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Transforming growth factor β suppresses β -catenin/wnt signaling and stimulates an adhesion response in human colon carcinoma cells in a Smad4/DPC4 independent manner

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Abstract

Transforming growth factor β (TGF β) suppresses the malignant phenotype of human colon carcinoma cells through diverse cellular pathways. Activation of β -catenin/Wnt signal pathway underlies the malignant phenotype of human colon carcinomas. The Smad family of signal transducing, sequence-specific transcription activators are mediators of TGF β signaling. In this report, we showed that TGF β suppressed the β -catenin/Wnt signal pathway in human colon carcinoma cells and stimulated an adhesion response in these cells in a Smad4/DPC4 independent manner. Smad/DPC4, however, was found to be linked to the growth inhibitory action of TGF β .

Keywords

TGF β ; Smads; β -catenin; TCF4; colon cancer

1. Introduction

Transforming growth factor β (TGF β) is a potent tumor suppressor in the early stages of colon carcinogenesis and loss of responsiveness to TGF β is regarded as an important underlying mechanism in the pathogenesis of colon cancer [1–4]. A few colon carcinoma cell lines established from differentiated primary human colon tumors have been shown to retain TGF β receptor expression and responsiveness to TGF β [5–9]. Carcinoma cells established from undifferentiated primary colon tumors, however, are usually unresponsive to TGF β [2, 5]. TGF β exerts a tumor suppressive effect on carcinoma cells derived from primary differentiated colon tumors *in vitro* and *in vivo* in athymic mice [1,5–9]. Thus, TGF β -responsive cells offer an opportunity in delineating the tumor suppressive signal pathways through which TGF β acts.

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TGF β elicits a multitude of diverse cellular responses from TGF β -responsive human colon carcinoma cells [5–9]. It restores normal growth control and blocks cell cycle progression by up-regulating the expression of cell cycle check point proteins [7,9]. It restores cell-matrix adhesion by inducing the synthesis of extracellular matrix adhesion molecules and integrin receptors for these molecules [10] and restores cell-cell adhesion by inducing the expression of the intercellular adhesion molecule E-cadherin [11]. The induction of cell-matrix and cell-cell adhesion by TGF β is collectively termed a TGF β mediated adhesion response [9]. Overall, TGF β functions to maintain the integrity of the colonic epithelium by eliciting the cellular signals to slow proliferation and allows proper cellular differentiation to occur. However, the intracellular signal pathways through which TGF β acts to elicit these diverse cellular responses is not thoroughly understood.

A family of cell-surface TGF β receptors mediates the signaling action of TGF β [12–14]. Loss of expression of these receptors or mutational defects in these receptors is a prominent mechanism of escape from the biologic control of TGF β in colon cancer [1–2]. The Smad family of signal transducing, sequence-specific transcription activators plays a critical role in transmitting TGF β signals from the cell surface to the nucleus and is linked to the growth-inhibitory pathway of TGF β in a variety of epithelial cells [15–18]. Upon ligand activation, TGF β type I and type II receptors form a heterotetrameric complex with activated serine/threonine kinase activities [19–20]. In mammalian cells, the receptor-regulated Smads (R-Smads 2 and 3) are direct targets of activated TGF β receptor. When phosphorylated by TGF β receptor, the R-Smads form a complex with Smad4/DPC4 and translocate to the nucleus to regulate the transcription of TGF β -responsive genes [19–20]. Smad4/DPC4 is regarded as a central mediator of Smad function and TGF β signaling in epithelial cells. Smad4/DPC4 is directly involved in the TGF β mediated induction of the 3TP-lux reporter gene, which contains a known TGF β inducible plasminogen activator inhibitor promoter [19]. Smads independent TGF β signal pathways, though not well delineated, have also been reported [21–22].

Activation of the β -catenin/Wnt signal pathway is an underlying mechanism in driving malignant progression and malignant cell behavior in epithelial cancer [4,23]. β -catenin/Wnt signaling is implicated in colon carcinogenesis and suppression of this signal pathway is associated with a reduction of cellular proliferation and the induction of cellular differentiation [23–24]. When Wnt is activated, β -catenin translocates to the nucleus, heterodimerizes with T-cell factor (TCF) DNA binding proteins to form a transcriptional activator complex which can then activate the transcription of a set of downstream genes which are responsible for driving the malignant phenotype [23–24]. Both TGF β and Wnt signaling pathways are thought to play critical roles in regulating the differentiation programs of the colonic epithelial cells.

