephosphorylation of Bad, activation of Bax, release of cytochrome *c*, and caspase activation. FTS-induced apoptosis of U87 cells was strongly attenuated by forced expression of survivin or by caspase inhibitors. These results show that resistance to apoptosis in glioblastoma multiforme can be abolished by a single Ras inhibitor, which targets both survivin, a critical inhibitor of apoptosis, and the intrinsic mitochondrial apoptotic machinery. [Mol Cancer Ther 2006;5(9):2337–47]

Introduction

Active Ras and phosphatidylinositol 3-kinase (PI3K)–dependent pathways, present in abundance in most glioblastoma multiformes (1, 2), contribute to maintenance of the malignant phenotype of these brain tumors by disrupting glioblastoma multiforme cell cycle arrest (3), increasing cell migration (4), enhancing glioblastoma multiforme cell survival (5), and promoting angiogenesis (6). The increased presence of active Ras-GTP in glioblastoma is secondary to mitogenic signals originating from activated receptor tyrosine kinases (7). Receptor-mediated activation of Ras signaling thus seems to be required for maintenance of the malignant phenotype of glioblastoma multiforme. Pharmacologic inhibitors of receptor tyrosine kinases (8) and Ras (9–11) or expression of dominant-negative Ras (2) inhibit glioblastoma multiforme cell growth, mainly by inhibiting Ras signaling to Raf/mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK and to PI3K/Akt.

Consistently with the complex Ras-associated phenotype of glioblastoma multiforme, we found that the Ras inhibitor farnesylthiosalicylic acid (FTS) inhibits U87 glioblastoma multiforme cell growth, induces cell death, and down-regulates hypoxia-inducible factor-1α (HIF-1α; ref. 9). These changes in the malignant phenotype were associated with inhibition of active Ras (K-Ras, N-Ras, and H-Ras isoforms) and attenuation of Ras signaling to ERK, PI3K, and Akt (9). By applying computational methods, in which clustering of gene expression profiles was combined with promoter sequence analysis to obtain global dissection of the transcriptional response to FTS, we found that the transcription factor E2F1 was the predominant Ras-dependent component associated with cell cycle arrest (10). Evidently, inhibition of Ras by FTS promoted proteasomal degradation of cyclin D1, with a concomitant decrease in phosphorylated retinoblastoma protein accompanied by down-regulation of E2F1 and decreased expression of key E2F1-regulated genes critical for cell cycle progression. U87 cell growth arrest induced by FTS was overridden by constitutive expression of E2F1 (10).

A related study with U87 and other glioblastoma multiforme cell lines showed that FTS causes disappearance of HIF-1α, which is followed by reduced expression of key glycolysis pathway enzymes and other HIF-1α-regulated genes (9). These changes lead to glycolysis shutdown and a dramatic reduction in ATP levels, resulting in a severe energy crisis (9). It thus seemed that the abovementioned FTS-induced death of U87 cells (9) might be attributable, at least in part, to loss of energy. Other experiments suggested, however, that cell death induced by FTS invokes additional mechanisms that may or may not be associated with energy loss. It was shown recently, for example, that down-regulation of HIF-1α by phosphorothioate antisense HIF-1α oligonucleotide causes apoptotic cell death in U87 cells (12). That study showed that the cytotoxic effect of

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the antisense HIF-1α is independent of p53 and can be attenuated by caspase inhibitors. In addition, a recent study showed that FTS induces caspase activation and apoptosis in glioblastoma cells (13). Interestingly, a recent study showed that inhibition of K-Ras expression in a K-Ras glioblastoma mouse model results in apoptotic tumor regression (14). However, the studies described above and our own experiments did not find the link between Ras inhibition in glioblastoma multiforme and the consequent activation of cell death programs.

In the present work, we show that a major effect of the blockage of Ras pathways in glioblastoma multiforme cells by FTS is the decrease in *survivin* gene transcripts. This effect was also apparent at the level of survivin protein, which was reduced to an almost undetectable amount by FTS or by expression of dominant-negative Ras. Consequently, U87 cells underwent caspase-dependent apoptotic cell death. The cells could be rescued from FTS-induced death by forced expression of survivin.

Materials and Methods

Cell Lines and Reagents

The glioblastoma cell lines used and their growth conditions [U87, U87-E2F1, DBTRG-05MG (20/20), LN229, and U373] have been described previously (10). U87 cells stably expressing survivin (U87-survivin cells) were established by transfection with pcDNA3-HA-survivin expression vector followed by G418 (1 mg/mL) selection. FTS was prepared as described (15). Cells were plated at a density of 2.5×10^5 in six-well plates for immunofluorescence assays and 1.5×10^6 in 10-cm dishes for all other assays. Drugs or vehicle (0.1% DMSO) was added to the cells 24 hours after plating. FTS, U0126, or LY294002 was added at the concentrations indicated. Hoechst dye 33258 was obtained from Sigma-Aldrich (St. Louis, MO); the enhanced chemiluminescence kit was from Amersham (Arlington Heights, IL); mouse anti-pan-Ras (antibody-3) and mouse anti-p53 (antibody-1) antibodies were from Calbiochem (La Jolla, CA); mouse anti-β-tubulin (AK-15) and mouse anti-phosphorylated ERK antibodies were from Sigma-Aldrich; rabbit anti-survivin (FL-142), rabbit anti-E2F1 (C-20), monoclonal mouse anti-E2F1 (KH95), and rabbit anti-caspase-3 (H-277) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-phosphorylated Akt (Ser^{473}; 4E2), rabbit anti-Bad, rabbit anti-phosphorylated Bad (Ser^{136}), rabbit anti-caspase-7, and mouse anti-caspase-9 (C9) antibodies were from Cell Signaling (Beverly, MA); mouse anti-β-catenin antibody was from BD Transduction Laboratories (San Jose, CA); mouse anti–cytochrome *c* (556433) was from BD PharMingen (San Diego, CA); rabbit anti-Bax NH_2-terminal antibody was from Upstate Biotechnology (New York, NY); mouse monoclonal anti-poly(ADP-ribose) polymerase antibody (SA-250) was from Biomol Research Laboratories (Plymouth Meeting, PA); mouse anti-HA-tag antibody (hybridoma supernatant) was a gift from Y. Henis (Tel Aviv University, Tel Aviv, Israel); peroxidase-goat anti-mouse IgG, peroxidase-goat anti-rabbit IgG, and peroxidase-donkey anti-goat IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA); LY294002 and U0126 were from Calbiochem; zVAD (Z-VAD-FMK) and Z-DEVD-FMK were from ICN Biomedicals (Aurora, OH); and QVD (Q-VD-OPH) was from MP Biomedicals (Aurora, OH).

Cell Proliferation and Cell Cycle Assays

For cell proliferation assays, we plated 1.5×10^3 cells in 96-well plates or 3.5×10^3 cells in 24-well plates. The cells were treated, 24 hours after plating, with different concentrations of FTS or with the vehicle (0.1% DMSO). Cell proliferation was determined after 3 days by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, which determines mitochondrial activity in living cells. The cells were incubated with 0.1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for 1 hour at 37°C and then lysed with 100% DMSO. Results were quantified by recording the absorbance at 570 to 630 nm. For fluorescence-activated cell sorting analysis, we plated 1×10^6 cells in 10-cm dishes and treated the cells with 75 μmol/L FTS 24 hours later. Control cells were treated with vehicle only. After 48 hours, the cells were collected and suspended in PBS containing propidium iodide (50 μg/mL; Sigma-Aldrich) and 0.05% Triton X-100 (BDH, Poole, United Kingdom) for nuclear staining and then analyzed with a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ).

