Transforming Growth Factor-ß1 Signaling Contributes to Caco-2 Cell Growth Inhibition Induced by 1,25-dihydroxyvitamin D₃

Anping Chen¶, Bernard H. Davis, Michael D. Sitrin, Thomas A. Brasitus and Marc Bissonnette*

RUNNING TITLE: 1,25(OH)₂D₃ sensitizes Caco-2 cells to growth inhibition by TGF-ß1

Gastroenterology Section, Department of Medicine, The University of Chicago MC4076, 5841 S. Maryland Ave., Chicago, IL 60637, U.S.A.

Tel: 773-702-8597
Fax: 773-702-2182
E-mail: mbissonn@medicine.bsd.uchicago.edu

*Corresponding author; ¶ current address: Department of Pathology, Department of Cellular Biology & Anatomy, Louisiana State University Health Sciences Center in Shreveport, 1501 Kings Highway, Shreveport, LA 71130

Key Words: TGF-ß; Calcitriol; IGF-II receptor; colon cancer chemoprevention; antiproliferative

ABBREVIATIONS
AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; IGF-IIIR, insulin-like growth factor-II receptor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; RPA, RNase protection assay; TGF-ß, transforming growth factor-beta.
ACKNOWLEDGMENT

The work presented was supported by National Institute of Health grants, including DK 39573 (T.A.B., M. D. S. and M. B.), CA 36745 (T.A.B. and M.B.), DK 47995 (A. C. and B. H. D.) and P30DK42086 (M. B. and T.A.B., Digestive Disease Research Core Center), as well as by the Samuel Freedman GI Cancer Laboratory at the University of Chicago.
ABSTRACT

The signal transduction pathways of transforming growth factor β1 (TGF-β1) and 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] have been shown to interact in several cell types. 1,25(OH)₂D₃ inhibits the growth of many cancer cells, including Caco-2 cells, a human colon cancer-derived cell line. Whereas the growth of normal colonic epithelial cells is inhibited by TGF-β1, most human colon cancer-derived cells, including Caco-2 and SW480 cells, are resistant to TGF-β1. The mechanisms underlying the antiproliferative actions of 1,25(OH)₂D₃ and the resistance to TGF-β growth inhibition, however, remain largely unknown. In the present studies, we observed that 1,25(OH)₂D₃ sensitized Caco-2 cells, as well as SW480 cells, to TGF-β1 growth inhibitory effects. Compared with 1,25(OH)₂D₃ alone, the combination of 1,25(OH)₂D₃ (100 nM) and TGF-β1 (2 ng/ml) caused a significant reduction in Caco-2 and SW480 cell numbers. In addition, we observed that the amount of active TGF-β1 was increased (~4-fold) by this secosteroid in conditioned media from Caco-2 cells. 1,25(OH)₂D₃ increased the expression of insulin-like growth factor II receptors (IGF-IIR) in Caco-2 cells, which facilitated activation of latent TGF-β1. 1,25(OH)₂D₃ was found to activate TGF-β signaling in Caco-2 cells. Furthermore, by using neutralizing antibodies to human TGF-β1, we demonstrated that this cytokine contributes to the secosteroid-induced inhibition of Caco-2 cell growth. 1,25(OH)₂D₃ was also found to enhance the type I TGF-β receptor mRNA and protein abundance in Caco-2 cells. Whereas the 1,25(OH)₂D₃-induced sensitization of Caco-2 cells to TGF-β1 was IGF-IIR-independent, the type I TGF-β receptor was required for this sensitization. Taken together, these studies have demonstrated that 1,25(OH)₂D₃ treatment of Caco-2 cells resulted in activation of latent TGF-β1, which was facilitated by the enhanced expression of IGF-IIR by this secosteroid. In addition, 1,25(OH)₂D₃ sensitized Caco-2 cells to growth inhibitory effects of TGF-β1, which also contributed to the inhibition of Caco-2 cell growth by this secosteroid.
INTRODUCTION

Colorectal cancer is a leading cause of cancer-related morbidity and mortality in the United States. Epidemiologic studies have found that the dietary intake of vitamin D and sunlight exposure are inversely associated with the risk of colon cancer (32). Studies from our laboratory and others have indicated that the active, hormonal form of vitamin D, 1,25(OH)₂D₃, and several of its analogues significantly inhibit colon cancer cell growth in vitro and in vivo (4, 16, 43). Prior studies from our group using Caco-2 cells have demonstrated that 1,25(OH)₂D₃ inhibits cell growth, enhances differentiation, and induces apoptosis (11, 16, 43), but many of the molecular mechanisms underlying these effects remain undefined.

In this regard, signaling pathways activated by 1,25(OH)₂D₃ and TGF-β are known to have relevant biological interactions in several non-colonic cell types, including synergistic inhibitory effects on cell growth (17, 21, 50). The TGF-β superfamily, like 1,25(OH)₂D₃, regulates a broad range of important cellular processes including proliferation, differentiation and apoptosis (38). Alterations in response to TGF-β stimulation are thought to play important roles in colon cancer development. Whereas the growth of normal colonic epithelial cells is inhibited by TGF-β1, most human colon cancer cells, including Caco-2 and SW480 cells, are resistant to the growth inhibitory effects of TGF-β1 (6, 49). With the exception of several mutations in TGF-β receptors and downstream signaling proteins (14, 31), which occur in a minority of colon tumors and colon cancer-derived cell lines, most sporadic colon cancers and cell lines have no identified molecular derangements responsible for their resistance to TGF-β growth inhibition.

TGF-β1 is synthesized and secreted in a latent, biologically inactive form, which must be activated before binding to TGF-β receptors. The insulin-like growth factor II receptor (IGF-IIR) is a multifunctional receptor with two distinct binding sites. One site binds to insulin-like growth factor II (IGF-II), leading to its degradation and, thereby regulating the bioavailability of extracellular IGF-II. The second site binds a variety of proteins bearing mannose-6-phosphate residues (28), including the inactive TGF-β1 precursor. An established physiological function of IGF-IIR is to facilitate the proteolytic activation of the inactive TGF-β precursor. TGF-β signaling is mediated by a network of transmembrane
serine/threonine kinase receptors, and their downstream signal transducing targets, the Smad proteins. This signaling is initiated by TGF-β binding to the type II TGF-β receptor (Tβ–RII), which then phosphorylates and activates the type I TGF-β receptor (Tβ–RI). These two receptors then form a heteromeric complex (probably a heterotetramer), which, in turn, phosphorylates regulatory Smad 2 and/or Smad 3 proteins. The latter proteins subsequently form a heteromeric complex with Smad 4 and migrate to the nucleus to regulate target gene expression. The Smad complexes often act in concert with other transcription factors, as well as with co-activators or co-repressors.