In this study, we showed that TGF β directly suppressed the β -catenin/Wnt signal pathway in TGF β -responsive human colon carcinoma cells by down-regulating the expression of β -catenin and TCF4 and suppressed the binding of β -catenin with TCF4 in the nuclei in a Smad4/DPC4 independent manner. TGF β also stimulated the induction of the matrix adhesion molecule fibronectin and the intercellular adhesion molecule E-cadherin in a Smad4/DPC4 independent manner. The growth inhibitory effect of TGF β , however, was found to be dependent on Smad4/DPC4.

2. Materials and methods

2.1. Cell culture and reagents, treatment with TGF β , cellular proliferation, enzyme linked immunosorbent assay (ELISA) and immunoblotting

Human colon carcinoma CBS cells derived from a differentiated primary human colon tumor were maintained in supplemental DMEM medium as previously described [23–24]. The medium of actively growing cells was replenished with medium containing TGF β 1 (10 ng/ml)

(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]. ELISA was used to measure the amount of fibronectin secreted into serum-free conditioned medium as described previously [9]. Immunoblotting was performed as previously described using commercially available polyclonal or monoclonal antibodies [8,11,24].

2.2. Transfection with flag-tagged dominant negative Smad4/DPC4 (DN-Smad) expression vector

A flag-tagged DN-Smad expression vector constructed in pcDNA3 was a generous gift from Dr. Luzhe Sun of the University of Texas at San Antonio. In this construct, the mutated DPC/Smad4 flag fragment was isolated from pCMV5/Flag-DPC4 [15] and subcloned into the HindIII/BamHI sites of pcDNA3. DN-Smad was transfected into cells using Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to protocols supplied by the manufacturer and selected for G418 resistance. Clones of G418 resistant cells were isolated by the use of cloning cylinders and stable transfectants established in cell culture. Immunoblottings, using an antibody against the flag epitope. (Sigma, St. Louis, Mo), were used to confirm the expression of exogenous DN-Smad.

2.3. 3TP Lux Luciferase reporter assay

Luciferase reporter assay was performed as described previously [23–24] using the 3TP-lux reporter gene [19] and a dual luciferase system (Promega, Madison, WI). All luciferase activities were normalized for transfection efficiency by co-transfection with pRL Renilla luciferase vector. Assays were performed in triplicates and the results represent the mean and standard error of the means of three independent experiments.

2.4. Confocal microscopy

In confocal microscopic analysis, primary antibodies used were mouse anti- β catenin (BD Biosciences, San Jose, CA) and rabbit anti-TCF-4 antibodies (Upstate, Billerica, MA). Secondary antibodies used were Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 555 goat anti-rabbit IgG. Images were examined with an Olympus FV500 confocal microscope equipped with FLUOVIEW version 4.3 software. β -catenin was stained green while TCF4 was stained red. Binding of β -catenin with TCF4 was stained yellow (merged image).

3. Results and Discussion

A flag-tagged dominant negative Smad4/DPC4 expression vector (DN-Smad) [15] was used to examine the linkage, if any, of the Smads pathway with that of the β -catenin/Wnt and adhesion signal pathways of TGF β . TGF β -mediated responses were determined in parental CBS cells and stable DN-Smad transfectants. Because the induction of 3TP-lux is absolutely dependent on functional Smads [19], TGF β was unable to induce 3TP-lux reporter gene activities in stable transfectants expressing the flag epitope (Figure 1A–B). Thus, the expression of DN-Smad blocked the Smad associated signal pathway of TGF β . TGF β is known to induce the expression and secretion of fibronectin and the expression of E-cadherin in TGF β -responsive human colon carcinoma cells [8,10]. We, therefore, determined if this aspect of the TGF β -mediated adhesion response was dependent on the Smad signal pathway. As shown in Figure 1C–E, blockade of this pathway by DN-Smad did not interfere with the ability of TGF β to induce the expression of fibronectin (Fig. 1C), the secretion of fibronectin (Fig. 1E) nor the induction of the expression of E-cadherin (Fig. 1D). About fibronectin secretion, there is no difference in the DN-Smad transfected clones by comparison with the parental control. However, a small difference in the induction of fibronectin expression in cell lysates prepared from the two transfectants was observed. The expression of fibronectin in one clone

