Transforming Growth Factor β Induces Apoptosis through Repressing the Phosphoinositide 3-Kinase/AKT/Survivin Pathway in Colon Cancer Cells

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Abstract

FET cells, derived from an early-stage colon carcinoma, are nontumorigenic in athymic mice. Stable transfection of a dominant-negative transforming growth factor β (TGFβ) type II receptor (DNRII) into FET cells that express autocrine TGFβ shows loss of TGFβ signaling and increased tumorigenicity in vivo indicating tumor suppressor activity of TGFβ signaling in this model. The ability of tumorogenic cells to withstand growth factor and nutrient deprivation stress (GFDS) is widely regarded as a key attribute for tumor formation and progression. We hypothesized that increased tumorigenicity of FET/DNRII cells was due to loss of participation of autocrine TGFβ in a “fail-safe” mechanism to generate cell death in response to this stress. Here, we document that loss of autocrine TGFβ in FET/DNRII cells resulted in greater endogenous cell survival in response to GFDS due to activation of the phosphoinositide 3-kinase (PI3K)/Akt/survivin pathway. Treatment of FET DNRII cells with a PI3K inhibitor (LY294002) inhibited Akt phosphorylation and reduced survivin expression resulting in increased apoptosis in FET/DNRII cells. We also show that exogenous TGFβ increased apoptosis in FET cells through repression of the PI3K/Akt/survivin pathway during GFDS. These results indicate that the PI3K/Akt/survivin pathway is blocked by TGFβ signaling and that loss of autocrine TGFβ leads to increased cell survival during GFDS through the novel linkage of TGFβ-mediated repression of survivin expression. Inhibition of survivin function by dominant-negative approaches showed that this inhibitor of apoptosis family member is critical to cell survival in the FET/DNRII cells, thus indicating the importance of this target for TGFβ-mediated apoptosis. [Cancer Res 2008;68(9):3152–60]

Introduction

Transforming growth factor β (TGFβ) is a group of multifunctional polypeptides that regulate a number of cellular processes through binding to TGFβ receptors. Three major types of TGFβ receptors, type I (RI), type II (RII), and type III (RIII), have been identified in most cells (1). After TGFβ binds to a heteromeric complex of RI and RII, RI is transphosphorylated by RII. The activated RI kinase then transmits signals through Smads to regulate transcription of target genes (2, 3).

Many studies indicate that TGFβ signaling can act either as a tumor promoter or a tumor suppressor. The tumor promoter function of TGFβ has been associated with its ability to induce an epithelial-to-mesenchymal transition (EMT), which confers resistance to the apoptotic effects of TGFβ (4–6). Phosphoinositide 3-kinase (PI3K)/Akt activation has been shown to be required for TGFβ-mediated EMT, cell survival, and migration (7). Recent work showed that TGFβ activates PI3K activity through association of TGFβ RI and the p85 subunit of PI3K in its tumor promoter function (8).

We and other investigators have shown that autocrine negative TGFβ mediates tumor suppressor activity in a variety of cancers, including colon cancers, and that loss of autocrine TGFβ activity leads to acquisition and progression of malignancy (9–15, 33). For example, ectopic expression of TGFβ decreases breast tumor formation in transgenic mice (16) and restoration of TGFβ receptors results in reduced tumorigenicity in many types of cancer cells (11, 14, 15, 17). On the other hand, elimination of autocrine TGFβ activity in colon cancer cells by antisense TGFβ transfection or inactivation of TGFβ RI by dominant-negative TGFβ RI (DNRII) transfection leads to increased tumorigenicity in athymic mice (12, 13, 33). These studies indicate that autocrine TGFβ activity plays an essential role in tumor suppression. Therefore, identifying the mechanism(s) by which tumor suppressor activity is controlled is crucial for our understanding of tumor progression and for developing therapeutic strategies.