We hypothesized that alterations in TGF-β signaling may mediate, at least in part, Caco-2 cell growth inhibition by vitamin D secosteroids. In the present studies, 1,25(OH)2D3 was observed to increase the amount of active TGF-β1 in the conditioned media from Caco-2 cells. Furthermore, this secosteroid sensitized Caco-2 cells to TGF-β1 growth inhibitory effects. The mechanisms for these effects of 1,25(OH)2D3 on TGF-β1 activation and signaling, their influence on Caco-2 cell growth, and their potential importance in the chemoprevention of colon cancer form the basis of this report.
MATERIALS AND METHODS

Chemicals and reagents: 1,25(OH)₂D₃ was purchased from Steroids LTD Laboratory (Chicago, IL). Active human TGF-ß1 was obtained from Promega (Madison, WI). Mannose-6-phosphate (Man-6-P) and glucose-6-phosphate (Glu-6-P) and other chemicals were of the highest purity available, and purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise indicated.

Cell culture, transfection and luciferase assay: Caco-2 and SW480 cells, each derived from human colonic carcinomas, were cultured at 37°C in 5% CO₂, in Dulbecco's modified Eagle's medium (DMEM), as previously described (11). Cells were treated with 1,25(OH)₂D₃, or vehicle (EtOH), for the indicated time and protected from fluorescent light. Sixty to eighty percent confluent cells (2-3 days post-plating), in 6-well cell culture plates, were transfected by lipofectAMINE™, following the protocol provided by the manufacturer (GIBCO BRL, Life Technology, Grand island, NY). Each transfection was performed in triplicate and repeated 3-4 times. The β-galactosidase expression plasmid pSV-β-gal (Promega, Madison, WI) was included to normalize for transfection efficiency. Luciferase assays were performed using a kit from Promega and following the protocol provided by the manufacturer. The luciferase activities of each transfection were expressed as relative units after normalization for transfection efficiency using β-galactosidase activities.

Plasmid constructs: The plasmids, pT7-TßRI and pT7-TßRII, used for generating single-stranded RNA probes complimentary to Tß-RI and Tß-RII for RNase protection assays, were generous gifts from Dr. Michael Centrella, Yale University (7). The plasmid p3TP-Luc is a TGF-ß-inducible luciferase reporter construct, containing the plasminogen activator inhibitor-1 (PAI-1) gene promoter, kindly provided by Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center, NY) (1, 5, 33).

RNA isolation and RNase protection assay (RPA): Total RNA was isolated by the TRI-Reagent, following the protocol recommended by the manufacturer (Sigma). A single-stranded RNA probe complimentary to IGF-IIR (411 bp) was generated as previously described (28, 48). To prepare RNA probes for Tß-RI and Tß-RII, pT7-TßRI was linearized with SmaI, and pT7-TßRII with EcoRI,
respectively (7). The 115 bp of 28S rRNA probe was used as an internal control (Ambion, Austin, TX). The anti-sense probes were synthesized and $^{32}$P-labeled by MAXIscript™ (Ambion). RPA was carried out with RPA II™ kits (Ambion) following the protocol provided by the manufacturer. The radioactivity in each band was measured by a phosphorimager (Molecular Dynamics, Sunnyvale, CA), as described previously (10, 11).

**Western blotting analysis:** Whole cell extracts were prepared from pre-confluent Caco-2 cells, and membrane proteins were prepared as described (2). Human colonic specimens were obtained from the Department of Surgical Pathology of the University of Chicago Hospitals. These included paired colon cancer samples and normal colonic mucosa dissected from the underlying layers from the same patient. Specimens were flash frozen in liquid nitrogen within 1 hr of resection. Tumor homogenates were prepared and stored at –70 °C until use. Western blotting analyses were performed as described (10, 11). Anti-Tß-RI and -RII antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and the anti-IGF-IIR serum was generously provided by Dr. Richard G. MacDonald of the University of Nebraska (29). Integrin beta1, a subunit of integrin receptors for the extracellular matrix (13), was used as an internal membrane protein control for the IGF-IIR Western blots.

**Cell proliferation assays:** Pre-confluent Caco-2 or SW480 cells (2-3 days post-plating) were incubated with indicated concentrations of active human TGF-ß1 (Promega), in the presence or absence of 1,25(OH)$_2$D$_3$ (100 nM) for 24 hr in serum-free DMEM. In other experiments, neutralizing anti-active TGF-ß1 antibodies (30 µg/ml) from Promega, control normal rabbit IgG, Man-6-P (100 µM) or Glu-6-P (100 µM) was added to serum-free DMEM 30 min prior to the addition of 1,25(OH)$_2$D$_3$. Cell growth was determined by counting cell numbers and/or by MTS assays (Promega). For MTS assays, all experiments were carried out in 96-well plates. Cell growth was analyzed by using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit following the protocol provided by the manufacturer.
TGF-β1 immunoassay (ELISA): After cell treatment, conditioned media was collected and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were assayed for the active form of TGF-β1 by a TGF-β1 E_{max} ImmunoAssay System (ELISA) (Promega) following the protocol provided by the manufacturer. This immunoassay system was designed for the sensitive and specific detection of biologically active TGF-β1. The antibody in the system does not recognize the TGF-β1 precursor. To determine the amount of total TGF-β1 in the conditioned media, samples were pretreated with 1N HCl for 15 min at room temperature prior to neutralization with 1 N NaOH, as suggested by the manufacturer. This procedure converts any latent TGF-β1 to the active form.