Statistical Analysis

All assays were done in triplicate or quadruplicate. Data were expressed as mean values ± SD. Statistical significance was determined by an unpaired Student's *t* test.

Western Immunoblotting and Ras-GTP Assays

To examine the effects of inhibitors on survivin, phosphorylated ERK, phosphorylated Akt, E2F1, p53, and β-catenin, we used lysates of drug-treated or control (vehicle treated) cells and did Western immunoblotting analysis or Ras-GTP assay (16). Ras-GTP was determined by the glutathione *S*-transferase–Ras-binding domain of Raf pull-down assay as described previously (16). Protein bands were visualized by enhanced chemiluminescence and quantified as described previously (16). All biochemical and immunoblotting assays were repeated at least thrice. Data are presented as mean values ± SD.

Immunofluorescence Assays

U87 glioblastoma cells were cultured on glass coverslips and incubated with FTS or vehicle as described above. Alternatively, the cells were transfected with vectors (total amount, 2.3 μg DNA) encoding green fluorescent protein (GFP), GFP-H-Ras(17N), pcDNA3-E2F1, or pcDNA3 (10) using the JetPEI reagent according to the manufacturer's instructions (Talron, Rehovot, Israel). The cells were fixed, permeabilized, and labeled 48 hours after drug treatment or transfection with anti-survivin antibody (5 μg/mL) and Cy3-conjugated donkey anti-rabbit secondary antibody (2 μg/mL), with anti–cytochrome *c* antibody (5 μg/mL) and Cy3-conjugated donkey anti-mouse

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secondary antibody (2 μg/mL), or with rabbit anti-Bax NH_2-terminal antibody and Cy3-conjugated donkey anti-rabbit secondary antibody (2 μg/mL) as described elsewhere (9). The cells were also labeled with Hoechst dye 33258 as described earlier (9). Fluorescence images of survivin, Bax, or cytochrome *c* (Cy3, red), cell nuclei (Hoechst, blue), and GFP or GFP-H-Ras(17N) (green) were collected using a fluorescence microscope (IX70, Olympus America, Melville, NY) as described previously (9).

Plasmids

The expression vectors pRSV-GFP-H-Ras(17N) and pcDNA3-E2F1 have been described previously (10). pcDNA3-HA-survivin plasmid was a gift from D.C. Altieri (University of Massachusetts Medical School, Worcester, MA).

Real-time PCR Analysis

Extracts of total RNA (1 μg) from cells treated for 48 hours with FTS (70 μmol/L) or vehicle (control) were reverse transcribed in a total volume of 20 μL using the iSCRIPT cDNA kit (Bio-Rad Laboratories, Hercules, CA). The cDNA samples were used for real-time PCR (SYBR Green PCR kit, Roche Diagnostics Mannheim, Germany). For the *survivin* gene, we used 5′-ACCACCGCATCTC-TAC3′ (forward primer) and 5′-TCCTCTATGGGGTCGT-3′ (reverse primer); for the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase*, we used 5′-CCAGAACATCA-TCCCTGC-3′ (forward primer) and 5′-GGAAGGCCATGC-CAGTGAGC-3′ (reverse primer). PCR amplifications were carried out using the LightCycler version 3.5 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. cDNA samples of control and FTS-treated cells (at least two dilutions for each) and cDNA standards were coamplified in the same reaction plate. The standard curve showed a linear relationship between the cycle threshold (C_T) values and the cDNA concentration. The relative expression of each gene was normalized using the expression levels of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* as standard.

Results

FTS Decreases the Level of Survivin Transcripts in U87 Cells

A previous study by our group showed that the Ras inhibitor FTS induces marked changes in gene expression, as evident from DNA microarray analysis of cell samples tested at zero time and after drug treatment for 24 and 48 hours or of vehicle-treated controls (9). In the earlier experiments, we focused on two groups of genes (i.e., HIF-1α-regulated and E2F1-regulated genes) that were down-regulated by the Ras inhibitor (9, 10). In the present study, we focused on a third group whose expression was altered by the FTS treatment (i.e., a group of genes that are associated with cell death mechanisms; Fig. 1A). Heat-map image of the 30 death-associated genes that were up-regulated and of the 23 death-associated genes that were down-regulated by the FTS treatment is shown in Fig. 1A. Within this group, the gene whose expression was most dramatically altered was *survivin* (BIRC5). At 24 and 48 hours of FTS treatment, *survivin* expression was found to be decreased by 2.3-fold (log ratio, −1.2) and 78.8-fold (log ratio, −6.3), respectively (Fig. 1A and B). In addition, there were alterations in the expression of transcription factors that control survivin transcription (17–19), including E2F1, p53, β-catenin, and TCF4 (Fig. 1B). Although we found additional transcriptional changes in death-associated genes (e.g., in Bax, caspase-1, caspase-4, caspase-5, SKIP3, TRADD, and TRAF1; Fig. 1A), the dramatic change observed in survivin expression and the changes in its regulators led us to suspect that the observed FTS-induced glioblastoma cell death (9) might involve mechanisms associated with survivin. Real-time PCR analysis validated the gene expression results and showed that FTS (75 μmol/L; 48 hours of treatment) induced a decrease of 26 ± 0.006–fold in the expression of *survivin* (Fig. 1C).

FTS or Dominant-Negative Ras Reduces Survivin in Glioblastoma Cells

Next, we examined whether the FTS-induced decrease in *survivin* expression was also manifested at the protein level. The effect of FTS on the amounts of survivin in U87 cells was determined by Western immunoblotting with anti-survivin antibody. The cells were treated with 75 μmol/L FTS and survivin was assayed at various times after the treatment (see Materials and Methods). To confirm anti-Ras activity, we assayed active Ras-GTP in the same samples. In agreement with earlier studies (9, 10), we found that FTS induced a time-dependent decrease in Ras-GTP (Fig. 1D); concomitant with this decrease, we found a time-dependent decrease in survivin protein (Fig. 1D). Importantly, in accord with the observed decrease in survivin mRNA (Fig. 1C), a 75% decrease in survivin protein was observed at 36 hours of FTS treatment, a time point at which the recorded sub-G_1 population of the cells was only 11% of the total cell population (9). This indicated that the decrease in survivin precedes death of the cells. Survivin levels continued to decrease, becoming almost undetectable at 48 hours of FTS treatment (Fig. 1D).

We next determined the effect of FTS on the amounts of survivin in three additional glioblastoma multiforme cell lines: DBTRG-05MG (20/20), LN229, and U373. The cells were incubated with 75 μmol/L FTS for 48 hours and then lysed and subjected to Western immunoblot analysis with anti-survivin antibody. Treatment with FTS resulted in a dramatic decrease in survivin in all cell lines, with the amounts of survivin recorded in 20/20, LN229, and U373 cells corresponding respectively to 15%, 2%, and 4% of that found in the untreated control cells (Fig. 2A). These results indicated that the effect of FTS on survivin is not restricted to U87 cells. Interestingly, U87 and 20/20 cells expressed wild-type p53, whereas LN229 and U373 cells expressed mutant p53. It therefore seemed that the FTS-induced down-regulation of survivin in glioblastoma multiforme might proceed by p53-independent mechanisms.