Apoptosis is an important aspect of tumor suppression. The PI3K pathway has been shown to play an important role in inhibition of apoptosis. Akt is an important downstream mediator of PI3K-initiated cell survival signaling and has a number of downstream substrates whose modification by the enzyme may contribute to malignant transformation (18). Recently, Akt activation has been linked to a member of inhibitor of apoptosis (IAP) family, survivin. It has been reported that Akt mediates survivin expression (19, 20). Survivin is inappropriately expressed in many types of malignancies, including colorectal, lung, gastric, and breast cancer (21–23). Survivin has the capacity to inhibit caspase-3, caspase-7, and caspase-9, and its overexpression leads to resistance to apoptosis caused by various apoptotic stimuli (24).

It has been shown that TGFβ induces apoptosis through a variety of pathways in different cell types. For example, Perlman et al. showed that TGFβ-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation in B-cell lymphomas and
mouse hepatocytes (25). Jang and colleagues showed that TGFβ induces apoptosis through Smad-mediated expression of the death-associated protein kinase in Hep3B cells (26). Moreover, TGFβ induces apoptosis through Smad-independent expression of the lipid phosphatase SHIP in hematopoietic cells (27). Activation of Akt has been shown to be necessary for protection from apoptosis induced by receptor saturating levels of exogenous TGFβ (28–30).

We show for the first time in this work that TGFβ induces apoptosis through repression of PI3K/Akt/survivin signaling and that autocrine TGFβ tumor suppressor activity is capable of repressing endogenous cellular activity of Akt in response to growth factor and nutrient deprivation stress (GFDS), thus indicating that there may be negative crosstalk between the TGFβ tumor suppressor and PI3K/Akt pathways.

FET, a colon carcinoma–derived cell line with autocrine TGFβ activity, was isolated from a well-differentiated early-stage cancer. FET cells form a small tumor nodule at the site of inoculation in athymic nude mice that does not progressively grow and then regresses over 3 to 5 weeks due to a high rate of apoptosis in the tumor (33, 31). The inability of these colon carcinoma–derived cells to generate progressive tumor growth in vivo suggests that, relative to the more progressed models, FET cells retain many normal growth controls, including inhibitory responsiveness to TGFβ. Moreover, they have normal colon epithelial cell differentiation characteristics, such as the ability to form transport domes in tissue culture (32). In contrast, as reported previously, FET cells stably transfected with DNRII (FET/DNRII) do not have autocrine TGFβ as a result of the inactivation of the receptor (33). FET/DNRII cells showed rapid and sustained tumor growth in vivo compared with the Neo control cells (33). Thus, it seems that loss of TGFβ tumor suppressor response is permissive for the development of inappropriate growth and survival responses by the FET cells beyond their initial immortalization. Taken together, these observations suggest that loss of TGFβ signaling enables the cells to withstand environmental stresses, such as growth factor and nutrient restriction that induce apoptosis in premalignant cells or early malignant stage cells. This study uses the FET Neo and DNRII cell model system to test the hypothesis that autocrine TGFβ signaling mediates GFDS-induced apoptosis. We show for the first time that abrogation of autocrine tumor suppressor TGFβ enables endogenous cell survival signaling in response to GFDS through a mechanism involving activation of the PI3K/Akt/survivin pathway. This link between loss of autocrine TGFβ activity and increased activation of Akt in the DNRII cells indicates that one aspect of autocrine TGFβ activity in its tumor suppressor context represses PI3K signaling and thereby tips the balance between cell survival and apoptotic signaling toward enabling apoptosis under stress conditions. Our results suggest that autocrine TGFβ plays an important safeguard during environmental stress by preventing the survival of premalignant and/or early-stage tumor cells during the period of stress before angiogenesis develops.

Materials and Methods

Cell lines and reagents. The FET/DNRII cells were obtained by transfection of DNRII into FET human colon carcinoma cells, whereas NEO control cells were generated by transfection of a control plasmid as described previously (33). FET Neo, FET/DNRII, GEO, and CBS colon carcinoma cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in SM medium [McCoy’s 5A serum-free medium (Sigma) with pyruvate, vitamins, amino acids, and antibiotics] supplemented with 10 ng/mL epidermal growth factor, 20 μg/mL insulin, and 4 μg/mL transferrin, as described previously (34). When the cells were under GFDS, they were cultured in SM medium in the absence of growth factor or serum supplements for the indicated time periods without medium changes in between.