Suppression of IGF-IIR and Tβ-RI protein expression by anti-sense oligonucleotides: These experiments were conducted as previously described (11). In brief, phosphorothioate-modified sense and anti-sense oligonucleotides were synthesized by Life Technology, Inc., (Grand Island, NY). The optimal concentrations to suppress the expression of these receptor proteins were determined by incubation of Caco-2 cells in DMEM with either anti-sense or sense oligonucleotides at concentrations between 0 to 100 µg/ml for 4 hr before the addition of 1,25(OH)_{2}D_{3}. After 24 hr incubation, the cells were harvested and the lysates were probed by Western blotting using anti-IGF-IIR or anti-Tβ-RI. To study the roles of IGF-IIR and Tβ-RI in the TGF-β1 sensitization induced by 1,25(OH)_{2}D_{3}, pre-confluent cells were pretreated with 50 µg/ml of either anti-sense or sense oligonucleotides 4 hr prior to the addition of 1,25(OH)_{2}D_{3} (100 nM) and exogenous active TGF-β1. In preliminary experiments we found this concentration of antisense for Tβ-R1 and and antisense for IGF-IIR caused maximum inhibition of protein expression for each of these receptors. The incubation was then continued for an additional 24 hr. Cell growth was determined by counting cell numbers and/or by MTS assays. The sequences for the anti-sense oligonucleotides used for the experiments were the following:

**anti-sense IGF-IIR oligonucleotides:** 5’-TCC TAG CTG AAC GGC CCG CAT-3’, (the first 21 nucleotides from the start codon of the human IGF-IIR gene (37)).

**anti-sense Tβ-RI oligonucleotides:** 5’-AGC AGC CGA CGC CGC CTC CAT-3’, (the first 21 nucleotides from the start codon of the Tβ-RI gene (20)).
**Immunocytochemistry:** Preconfluent Caco-2 cells in 12-well plates (2-3 days post-plating) were pretreated with or without anti-sense IGF-IIR oligonucleotides (50 µg/ml) 4 hr prior to the addition of 1,25(OH)₂D₃ (100 nM) for an additional 24 hr. Cells treated with vehicle (0.08% EtOH) for 24 hr were used as a control. Cells were fixed with 100% methanol. After rinsing and blocking with PBS/1% BSA, cells were labeled with anti-IGF-IIR serum (1:100). As a negative control, cells were labeled with normal (non-immune) rabbit IgG. The cells were immunostained using biotinylated secondary antibodies and the ABC kit from Vector laboratories, Inc. (Burlingame, CA).

**Statistical analysis:** Differences between means were evaluated using an unpaired Student's t-test (p<0.05 considered as significant). Where appropriate, comparisons of multiple treatment conditions with controls were analyzed by ANOVA with the Dunnett's test for post hoc analysis.
RESULTS

1,25(OH)₂D₃ sensitizes Caco-2 cells to the growth inhibitory effects of TGF-β1

Our previous studies demonstrated that 1,25(OH)₂D₃ significantly inhibited Caco-2 cell growth in a dose-dependent manner (10⁻¹⁰ - 10⁻⁷ M), with a maximal inhibition at 100 nM (16), and no toxicity up to 300 nM as assessed by lactate dehydrogenase (LDH) release (41). Based on these observations, we chose to study pre-confluent Caco-2 cells treated with 1,25(OH)₂D₃ at 100 nM for these experiments. Caco-2 cells were chosen to evaluate the potential ability of 1,25(OH)₂D₃ to sensitize colon cancer cells to the growth inhibitory effects of TGF-β1. Compared with cells without any treatment, 1,25(OH)₂D₃ alone (i.e. TGF-β1 = 0 ng/ml) significantly reduced Caco-2 cell numbers (Fig. 1). As shown in Fig. 1, in the absence of 1,25(OH)₂D₃, Caco-2 cells were resistant to the growth inhibitory effects of exogenous active TGF-β1 (0.1-4 ng/ml) alone. In contrast, in the presence of 1,25(OH)₂D₃ (100 nM), TGF-β1, in a dose-dependent manner, further significantly reduced Caco-2 cell numbers. The optimal TGF-β1 growth inhibitory dose was 2-2.5 ng/ml (Fig. 1). We also assessed whether another TGF-β resistant human colon cancer cell line, SW480, could also be sensitized by 1,25(OH)₂D₃ to TGF-β growth inhibition (Fig. 1). In agreement with previous reports (6, 49), exogenous active TGF-β1 alone had no effect on SW480 cell growth (data not shown). In contrast, in the presence of 1,25(OH)₂D₃ (100 nM), exogenous active TGF-β1 caused a significant dose-dependent reduction in cell numbers of SW480 cells. As in the case of Caco-2 cells, maximal inhibition was seen at 2.5 ng/ml and was somewhat reduced at higher concentrations. These results indicated that SW480 cells, like Caco-2 cells, were sensitized by 1,25(OH)₂D₃ to the growth inhibitory effects of TGF-β1.

1,25(OH)₂D₃ increases the amount of active TGF-β1 in Caco-2 conditioned media

To determine the potential ability of 1,25(OH)₂D₃ to alter the abundance of TGF-β1 in Caco-2 cells, conditioned media from these cells, treated with 1,25(OH)₂D₃ (100 nM) or vehicle for 24 hr, were analyzed for both the total amount of TGF-β1 and the active form of this cytokine by immunoassays.
(ELISA) as described in “Methods”. Our results indicated that, while 1,25(OH)₂D₃ did not change the total amount of TGF-β1, this secosteroid increased the amount of active TGF-β1 by ~4-fold in Caco-2 conditioned media (Fig. 2). Prior studies demonstrated that mannose 6-phosphate (Man-6-P) prevented the activation of latent TGF-β1 by blocking the binding of the pro-form of TGF-β1 to IGF-IIR (12). In agreement with these findings, we observed that the 1,25(OH)₂D₃-induced increase in active TGF-β1 in Caco-2 conditioned media could be blocked by Man-6-P (100 µM) (Fig. 2), but not by glucose 6-P (data not shown), suggesting the involvement of IGF-IIR in this process.

