Restoration of Transforming Growth Factor-β Receptor II Expression in Colon Cancer Cells with Microsatellite Instability Increases Metastatic Potential * in Vivo*

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Microsatellite instability (MSI), which occurs in 15% of colorectal cancer, has been shown to have a lower incidence of metastasis and better patient survival rates compared with microsatellite stable colorectal cancer. However, a mechanistic understanding of the basis for this difference is very limited. Here, we show that restoration of TGF-beta signaling by re-expression of TGF-beta receptor II in MSI colon cancer cells increased PI3K/AKT activation, conferred resistance to growth factor deprivation-mediated apoptosis in vitro, blocked the prosurvival and promotility effects of TGF-beta in vivo, increased metastasis in an orthotopic model of colon cancer, and indicates that TGF-beta mediates tumor suppressor activity in a variety of colon cancers, and that loss of autocrine TGF-beta signaling in MSI colorectal cancer patients may be associated with significantly improved survival in MSI colorectal cancer patients.

Colorectal cancer is the second leading cause of cancer mortality in the United States (1), with the majority of deaths from metastatic disease. DNA microsatellite instability (MSI) occurs in 15% of colorectal cancer (2). It has been reported that patients with MSI colorectal carcinomas have decreased likelihood to develop metastasis and better survival rates compared with those with microsatellite stable tumors (3–7). The molecular basis for the survival advantage in MSI tumors is not well understood.

TGF-beta factors are a group of multifunctional proteins that regulate many cellular processes through binding to TGF-beta receptors. Three major types of TGF-beta receptors, type I (RI), type II (RII), and type III (RIII), have been identified in most cells (8). TGF-beta RI is transphosphorylated and activated by TGF-beta RII after TGF-beta binds to a heteromeric complex of TGF-beta receptors. The activated TGF-beta RI kinase then transmits a signal through Smad proteins to regulate transcription of target genes (9, 10).

This article has been withdrawn by the authors. Part of the AKT immunoblot in Fig. 3A was used as actin in Fig. 1A (right, HCT116 wt). The actin immunoblots in Figs. 1B, 3A, and 4E have overlapping lanes. The actin immunoblots in Figs. 4 (A, C, and D) and 5C have overlapping lanes. Figs. 1B, 3A, and 5 (A and C) have undeclared gel splices.
TGFβ increased AKT phosphorylation under GFDS and that inhibition of PI3K/AKT activation by LY294002 reversed TGFβ-mediated protection from GFDS-induced apoptosis as well as TGFβ-mediated promotion of motility. We have also identified Bim, a pro-apoptotic protein, as a downstream effector of TGFβ signaling in cell survival. Further studies showed that TGFβ reduced E-cadherin expression, which contributed to increased cell survival under GFDS in colon cancer cells. Finally, re-expression of TGFβ RII in MSI colon cancer cells increased metastatic colonization in the liver in an orthotopic model in vivo. Taken together, our results demonstrate that TGFβ RII is a survival and metastasis promoter, the loss of which provides MSI colon cancer patients a survival advantage.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The human colon carcinoma cell lines HCT116, HCT116 wt, DLD1, and RKO were cultured at 37 °C in a humidified incubator with 5% CO2 in McCoy’s 5A serum-free medium supplemented with 10% fetal bovine serum or 10 ng/ml epidermal growth factor, 20 μg/ml insulin, and 4 μg/ml transferrin (28). When cells were subjected to growth factor and nutrient deprivation stress, they were cultured in McCoy’s 5A serum-free medium in the absence of growth factor or serum supplements.

Antibodies for caspase-3, AKT, phosphorylated Akt (Ser473), Bim, phosphorylated Smad3, and Slug were obtained from Cell Signaling Technology (Beverly, MA). Antibodies for phosphorylated Smad2 and anti-vimentin (clone VIM 3B4) antibodies was from Millipore (Billerica, MA). Anti-TGFβRII antibody was from Abcam (Cambridge, MA). Anti-E-cadherin antibody was a gift from Dr. Masatoshi Takeichi (RIKEN Center for Developmental Biology, Kobe, Japan). Anti-actin antibody was from Amersham Biosciences.