Antibodies for caspase-3, caspase-8, caspase-9, poly(ADP-ribose) polymerase (PARP), Akt, and phosphorylated Akt (Ser473) were obtained from Santa Cruz and Research Diagnostics, Inc., respectively. Actin antibody was from Sigma, pSmad2 antibody was from Chemicon. Caspase inhibitor ZVAD-fmk, PI3K inhibitor LY294002, and TGFβ RI inhibitor were obtained from Calbiochem.

PI3K assay. The cells were washed with PBS and lysed in lysis buffer [137 mmol/L NaCl, 20 mmol/L Tris (pH 7.4), 1 mmol/L MgCl2, 1 mmol/L CaCl2, 1% NP40, 100 μmol/L NaVO3, and 1 mmol/L phenylmethylsulfonyl fluoride]. Protein concentrations were determined by bichinchoninic acid protein assay reagent (Pierce). Lysates (400 μg of protein) were incubated with p85 antibody (Upstate) at 4°C overnight, followed by further incubation with protein A–agarose for 2 h. Immune complexes were washed twice with each wash buffer [PI3K wash 1, PBS 1% NP40/NaVO3; PI3K wash 2, 100 mmol/L Tris (pH 7.4)/5 mmol/L LiCl/NaVO3; PI3K wash 3, 10 mmol/L Tris (pH 7.4)/150 mmol/L NaCl/5 mmol/L EDTA/NaVO3]. After the last wash was removed, PI3K assays were performed as described previously (35). Briefly, samples were resuspended in 50 μL of PI3K buffer [20 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, and 0.5 mmol/L EDTA], and 10 μg of phosphatidylinositol were added. After 10 min at room temperature, 10 μCi of [32P]ATP and MgCl2 to a final concentration of 20 μmol/L were added. After 10 min at room temperature, lipids were extracted, first with 150 μL of CHCl3/methanol/HCl (10:20:0.2) and 100 μL of pure CHCl3. The second extraction used 80 μL of methanol/1N HCl (1:1). Samples were spotted on 1% potassium oxalate–treated TLC plates (Analtech) and developed in CHCl3/methanol/NH4OH/H2O (129:14:15:21).

Western blot analysis. Cells were lysed in NP40 lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% NP40, 50 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, 25 μg/mL aprotinin, 25 μg/mL trypsin inhibitor, and 25 μg/mL leupeptin] at 4°C. The supernatants were cleared by centrifugation. Protein (30–100 μg) was fractionated on a 10% acrylamide denaturing gel and transferred onto a nitrocellulose membrane (Life Science, Amersham) by electroblotting. The membrane was blocked with 5% nonfat dry milk in TBST [50 mmol/L NaCl, 0.05% Tween 20] and washed in TBST. The proteins were detected by the enhanced chemiluminescence system (Amersham).

DNA synthesis assays. [3H]Thymidine incorporation was used to determine cell proliferation of FET Neo and DNRII cells after TGFβ treatment. The cells were seeded in six-well plates and treated with 5 ng/mL TGFβ or TGFβ RI inhibitor (100 mmol/L) for 24 to 48 h. Apoptosis assays were performed using a DNA fragmentation ELISA kit as described in the manufacturer’s protocol (Invitrogen). Statistical analyses were done using Student’s t test. Ps of <0.01 were considered significant.