1,25(OH)₂D₃ enhances the expression of IGF-II receptors in Caco-2 cells

As noted earlier, the IGF-IIR facilitates the activation of TGF-β1 by binding the precursor of this cytokine and subsequently releasing the inhibitory mannose-6-P containing latency peptide (12, 15, 22). Prior studies have demonstrated that Caco-2 cells express functional IGF-IIR (18). Since 1,25(OH)₂D₃ significantly increased the active form of TGF-β1 in the conditioned media of Caco-2 cells and Man-6-P could prevent this increase (Fig. 2), it was plausible that 1,25(OH)₂D₃ might induce the expression of IGF-IIR in these cells. To study this possibility, we examined the effect of 1,25(OH)₂D₃ on the expression of IGF-IIR in Caco-2 cells. 1,25(OH)₂D₃ significantly increased the steady state levels of IGF-IIR mRNA by 3-fold in Caco-2 cells as demonstrated by RNase protection assays (Fig. 3A & B). Additional experiments were performed to assess the effects of 1,25(OH)₂D₃ on IGF-IIR membrane protein expression. Pre-confluent Caco-2 cells were treated with 1,25(OH)₂D₃ (100 nM) or EtOH for 24 hr. 1,25(OH)₂D₃ significantly enhanced the expression of membrane IGF-IIR proteins by approximately 5.5-fold in Caco-2 cells (Fig. 3C & D).

TGF-β1 contributes to the 1,25(OH)₂D₃-induced inhibition of Caco-2 cell growth

Having established that 1,25(OH)₂D₃ increased the active form of TGF-β1, we asked if immune neutralization of TGF-β1 would alter the ability of this secosteroid to inhibit Caco-2 cell growth. Caco-2 cells in serum-free DMEM were pretreated with anti-TGF-β1 antibodies (α-TGF-β1, 30 µg/ml) or non-immune serum (NRIgG) for 30 min prior to the addition of this secosteroid. As shown in Fig. 4,
pretreatment of cells with anti-TGF-β1 antibodies, but not non-immune serum (NRIgG), significantly reduced the growth inhibition of Caco-2 cells by 1,25(OH)_{2}D_{3} from 26% to 15%. These results, thus, suggested an autocrine/paracrine role for TGF-β1, and supported our hypothesis that TGF-β1 mediates, at least in part, the antiproliferative effects of this secosteroid in these cells. To further evaluate the role of the activation of latent TGF-β1 in the 1,25(OH)_{2}D_{3}-induced inhibition of cell growth, Caco-2 cells in serum-free DMEM were pretreated with Man-6-P (100 µM) or Glu-6-P (100 µM) prior to the addition of 1,25(OH)_{2}D_{3}. As shown in Fig. 4, Man-6-P, but not Glu-6-P, reduced the inhibition of Caco-2 cell growth induced by 1,25(OH)_{2}D_{3} from 26% to 16%. These studies indicated that activation of latent TGF-β1, facilitated by IGF-IIIR, played an important role in the inhibition of Caco-2 cell growth induced by this secosteroid. Increasing the concentrations of neutralizing anti-TGF-β1 antibodies, or Man-6-P, did not further limit this growth inhibition by 1,25(OH)_{2}D_{3}, suggesting that other TGF-β-independent mechanisms also contribute to the inhibition of cell growth by this secosteroid. It should be emphasized, however, that additional (exogenous) active TGF-β1 alone failed to inhibit the growth of these cells in the absence of 1,25(OH)_{2}D_{3}. These results indicate, therefore, that TGF-β1 sensitization must involve more than an increased conversion of the latent to the active form of this cytokine.

1,25(OH)_{2}D_{3} activates TGF-β signaling in Caco-2 cells

To assess the ability of 1,25(OH)_{2}D_{3} to activate TGF-β signaling in Caco-2, cells were transiently transfected with a TGF-β1-inducible luciferase reporter plasmid p3TP-Luc. This luciferase reporter plasmid contains the promoter for the plasminogen activator inhibitor-1 (PAI-1) gene, which includes TGF-β response elements (1, 5, 33). After transfection, cells were treated with the indicated agents as described in Fig. 5 and luciferase activities in the treated cells were determined. As shown in Fig. 5, compared to EtOH as control, 1,25(OH)_{2}D_{3} significantly increased the luciferase activity by 4.9-fold in cells transfected with p3TP-Luc. Man-6-P abrogated the ability of 1,25(OH)_{2}D_{3} to increase the luciferase activity in these cells. Not surprisingly, TGF-β1 alone did not alter luciferase activity in Caco-2 cells. However, in the presence of 1,25(OH)_{2}D_{3}, TGF-β1 significantly increased luciferase activity by an additional 45%, compared to 1,25(OH)_{2}D_{3} treatment alone. Man-6-P pretreatment could not block the
increase in luciferase activity in the cells treated with both 1,25(OH)₂D₃ and exogenous active TGF-β1. This result is not surprising since Man-6-P prevents the conversion of precursor TGF-β1 to the active form, but does not inhibit the activated species. Taken together, these results indicate that 1,25(OH)₂D₃ confers competence to transcriptional activation of target genes by TGF-β1 in Caco-2 cells, which is likely involved in the ability of this secosteroid to sensitize Caco-2 cells to TGF-β1 growth inhibition.

1,25(OH)₂D₃ increases the abundance of Tβ-RI protein and mRNA in Caco-2 Cells

As noted earlier, TGF-β signaling is initiated by TGF-β binding to Tβ-RII, which phosphorylates and activates Tβ-RI. The signaling subsequently passes to target genes through Smad proteins. As shown in Fig. 1, treatment with 1,25(OH)₂D₃ sensitized Caco-2 and SW480 cells to growth inhibition by TGF-β1. We postulated that this 1,25(OH)₂D₃-induced sensitization might arise, at least in part, by up-regulation of TGF-β receptor expression. To address this hypothesis, total mRNA and protein extracts were prepared from Caco-2 cells treated with 1,25(OH)₂D₃ for the indicated time. RNase protection assays (RPA) indicated that 1,25(OH)₂D₃ stimulated an increase in the steady state levels of Tβ-RI mRNA, but not Tβ-RII mRNA, by 1.4-, 2.3-, 2.9-, and 2.8-fold after 3, 6, 16 and 24 hr treatment, respectively, compared with control (0 hr) (Fig. 6A & B). As assessed by Western blotting analyses, 1,25(OH)₂D₃ treatment for 24 hr significantly increased the protein abundance of Tβ-RI by 2.5-fold (Fig. 6C), but not Tβ-RII (data not shown), in Caco-2 cells.