Plasmids, siRNA Transfection, and Retroviral Infections—Human TGFβRII cDNA was cloned into a pBABE-based retroviral expression vector targeting human E-cadherin (ATCGA-3’). Anti-TGFβRII siRNA was cloned into PZ2 Targetplus siRNA vector and retroviral infections were performed using Dharmafect 1 reagent (Dharmacon). The viruses were concentrated and used to infect HCT116 wt-RII cells (Clontech) with 10% serum. The supernatants were harvested for Western blotting or plated in a 96-well plate for ELISAs (Roche Applied Science) according to the manufacturer’s protocol. Statistical analyses were performed using Student’s t test.

In Vivo Orthotopic Model and Immunohistochemistry—Orthotopic implantation was performed as described previously (26). Briefly, exponentially growing GFP-labeled cells (5 × 10⁶ cells) were inoculated subcutaneously into BALB/c nude mice. Once xenografts were established, they were excised and minced into 1-mm³ pieces. Two of these pieces were then subserosally implanted into the cecum of other BALB/c nude mice. 28 days post-implantation, animals were killed. Organs were explanted and imaged. Tissues were then processed and embedded in paraffin. Slides were cut for hematoxylin/eosin Ki67 staining (Dako Corp.) and for terminal nucleotidyltransferase-mediated nick end labeling (TUNEL) assays (Apoptag, Oncor, Gaithersburg, MD). The apoptosis and proliferation were determined quantitatively by counting the number of positively stained cells for TUNEL and Ki67, respectively, per field at ×40 magnification. Three histologically similar fields were randomly selected from each slide for analysis. p values were calculated using Student’s t test.

RESULTS

Restored TGFβ Signaling Protects Colon Cancer Cells from GFDS-induced Apoptosis—HCT116 cells have inactivated TGFβ RII due to MSI-associated mutations (24). The cell
TGFβ Signaling Promotes Metastasis in MSI Colon Cancer

FIGURE 1. Restoration of TGFβ responsiveness increases cell survival capacity under GFDS. A) Cells were transduced into HCT116 and HCT116 wt cells. Expression of exogenous TGFβ RII (left panels) and phosphorylated Smad2 (right panels) was determined in HCT116 cells under GFDS for 4 days and in HCT116 wt cells, parental diploid HCT116 cells were included for in vitro experiments. Wild-type TGFβ wt cells, parental diploid HCT116 cells were included for observation. With the presence or absence of TGFβ RII, expression of TGFβ and/or TGFβ RI kinase inhibitor (RI inh.) under GFDS for 3 days, parental HCT116 and HCT116 wt cells from GFDS-induced apoptosis as shown by decreased caspase-3 cleavage, whereas little effect was observed in vector-expressing cells (Fig. 2A). The addition of exogenous TGFβ did not further reduce caspase-3 cleavage in these cells (Fig. 2A), suggesting that DLD1 cells have very strong endogenous TGFβ signaling. Similar to HCT116 and HCT116 wt cells, TGFβ protected TGFβ RII-expressing RKO cells from GFDS-induced apoptosis as shown by decreased caspase-3 cleavage, whereas little effect was observed in vector-expressing cells (Fig. 2B).

To ensure that TGFβ-mediated cell survival is not specific to HCT116 cells and their derivative cells, TGFβ RII was re-expressed in two other colon cancer cell lines with MSI, DLD1 and RKO (24, 32). Restoration of TGFβ RII expression in DLD1 cells protected them from GFDS-induced apoptosis as reflected by significantly reduced caspase-3 cleavage (Fig. 2A). The addition of exogenous TGFβ did not further reduce caspase-3 cleavage in these cells (Fig. 2A), suggesting that DLD1 cells have very strong endogenous TGFβ signaling. Similar to HCT116 and HCT116 wt cells, TGFβ protected TGFβ RII-expressing RKO cells from GFDS-induced apoptosis as shown by decreased caspase-3 cleavage, whereas little effect was observed in vector-expressing cells (Fig. 2B).