DNA synthesis assays. [3H]Thymidine incorporation was used to determine cell proliferation of FET Neo and DNRII cells after TGFβ treatment. The cells were seeded in six-well tissue culture plates and grown to 70% to 80% confluence. The cells were then stressed by deprivation of growth factors for the indicated times (24–72 h) or treated with 5 ng/mL TGFβ or TGFβ RI inhibitor (100 mmol/L) for 24 to 48 h. Apoptosis assays were performed using a DNA fragmentation ELISA kit as described in the manufacturer’s protocol (Invitrogen). Statistical analyses were done using Student’s t test. Ps of <0.01 were considered significant.
Ecotopic expression of the wild-type and dominant-negative survivin. Survivin dominant-negative constructs C84A (36) and D53A (37) were generated by site-directed mutagensis and subcloned into a pBabe-based retroviral vector. The 293GP packaging cells (Clonetech) were cotransfected with pVSV-G and empty retroviral vector or retroviral vectors containing the wild-type survivin cDNA, survivin dominant-negative C84A or D53A cDNA. The viruses were harvested 48 h later and used to infect FET DNRII cells.

Results

TGFβ signaling inhibits PI3K activity and increases apoptosis in FET cells during GFDS. It has been reported that TGFβ increases PI3K activity through the binding of TGFβRI to the p85 subunit of PI3K in Cos 7 cells (8). However, other results indicate that autocrine TGFβ signaling inhibits the PI3K pathway under stress conditions that select for the malignant phenotype in vivo. To test this hypothesis, we determined whether exogenous TGFβ treatment inhibits PI3K activity in colon cancer cells in a concentration-dependent manner. We treated TGFβ-sensitive FET cells (38) with exogenous TGFβ. As shown in Fig. 1A and B, exogenous TGFβ inhibited PI3K activity and Akt phosphorylation in FET cells. Quantitation of repeat experiments showed that 5 ng/mL TGFβ inhibited ~50% of PI3K activity (Supplementary Fig. S1A) and 60% of Akt phosphorylation in FET cells after 24 hours of treatment (Supplementary Fig. S1B).

Survivin belongs to the IAP family, and it has been reported that Akt mediates survivin expression (19, 20); therefore, we determined whether TGFβ-mediated inhibition of PI3K results in diminished expression of survivin as well. As shown in Fig. 1B, exogenous TGFβ reduced survivin expression in a concentration-dependent manner. Quantitation of repeat experiments showed that survivin expression in FET cells was inhibited by >50% after 24 h of TGFβ (5 ng/mL) treatment (Supplementary Fig. S1B). Thus, these experiments provide an indication that, instead of activating PI3K, TGFβ tumor suppressor signaling inhibits PI3K activity and phosphorylation of Akt in FET cells with concomitant inhibition of the IAP family protein, survivin.

Given the importance of the PI3K/Akt/survivin pathway in apoptosis/cell survival, we next determined whether exogenous TGFβ could induce apoptosis in FET cells during GFDS. FET cells were treated with TGFβ (5 ng/mL) while deprived of growth factors. Apoptosis was measured by DNA fragmentation assays. TGFβ treatment increased apoptosis by 2.5-fold in FET cells after 24 hours of growth factor deprivation (Fig. 1C). Exogenous TGFβ...
also enhanced PARP cleavage in a time-dependent and concentration-dependent manner during growth factor deprivation (Fig. 1D). These observations indicated that exogenous TGFβ enhanced apoptosis in FET cells during GFDS.

**Caspase activation in FET cells by TGFβ treatment.** There are two principle pathways to apoptosis called the mitochondrial “intrinsic” pathway and the transmembrane “extrinsic” pathway. Caspase-9 is activated by release of cytochrome c from mitochondria (intrinsic pathway), whereas caspase-8 is activated through death membrane receptors (extrinsic pathway; ref. 39). To determine which caspases were activated in FET cells after TGFβ treatment, Western blots were performed to detect caspase cleavage using specific antibodies against caspase-8 and caspase-9, respectively. There was a significant increase of cleaved caspase-9 fragments in FET cells after 24 hours of TGFβ treatment (Fig. 2A). Caspase-8 was not activated by TGFβ treatment in FET cells (Fig. 2C). There was also a significant increase of cleavage of caspase-3, a downstream effector caspase, after 24 hours of TGFβ treatment in FET cells (Fig. 2A). In addition, exogenous TGFβ increased cleavage of caspase-9 and caspase-3 in a concentration-dependent manner (Fig. 2B). These results suggested that exogenous TGFβ-induced apoptosis during GFDS occurs through activation of caspase-9 and caspase-3 in FET cells. To confirm the role of caspase activation in TGFβ-induced apoptosis, the caspase inhibitor ZVAD-fmk was used. As shown in Fig. 2D, ZVAD-fmk efficiently inhibited caspase-3 activation, as well as PARP cleavage induced by TGFβ treatment. This indicated that caspase activation was critical to TGFβ-induced apoptosis in FET cells. Inhibition of caspases is thought to contribute to protection from apoptosis by survivin (40).

