Activation of TGF-β-Smad signaling pathway following polyamine depletion in intestinal epithelial cells

Lan Liu,1,2 Rachel Santora,1,2 Jaladanki N. Rao,1,2 Xin Guo,1,2 Tongtong Zou,1,2 Huifang M. Zhang,1,2 Douglas J. Turner,1,2 and Jian-Ying Wang1,2,3

Departments of 1Surgery and 3Pathology, University of Maryland School of Medicine, and 2Baltimore Veterans Affairs Medical Center, Baltimore, Maryland 21201

Submitted 1 April 2003; accepted in final form 9 July 2003

The epithelium of the intestinal mucosa has the most rapid turnover rate of any tissue in the body, and its integrity depends on the balance among cell proliferation, growth arrest, and apoptosis (26, 37). Intestinal epithelial cells continuously regenerate in the proliferating zone within the crypts and differentiate as they migrate up the luminal surface of the colon and villous tips in the small intestine (13, 14). Mature differentiated cells at the luminal surface and villous tips are quickly lost through the process of apoptotic cell death and are replaced by new cells. This rapid dynamic turnover rate of intestinal epithelial cells is highly regulated and critically controlled by numerous factors, including cellular polyamines (27, 30, 48). The natural polyamines spermidine and spermine and their precursor putrescine are ubiquitous organic cations of low molecular weight in eukaryotic cells and are intimately involved in distinct cellular functions (30, 45). A series of observations from our previous studies (19, 34, 35, 48, 49) and others (27, 30) have shown that normal intestinal mucosal growth depends on the superfamily signals from the cell surface to the nucleus (1, 54). On the basis of distinct functions, Smads are grouped into three classes: the receptor-regulated Smads (R-Smads) Smad2 and Smad3; the common Smad (co-Smad) Smad4; and the inhibitory Smads (I-Smads) Smad6 and Smad7 (10, 12, 24, 29). All TGF-β family members, including TGF-β, activins, and bone morphogenetic proteins, use type I and type II transmembrane serine/threonine kinase receptors in a variety of cell types (1, 10, 12, 29, 32). On ligand binding, the activated type II kinase phosphorylates the type I receptor, which subsequently phosphorylates the R-Smads on a COOH-terminal SSXS motif. This induces dissociation of the R-Smad from the receptor, stimulates the assembly of a heteromeric complex between the phosphorylated R-Smads and Smad4, and results in the nuclear accumulation of this heteromeric Smad3-Smad4 complex (4, 10, 29). In the nucleus, Smads bind to a specific DNA site (GTCTAGAC) and cooperate with various transcription factors in regulating target gene expression (5, 23, 51).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
ploy of polyamines to the dividing cells within the crypts and that polyamines, either synthesized endogenously or supplied luminally, are absolutely required for epithelial cell division. Increasing cellular polyamines stimulates intestinal epithelial cell renewal, and decreasing cellular polyamines inhibits cell proliferation both in vivo and in vitro.

We (34) have recently demonstrated that depletion of cellular polyamines induces the activation of TGF-β gene expression through posttranscriptional regulation and that increased gene product, TGF-β, plays a critical role in the inhibition of normal intestinal epithelial cell proliferation. Polyamine-deficient cells also highly express the TGF-β type I receptor, which is associated with increased sensitivity to growth inhibition when these cells are exposed to exogenous TGF-β (40). The current study went further to determine the possibility that Smad proteins are the downstream intracellular effectors of activated TGF-β/TGF-β receptor signaling in polyamine-deficient cells. First, we examined whether polyamine depletion by inhibition of ornithine decarboxylase, the rate-limiting enzyme in the biosynthesis of polyamines, increased Smad activity in normal intestinal epithelial cells (the IEC-6 line). Second, we examined whether this increased Smad activity following polyamine depletion specifically activated Smad-dependent promoters. Third, we determined whether the observed Smad activation resulted from increased expression of TGF-β in polyamine-deficient cells. Some of these data have been published previously in abstract form (25).

MATERIALS AND METHODS

Chemicals and supplies. Disposable cultureware was purchased from Corning Glass Works (Corning, NY). Tissue culture media and diazylated fetal bovine serum (dFBS) were obtained from Invitrogen (Carlsbad, CA), and biochemicals were from Sigma (St. Louis, MO). The double-stranded oligonucleotides used in EMSA and specific antibodies against Smad3 and Smad4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). α-Difluoromethylornithine (DFMO) was purchased from Ilex Oncology (San Antonio, TX). The [α-32P]dCTP (3,000 Ci/mmole) was purchased from Amersham (Arlington Heights, IL).