Expression of both Tβ-RI and RII are significantly reduced in human colonic carcinomas and Caco-2 cells

Since 1,25(OH)₂D₃ up-regulated the expression of Tβ-RI in Caco-2 cells and sensitized these cells to TGF-β1 inhibition, it was of interest to compare the expression of TGF-β receptors in normal colon to that of colonic cancer and colon cancer-derived Caco-2 cells. To evaluate the expression levels of Tβ-RI and RII, protein extracts prepared from colonic carcinomas and corresponding normal colonic mucosa, as well as Caco-2 cells, were analyzed by Western blot analyses. Compared with matched normal colonic mucosa (Fig. 7A, Lane 1 & 2), expression levels of both Tβ-RI and Tβ-RII were significantly reduced by
approximately 30% and 55%, respectively, in human colonic tumors (Fig. 7A, Lane 3 & 4). In addition, compared with normal colonic mucosa (Lane 1 & 2), expression of both Tß-RI and RII in Caco-2 cells (Fig. 7A, Lane 5 & 6) were also markedly reduced. Tß-RI expression was induced by 1,25(OH)2D3 in Caco-2 cells by 2.3 fold (Fig. 7A, Lane 7 & 8). In contrast, Tß-RII was not altered by 1,25(OH)2D3 (Fig. 7A, Lane 7 & 8).

**Tß-RI is required for the TGF-ß1 sensitization of Caco-2 cells induced by 1,25(OH)2D3**

In initial experiments, we demonstrated that anti-sense Tß-RI or IGF-IIR oligonucleotides at 40-60 µg/ml significantly and specifically inhibited the expression of Tß-RI and IGF-IIR proteins, respectively, in Caco-2 cells (Fig. 8A & B). In addition, we examined the effect of anti-sense IGF-IIR oligonucleotides on IGF-IIR protein expression detected by immunocytochemical staining (Fig. 9). Compared with the cells treated with 1,25(OH)2D3 alone, pretreatment of cells with anti-sense IGF-IIR oligonucleotides significantly reduced the level of IGF-IIR protein staining induced by 1,25(OH)2D3, demonstrating that IGF-IIR protein is significantly inhibited by anti-sense IGF-IIR oligonucleotides in Caco-2 cells. Using these anti-sense oligonucleotides, we next evaluated the roles of these receptors in the ability of 1,25(OH)2D3 to inhibit Caco-2 cell growth and to sensitize these cells to the antiproliferative effect of TGF-ß1. Caco-2 cells were pretreated with either anti-sense Tß-RI or IGF-IIR oligonucleotides (50 µg/ml) 4 hr prior to the addition of the indicated doses of active TGF-ß1 and 1,25(OH)2D3 (100 nM) for an additional 24 hr. As shown in Fig. 8C, anti-sense Tß-R1 oligonucleotides significantly reduced growth inhibition of Caco-2 cells by 1,25(OH)2D3 from 25% to 16%, and abrogated the previously observed secosteroid-induced sensitization of these cells to TGF-ß1. These results indicated that Tß-RI significantly contributes to the 1,25(OH)2D3-induced inhibition of Caco-2 cell growth. Furthermore, as expected, this receptor is required for 1,25(OH)2D3 to confer responsiveness of Caco-2 cells to the antiproliferative effect of TGF-ß1. Down-regulation of IGF-IIR in Caco-2 cells by anti-sense IGF-IIR oligonucleotides also significantly reduced the growth inhibition by 1,25(OH)2D3 from 25% to 14% (Fig. 8C). This is in agreement with the role of IGF-IIR in the activation of latent TGF-ß. Not
surprisingly, however, anti-sense IGF-IIR oligonucleotides were unable to block the secosteroid-induced sensitization of Caco-2 cells to the growth inhibitory effects of exogenous active TGF-β1 (Fig. 8C).
DISCUSSION

In this paper we report for the first time two important interactions of 1,25(OH)₂D₃ with the TGF-β1 signaling system that explain, at least in part, the antiproliferative effect of this secosteroid in Caco-2 cells. We found that 1,25(OH)₂D₃ (100 nM) increased the abundance of active TGF-β1 by stimulating the expression of IGF-IIR in Caco-2 cells. In addition, we observed that 1,25(OH)₂D₃ (100 nM) sensitized Caco-2 cells, as well as SW480 cells, to the growth inhibitory effects of TGF-β1.

Although the growth of normal epithelial cells, including colonocytes, is inhibited by TGF-β1 (39), primary colon neoplasms and most colon cancer cell lines are typically resistant to TGF-β1 (8). Progression from colonic adenoma to carcinoma is accompanied by increasing resistance to TGF-β-induced growth inhibition (30). With the exception of several specific mutations in TGF-β receptors or downstream Smad proteins that occur predominantly in tumors with DNA mismatch repair defects, the majority of sporadic colon cancers and colon cancer cell lines have no identified molecular derangements that explain their resistance to TGF-β growth inhibition (19).

TGF-β1 is secreted by cells in a latent, biologically inactive form, which must be activated before binding to TGF-β receptors. IGF-IIR binds and facilitates the proteolytic activation of the inactive TGF-β precursor (12, 22). In the present study, total TGF-β1 secreted by Caco-2 cells was not affected by 1,25(OH)₂D₃, however, the abundance of active TGF-β1 was increased. In several non-colonic cell lines 1,25(OH)₂D₃ was reported to increase total TGF-β secretion (23, 51), suggesting that the effects of 1,25(OH)₂D₃ on TGF-β1 gene expression and activation are cell-type specific. 1,25(OH)₂D₃ enhanced the protein and mRNA expression of IGF-IIR in Caco-2 cells, as demonstrated by immunocytochemical and Western blotting studies and by RNase protection assays, respectively. Moreover, treatment of Caco-2 cells with Man-6-P abolished the increase in active TGF-β1, which further supports our assumption that the up-regulation of IGF-IIR expression was responsible for the increase in active
TGF-β1 by 1,25(OH)₂D₃. We have demonstrated that induction of the IGF-IIR and subsequent TGF-β1 activation in Caco-2 cells is responsible, at least in part, for the 1,25(OH)₂D₃-induced growth inhibition of these cells. Down-regulation of IGF-IIR by anti-sense oligonucleotides, blockage of latent TGF-β binding to IGF-IIR by Man-6-P, or neutralization of TGF-β1 by antibodies, diminished the anti-proliferative effects of 1,25(OH)₂D₃ in Caco-2 cells, demonstrating that this secosteroid stimulated the activation of TGF-β, which then acted by an autocrine/paracrine mechanism to inhibit growth. These results suggested that IGF-IIR indirectly contribute to the 1,25(OH)₂D₃-induced Caco-2 cell growth inhibition by facilitating activation of latent TGF-β. We do not believe, however, that there is a direct requirement for IGF-IIR in the 1,25(OH)₂D₃-induced sensitization of Caco-2 cells to TGF-β inhibitory effects. In this regard, anti-sense IGF-IIR oligonucleotides were unable to block the secosteroid-induced sensitization of Caco-2 cells to the growth inhibitory effects of exogenous active TGF-β1 (Fig. 8C).