**Abrogation of autocrine TGFβ signaling results in resistance to GFDS-induced apoptosis.** We next explored whether endogenous TGFβ activity plays a role in GFDS-induced apoptosis because it has been shown that endogenous TGFβ activity, rather than responsiveness to exogenous TGFβ, is a key determinant of tumor progression in colon cancer cells (12–14). We took advantage of the FET Neo and DNRII cell model to address this question. FET/DNRII cells, FET cells that have been stably transfected with DNRII, do not have autocrine TGFβ nor do they respond to exogenous TGFβ as a result of the inactivation of TGFβRII (33). This is reflected by the lack of Smad2 phosphorylation in DNRII cells after TGFβ treatment, whereas Smad2 was significantly phosphorylated in FET control cells transfected with empty vector (FET Neo), indicating that TGFβ signaling was abrogated in the DNRII cells (Fig. 3A). DNRII cells were resistant to TGFβ-induced growth inhibition compared with the Neo control cells, further confirming the lack of TGFβ signaling in the transfected cells (Fig. 3B).

Comparison of FET Neo cells and their autocrine TGFβ-null DNRII counterpart showed that PI3K activity decayed rapidly in the Neo cells during GFDS, whereas it remained unchanged for up

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**Figure 2.** Exogenous TGFβ-induced apoptosis through caspase activation. A and B, FET cells were deprived of growth factors while treated with TGFβ (5 ng/mL) for 0, 14, and 24 h (A) or treated with different concentrations of TGFβ (B) for 24 h after growth to 70% confluence. Cells were harvested, and Western blot analysis was performed with anti-caspase-9 and caspase-3 antibodies. α-Tubulin was used as a loading control. C, FET cells were deprived of growth factors while treated with TGFβ (5 ng/mL) for 24 and 48 h after growth to 70% confluence. Cells were harvested, and Western blot analysis was performed with anti-caspase-8 antibody, α-Tubulin was used as a loading control. D, FET cells were deprived of growth factors while treated with TGFβ (5 ng/mL) in the absence or presence of caspase inhibitor ZVAD-fmk (50 μmol/L) for 24 h. Cells were harvested, and Western blot analysis was performed with anti-caspase-3 and anti-PARP antibodies. Actin was used as a loading control. Representatives of three different experiments.
to 48 hours in the DNRII cells under GFDS (Fig. 3C). Quantitation of repeat experiments showed that PI3K activity was reduced by ~20% and 50% in FET Neo cells after being deprived of growth factors for 24 and 48 hours, respectively, whereas no significant changes of PI3K activity was detected in DNRII cells under GFDS (Supplementary Fig. S2A). Consequently, phosphorylated Akt was low in the FET Neo cells and continued to decrease dramatically when the cells were growth factor deprived for 24 and 48 hours. In contrast, FET/DNRII cells that had lost autocrine TGFβ expressed phosphorylated Akt at higher and more sustained levels than FET Neo cells (Fig. 3D), indicating that loss of autocrine TGFβ activity relieved inhibitory constraints on Akt activation in the DNRII cells. In addition, survivin expression decreased significantly during GFDS in the Neo cells but not in the DNRII cells (Fig. 3D). Quantitation of repeat experiments showed that Akt phosphorylation was inhibited by ~30% and 60% in FET Neo cells after being deprived of growth factors for 24 and 48 hours, respectively, whereas survivin expression was reduced by 40% and 55% after being deprived of growth factors for 24 and 48 hours, respectively. No significant changes of both Akt phosphorylation and survivin expression were detected in DNRII cells under GFDS (Supplementary Fig. S2B). These results indicated that, in its tumor suppressor context, autocrine TGFβ activity represses Akt activation and survivin expression, thereby tipping the balance between cell survival and apoptotic signaling toward apoptosis. This was further confirmed in two other TGFβ-responsive colon cancer cell lines CBS and GEO. Consistent with increased PARP cleavage, phosphorylation of Akt and expression of survivin decreased significantly during GFDS in these cell lines in a similar manner to FET cells (data not shown).