TGFβ Activates the PI3K/AKT Pathway and Promotes Cell Survival in a Smad2/3-dependent Manner—Because PI3K/AKT is a major survival pathway in colon cancer cells, we next determined the effect of TGFβ on AKT activation. Phosphorylation of AKT at Thr308 and Ser473 leads to its kinase activation (33). As shown in Fig. 3A, TGFβ treatment increased the levels of phosphorylation of AKT at Ser473 in HCT116 wt-RII cells under GFDS, whereas it has little effect on AKT phosphorylation in HCT116 wt-V control cells. Functionally, targeting PI3K/AKT with a potent PI3K inhibitor (LY294002) reversed

PI3KCA (designated HCT116 wt), which have only the PIK3CA allele (29). The reason to choose the model we chose to use in the study is that the very strong endogenous TGFβ RI kinase, which offers a bigger window to observe reduced malignancy in vivo results. To ensure that TGFβ-mediated cell survival is not specific to HCT116 cells and their derivative cells, TGFβ RII was re-expressed in two other colon cancer cell lines with MSI, DLD1 and RKO (24, 32). Restoration of TGFβ RII expression in DLD1 cells protected them from GFDS-induced apoptosis as reflected by decreased caspase-3 cleavage, whereas little effect was observed in vector-expressing cells (Fig. 2A). The addition of exogenous TGFβ did not further reduce caspase-3 cleavage in these cells (Fig. 2A), suggesting that DLD1 cells have very strong endogenous TGFβ signaling. Similar to HCT116 and HCT116 wt cells, TGFβ protected TGFβ RII-expressing RKO cells from GFDS-induced apoptosis as shown by decreased caspase-3 cleavage, whereas little effect was observed in vector-expressing cells (Fig. 2B).

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down-regulation of Bim expression is PI3K-dependent. Therefore, we hypothesized that TGFβ increases cell survival through down-regulation of Bim expression. To test this hypothesis, we knocked down Bim expression in HCT116 wt-RII cells using a siRNA pool. As a result, Bim expression was significantly reduced in Bim siRNA-transfected cells compared with cells transfected with a nonspecific control siRNA (Fig. 4D). When subjected to GFDS, Bim knockdown cells were more resistant to GFDS-induced apoptosis than the control cells as reflected by reduced caspase-3 cleavage (Fig. 4E). DNA fragmentation assays showed that apoptosis under GFDS was reduced by 40% in Bim siRNA-transfected cells compared with control siRNA-transfected cells (*, p < 0.02) (Fig. 4F). These results indicate that reduction of Bim expression increases the resistance of HCT116 wt-RII cells to GFDS-induced apoptosis. Taken together, our results demonstrate that TGFβ signaling down-regulates expression of Bim, which leads to increased cell survival under stress conditions.

**TGFβ Signaling Inhibits EMT**—TGFβ signaling has been shown to inhibit EMT in different cell types (38, 39). To determine whether TGFβ signaling induces EMT in HCT116 wt-RII cells, expression of E-cadherin, vimentin, and Slug was examined in the presence or absence of TGFβ (Fig. 5A). Treatment with TGFβ for 4 days significantly induced E-cadherin expression in HCT116 wt-RII cells. EMT has been associated largely with invasion/motility (40). However, its role in cell survival is not very clear. To determine whether TGFβ-induced EMT contributes to resistance to GFDS-induced apoptosis, E-cadherin was knocked down in HCT116 wt-RII cells by transfecting a shRNA construct into the cells. Expression of E-cadherin was significantly reduced in E-cadherin knockdown cells (Fig. 5B). When subjected to GFDS, E-cadherin knockdown cells were more resistant to GFDS-induced apoptosis than the control cells as reflected by reduced caspase-3 cleavage (Fig. 4C). In addition, treatment with TGFβ under GFDS decreased caspase-3 cleavage significantly in the control cells, whereas it caused a slight decrease in caspase-3 cleavage in E-cadherin knockdown cells (Fig. 4C). These results were further confirmed by DNA fragmentation assays, which showed that E-cadherin knockdown cells displayed 35% reduction of apoptosis under GFDS compared with control shRNA-transfected cells (**, p < 0.003) and that TGFβ treatment did not further decrease apoptosis in these cells (Fig. 5D). Furthermore, overexpression of E-cadherin in HCT116 wt-RII cells abolished the protective effect of TGFβ against GFDS-induced apoptosis (Fig. 5E and F). These results indicate that TGFβ protects HCT116 wt-RII cells from GFDS-induced apoptosis through down-regulation of E-cadherin and suggest that EMT plays an important role in aberrant cell survival of cancer cells under stress conditions.
**TGFβ Promotes Cell Motility through the PI3K/AKT Pathway**—In addition to cell survival, TGFβ has been shown to promote cell motility in many cancer cell types (41). To determine the effect of TGFβ on cell motility in colon cancer cells, Transwell assays were performed in the presence or absence of TGFβ. As shown in Fig. 6A (left panel), HCT116 wt-RII cells