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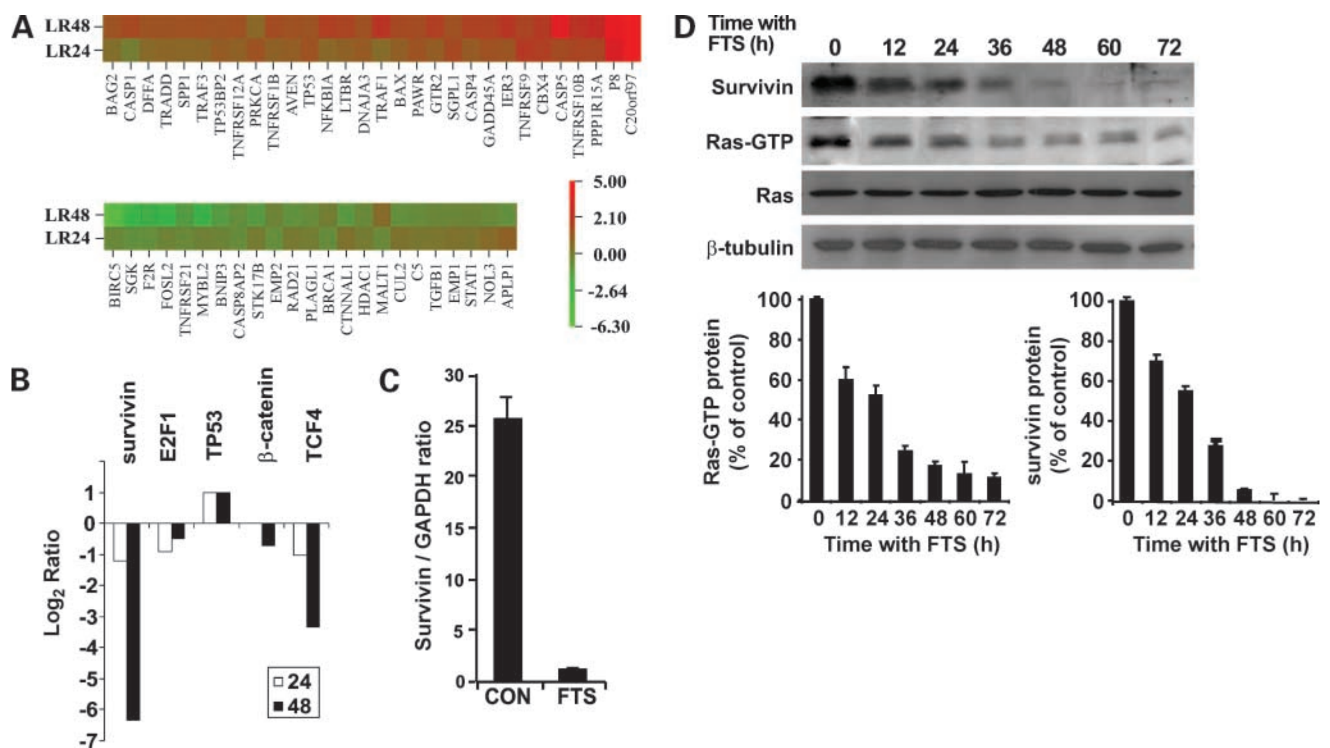

Figure 1. Down-regulation of survivin expression in U87 cells by the Ras inhibitor FTS. **A,** heat-map image depicts changes in death-associated gene expression following FTS treatment. Levels of expression of the indicated genes were determined by microarray analysis of control and FTS-treated cells (described in http://www.eng.sheba.co.il/genomics). *Bottom, right,* base-two logarithmic ratios of mRNA hybridization signal intensities of FTS-treated cell samples (75 μmol/L FTS for 24 or 48 h) and mRNA hybridization signals of control cell samples at 24 (*LR24*) and 48 (*LR48*) h (color code). The map was generated using the clustered image map miner tool (http://www.discover.nci.nih.gov). **B,** effects of FTS on transcription of survivin and its transcriptional regulators. Data generated as detailed above are presented as logarithmic ratios. **C,** real-time reverse transcription-PCR analysis of survivin transcripts in control and FTS-treated cells (75 μmol/L FTS; 48 h). The level of survivin transcripts was normalized to the expression of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; a housekeeping gene). Data are expressed as ratios of survivin to glyceraldehyde-3-phosphate dehydrogenase. **D,** FTS induces a time-dependent decrease in survivin protein. Cells were incubated with 75 μmol/L FTS for the indicated times and then lysed and subjected to Western immunoblotting with anti-survivin antibody. Lysates were also subjected to the Ras-GTP – Ras-binding domain pull-down assay followed by Western immunoblotting with anti-Ras antibody. Samples of total cell lysates were also immunoblotted with pan anti-Ras and anti-β-tubulin antibodies (loading control). Immunoblots of a typical experiment. Results of densitometric analysis. *Columns,* mean (n = 3); *bars,* SD.

We next examined whether the FTS-induced decrease in survivin was a consequence of FTS-induced growth arrest or of inhibition of Ras-dependent pathways that control survivin expression (20). We did this by attenuating the growth of U87 cells by lowering serum concentrations and assaying survivin in the growth-attenuated cells. Under the conditions used in these experiments, no cell death was observed. Growth of U87 cells in 1% serum was, however, reduced by ~2-fold relative to their growth in 20% serum (Fig. 2B). Despite this reduction in the rate of cell proliferation, the survivin levels remained relatively high (Fig. 2B), suggesting that survivin expression in U87 cells may not be dependent on the rate of U87 cell growth. Thus, FTS-induced inhibition of cell growth (10) is evidently not more than partially responsible, if at all, for the drug-induced decrease in survivin (Fig. 1C). This is consistent with earlier studies, which showed that survivin expression induced by active H-Ras is independent of cellular proliferation (20).

We next examined the effect of Ras inhibition on survivin by using an independent approach to inhibit Ras [i.e., by the use of GFP-tagged H-Ras(17N), a dominant-negative Ras isoform]. U87 cells were transfected with GFP-H-Ras(17N) or with GFP vector as a control. The cells were then stained with rabbit anti-survivin antibody and then with Cy3-conjugated anti-rabbit antibody. Typical dual fluorescence images (shown in Fig. 2C) show that, compared with the levels of survivin (red, Cy3 fluorescence) in both the cytoplasm and the nucleus of the control GFP-expressing cells (green fluorescence), the corresponding levels in the cells expressing GFP-H-Ras(17N) were much lower. GFP-H-Ras(17N) transfectants with no nuclear survivin accounted for as much as 80% of the total number of GFP-H-Ras(17N)-expressing cells compared with only 20% for the control GFP transfectants (Fig. 2C). Taken together, these experiments showed that inhibition of Ras by two independent methods [i.e., FTS treatment and GFP-H-Ras(17N) expression] leads to down-regulation of survivin in glioblastoma multiforme cells.

Inhibitors of Ras Pathways Mimic the Effect of FTS on Survivin in U87 Cells

Because both the Ras/Raf/MEK/ERK pathway and the Ras/PI3K/Akt/glycogen synthase kinase-3 pathway

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participate in the regulation of survivin expression (20), we next examined the effects of inhibitors of these pathways on the levels of survivin in U87 cells. The cells were incubated for 24 hours with the MEK inhibitor U0126 or with the PI3K inhibitor LY294002 and then assayed for phosphorylated ERK, phosphorylated Akt, and survivin. As expected, U0126 (15 or 30 μmol/L) decreased phosphorylated ERK and LY294002 (20 or 40 μmol/L) decreased phosphorylated Akt (Fig. 3). Both inhibitors also induced a concentration-dependent decrease in survivin. Evidently, at the higher drug concentrations, survivin expression was completely abolished (Fig. 3). These results, along with earlier demonstrations that FTS treatment of a variety of glioblastoma multiforme cell lines inhibits the activation of ERK and Akt (9, 13), strongly suggested that the FTS-induced inhibition of *survivin* expression is associated with down-regulation of the Raf/MEK/ERK and PI3K/Akt/glycogen synthase kinase-3 Ras pathways.

