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Switch of transforming growth factor β function from tumor suppression to stimulation in adenomatous polyposis coli (APC) knocked-down human colon carcinoma cells

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Abstract

TGF β exerts a potent tumor-suppressive effect in the human colon carcinoma CBS and Moser cells. However, TGF β can also function as a tumor promoter. The mechanisms underlying the tumor promoting effect of TGF β is not understood. Both the CBS and Moser cells were found to express mutant (truncated) APC. Expression of this form of APC did not interfere with the tumor-suppressive function of TGF β . However, when APC expression was knocked down in these cells, TGF β function switched from that of tumor suppression to that of tumor promotion. TGF β stimulated cellular invasion and anchorage-independent growth in APC knocked-down cells. Knocking down APC expression abrogated the ability of TGF β to induce the expression of the tumor suppressor E-cadherin and the cyclin dependent kinase inhibitor p21/Waf1. TGF β now stimulated the constitutive TCF transcriptional activation activity associated with the β -catenin/Wnt pathway in the APC knocked-down cells. Thus, the level of APC expression determined the type of TGF β function in these human colon carcinoma cells.

Keywords

TGF β ; tumor promotion; tumor suppression; APC; β -catenin/Wnt; colon cancer

1. Introduction

Transforming growth factor β (TGF β) is a potent regulator of epithelial cell proliferation and differentiation and function as a tumor suppressor in the early stages of colon carcinogenesis [1–4]. TGF β maintains the integrity of the colonic epithelium by maintaining cell-matrix and cell-cell adhesion, activating the cellular signals to inhibit proliferation and induce cellular differentiation [4–13]. TGF β mediates its action through a family of cell-surface receptors [12–14]. Thus, loss of expression of TGF β receptors or mutational defects in these receptors is a prominent mechanism of escape from TGF β -mediated growth and differentiation control in the progression of colon cancer [1–2]. Paradoxically, TGF β can also act as a tumor promoter and stimulates malignant progression and malignant cell behavior at the later stages of

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carcinogenesis. The tumor promoting effects of TGF β have been reported in a variety of epithelial cancer [15–20]. The underlying mechanisms by which TGF β switches from a tumor-suppressive to that of a tumor promoting function in the progression of colon cancer, however, is not known.

A few human colon carcinoma cell lines that were originally developed from differentiated primary human colon tumors retain functional TGF β receptors and signaling mechanisms [4–13]. TGF β exerts a growth-inhibitory and differentiation-inducing effect in these cells [3–13]. The CBS and Moser cell lines are examples of such cell lines developed from differentiated human colon tumors that retain TGF β receptor expression and function and respond to the tumor-suppressive effects of TGF β [1–2,4–13]. On the other hand, carcinoma cells established from undifferentiated primary colon tumors are usually unresponsive to TGF β with mutational defects in TGF β receptors [2,5]. TGF β elicits diverse cellular responses from the CBS and Moser colon carcinoma cells [4–13]. TGF β restores normal growth control and blocks cell cycle progression by inducing the expression of the cyclin dependent kinase inhibitor p21/Waf1 [7,9]. It restores cellular adhesion by inducing the synthesis of matrix adhesion molecules and the expression of the tumor suppressor and intercellular adhesion molecule E-cadherin [9–11]. More over, TGF β directly suppresses the β -catenin/Wnt signaling pathway in these cells, a key underlying mechanism in driving malignant progression not only in colon cancer but in a variety of epithelial cancer [4,21,24–25].

The Adenomatous polyposis coli (APC) gene is viewed as the gene for colorectal cancer because germ line mutation in the APC gene causes familial adenomatous polyposis and APC mutations are found in most sporadic colorectal cancer [26]. APC is multifunctional; its function include cellular adhesion, migration, apoptosis, chromosomal segregation at mitosis and β -catenin/wnt signaling [26]. APC protein complexes with β -catenin and leads to its degradation; prevents its binding to TCF4 and attenuates the Wnt signal pathway. Loss of APC function through mutations is regarded as a crucial event in the early transformation of the colonic epithelium [26].

Mutations in the APC gene results in the expression of truncated proteins [27–28]. Interestingly, in this report, we found that both the CBS and Moser cell lines expressed a truncated form of APC. However, when we knocked down APC expression by shRNA targeting the APC in these cells, the function of TGF β now switched from that of a tumor suppressor to that of a tumor promoter. In the APC knocked-down cells, TGF β now stimulated cellular invasion, growth in soft agarose (anchorage independent growth) and induced the activation of β -catenin/Wnt signal pathway. In the APC knocked-down cells, TGF β was unable to induce the expression of E-cadherin and the induction of p21/Waf1 was significantly attenuated. It is concluded that the level of APC protein expression may determine the type of TGF β function in these colon carcinoma cells.