was induced by TGF β to a higher level by comparison to the other. Because the CBS line (like most cell lines) is not homogeneous in nature, it is likely that these two clones may represent two slightly different phenotype; one is a better responder to TGF β in terms of the induction of fibronectin expression. Overall, this aspect of the TGF β -mediated adhesion response was elicited independently of Smads. We have previously shown that protein kinase C (PKC) α is a postreceptor focal point in the induction of an adhesion response by TGF β [9]. It is concluded that the induction of an adhesion response by TGF β in these colon carcinoma cells is dependent on PKC α but independent of Smads.

We next determined if TGF β could directly modulate the β -catenin/Wnt signal pathway and if Smads were involved in this action of TGF β . TGF β in the concentration of 10 ng/ml and treatment for 24 hours induces a maximal response in terms of the suppression of β -catenin. This protocol was used to ascertain if DN-Smad would block the suppression of β -catenin. Confocal microscopic analysis showed that treatment of the CBS cells with TGF β resulted in a down-modulation of the expression of both β -catenin and TCF4 with a concurrent reduction of β -catenin binding to TCF4 in the nuclei (Figure 2A). Expression of DN-Smad did not interfere with the ability of TGF β to down-regulate the expression of both β -catenin and TCF4 (Figure 2B–C). These results showed that TGF β modulates this aspect of the β -catenin/Wnt signal pathway independently of Smads.

The anti-proliferative action of TGF β in epithelial cells has been shown to be intimately associated with the Smads signal pathway and the cyclin dependent kinase inhibitor p21/Waf1 (9,25). TGF β is known to inhibit cellular proliferation and induces the expression of p21/Waf1 in TGF β -responsive human colon carcinoma cells [9]. Therefore, in a final series of experiments, we determined the role of Smads, if any, in the anti-proliferative action of TGF β and its ability to induce the expression of p21/Waf1. As shown in Figure 3, TGF β is a potent growth inhibitor and its growth-inhibitory effect could be detected as early as 24 hours after plating of the CBS cells (Fig. 3B). At day four, the growth of the TGF β -treated CBS cells was reduced by 50% when compared to untreated controls (Fig. 3B). The DN-Smad transfectants showed a slightly higher rate of proliferation by comparison to the parental CBS cells. Interestingly, these transfectants were still susceptible to the growth inhibitory effect of TGF β but to a much lesser extent by comparison to the parental CBS cells (Figure 3B). Treatment of the CBS cells with TGF β resulted in a good induction of p21/Waf1 (Figure 3A). Surprisingly, in the DN-Smad transfectants, a higher endogenous level of p21/Waf1 expression was observed by comparison to the parental CBS cells (Figure 3A). Treatment of the DN-Smad transfectants with TGF β had no significant effect on the expression of p21/Waf1 in these cells (Figure 3A).

Smad4/DPC4 is considered a tumor suppressor and its function has been considered a requirement for TGF β signaling. About its tumor suppressive function, blockade of Smad4 in the CBS cells led to an increase in the rate of proliferation and a decrease in the anti-proliferative response to TGF β . It is likely that these changes, over time, may constitute one of the many mechanisms underlying carcinogenesis and malignant progression in the colon. There is considerable amount of evidence to suggest that contingent on the cell type, multiple different intracellular signal pathways exist for TGF β [9,21,26]. It is also likely that within an epithelial phenotype and contingent on the stage of cellular differentiation, TGF β may utilize different signal pathways and elicits different cellular responses. For example in colon adenoma (pre-malignant) cells, TGF β elicits a potent growth inhibitory response (80% growth inhibition) in an absolute Smad4 independent manner [21] while in the CBS cells (differentiated carcinoma), inactivating Smad4 only down-modulated the growth inhibitory response to TGF β . It is unlikely that the Smad4/DPC 4-independent mode of action of TGF β is not uniquely associated with the CBS cells but also extends to other TGF- β -responsive colon carcinoma cell lines derived from differentiated or moderately differentiated human colon tumors. TGF β elicits a

very similar (CBS-like) cellular responses from this group of cells but not from cells derived from undifferentiated tumors [4–9].