Ras-Dependent Regulators of Survivin Are Affected by FTS in U87 Cells

As pointed out above, the expression of several regulators of survivin transcription, all of which are reportedly controlled by Ras signaling pathways (17–19), were altered by FTS in U87 cells (Fig. 1B). These regulators include E2F1 (17), p53 (18), and β-catenin (19). E2F1 binds to the survivin promoter and acts as a positive regulator of *survivin* expression (17). Studies by our group showed that FTS down-regulates E2F1 transcriptional activity (10). It was therefore reasonable to postulate that FTS down-regulates survivin by an E2F1-dependent mechanism. To investigate this possibility, we used U87 cells with forced E2F1 expression (U87-E2F1 cells), in which the high levels of E2F1 prevented FTS from inhibiting cell cycle progression (10). Unlike in U87 cells, in which the Ras-controlled E2F1 expression is disrupted by FTS (see also Fig. 4A, *top*; ref. 10), in U87-E2F1 cells, FTS had only a minor effect on the levels of E2F1 (Fig. 4A). We were therefore able to compare the effects of FTS on survivin levels in U87 cells and in U87-E2F1 cells, in which the amounts of E2F1 are not altered by FTS.

Two types of experiments were done. First, we determined the amounts of survivin by Western immunoblotting with anti-survivin antibody. As expected (17), the forced expression of E2F1 caused marked up-regulation of survivin (Fig. 4B). Treatment with 75 μmol/L FTS for

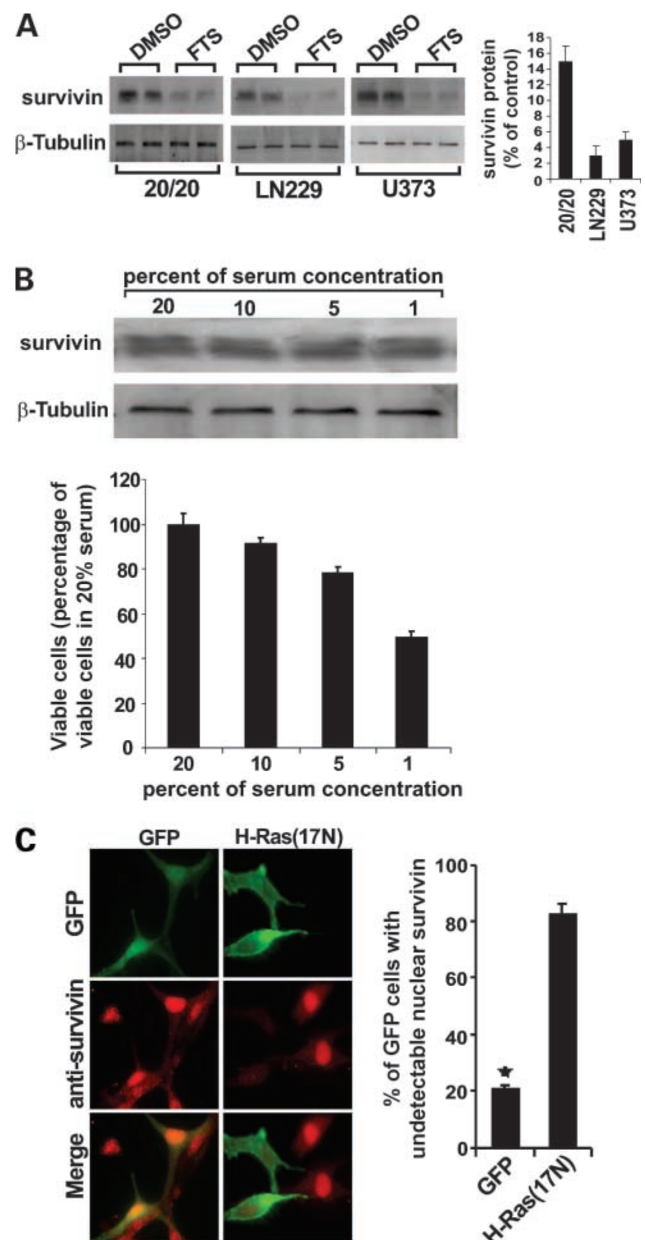

Figure 2. Ras inhibition by FTS or dominant-negative Ras down-regulates survivin. **A,** U373, 20/20, and LN229 glioblastoma multiforme cells were grown with or without 75 μmol/L FTS for 48 h and then processed for the determination of survivin by immunoblotting as described in Fig. 1D. Samples of total cell lysates were also immunoblotted with anti-β-tubulin antibody as a loading control. Results of a representative experiment, all of which were done at least thrice. *Right,* analysis of the immunoblots by densitometry. *Columns,* mean (n = 3); *bars,* SD. **B,** survivin expression remains relatively high in U87 cells grown under growth-limiting conditions. Cells were cultured for 3 d in DMEM containing 20%, 10%, 5%, or 1% FCS. The cultures were then subjected to the assay of survivin in cell lysates or determination of viable cells. *Top,* total lysates were subjected to Western blot analysis with antibodies against survivin and β-tubulin as a loading control; *bottom,* the apparent number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are expressed in terms of viable cells in each of the specified conditions relative to that determined in cultures grown with 20% FCS. *Columns,* mean (n = 6); *bars,* SD. **C,** dominant-negative Ras down-regulates survivin in U87 glioblastoma multiforme cells. U87 cells were transfected with 2 μg GFP-H-Ras(17N) or with 0.3 μg GFP and 2 μg empty pcDNA3 vector (control). The cells were fixed and permeabilized 48 h later. They were then labeled with anti-survivin antibody and visualized under a fluorescence microscope as described in Materials and Methods. *Left,* typical dual images of fluorescence-labeled survivin (Cy3; *red*) and of the GFP fluorescence (*green*); *right,* the percentage of GFP-labeled cells with undetectable nuclear survivin was calculated for the GFP control and for the GFP-H-Ras(17N) transfectants. *Columns,* mean of five samples in each case, with 100 cells per sample scored for low levels of nuclear survivin; *bars,* SD. *, $P < 0.005$, significantly different from control, Student's t test.

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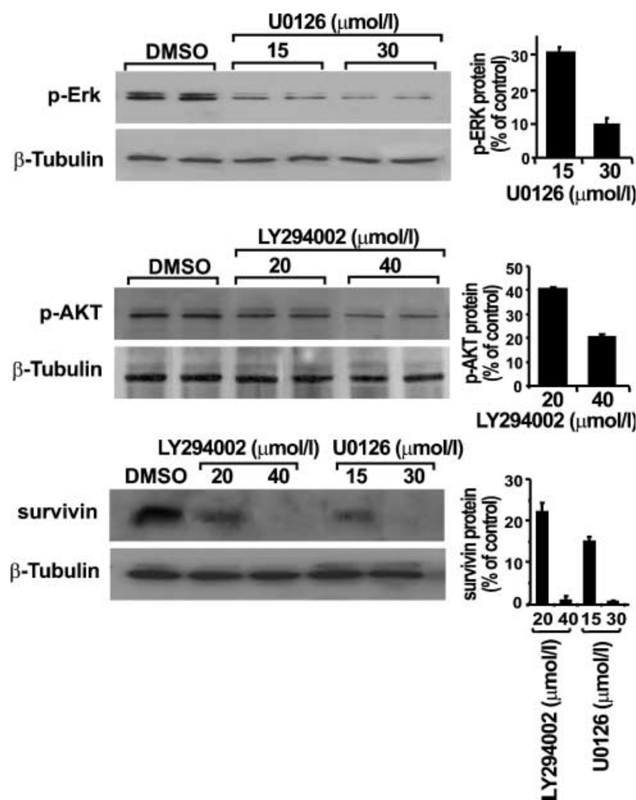

Figure 3. Inhibitors of PI3K and of MEK1 decrease survivin in U87 cells. U87 cells were grown for 24 h in the presence and absence of MEK1 inhibitor U0126 (15 or 30 μmol/L) or of the PI3K inhibitor LY294002 (20 or 40 μmol/L). Cell lysates were subjected to Western blot analysis using anti-survivin, anti-phosphorylated Akt, and anti-phosphorylated ERK antibodies or anti-β-tubulin antibodies as described in Fig. 1D. Immunoblots of representative experiments, each done thrice. *Right,* densitometric analysis of the immunoblots. *Columns,* mean (n = 3); *bars,* SD.