2. Materials and methods

2.1. Cell culture and reagents, treatment with TGF β and immunoblottings

Human colon carcinoma CBS and Moser cells were maintained in supplemental DMEM medium containing 5% fetal bovine serum as previously described [24–25]. The medium of actively growing cells was replenished with medium containing 10 ng/ml TGF β (R& D, Minneapolis, MN) for the time periods as indicated in the figure legends. Cellular proliferation was determined in 24 well culture plates by counting the cells with the aid of a hemocytometer as previously described [9].

Immunoblottings were performed essentially as previously described [11]. Total cell lysates were prepared in lysate buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1.0 mM EDTA, 0.5% NP40,

0.5% Triton X-100, 2.5 mM sodium orthovanadate, 10 µl/ml protease inhibitor cocktail (EMD chemicals Inc., San Diego, CA) and 1 mM PMSF). Protein concentrations were determined by the Bio-Rad dye binding assay (Bio-Rad, Hercules, CA). For the analysis of E-cadherin and p21/Waf1 expression, cellular proteins were fractionated by reducing 10 % SDS-PAGE while the analysis of APC was performed in 4% reducing SDS-PAGE. Fractionated proteins were electrophoretically transferred onto nitrocellulose membranes, blocked (5% nonfat dried milk in 1× TBS buffer containing 0.1% Tween 20) and immunostained with either primary anti-E-cadherin (Zymed Laboratories, Carlton Court, San Francisco, CA), anti-p21/Waf1 (Cell Signaling Technology, Danvers, MA) or anti-APC antibodies (Abcam Inc., Cambridge, MA). Horseradish peroxidase–conjugated secondary antibodies in conjunction with chemiluminescence detection and the FUJIFILM LAS-3000 system (Fujifilm Life Science, Stamford, CT) were used to visualize the binding of the primary antibodies to immobilized proteins of interest on the membranes. β-actin expression (using anti-human β-actin antibody (Abcam Inc., Cambridge, MA)) was used as internal controls for equal protein loading.

2.2. Construction of shRNA expression vector (shRNA-APC) targeting APC

Transfection, selection and development of stable transfectants—shRNA-APC expression vector was constructed in pRNA-U6.1/Hygro under the control of U6 promoter and contains a hygromycin-resistant gene for the selection of stable transfectants for hygromycin resistance. shRNA-APC was purchased from GenScript (Piscataway, NJ). The target sequence 5'-GGATCCCGCTGGTATTACGCT CAACTTCTTGATATCCGGAAGTTGAGCGTAATACCAGTTTTTTCCAAAAGCTT-3' was designed with the company's shRNA Target Finder and Design Tool. The underlined sequence represents the target sequence of APC inserted into pRNA-U6.1/Hygro. pRNA-U6.1/Neo/Ctrl (GenScript) with a scrambled insert was used as a control vector in transfection experiments.

Plasmid shRNA-APC or control vector was transfected into CBS or Moser carcinoma cells using Lipofectamine™ 2000 (Invitrogen Life Technologies, Frederick, MD) according to the manufacturer's protocol. Stable transfectants were established by selection of the cells with 200 µg/ml of hygromycin B or 500 µg/ml of G418. Clones of stable transfectants were obtained by the use of cloning cylinders. Transfectants were routinely cultured in SMEM medium containing 50 µg/ml of hygromycin B or 200 µg/ml of G418.

2.3. RT-PCR

RT-PCR analysis was performed to determine the expression of shRNA insert targeting the APC gene. Total RNA was isolated using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA). Reverse transcription of the RNA to single-strand cDNA was then performed using Oligo d(T) 16 primer and MuLV Reverse Transcriptase supplied in the RNA PCR Core Kit (Applied Biosystems, Foster City, CA). PCR was performed using PLATINUM Taq DNA Polymerase (Invitrogen) in a gene Amp 9700 PCR system (Perkin-Elmer, Norwalk, CT). PCR products were detected by agarose gel electrophoresis. Primers of shRNA-APC insert were designed as follows: sense, 5'-GGATCCCGCTGGTATTACG-3'; antisense, 5'-AAGCTTTTGGAAAAAAGCTG-3'.

2.4. Constitutive TCF transcriptional Activation Activity (CTAA)

This assay was performed as described previously [24]. CTAA was measured by dual luciferase reporter assays (Promega, Madison, WI) using the constructs pTOPFLASH and pFOPFLASH and as a ratio of luciferase activity from the pTOPFLASH vector to the pFOPFLASH vector. All luciferase activities were normalized for transfection efficiency by co-transfection with pRL Renilla luciferase vector.