To characterize the relationship between autocrine TGFβ tumor suppressor activity and cell survival signaling, we next determined whether FET Neo cells and DNRII cells responded in the same way to growth factor deprivation-induced stress. The DNRII cells and Neo control cells were deprived of growth factors for 24, 48, and 72 hours, and apoptosis was measured by DNA fragmentation assays. As shown in Fig. 4A, there was a significant increase of apoptosis (up to 8-fold) in FET Neo cells compared with FET/DNRII cells (up to 2.5-fold) during GFDS (P < 0.01). In addition, PARP cleavage and caspase activation were measured by Western blot assays. The appearance of cleaved PARP fragments was observed in the Neo cells after deprivation of growth factors for 24 hours, whereas it was not seen in the DNRII cells until 48 hours after growth factor deprivation (Fig. 4B). There was a significant increase of cleavage of caspase-9 and caspase-3 after 48 hours of growth factor deprivation in FET Neo cells but not in FET/DNRII cells (Fig. 4B). These results indicated that the DNRII cells were more resistant to GFDS-induced apoptosis than the Neo control cells. They also suggest that autocrine TGFβ plays an important role in GFDS-induced apoptosis.

To further confirm the role of autocrine TGFβ in GFDS-induced apoptosis, we treated FET cells with a TGFβ RI inhibitor (Calbiochem) to abrogate TGFβ signaling. The RI inhibitor is a cell-permeable ATP-competitive inhibitor of TGFβ RI kinase. It has been shown to be potent and selective for inhibition of TGFβ signaling (41). As shown in Fig. 5A, the RI inhibitor effectively
inhibited Smad2 phosphorylation induced by TGFβ treatment, indicating that TGFβ signaling was blocked by the RI inhibitor. We next explored whether inhibition of TGFβ signaling would restore Akt phosphorylation and survivin expression in FET cells. We found that treatment of FET cells with 100 nmol/L RI inhibitor led to increased Akt phosphorylation and survivin expression during GFDS (Fig. 5A and B, lanes 1 and 3). Quantitation of repeat experiments showed that Akt phosphorylation and survivin expression were increased by ~1.6-fold and 1.4-fold, respectively, in FET cells after treatment with the RI inhibitor (Supplementary Fig. S3A). The RI inhibitor not only increased survivin expression in the absence of exogenous TGFβ, it also antagonized exogenous TGFβ-mediated inhibition of survivin expression (Fig. 5A), suggesting that TGFβ plays a role in regulation of survivin expression.

**Figure 5.** TGFβ RI inhibitor protected FET cells from GFDS-induced apoptosis. **A,** FET cells were deprived of growth factors while treated with TGFβ (5 ng/mL) in the absence or presence of TGFβ RI inhibitor (100 nmol/L) for 24 h. Cells were harvested, and Western blot analysis was performed with anti-pSmad2 and anti-survivin antibodies. Actin was used as a loading control. **B,** FET cells were deprived of growth factors while treated with TGFβ RI inhibitor (100 nmol/L) for 24 and 48 h. Cells were harvested, and Western blot analysis was performed with anti-pAkt (Ser473) and anti-Akt antibodies. Actin was used as a loading control. **C,** FET cells were deprived of growth factors while treated with 100 nmol/L TGFβ RI inhibitor for 48 h, and DNA fragmentation assays were performed as described in Materials and Methods. Representatives of three different experiments.
perhaps, through its effects on Akt. As a result, the RI inhibitor protected FET cells from GFDS-induced apoptosis as reflected by DNA fragmentation assays (Fig. 5C). Of note, the RI inhibitor did not have any effect on mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) activation (data not shown), indicating the specificity of the inhibitor. Taken together, these results indicated that autocrine TGFβ mediates GFDS-induced apoptosis through suppression of the PI3K/Akt/survivin pathway.