48 hours, however, caused a marked decrease in survivin not only in U87 cells but also in U87-E2F1 cells (Fig. 4B). Second, we cotransfected U87 cells with a 6-fold excess of E2F1 and GFP (to distinguish between transfected and nontransfected cells) and then did immunofluorescence analysis of controls and FTS-treated cells using anti-survivin antibody. The results of these experiments showed that there was more survivin (red fluorescence) in the nuclei of cells expressing excess E2F1 (cells labeled with green fluorescence) than in the nontransfected cells (without green fluorescence; Fig. 4C). The percentage of E2F1-expressing cells with relatively large amount of nuclear survivin was 89% and that of the nontransfected cells was 55% (Fig. 4C). Here too, treatment with FTS strongly decreased survivin both in cells overexpressing E2F1 and in the nontransfected cells (Fig. 4C). Taken together, these experiments showed that FTS induces down-regulation of survivin even under conditions, in which it does not cause a significant reduction in E2F1. It therefore seems that the effect of FTS on survivin expression might be mediated by its effect not only on E2F1 but also on other regulators of survivin.

Next, we examined the effects of FTS on two additional regulators of survivin: p53, which inhibits *survivin* gene expression (18), and β-catenin, which positively regulates *survivin* gene expression (19). We treated U87 cells with 75 μmol/L FTS for 24, 48, or 72 hours and then used Western immunoblotting, using anti-p53 and anti-β-catenin antibodies, to determine the amounts of p53 and β-catenin. The p53 protein showed a 2-fold increase 24 hours after FTS treatment and remained at this relatively high level 48 and 72 hours after treatment (Fig. 4D). This finding suggested that p53 could potentially be involved in the drug-induced down-regulation of survivin. The amount of β-catenin was not altered 24 hours after the treatment with FTS (Fig. 4D), a time point at which survivin levels were already markedly decreased (by 40%; Fig. 1D). However, a decrease in β-catenin was observed 48 hours after the FTS treatment (Fig. 4D). Thus, the early effect of FTS on survivin does not seem to be associated with β-catenin regulation.

FTS-Induced Apoptotic Cell Death in U87 Cells Is Blocked by Constitutive Expression of Survivin

Next, we examined whether the FTS-induced down-regulation of survivin is associated with the drug-induced death of U87 cells. For this purpose, we established U87 cell lines with forced expression of HA-tagged survivin and compared the effect of FTS on the death of U87 and of U87-survivin cells. Two U87-survivin cell lines were established, the one overexpressing HA-survivin (U87-survivin-H; Fig. 5A, *top, left* and *right*) and the other expressing HA-survivin at levels comparable with the levels of endogenous survivin (U87-survivin-L; Fig. 5A, *bottom, left*). Immunofluorescence analysis with anti-survivin antibody showed strong nuclear localization of survivin in the U87-survivin-H cells and no effect of FTS (75 μmol/L; 48 hours) on this localization or on the total amount of survivin in the cells (Fig. 5A, *bottom*). These results were confirmed by Western immunoblot analysis, which showed that the FTS treatment did not affect the amounts of the abundant HA-tagged survivin in these cells (Fig. 5A, *top*). As expected, the treatment caused a marked decrease in endogenous survivin in both U87 and U87-survivin-H cells (Fig. 5A, *top*). FTS treatment caused the death of U87 cells (>40% of the cells exhibited a subdiploid DNA content, sub-G_1 phase) but not of U87-survivin-H cells (7.5% dead cells; Fig. 5B, *left*). Consistent with these observations, live cells accounted for 40% of the FTS-treated U87 cells but 90% of the U87-survivin-H cells (Fig. 5B, *right*). Similar results were obtained with U87-survivin-L cells, which were partially protected from FTS-induced cell death (Fig. 5B, *left* and *right*). This showed that maintenance of HA-survivin at levels comparable with those of wild-type survivin is sufficient to attenuate the FTS-induced death of U87 cells. Taken together, these results then showed that the death of U87 cells induced by FTS is overridden by forced expression of survivin, suggesting that the down-regulation of survivin by FTS is associated with the drug-induced cell death.

To further substantiate this suggestion, we studied the effect of FTS on apoptosis in U87 cells because survivin,

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like other members of the inhibitor of apoptosis protein family of proteins, can act as an antiapoptotic factor, which, *inter alia*, inhibits caspase-3 and caspase-7 and was shown to inhibit caspase-9-dependent apoptosis (21–24). U87 cells were treated with 75 μmol/L FTS for 48 hours and then double stained with Hoechst to label the cell nuclei (blue) and with rabbit anti-survivin antibody followed by Cy3-conjugated anti-rabbit antibody (red, Cy3 fluorescence). Typical dual fluorescence images (Fig. 6A) show Hoechst staining of round intact nuclei of the control cells with large amounts of survivin. In marked contrast, Hoechst staining of the FTS-treated cells showed fragmented and condensed nuclei (and smaller amounts of survivin) typical of apoptotic nuclei (Fig. 6A). Quantitative analysis of the results showed that FTS induced an increase of 80% in apoptotic nuclei and a decrease of 85% in survivin (Fig. 6A). These experiments suggested that FTS induces apoptotic death of U87 cells.

To further support this possibility, we examined the effect of caspase inhibitors on the FTS-induced death of U87 cells. The results showed that zVAD (Z-VAD-FMK), a broad-spectrum inhibitor of caspases, or QVD (Q-VD-OPH), a pan-caspase inhibitor, exerted a marked protective effect against the FTS-induced death of U87 cells (Fig. 6B). Evidently, in the presence of these inhibitors, some 60% to 70% of cells survived. Treatment of the cells with a specific