In addition, the IGF-IIR may affect cell growth by regulating the bioavailability of extracellular IGF-II (12, 22). As a potent mitogenic polypeptide homologous to insulin, IGF-II, which is secreted by Caco-2 cells, binds to IGF-I receptors and stimulates cell growth (3). In contrast, binding to the IGF-IIR, which does not transduce a mitogenic signal, results in an accelerated degradation of IGF-II and thereby a reduction in cell growth (28, 34). Hence, the enhanced expression of the IGF-IIR by 1,25(OH)₂D₃ would be expected to reduce the bioavailability of mitogenic IGF-II, and thereby contribute to the growth inhibition of Caco-2 cells caused by this secosteroid. Our results demonstrated that when IGF-IIR expression was blocked by IGF-IIR antisense oligonucleotides, large doses of exogenous TGF-β1 plus 1,25(OH)₂D₃ failed to inhibit growth to the same extent as cells without the antisense treatment. This observation suggests that the antisense oligonucleotides, which mediated down-regulation of IGF-IIR, also positively influenced growth by a TGF-β1 independent mechanism, possibly by increasing the bioavailability of mitogenic IGF-II.

Signaling pathways induced by 1,25(OH)₂D₃ and TGF-β1 have been shown to interact in several non-colonic cell types, resulting in increased TGF-β1 release (17, 23), enhanced TGF-β receptor...
expression (21, 50, 51), decreased cell growth (17, 23), and enhanced differentiation (35). In the present study, although TGF-β1 alone had no effect on cell growth, the combination of 1,25(OH)_{2}D_{3} and TGF-β1 caused significantly more growth inhibition than 1,25(OH)_{2}D_{3} alone. This indicated that 1,25(OH)_{2}D_{3} sensitized these cells to the growth inhibitory effects of TGF-β1.

In this regard, our studies have demonstrated that the levels of both Tβ-RI and RII were significantly and comparably reduced in Caco-2 cells and in human colonic tumors, compared with normal colonic mucosa. 1,25(OH)_{2}D_{3} increased the expression of Tβ-RI mRNA and protein by about 2.5-fold in Caco-2 cells, but did not change the level of Tβ-RII. Results from experiments with Tβ-RI anti-sense oligonucleotides indicated that this receptor contributed to the 1,25(OH)_{2}D_{3}-induced inhibition of cell growth, and not surprisingly, was required for the sensitization of these cells to growth inhibition by exogenous TGF-β1. Interestingly, another human colon carcinoma-derived cell line, GEO cells, are also insensitive to TGF-β1 and express low levels of Tβ-RI mRNA (47). Stable transfection of Tβ-RI, but not Tβ-RII, cDNA increased TGF-β1 binding and resulted in increased growth inhibition by exogenous TGF-β1 (47), indicating that the low level of Tβ-RI was a limiting factor for the growth-inhibitory effects of TGF-β1 in those cells. Other studies have suggested a major role of Tβ-RII in the regulation of gene expression, while Tβ-RI appears to mainly transduce regulatory effects of TGF-β1 on cell growth (27, 40). Thus, it is likely that the up-regulation of Tβ-RI by 1,25(OH)_{2}D_{3} contributed, at least in part, to the sensitization of Caco-2 cells to the growth-inhibitory effect of TGF-β caused by this secosteroid. Maximal growth inhibition occurred at 2.5 ng/ml. The somewhat reduced inhibition at higher TGF-β concentrations that others have also observed (36), may reflect a non receptor mediated effect.

In addition, vitamin D signaling has been found to interact with Smad proteins in other cell types. Transcriptionally active complexes containing the vitamin D receptor (VDR) and Smads have been identified (53). Smad proteins have been reported to activate or inhibit VDR transactivating ability (52). Studies are in progress in our laboratory to elucidate the roles of Smad proteins in the TGF-β sensitization of Caco-2 cells by 1,25(OH)_{2}D_{3}.
The mechanisms by which 1,25(OH)₂D₃ up-regulates IGF-IIR and Tß-RI expression remain unsolved. Our prior studies demonstrated that 1,25(OH)₂D₃ induced the activation of the transcription factor AP-1 in Caco-2 cells (11). It is possible that AP-1, activated by this secosteroid, might mediate the up-regulation of IGF-IIR and Tß-RI gene expression, since putative AP-1 binding sites are present in the promoter regions of both genes (20, 26). Alternatively, these promoters may contain vitamin D receptor response elements. Additionally, the c-Jun component of AP-1 may interact with Smad proteins in regulating gene expression as described by others (25, 46).

With regard to sensitization to TGF-β1 growth inhibition by other agents, previous studies have demonstrated that another differentiating agent, the short chain fatty acid butyrate, like 1,25(OH)₂D₃, induced the maturation, and inhibited the cell growth, of several colon cancer cell lines, including Caco-2 cells (24, 42). Similar to our observation with 1,25(OH)₂D₃, butyrate was found to sensitize these cells to growth inhibition by TGF-β1 (24, 42). It was suggested that butyrate might alter the expression of proteins that mediate TGF-β1 responses in these cells, but the signal transduction elements involved in these processes were not identified. It bears emphasis that although the additional inhibition of Caco-2 cell growth, induced by the combination of TGF-β1 and 1,25(OH)₂D₃ in this study, or by butyrate (24, 42), is relatively modest, its impact on tumor progression may be considerable. Mathematical models of tumorigenesis have suggested, for example, that even limited decreases in their proliferative rates may ultimately result in significant inhibition of tumor growth (44, 45).