In summary, we have shown that in the human colon carcinoma CBS cells, TGF β down-modulated the expression and interaction of β -catenin and TCF4 in a Smad4/DPC4 independent manner. The TGF β induced adhesion response was also found to be independent of Smad4/DPC4 while inactivation of Smad4/DPC4 down-modulated the growth inhibitory effect of TGF β in these cells.

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Abbreviations

TGFβ, transforming growth factorβ;.

Fig. 1. Effect of DN-Smad on the induction of 3TP-lux reporter gene activities, fibronectin expression/secretion and E-cadherin expression. A flag-tagged dominant negative Smad4/DPC4 expression vector constructed in pcDNA3 was transfected into CBS cells and stable transfectants were established in culture as described in Methods. (A) Immunoblot analysis of flag epitope expression in DN-Smad transfectants. Lane 1, parental CBS cells; lanes 2–6, DN-Smad transfectants. (B) 3TP-lux reporter luciferase activities in parental CBS and DN-Smad transfectants. (C and D) Immunoblot analysis of fibronectin and E-cadherin expression, respectively. Lanes 1 and 2, parental CBS untreated control and CBS cells treated with TGF β , respectively (10 ng/ml for 24 hours). Lanes 3–4, DN-Smad stable transfectant clone 1

untreated control and DN-Smad clone1 treated with TGF β , respectively. Lanes 5–6, DN-Smad stable transfectant clone2 untreated control and DN-Smad clone2 treated with TGF β , respectively. (E) Secretion of fibronectin in response to TGF β treatment. Fibronectin secreted into 24 hour serum free conditioned medium was assayed by ELISA [9]. Dotted bars, untreated controls. Solid bars, cells were treated with TGF β (10 ng/ml) for 24 hours. DN-Smad, clones of stable transfectants transfected with DN-Smad vector.

Fig. 2. Effect of DN-Smad on TGF β down-regulation of β -catenin and TCF4 expression. (A) Confocal microscopic analyses of β -catenin and TCF4 expression. For confocal microscopic analyses, primary antibodies used were mouse anti- β catenin and rabbit anti-TCF-4 antibodies.

Secondary antibodies used were Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 555 goat anti-rabbit IgG. Images were examined with an Olympus FV500 confocal microscope equipped with FLUOVIEW version 4.3 software. B-catenin was stained green while TCF4 was stained red. Binding of β -catenin with TCF4 was stained yellow (merged image). (B–C) Immunoblot analysis of β -catenin and TCF4 expression, respectively. Lanes 1 and 2, CBS untreated control and CBS treated with TGF β , respectively (10 ng/ml for 24 hours). Lanes 3–4, DN-Smad stable transfectant clone1 untreated control and DN-Smad clone1 treated with TGF β , respectively. Lanes 5–6, DN-Smad stable transfectant clone 2 untreated control and DN-Smad clone 2 treated with TGF β , respectively.

Fig. 3. Effect of DN-Smad on TGF β induction of p21/Waf1 and TGF- β mediated growth inhibition. (A) TGF β induction of p21/Waf1. Immunoblot analyses of p21/Waf1 expression. Lanes 1 and 2, CBS untreated control cells and CBS treated with TGF β , respectively (10 ng/ml for 24 hours). Lanes 3–4, DN-Smad stable transfectant clone1 untreated control and DN-Smad clone1 treated with TGF β , respectively. Lanes 5–6, DN-Smad stable transfectant clone 2 untreated control and DN-Smad clone 2 treated with TGF β , respectively. (B) TGF β and cellular proliferation. Cellular proliferation was determined in 24 well culture plates by counting the cells with the aid of a hemocytometer (9). Each point represents the mean and standard error of triplicate determinations. Hollow bars, cells cultured without TGF β . Solid bars, cells cultured with TGF β (10 ng/ml). DN-Smad, clones of stable transfectants transfected with DN-Smad vector.