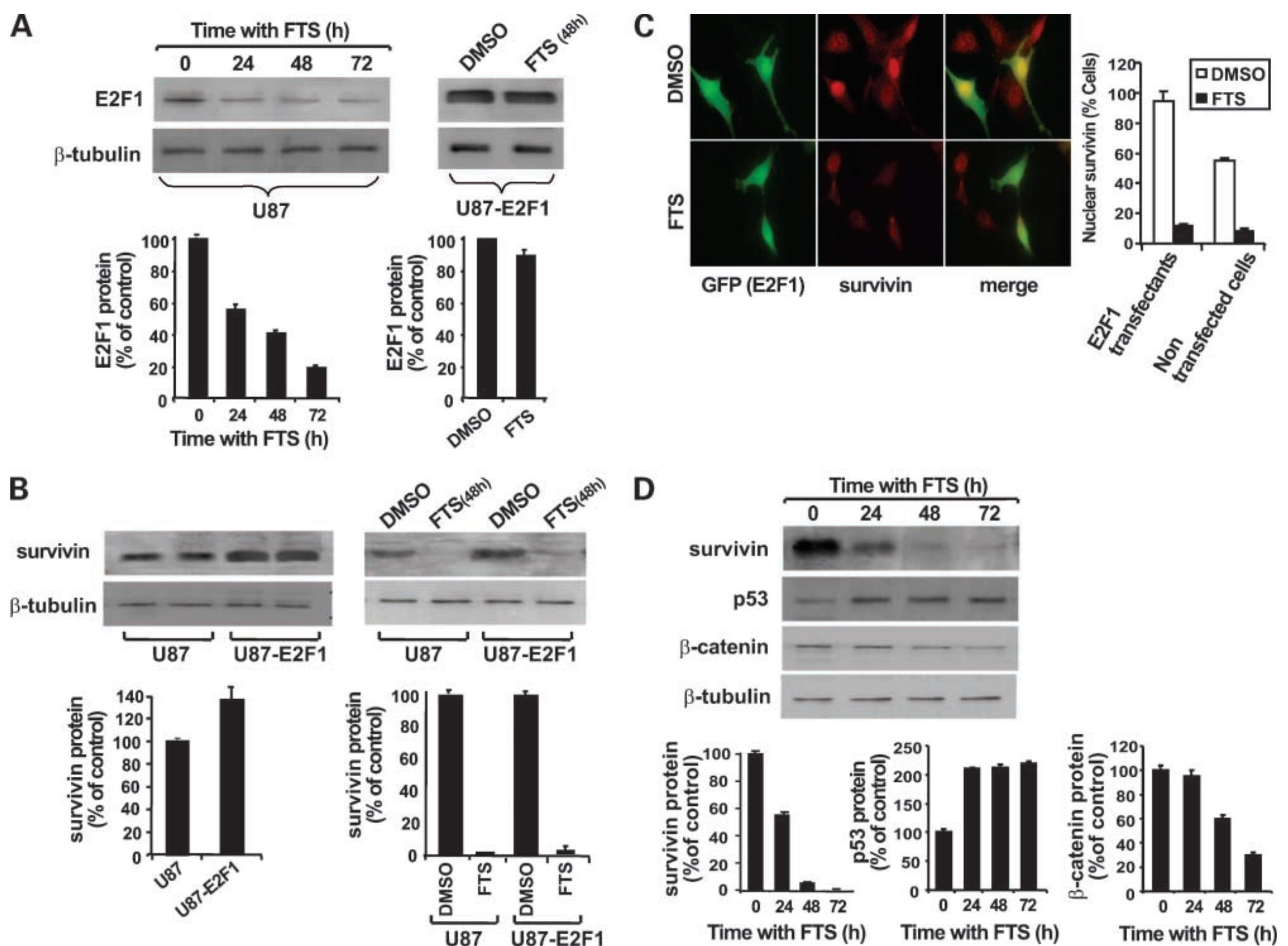

Figure 4. Ras-dependent regulators of survivin are affected by FTS in U87 cells. **A** and **B,** constitutive expression of E2F1 does not prevent the FTS-induced down-regulation of survivin. U87 cells and U87 cells constitutively expressing E2F1 (U87-E2F1) were incubated with or without 75 μmol/L FTS for the indicated periods (**A**) or for 48 h (**B**) and then lysed and subjected to Western immunoblot analysis with anti-E2F1 or anti-survivin antibody as described in Fig. 1D. Immunoblots of representative experiments, all of which were done thrice. *Bottom,* densitometric analysis of the immunoblots. *Columns,* mean (n = 3); *bars,* SD. **C,** transient expression of E2F1 in U87 cells does not prevent the FTS-induced decrease in survivin. U87 cells were cotransfected with E2F1 and GFP (6-fold excess of E2F1 over GFP). The cells were treated, 24 h after transfection, with 75 μmol/L FTS (for 48 h) and then fixed and labeled with anti-survivin antibody as described in Fig. 2C. Dual images of fluorescence labeled survivin (Cy3; *red*) and of GFP fluorescence (*green*), which specifies the E2F1 transfectants. *Right,* numbers of cells with nuclear survivin relative to the total number of E2F1-overexpressing cells (GFP labeled) and the number of cells with nuclear survivin relative to the total number of nontransfected cells (not labeled with GFP). *Columns,* mean of 100 cells scored in each category; *bars,* SD. **D,** time-dependent effects of FTS on the amounts of survivin and p53 and β-catenin in U87 cells. U87 cells were incubated with or without 75 μmol/L FTS for the indicated times and then subjected to Western blot analysis with anti-survivin, anti-p53, and anti-β-catenin antibodies as described in Fig. 1D. Immunoblots of a typical experiment. *Bottom,* densitometric analysis of the immunoblots. *Columns,* mean (n = 3); *bars,* SD.

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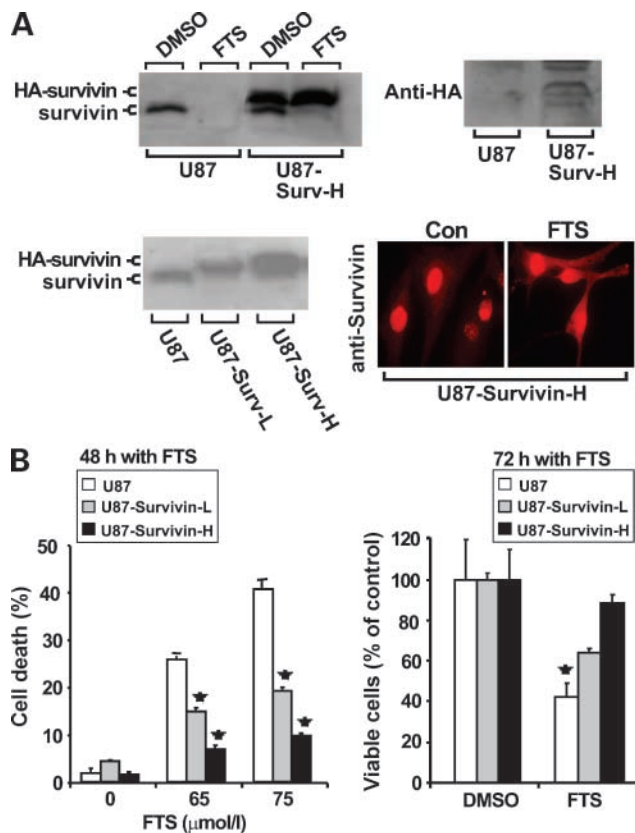

Figure 5. Forced expression of survivin overrides FTS-induced death of U87 cells. **A,** U87 cells and U87 cells constitutively expressing relatively high or relatively low levels of HA-tagged survivin (U87-survivin-H and U87-survivin-L cells, respectively) were treated for 48 h with 75 μmol/L FTS or left untreated and then subjected either to Western blot analysis with anti-survivin (*top, left* and *bottom, left*) or anti-HA antibody (*top, right*) or to immunofluorescence analysis with anti-survivin antibody (*bottom, right*). **B,** U87 (*white columns*), U87-survivin-H (*black columns*), and U87-survivin-L (*gray columns*) cells were treated with FTS as in (**A**) and then subjected to determination of cell death (sub-G_1 population) by fluorescence-activated cell sorting analysis [the data are expressed in terms of sub-G_1 population as a percentage of the total cell population (*left*)] and to the determination of live cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method [FTS-treated cells expressed as a percentage of untreated cells (*right*)]. *Columns,* mean (n = 3); *bars,* SD. *, $P < 0.05$, significantly different from control, Student's t test.