Our previous studies demonstrated that 1,25(OH)₂D₃ causes a decrease in cell numbers without a specific change in the distribution of cells in G₁, S or G₂/M. Specifically, we found that this secosteroid causes an increased doubling time (41). In the previous report, however, we did not examine the effects of TGF-β antibodies on Caco-2 cell growth inhibition by 1,25(OH)₂D₃. We have also previously shown that 1,25(OH)₂D₃ increases apoptosis in Caco-2 cells (9). In this communication we now demonstrate that the antiproliferative effects of 1,25(OH)₂D₃ are mediated in part by sensitization of Caco-2 cells to growth inhibition by TGF-β. It is likely that both apoptosis and increased doubling time contribute to the antiproliferative effects of 1,25(OH)₂D₃. The increased doubling time may reflect a generalized cell cycle
slowing in addition to increased apoptosis. Additional studies, beyond the scope of this manuscript, will be required to characterize the selective contributions of these two antiproliferative pathways (i.e. apoptosis and generalized slowing of the cell cycle) to Caco-2 cell growth inhibition by the combination of TGF-β and 25(OH)₂D₃.

In summary, our present studies have demonstrated that 1,25(OH)₂D₃ up-regulated the expression of IGF-IIR in Caco-2 cells, which accounted for an increase in the amount of active TGF-β1 in the conditioned media of these cells. The increased active TGF-β1 mediated, at least in part, the 1,25(OH)₂D₃-induced inhibition of cell growth. In addition, our studies have shown that 1,25(OH)₂D₃ activated TGF-β signaling in Caco-2 cells, and induced the sensitization of Caco-2 cells to the growth inhibitory effects of TGF-β1. Furthermore, this secosteroid increased the expression of Tβ-RI in Caco-2 cells, which played an important role in the 1,25(OH)₂D₃-induced inhibition of their growth, as well as the sensitization of these cells to TGF-β growth suppression. These studies have, therefore, provided novel insights into the mechanisms by which 1,25(OH)₂D₃ inhibits colon cancer cell growth. Increased understanding of TGF-β resistance in Caco-2 cells, which do not have identified mutations in TGF-β signaling, may elucidate more general mechanisms by which sporadic colon cancers escape TGF-β growth inhibition. It will be of interest to characterize the cis- and trans-activating elements involved in the up-regulation of IGF-IIR and Tβ-RI genes by 1,25(OH)₂D₃ in neoplastic colonic cells. Additional studies will be required to further characterize the interactions of TGF-β and MAPK signaling pathways, both of which are activated by 1,25(OH)₂D₃ and involved in the alterations in growth phenotype induced by this secosteroid (11). The pleiotropic actions of 1,25(OH)₂D₃ on critical signaling pathways that regulate the growth of colonic cells suggest that this secosteroid is a potentially potent and novel chemopreventive agent against colonic cancer.
FIGURE LEGENDS

Figure 1. 1,25(OH)2D3 sensitizes Caco-2 and SW480 cells to the growth inhibitory effects of TGF-ß1. Caco-2 and SW480 cells (2-3 days post-plating) were treated for 24 hr with the indicated doses of active TGF-ß1 in the absence (TGF-ß alone), or presence of 1,25(OH)2D3 (100 nM). Cell growth was determined by counting cell numbers. Values are expressed as means ± S.D. (n=6). * p< 0.05, compared with SW480 cells treated with 1,25(OH)2D3 alone. † p< 0.05, compared with Caco-2 cells treated with 1,25(OH)2D3 alone. Error bars, if not indicated, were contained within the data points.

Figure 2. 1,25(OH)2D3 increases the active form of TGF-ß1 in the conditioned media of Caco-2 cells. Aliquots of conditioned media from pre-confluent Caco-2 cells, treated with 1,25(OH)2D3 (100 nM) or vehicle (EtOH) in the presence or absence of Man-6-P for 24 hr, were analyzed for total and active TGF-ß1 by a TGF-ß1 ELISA (See "METHODS" for details). Values are expressed as means ± S.D. (n=6). * p < 0.05, compared with active TGF-ß1 in media from vehicle (EtOH)-treated cells.

Figure 3. 1,25(OH)2D3 increases the IGF-IIR expression in Caco-2 cells. Caco-2 cells were exposed to 1,25(OH)2D3 (100 nM) for the indicated time. Total RNA or membrane proteins were prepared for the RNase protection assays (RPA), or Western blotting analyses, respectively, as described in "METHODS". A. Representative IGF-IIR RPA. Fifteen micrograms of total RNA per sample were analyzed. Human 28S rRNA was used as the internal control to normalize for total RNA loading. The protected IGF-IIR and 28S rRNA are indicated on the right. B. Quantitation of IGF-IIR RPA with means ± S. D. from four independent experiments. * p<0.05, compared with untreated control. C. Representative Western blotting analyses of membrane IGF-IIR. Integrin β1, a subunit of extracellular matrix receptors, was used as an internal membrane protein control (13). D. Quantitation of membrane IGF-IIR protein, normalized to integrin β1, with means ± S. D. from three independent experiments. * p<0.05, compared with untreated control.
Figure 4. TGF-β1 contributes to the 1,25(OH)$_2$D$_3$-induced inhibition of Caco-2 cell growth. Caco-2 cells (2-3 days post-plating) were treated with EtOH or active TGF-β1 (2 ng/ml) in the presence or absence of 1,25(OH)$_2$D$_3$, or with 1,25(OH)$_2$D$_3$ plus anti-TGF-β1 antibodies (α-TGF-β1, 30 µg/ml), normal rabbit IgG (NRIgG), Man-6-P (100 µM), or Glu-6-P(100 µM). α-TGF-β1, Man-6-P or Glu-6-P was added prior to the addition of 1,25(OH)$_2$D$_3$. Cell growth was determined by counting cell numbers. Values are expressed as means ± S.D. (n=6). * p < 0.05, compared with ETOH-treated cells. ‡ p < 0.05, compared with 1,25(OH)$_2$D$_3$ alone.

Figure 5. 1,25(OH)$_2$D$_3$ activates TGF-β1 signaling. Pre-confluent Caco-2 cells (2-3 days post-plating) were transiently transfected with the TGF-β-inducible luciferase reporter plasmid p3TP-Luc. The β-galactosidase expression plasmid pSV-β-gal (Promega) was co-transfected to normalize transfection efficiency. After transfection, cells were treated with 1,25(OH)$_2$D$_3$, or ethanol (EtOH), plus or minus active TGF-β1 (2 ng/ml) for an additional 36 hr. Where indicated, Man-6-P (100 µM) was added 3 hr prior to the addition of 1,25(OH)$_2$D$_3$. Luciferase activities were determined by Luciferase Assay (Promega). The luciferase activities of each transfection were expressed as relative units after normalization for transfection efficiency from β-galactosidase activity. Values are expressed as means ± S.D. (n=6). * p < 0.05, compared with EtOH-treated cells; ** p < 0.01, compared with cells treated with 1,25(OH)$_2$D$_3$ alone.