2.5. Effect of TGF β on Cellular invasion and anchorage independent growth

These assays were performed as previously described [29]. The effect of TGF β on cellular invasion was determined by using porous matrigel-coated invasion chambers (Corning, Corning, NY). Fifty thousand TGF β -treated cells (exposed to TGF β for 48 h) or untreated control cells were seeded into the top compartments of the invasion chambers. The chambers were then incubated in a humidified CO₂ incubator at 37°C for 3 days. The number of cells invaded into the bottom compartments of the invasion chambers was determined by counting with the aid of a hemocytometer. Triplicate determinations were performed in each experiment. The value shown represents the mean $\pm$ standard error of three experiments.

The effect of TGF β on anchorage-independent growth was determined in 0.35% low melting agarose in culture medium containing 10% fetal bovine serum. Five thousand TGF β -treated (exposed to TGF β for 48 h) were seeded into 60 $\times$ 15 mm culture dishes containing 10 ng/ml of TGF β while untreated control cells were seeded into similar dishes without TGF β . Soft agarose dishes were then incubated in a humidified CO₂ incubator at 37° for 10 days. Colonies were stained with p-iodonitrotetrazolium violet and the number of colonies formed was counted manually with the aid of a magnifying glass. Each value shown represents the mean $\pm$ standard error of triplicate determinations.

3. Results and Discussion

HCT116 human colon cancer cell line express the wild type APC protein with a molecular size of about 312 kDa while the SW480 cell line express a mutant (truncated) form of the protein with a molecular size of about 147 kDa [27]. We, therefore, determined the size of the APC protein in the CBS and Moser cells by immunoblottings and compared its size to that of the HCT116 and SW480. As expected, the HCT116 expressed the wild type APC while the SW480 expressed a truncated form of the protein (Fig. 1). Surprisingly, both the CBS and Moser cells expressed a truncated form of APC protein with a molecular size similar to that of SW480 cells (Fig. 1). This data suggest that the expression of mutant APC protein in the CBS and Moser cells does not interfere with the tumor-suppressive function of TGF β .

In order to delineate the role of APC in TGF β function, we used a shRNA expression vector targeting the APC to disrupt its expression in the CBS and Moser cells. We then determined the consequences of APC knocked down on the cellular responses to TGF β . The shRNA vector was transfected into the CBS and Moser cells and stable clones of transfectants from each cell line (clones 4 and 7 from CBS and clones 1 and 3 from Moser) were used to evaluate the cellular responses to TGF β . These shRNA transfected clones were chosen because they expressed the exogenous APC targeting sequence with a substantial knocked down in the expression of APC protein (Fig. 2). A scrambled vector was used as control in transfection experiments. None of the control transfected clones showed any decrease in APC expression and alteration in their cellular responses to TGF β by comparison with their parental cells. Therefore, the results obtained with the control transfectants are not shown.

As expected, TGF β induced the expression of E-cadherin and p21/Waf1 in the CBS and Moser cells (Fig. 3). However, in the APC knocked-down cells (both CBS and Moser), TGF β was unable to induce the expression of the tumor suppressor E-cadherin (Fig. 3). P21/Waf1 is regarded as a differentiation marker in colon carcinomas and the induction of p21/Waf1 expression by TGF β in the CBS and Moser cells is intimately associated with the induction of a more benign or differentiated phenotype in these cells [7,9,21]. The ability of TGF β to induce the expression of p21/Waf1 in their APC knocked-down counterparts, however, was significantly attenuated (Fig. 3).

We next determined if knocking down the expression of APC would alter the effect of TGF β on CTAA. CTAA was measured as dual luciferase reporter assays using the constructs pTOPFLASH and pFOPFLASH [24]. As expected, treatment of the CBS and Moser cells led to a significant down-modulation of CTAA (Fig. 4). By contrast, TGF β stimulated CTAA in the APC knocked-down cells (Fig. 4). This data shows that when the expression of APC was knocked down, TGF β actually stimulates the Wnt signal pathway rather than suppressing it. Because activation of the Wnt pathway is an underlying mechanism in driving malignant cell behavior [4,21,24–25], we next determined the effect of TGF β on cellular invasion and the propensity to grow in soft agarose (anchorage-independent growth). TGF β inhibited the invasive capability of the CBS and Moser cells but stimulated the invasive capability of their APC knocked-down counterparts (Fig. 5). Likewise, TGF β inhibited the anchorage-independent growth of CBS and Moser cells but stimulated their propensity to grow in soft agarose in their APC knocked-down counterparts (Fig. 6).