caspase-3 inhibitor (Z-DEVD-FMK) also resulted in their protection from FTS-induced cell death but to a smaller extent than that observed with the general caspase inhibitors (Fig. 6B). Taken together, these results suggested that FTS induces the caspase-dependent apoptotic death of U87 cells. Consistent with this possibility, we found that FTS induces cleavage of procaspase-3 and procaspase-7 and of their upstream activator procaspase-9, resulting in the appearance of the corresponding cleavage products, which are known as active caspases (Fig. 6C). In addition, we found that FTS induces cleavage of poly(ADP-ribose) polymerase, one of the death substrates that is cleaved by caspase-3 (Fig. 6C). Importantly, the FTS-induced cleavage of the caspases and of poly(ADP-ribose) polymerase was completely blocked by forced expression of survivin (Fig. 6C). Because release of cytochrome *c* from the mitochondria is essential for apoptosome formation, which leads to the activation of caspase-9, we next examined the effect of FTS on the proapoptotic molecules Bad and Bax, known to promote cytochrome *c* release. Bad is an ''enabler'' of cytochrome *c* release because it neutralizes Bcl2 or Bcl-xL, and the neutralization then enables Bax to activate cytochrome *c* release from the mitochondria (25). Bad is active in its nonphosphorylated form and is inactivated by Akt-mediated phosphorylation on Ser^{136} (26). Consistent with the finding that FTS induces inhibition of PI3K activation and phosphorylation of Akt (9), we observed a decrease in phosphorylated Bad (Ser^{136}) in U87 cells that were incubated with 75 μmol/L FTS for 48 and 72 hours (Fig. 6C). We then did immunofluorescence experiments in the FTS-treated U87 cells using an anti-Bax NH_2-terminal antibody that recognizes the NH_2 terminus of the open, active conformer of Bax, leading to release of cytochrome *c* from the mitochondria (27). The results of these experiments showed positive staining with the anti-Bax NH_2-terminal antibody, indicating that FTS induces activation of Bax (Fig. 6D). Consistent with the observed activation of Bax and Bad, we found that FTS induces cytochrome *c* release from the mitochondria of U87 cells (Fig. 6D).

Discussion

The results of this study showed that the Ras inhibitor FTS causes a decrease in the level of *survivin* transcripts in U87 glioblastoma multiforme cells (Fig. 1B and C), with a concomitant marked decrease in survivin protein (Fig. 1D). Decrease in *survivin* gene transcripts, together with the short half-life of survivin (which is rapidly degraded by the proteasome; ref. 28), can explain the observed reduction in survivin in FTS-treated U87 cells. Consistent with the notion that the decrease in survivin in FTS-treated U87 cells is mainly, if not entirely, attributable to decrease in the levels of survivin mRNA are the results obtained with U87-survivin cells, in which survivin expression is not under the control of transcription factors that bind to the survivin promoter. In these cells, FTS induced down-regulation of the endogenous but not of the forcibly expressed survivin (Fig. 5A). The effect of FTS on survivin was mimicked by dominant-negative Ras (Fig. 2C) and by inhibitors of the Raf/MEK/ERK and the PI3K/Akt pathways (Fig. 3).

These results are consistent with the conclusion that the observed FTS-induced inhibition of Ras and the consequent inhibition of activation of the Ras-dependent ERK and Akt (Fig. 1; ref. 9) are associated with the drug-induced down-regulation of survivin expression in U87 cells. Other studies showed, in addition, that MEK and PI3K inhibitors induce down-regulation of survivin in other cell lines (20). In line with this assertion, earlier studies showed that the major factors regulating *survivin* transcription positively (i.e., E2F1 and β-catenin; refs. 17, 19) or negatively (i.e., p53;

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ref. 18) are controlled by these Ras pathways. Moreover, in agreement with previous results in other tumor cell lines (29) and in glioblastoma multiforme (10), we showed that FTS induces down-regulation of E2F1 and up-regulation of p53 (Fig. 4A and D). In addition, FTS induced down-regulation of β-catenin (Fig. 4D). It seems, however, that neither of these regulators by itself is essential for down-regulation of survivin by FTS. This is because (*a*) FTS down-regulated survivin in glioblastoma multiforme cells with or without p53 mutation (Fig. 2A), (*b*) FTS down-regulated survivin expression in U87 cells with forced E2F1 expression (Fig. 4B), and (*c*) FTS down-regulation of survivin preceded the decrease in β-catenin (Fig. 4D). Thus, apparently, a Ras-regulated factor, as yet unknown, controls *survivin* transcription in glioblastoma multiforme.

Survivin has been implicated in the inhibition of apoptosis and is associated with reduced apoptotic index in human tumor cells (30). Survivin was found to be up-regulated in various human cancers, including neuroblastomas (31), bladder cancer (32), colon/colorectal cancer (33), gastric cancer (34), hepatocellular carcinomas (35), and human gliomas (36). Survivin, like other mammalian inhibitor of apoptosis proteins (37), can bind to caspase-3 and caspase-7 (21). Studies with a transgenic mouse model that selectively expresses survivin in the skin confirmed that this protein inhibits the intrinsic, caspase-9-dependent apoptotic pathway (22). Accumulating data point to the participation of survivin both in cell cycle progression during mitosis through its association with the mitotic spindle (38) and in blocking of apoptosis through its inhibitory effects on caspase-3, caspase-7, and caspase-9