The PI3K/Akt/survivin pathway is critical for increased survival in the DNRII cells. Loss of TGFβ signaling enables the DNRII cells to have relatively high and sustained Akt activation during GFDS, which might be responsible for their resistance to GFDS-induced apoptosis. To show that PI3K/Akt is critical for enhanced survival in the DNRII cells, we used the potent PI3K inhibitor LY294002 to block the enzyme activity. LY294002 was added to FET/DNRII cells when they were deprived of growth factors. LY294002 inhibited Akt activation in a concentration-dependent manner (Fig. 6A), indicating that the PI3K pathway was blocked by LY294002. Blockade of PI3K/Akt activation resulted in decreased survivin expression (Fig. 6A), which is consistent with previous reports that PI3K/Akt mediates survivin expression (19, 20). Quantitation of repeat experiments showed that 48-hour treatment of LY294002 (25 μM) inhibited Akt phosphorylation and survivin expression by ~50% and 60%, respectively, in FET/DNRII cells (Supplementary Fig. S3B). Activation of MAPK and JNK was determined after LY294002 treatment to show the specificity of the inhibitor. As a result, LY294002 showed that 48-hour treatment of LY294002 (25 μM) inhibited Akt activation in a concentration-dependent manner after LY294002 treatment to show the specificity of the inhibitor. As a result, LY294002 had no effect on their activation (data not shown). We next determined the effect of LY294002 treatment on apoptosis induced by GFDS. DNA fragmentation assays showed that treatment with LY294002 increased apoptosis by 2-fold at 24 hours and by 2.8-fold at 48 hours after deprivation of growth factors in FET DNRII cells (Fig. 6B, top). PARP cleavage, caspase-3, and caspase-9 activation were also increased in FET/DNRII cells in a concentration-dependent manner after LY294002 treatment (Fig. 6B, bottom). Forced expression of survivin protected FET/DNRII cells from LY294002-induced apoptosis during GFDS (Fig. 6C). Furthermore, survivin dominant-negative constructs C84A (36) and D53A (37) sensitized FET/DNRII cells to GFDS-induced apoptosis (Fig. 6D). These results indicate that inhibition of the PI3K/survivin pathway restored the sensitivity of the DNRII cells to GFDS-induced apoptosis and that PI3K/Akt/survivin signaling plays an essential role in the resistance of DNRII cells to GFDS-induced apoptosis. In summary, our results show that autocrine TGFβ tumor suppressor is responsible for GFDS-induced apoptosis by inhibiting the PI3K/Akt/survivin pathway. Loss of this repressive function enables the induction of inappropriate endogenous cell survival signaling (i.e., PI3K/Akt/survivin pathway), which leads to increased survival in response to cellular stress. This could play an important role in both tumor initiation and progression.