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**FIGURE 3.** TGFβ-mediates protection of colon cancer cells from GFDS-induced apoptosis is dependent upon activation of the PI3K/AKT pathway and expression of Smad2 and Smad3. A, AKT expression and phosphorylation (p) were determined in HCT116 wt-V and HCT116 wt-RII cells under GFDS in the presence or absence of TGFβ for 3 days. B, DNA fragmentation assays were performed in HCT116 wt-RII cells treated with TGFβ and/or LY294002 (LY) under GFDS for 3 days. The data are presented as the mean ± S.D. of triplicate experiments. *, p < 0.004; **, p < 0.002. C, expression of Smad proteins and phosphorylated Smad proteins (pSmad) in HCT116 wt-RII cells transfected with empty vector (pSR), Smad2 shRNA (sh-S2), Smad3 shRNA (sh-S3), or both shRNAs (sh-S2+3). D, DNA fragmentation assays were performed in cells under GFDS for 3 days in the presence or absence of TGFβ. The data are presented as the mean ± S.D. of triplicate experiments. *, p < 0.005.

**FIGURE 4.** TGFβ down-regulates the pro-apoptotic protein Bim, which contributes to increased cell survival under GFDS. Expression of Bim was determined in HCT116 wt-V and HCT116 wt-RII cells treated with TGFβ (A), in HCT116 wt-RII cells transfected with empty vector (pSR) or Smad2 and Smad3 shRNAs (sh-S2+3) followed by TGFβ treatment (B), or in HCT116 wt-RII cells treated with TGFβ in the presence or absence of LY294002 (LY). C, expression of Bim was significantly reduced in Bim siRNA-transfected HCT116 wt-RII cells compared with nonspecific siRNA-transfected cells (NC). D, the levels of cleaved caspase-3 were examined in Bim siRNA- or nonspecific siRNA-transfected cells under GFDS for 3 days. E, DNA fragmentation assays were performed in Bim siRNA- or nonspecific siRNA-transfected cells (control (Ctr)) under GFDS for 3 days. The data are presented as the mean ± S.D. of triplicate experiments. *, p < 0.02.
displayed an increase in the motility of >2-fold compared with HCT116 wt-V control cells (*, p < 0.006). The addition of exogenous TGFβ increased the cell motility of HCT116 wt-RII cells by 2-fold (**, p < 0.01) (Fig. 6A, left panel). Although parental HCT116 cells expressing TGFβ RII (designated HCT116-RII) showed similar motility compared with vector control cells (designated HCT116-V), exogenous TGFβ treatment did increase the motility of HCT116-RII cells by >50% (**, p < 0.01) (Fig. 6A, right panel). To confirm the effect of TGFβ, a TGFβ RI kinase inhibitor was used in the Transwell assays. Treatment of HCT116 wt-RII cells with the TGFβ RI inhibitor abolished the promoting effect of TGFβ on cell motility (*, p < 0.01) (Fig. 6B). Of note, the addition of the TGFβ RI inhibitor alone reduced the motility of HCT116 wt-RII cells, confirming the effect of endogenous TGFβ in these cells. These results indicate that restoration of TGFβ signaling in HCT116 and HCT116 wt cells increases their motility. To determine whether TGFβ promotes motility through activation of the PI3K pathway, cells were treated with LY294002. LY294002 abrogated promotion of motility by TGFβ (*, p < 0.007) (Fig. 6C), indicating that TGFβ signals through PI3K to increase cell motility.