We had observed a lower steady state level of CTAA in the APC knocked-down clones by comparison to the parental cell lines. This probably reflects on the heterogeneity of the parental cell lines, which contain cells with different steady state level of CTAA. It is likely that clones with lower CTAA by comparison to the average steady state level of the parental cell lines were isolated during the course of our experiments. Immunocytochemical analysis was performed to determine if TGF β facilitated the accumulation of β -catenin in the nuclei of APC knocked-down cells. Treatment of the APC knocked-down cells with TGF β did not lead to an increase in the accumulation of β -catenin in nuclei (not shown). It is likely that in the APC knocked-down cells, TGF β promoted the binding of β -catenin already in the nuclei to TCF4. We hypothesize that at least one mechanism behind the action of TGF β in promoting malignant cell behavior is the activation of the wnt signal pathway. Other mechanisms could also be involved in the tumor-promoting action of TGF β such as the activation of Akt as reported in prostate cancer cells [19].

In summary, both the TGF β responsive CBS and Moser cells were found to express a truncated form of APC which did not interfere with the tumor suppressive function of TGF β . However, when APC expression was knocked down, TGF β function switched from that of tumor suppression to that of tumor promotion. In APC knocked-down cells, TGF β stimulated (rather than suppressed) malignant cell behavior. In APC knocked down cells, TGF β was unable to induce the tumor suppressor E-cadherin nor the differentiation marker p21/Waf1 and stimulated (rather than suppressed) CTAA of the Wnt signal pathway.

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Abbreviations

TGFβ	transforming growth factor β
CTAA	constitutive TCF transcriptional Activation Activity

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Fig. 1. Immunoblot analysis of APC expression. Cellular lysates were fractionated by 4% reducing SDS-PAGE and blotted with anti-APC antibodies as described in Methods. Lanes 1, SW480; 2, HCT116; 3, CBS and 4, Moser.

Fig. 2.

Expression of shRNA targeting the APC knocked down the expression of APC protein. (A) RT-PCR analysis of shRNA targeting the APC. Lanes 1, molecular weight ladder; 2, parental CBS cells; 3–4, CBS transfectants clone 4 and 7, respectively; 5, parental Moser; 6–7, Moser transfectants clone 1 and 3, respectively. (B) immunoblot analysis of APC expression in CBS and its shRNA transfectants, Lanes 1, parental CBS; 2–3, transfectants clone 4 and 7, respectively. (C) immunoblot analysis of APC expression in Moser and its shRNA transfectants, Lanes 1, parental Moser; 2–3, transfectants clone 1 and 3, respectively.

Fig. 3.

Immunoblot analysis of the effect of knocking down APC expression on TGF β induction of the expression of E-cadherin and p21/Waf1. Cells were treated with TGF β for 24 h and the expression of these molecules was compared to that of the untreated controls. (A) CBS cells. Lanes 1–2, parental CBS and CBS cells treated with TGF β , respectively; 3–4, CBS shRNA transfectant clone 4 untreated and treated with TGF β , respectively; 5–6, CBS shRNA transfectant clone 7 untreated and treated with TGF β , respectively. (B) Moser cells. Lanes 1–2, untreated parental Moser and Moser treated with TGF β , respectively; 3–4, Moser shRNA transfectant clone 1 untreated and treated with TGF β , respectively; 5–6, Moser shRNA transfectant clone 3 untreated and treated with TGF β , respectively.

Fig. 4.

Effect of knocking down APC expression on TGF β modulation of CTAA. CTAA was measured by dual luciferase reporter assays as described in Methods. Hollow bars, untreated controls. Solid bars, cells treated with TGF β for 24 h. CL, clone of shRNA transfectant. Error bars represent the mean and SE of triplicate determinations.

Fig. 5. Effect of knocking down APC expression on TGFβ modulation of cellular invasion. This assay was performed using matrigel-coated invasion chambers as described in Methods. (A) CBS cells. (B) Moser cells. Hollow bars, untreated controls. Solid bars, cells treated with TGFβ for 48 h before seeding into the invasion chambers. CL, clone of shRNA transfectant. Error bars represent the mean and SE of triplicate determinations.

Fig. 6. Effect of knocking down APC expression on TGF β modulation of anchorage-independent growth. Soft agarose assays were performed as described in Method. (A) CBS cells. (B) Moser cells. Hollow bars, untreated controls. Solid bars, cells treated with TGF β for 48 h before seeding into the soft agarose dishes. CL, clone of shRNA transfectant. Error bars represent the mean and SE of triplicate determinations.