Discussion

The autocrine hypothesis was introduced originally for positive growth factors, such as TGFα, to account for the growth advantage of malignant cells over their normal counterparts (42). On the other hand, TGFβ has been shown to be an autocrine-negative growth factor, as evidenced by stimulation of growth of several cell lines treated with TGFβ-neutralizing antibody (11–14). Therefore, loss of autocrine TGFβ could also provide malignant cells a growth advantage. Previously, we showed that repression of autocrine TGFβ activity by TGFβ antisense transfection led to a robust malignant phenotype as reflected by the ability to form large xenografts compared with the control cells that failed to grow progressively in vivo due to the high intrinsic apoptosis. Importantly, the cells targeted by this antisense approach retained the ability to respond to any exogenous TGFβ available because the TGFβ receptors were still intact (12, 13). Moreover, inactivation of TGFβ RI also led to increased tumorigenicity in athymic mice (33). In contrast, restoration of autocrine-negative TGFβ activity by replacingmutationally inactivated TGFβ RI with the wild-type RI resulted in reduced cell proliferation and diminished tumorigenicity (14, 15). However, responsiveness to growth inhibition by exogenous TGFβ in tissue culture was not reestablished, indicating that endogenous TGFβ activity was sufficient for tumor suppression (14, 17). Taken together, this series of studies indicated that autocrine-negative TGFβ activity rather than exogenous TGFβ is necessary and sufficient to deter malignant progression in human colon carcinoma cells in vivo. However, the mechanisms underlying autocrine TGFβ tumor suppressor activity in relation to apoptosis remain largely unknown. Therefore, exploration of the mechanisms by which autocrine TGFβ induces apoptosis is important for our understanding of the carcinogenic process and malignant progression.

The present study suggests that endogenous TGFβ activity acts as an intrinsic fail-safe system to initiate cell death and prevent malignant progression through inappropriate activation of the PI3K/AKT pathway.

Several studies have shown that exogenous TGFβ induces apoptosis in a variety of cell types through different mechanisms (25, 26, 45). However, thus far, there are no studies characterizing autocrine-negative TGFβ activity in cell survival. We have made the novel observation that endogenous cellular TGFβ signaling increases apoptosis during GFDS and that loss of autocrine TGFβ in FET DNRII cells resulted in increased PI3K/Akt activation and survivin expression, as well as resistance to GFDS-induced apoptosis. This mechanism of inducing apoptosis by suppressing cell survival signaling is different from mechanisms that induce apoptotic signaling in other cell systems, for example, TGFβ induces apoptosis by DPC4 through stress-activated protein kinase/JNK signaling pathway in pancreatic carcinoma (46). Moreover, it has also been shown that extracellular signal-regulated kinase, p38, and c-Src kinases are involved in TGFβ-induced apoptosis in FaO hepatoma cells (47, 48). Taken together with its role in malignant progression, the effect of autocrine-negative TGFβ on apoptosis provides another important mechanistic basis for its tumor suppressor activity. The linkage of this apoptotic mechanism in human colon cancer cell lines to repression of survivin expression may be of significance, because survivin, which is generally not expressed in differentiated cells, is strongly associated with poor prognosis in colon cancer (49).

It has been recently reported that, in Cos7 cells, exogenous TGFβ activates PI3K through a mechanism by which TGFβ receptor RI binds to the p85 subunit of PI3K and activates the kinase (8). The activation of the PI3K pathway by TGFβ is associated with tumor-promoting effects of TGFβ including its ability to induce an EMT, which confers resistance to the apoptotic effects of TGFβ (4–6). PI3K/Akt activation has been shown to be required for TGFβ-mediated EMT, cell survival, and migration (7, 50). In contrast, our study shows that TGFβ also represses PI3K/Akt activation, which contributes to its tumor suppressor function.
There is a fine balance between cell survival and apoptotic signaling, which is maintained by oncogenes and tumor suppressors. Activation of oncogenes (i.e., PI3K) and/or inactivation of tumor suppressors (i.e., TGFβ) are involved in promoting cell survival during tumorigenicity and metastasis. The ability of malignant cells to withstand environmental stresses is considered a key factor in tumor development and progression (51). Escape from TGFβ-mediated apoptosis may contribute to tumor progression and metastasis through an enhanced survival capacity under conditions of poor blood supply or in the initial formation of metastatic colonies. Because environmental restriction on growth seems to be common in solid tumors, such as colon carcinoma, a mechanism for escape from an autocrine-negative control that enables cell survival signaling would be highly advantageous to malignant cells. Here, we have shown that abrogation of autocrine TGFβ enables increased PI3K/Akt activation in FET/DNRII cells under GFDS, which shifts the balance toward survival, and this is the basis for resistance of the cells to GFDS-induced apoptosis.

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