Restoration of TGFβ Signaling in HCT116 wt Cells Increases Metastasis in an Orthotopic Model—Because cell survival and motility are two important determinants of metastasis (42, 43), we next used an orthotopic model to determine the effect of restoring TGFβ signaling on metastasis of MSI colon cancer
cells. HCT116 wt-V and HCT116 wt-RII cells were stably transfected with GFP and characterized in the orthotopic model as described previously (26).

In vivo studies showed that animals implanted with xenografts formed by HCT116 wt-V and HCT116 wt-RII cells demonstrated 100% primary growth at the site of implantation with clear invasion of the bowel upon histological evaluation (Fig. 7A, upper panels). However, compared with HCT116 wt-V cells, orthotopic implantation of HCT116 wt-RII cells gave rise to a significantly increased incidence of metastatic localization to the liver (Table 1). HCT116 wt-RII orthotopic tumors generated liver metastases in ~94% of the animals compared with 50% for HCT116 wt-V orthotopic tumors. Moreover, fluorescence imaging of explanted liver showed a remarkable increase in the numbers of liver metastases in the animals implanted with HCT116 wt-RII cells relative to control animals (Fig. 7B, upper panels). Quantitation of liver metastatic loci indicated a >10-fold difference in the numbers of liver metastases between these two groups of animals (*, p < 0.0005) (Fig. 7B, lower panel). The presence of metastatic disease was confirmed by microscopic histological analysis (Fig. 7A, lower panels). These results indicate that restoration of TGFβ signaling by re-expression of TGFβRII increases metastasis of HCT116 wt cells in vivo. To determine whether TGFβ-mediated cell survival is associated with metastatic potential in vivo, TUNEL assays

**TABLE 1**

| Cell line         | Primary tumors | Liver metastasis |
|-------------------|----------------|------------------|
| HCT116 wt-V       | 10/10 (100%)   | 5/10 (50%)       |
| HCT116 wt-RII     | 17/17 (100%)   | 16/17 (94%)      |
were performed in primary tumors. TUNEL staining of primary tumors showed that there were significantly fewer apoptotic cells in the tumors of HCT116 wt-RII cells (~1% positive cells) than in those of HCT116 wt-V cells (>50% positive cells) (Fig. 7, C and D, upper panels). Meanwhile, Ki67 staining showed that tumors of HCT116 wt-RII cells had fewer proliferative cells than those of HCT116 wt-V cells (30% versus 65%) (Fig. 7, C and D, lower panels). Although restoration of TGFβ signaling in HCT116 wt cells decreased cell proliferation in primary tumors by ~2-fold, it increased cell survival by almost 50-fold. These data are fully consistent with cell survival as a key factor in determining metastasis. Taken together, these in vivo results demonstrate an important role for TGFβ signaling in distant metastatic colonization of MSI colon cancer cells, providing a molecular mechanism of the favorable outcome in MSI colorectal cancer patients.

**DISCUSSION**

TGFβ signaling plays a dual role in tumorigenesis. It elicits tumor-suppressive functions in tumor initiation, whereas it enhances tumor progression and metastasis at later stages, attributed to its ability to protect cancer cells from stress-induced apoptosis, induce EMT, and promote cell migration and invasion (11). TGFβ/RII is mutated in up to 90% of colon cancers with MSI (24). Mutations of TGFβ/RII have been implicated to be associated with favorable prognosis and better survival in patients with MSI colon cancers (25). We have shown previously that TGFβ/RII signaling promotes metastasis to the liver in an orthotopic tumor model. Our studies indicate that TGFβ/RII is at least one of the molecular determinants of the dual functions of TGFβ in colon cancer cells. Therefore, we do not exclude the possibility that other molecules are involved in the metastasis-promoting function of TGFβ in these cells. More studies are needed to determine the mechanisms of colon cancer progression and metastasis.

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