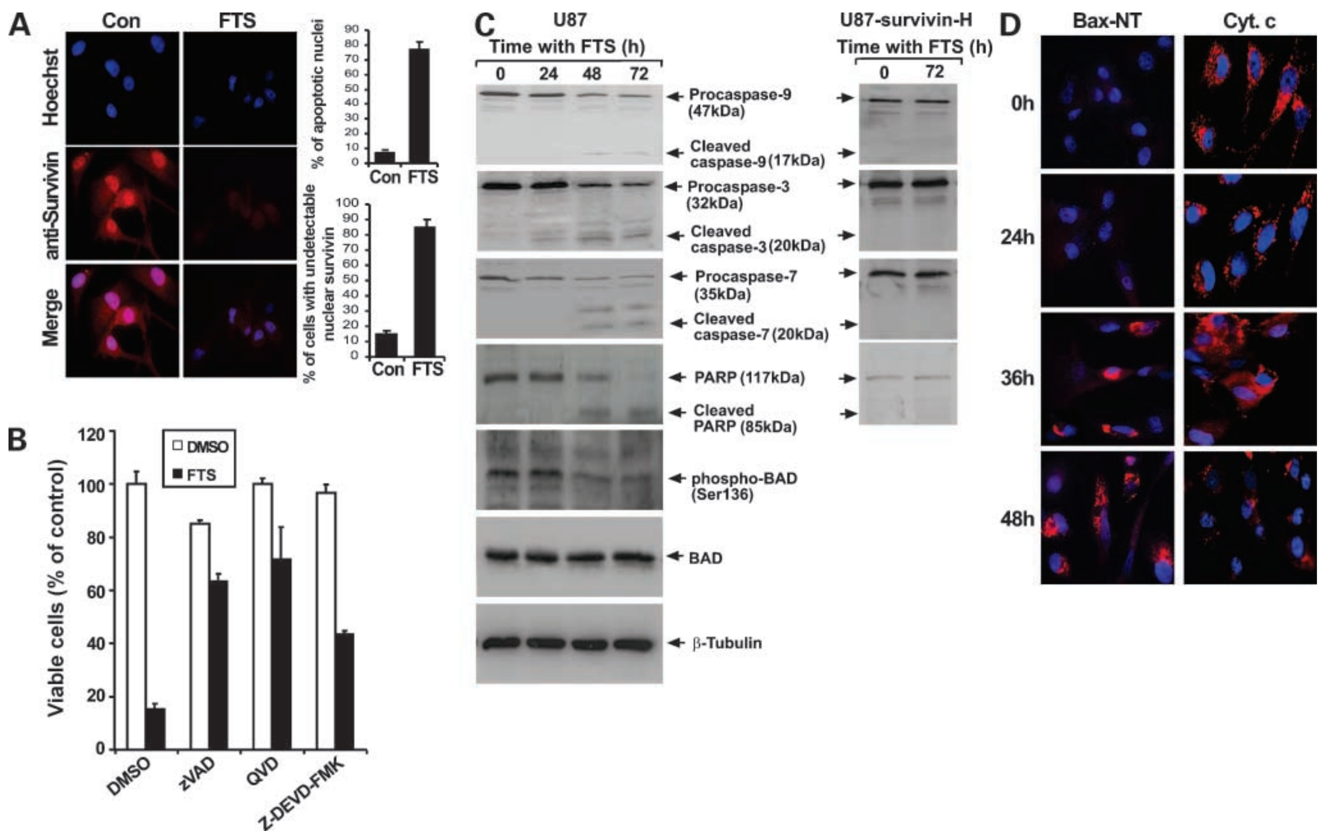

Figure 6. FTS induces caspase-dependent apoptotic cell death of U87 cells. The cells were incubated with or without 75 μmol/L FTS for 72 h or as indicated and then subjected to the assays as specified. **A,** cells were stained with Hoechst and with anti-survivin antibody as described in Fig. 2C. Dual images of fluorescence-labeled survivin (Cy3; *red*) and of the Hoechst-stained nuclei (*blue*) recorded in a typical experiment. *Right,* statistical analysis of the data. *Top,* number of cells with apoptotic nuclei expressed as a percentage of the total number of cells; *bottom,* number of cells with no nuclear survivin expressed as a percentage of the total number of cells as in Fig. 2C. *Columns,* mean (n = 100 cells); *bars,* SD. **B,** caspase inhibitors attenuate FTS-induced death of U87 cells. Two hours before treatment with FTS, the cells were treated with either the vehicle or the caspase inhibitors zVAD (20 μmol/L), QVD (20 μmol/L), or Z-DEVD-FMK (100 μmol/L). Cell viability was then determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Data are presented as percentages of control treatment (without drugs). *Columns,* mean (n = 3); *bars,* SD. **C,** FTS induces cleavage of procaspases and poly(ADP-ribose) polymerase (*PARP*) and a decrease in phosphorylated Bad. Lysates of control and FTS-treated U87 or U87-survivin-H were subjected to Western blot analysis with anti-caspase-9, anti-caspase-3, anti-caspase-7, anti-poly(ADP-ribose) polymerase, anti-phosphorylated Bad, anti-Bad, and anti-β-tubulin antibodies as described in Fig. 1D. Immunoblots of a typical experiment. Similar results were obtained in three independent experiments. **D,** FTS induces Bax activation and cytochrome *c* release. Cells were incubated for the indicated times time with or without FTS and then labeled with Hoechst (*blue*) and anti-Bax NH_2-terminal antibody (*red*) or with Hoechst and anti – cytochrome *c* antibody (*red*). (*red*). Typical dual fluorescence images. Similar results were obtained in three independent experiments.

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(23, 24). Our results showed that the down-regulation of survivin by FTS in U87 cells is accompanied by caspase-mediated apoptotic cell death (Figs. 5 and 6), which is rescued by forced expression of survivin (Fig. 5). These results strongly suggested that the FTS-induced apoptosis in U87 cells is mediated, at least in part, by deregulation of survivin and facilitation of the intrinsic caspase-dependent pathways. Caspase inhibitors indeed attenuated the FTS-induced death of U87 cells (Fig. 6B). Consistent with this notion, previous studies showed that dominant-negative (T34A) survivin (39) or survivin antisense oligonucleotides (40) induce caspase-dependent apoptosis in glioblastoma multiforme. Down-regulation of survivin in other human tumor cell lines also resulted in increased apoptosis (41). From all of the above, it seems that inhibition of Ras can override deregulated survivin functions in cancer cells, thereby relieving the survivin-associated blockage (''braking'') of cell death.

The relief of survivin inhibition of intrinsic apoptotic pathways under certain circumstances or a genetic background seems to be sufficient to facilitate tumor cell death (42). It is most likely, however, that this effect operates because it is accompanied by mechanisms promoting activation of death pathways or preventing survival signals. Such mechanisms can operate both extracellularly and intracellularly. It is important to note that, because active Ras controls multiple targets, including intrinsic apoptotic pathways, the Ras inhibition in glioblastoma multiforme that leads to cell death (Figs 5 and 6) results both in inhibition of survivin expression and in activation of intrinsic apoptotic pathways. Inhibition of Ras and its pathways in U87 cells (9) was indeed accompanied by activation of Bad and Bax, leading to release of cytochrome *c* from the mitochondria (Fig. 6). Interestingly, microarray analysis enabled us to detect an increase (2.6-fold) in Bax in FTS-treated U87 cells.[3]

It should be emphasized that inhibition of Ras by FTS or by dominant-negative Ras leads to proteasomal degradation of HIF-1α (9) and at the same time to inhibition of *survivin* transcription. The loss of HIF-1α has numerous consequences, including glycolysis shutdown and promotion of cell death. Indeed, ATP production is dramatically reduced in FTS-treated U87 cells (9). Recent experiments showed that inhibition of HIF-1α by phosphorothioate antisense HIF-1α oligonucleotide induces p53-independent apoptosis in U87 cells (12). Other studies showed that knockout of HIF-1α gene expression via antisense HIF-1α or by siRNA treatment induces apoptosis of human squamous cell carcinoma (43). These effects might be associated with the lack of glycolysis in tumors cells, in which HIF-1α was down-regulated; recent experiments showed that direct inhibition of glycolysis in human lymphoma and leukemia cells caused dephosphorylation of Bad, leading to translocation of Bax to the mitochondria, loss of mitochondrial membrane integrity, release of cytochrome *c*, and activation of apoptotic cascades (44). Such a mechanism whereby mitochondrial membrane integrity is lost may also operate in FTS-treated U87 cells, in which FTS down-regulates HIF-1α and blocks glycolysis. Consistent with this possibility, we showed that FTS inhibits PI3K and Akt phosphorylation, inhibits Bad phosphorylation, and induces Bax translocation to the mitochondria (Fig. 6). In addition, we showed earlier that FTS down-regulates expression the HIF-1α-controlled glucose transporter Glut1 (9). Glut1 was shown to prevent metabolism-sensitive conformational changes in Bax and thus to inhibit cell death (45). Other studies showed that expression of HIF-1α closely correlates with expression of survivin (46, 47). Thus, reduced expression of HIF-1α seems to induce Bax activation and at the same time to reduce expression of survivin. More experiments are needed to tell whether the FTS-induced down-regulation of HIF-1α (9) and of survivin are dependent events.

In conclusion, we propose that the strong apoptotic cell death induced by FTS in glioblastoma multiforme could be the result of both relief of the apoptosis ''brake'' (survivin) and induction of intrinsic apoptotic pathways possibly associated with the disappearance of HIF-1α (9). The results described here nonetheless provide a strong rationale for the potential use of FTS as treatment modality for glioblastoma multiforme.

[3] http://www.eng.sheba.co.il/genomics.

Acknowledgments

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