Figure 6. 1,25(OH)$_2$D$_3$ increases the steady state levels of Tβ-RI mRNA and protein in Caco-2 Cells. Caco-2 cells in duplicate were treated with 1,25(OH)$_2$D$_3$ (100 nM) for the indicated time. Total proteins or RNA were prepared for Western blotting analyses or RPA, respectively. A. Representative RPA for Tβ-RI and Tβ-RII and human 28S rRNA as the internal control to normalize for total RNA loading. B. Quantitation of Tβ-RI and Tβ-RII mRNA with means ± S. D. from three independent experiments, each in duplicate. * p < 0.05, compared with control (0 hr). C. Representative Western blots of Tβ-RI and an internal control of β-actin.
Figure 7. Expression levels of both Tβ-RI and RII are reduced in human colonic cancers and Caco-2 cells. Protein lysates were prepared from six separate human colonic tumors and corresponding normal colonic mucosa, as well as from Caco-2 cells treated with or without 1,25(OH)2D3 (100 nM) for 24 hr. A. Western blots of Tβ-RI, Tβ-RII in two representative tumors and corresponding control mucosa as well as treated and untreated Caco-2 cells. β-actin was used as an internal control. B. Quantitation of Tβ-RI and Tβ-RII proteins with means ± S. D. (n= 6 human colonic tumors and matched normal colonic mucosa). Caco-2 cells were plated in duplicate (n=3, independent experiments). * p < 0.05, compared with normal mucosa; ** p < 0.02, compared with untreated Caco-2 cells (control).

Figure 8. Anti-sense Tβ-R1 oligonucleotides reduced the 1,25(OH)2D3–induced inhibition of cell growth and completely abolished the sensitization of Caco-2 cells to TGF-β1 induced by this secosteroid. Caco-2 cells (2-3 days post-plating) were incubated in DMEM with 1,25(OH)2D3 alone, or containing the indicated anti-sense or sense oligonucleotides (see details described in "METHODS"). A. Representative Western blots of cells treated with 1,25(OH)2D3 alone, or with either antisense or sense Tβ-RI oligonucleotides at the indicated concentrations for 24 hr. B. Representative Western blots of cells treated with 1,25(OH)2D3 alone, or with either antisense or sense IGF-IIR oligonucleotides at the indicated concentrations for 24 hr. C. Proliferation assays of cells pretreated with or without 50 µg/ml sense, or anti-sense Tβ-RI, or anti-sense IGF-IIR oligonucleotides for 4h. Cells were then incubated with the indicated concentrations of active TGF-β1 alone, or with 1,25(OH)2D3 (100 nM) for 24 hr. Cell growth was evaluated by MTS assays. Values are expressed as means ± S.D. (n=6). * p < 0.05, compared with cells treated with 1,25(OH)2D3 alone (TGF-β1 = 0 ng/ml).

Figure 9. Anti-sense IGF-IIR oligonucleotides significantly reduces the protein expression of IGF-IIR induced by 1,25(OH)2D3 in Caco-2 cells. Caco-2 cells (2-3 days post-plating) were pretreated with or without anti-sense IGF-IIR oligonucleotides (50 µg/ml) 4 hr prior to the addition of
1,25(OH)₂D₃ (100 nM) for 24 hr prior to fixation. Cells were labeled with anti-IGF-IIR antibodies (1:100) and immunostained by the biotinylated secondary antibodies and ABC kit from Vector Laboratories. Representative examples are shown here from three independent experiments (original magnification, x 100). Control samples, stained with non-immune serum, were negative (data not shown).

A. Cells treated with 0.08% EtOH as a negative control;  
B. Cells treated with 1,25(OH)₂D₃;  
C. Cells pretreated with anti-sense IGF-IIR oligonucleotides (α-sense oligoes) prior to the addition of 1,25(OH)₂D₃
REFERENCE


Active TGF-β1 (ng/ml)

Cell numbers (x 1000)

**Caco-2 (TGF-β1 alone)**

**SW480 [+ 1,25(OH)_{2}D_{3}]**

**Caco-2 [+ 1,25(OH)_{2}D_{3}]**

Fig. 1
**Fig. 3**

A. Relative levels of IGF-IIR mRNA over time (0h, 6h, 16h, 24h).

B. Bar graph showing relative levels of IGF-IIR mRNA at different time points.

C. Western blot analysis showing membrane IGF-IIR and Integrin β1.

D. Bar graph showing relative abundance of IGF-IIR protein at different time points.
Fig. 4

Cell numbers (x 1000/ml)

- Ctr
- TGF-β1
- 1,25 (OH)₂D₃

TGF-β1  α-TGF-β1  NRIgG  Man-6-P  Glu-6-P

* -26%
‡‡ -27%
‡ -27%
-15%
-16%
-40%
-27%
Luciferase activities (relative units)

Fig. 5
A.  

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>3h</th>
<th>6h</th>
<th>16h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RII</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.  

Levels of mRNA (arbitrary units)

- RI
- RII

Levels over time (hr):

- 0
- 5
- 10
- 15
- 20
- 25

C.  

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>6h</th>
<th>16h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-βRI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6
A.

<table>
<thead>
<tr>
<th></th>
<th>Mucosa</th>
<th>Tumor</th>
<th>Control</th>
<th>1,25(OH)$_2$D$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tβ-RI</strong></td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td><strong>Tβ-RII</strong></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td><strong>β-actin</strong></td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
</tbody>
</table>

1 2 3 4 5 6 7 8

B.

![Graph](image13)

Fig. 7
A. anti-sense oligoes          sense oligoes (Tβ-R1)
   0  20  40  60            0  20  40  60 (µg/ml)

B. anti-sense oligoes          sense oligoes (IGF-IIR)
   0  20  40  60            0  20  40  60 (µg/ml)

C. Cell Growth Inhibition (% control)

Fig. 8
Fig. 9

ctr                        1,25(OH)\textsubscript{2}D\textsubscript{3}                        \(\alpha\)-sense oligoes + 1,25(OH)\textsubscript{2}D\textsubscript{3}