Reporter plasmids and recombinant adenovirus construction. Three Smad-dependent reporters used in this study were derived from the pGL3 promoter luciferase vector (Promega, Madison, WI). The pJun-Luc construct containing the −79 to +170 sequence of the human c-Jun promoter with three copies of connective Smad-binding element (SBE) was a gift from Dr. X. F. Wang (Duke University School of Medicine, Durham, NC) (51). The pPAI1-Luc construct contains the −800 to +22 sequence of the type 1 plasminogen activator inhibitor (PAI-1) gene promoter that has three copies of separated SBE (gift of Dr. Z. J. Sun, Stanford University School of Medicine, Palo Alto, CA) (9, 43). The reporter plasmid pSBE4-Luc containing the synthetic sequence with four copies of connective SBE is a gift from Dr. J. Duyster (Technical University of Munich, Munich, Germany) (44, 54). A recombinant adenovirus expressing a dominant negative mutant Smad4 (AdMSmad4) was a generous gift from Dr. D. M. Simeone (University of Michigan, Ann Arbor, MI) (41). The dominant-negative Smad4 construct was bluntly ligated into the EcoRV site of the shuttle vector (pAdTrack), which has an independent cytomegalovirus (CMV)-driven green fluorescent protein expression construct as a marker. Recombinant adenoviral plasmids were packaged into infectious adenoviral particles by transfecting human embryonic kidney 293 cells by using LipofectAMINE PLUS reagent. The adenoviral particles were propagated in 293 cells and purified on cesium chloride ultracentrifugation. Titers of the introduced genes by fluorescent microscopy. pAdeno-X, which was the recombinant replication-incompetent adenovirus carrying no cDNA insert (AdNull), was grown and purified as described above and served as a control adenovirus. Cells were infected by various concentrations of AdMSmad4 or AdNull, and cell samples were collected for various measurements 72 h after the infection.

Cell culture. The IEC-6 cell line was purchased from the American Type Culture Collection at passage 13. The cell line was derived from normal rat intestinal crypt cells and was developed and characterized by Quaroni et al. (38). Stock culture supernates were measured by Western blotting analysis, immunohistochemical staining, and EMSA, respectively.

The second series of studies was to determine whether polyamine depletion by treatment with DFMO increased Smad activity in IEC-6 cells. The general protocol of the experiments and methods were similar to those described previously (34, 40). Briefly, IEC-6 cells were plated at 6.25 × 104 cells/cm2 and grown in control medium (DMEM plus 5% dFBS, 10 μg/ml insulin, and 50 μg/ml gentamicin sulfate) or the DMEM medium containing 5 mM DFMO or DFMO plus 5 μM spermidine for 4, 6, and 8 days. The Smad3 and Smad4 protein levels, their cellular distribution, and Smad sequence-specific DNA-binding activity were measured by Western blotting analysis, immunohistochemical staining, and EMSA, respectively.

The second series of studies was to determine whether observed activation of Smads following polyamine depletion had the transcriptional activity in IEC-6 cells. Three widely accepted luciferase reporters of Smad-dependent promoters were used in this study, and the activity of luciferase reporter was measured in cells grown in the DMEM medium containing DFMO alone or DFMO plus spermidine for 6 days.

The third series of studies was to define the relationship between increased expression of TGF-β and Smad activation in polyamine-deficient cells. Functions of TGF-β were examined by using immunoneutralizing anti-TGF-β antibody or antisense TGF-β oligodeoxyribonucleotides. Cells were initially treated with DFMO for 3 days, exposed to the media containing DFMO plus either anti-TGF-β antibody or antisense TGF-β oligodeoxyribonucleotides for 24 h, and then transgenic with various luciferase reporter constructs. Levels of Smad3 and Smad4 proteins and their transcriptional activity were measured 48 h after transfection.

Measurement of TGF-β content. The levels of TGF-β in culture supernates were measured with the use of the TGF-β1 ELISA system (Promega). After cells in 30-mm dishes were grown in the presence or absence of DFMO for various times, the monolayer of cells was washed once with Dulbecco’s PBS, and then 1 ml of fresh medium was added. The medium was collected following 12 h of further culture, and the content of
TGF-β was measured according to the manufacturer’s instructions. Cells were dissociated in 0.5 ml of 0.5 N NaOH at 37°C in humidified air for 90 min. The protein content of an aliquot of cell lysate was determined by the method described by Bradford (2). The levels of TGF-β content were normalized by protein and expressed as picograms per milliliter per milligram of protein.

**Western blot analysis.** Cell samples, placed in SDS sample buffer, were sonicated and then centrifuged (2,000 rpm) at 4°C for 30 min. The supernatant from cell samples was boiled for 10 min and then subjected to electrophoresis on 10% acrylamide gels according to Laemmli (16). After the transfer of protein onto nitrocellulose filters, the filters were incubated for 1 h in 5% nonfat dry milk in 1× PBS-Tween 20 [PBS-T; 15 mM Na2HPO4, 80 mM Na2HPO4, 1.5 M NaCl, pH 7.5, and 0.5% (vol/vol) Tween 20]. Immunological evaluation was performed by 1 h in PBS-T buffer containing 0.2 μg/ml of the specific antibody against Smad3 or Smad4 protein. The filters were subsequently washed with 1× PBS-T and incubated for 1 h with the second antibody conjugated to peroxidase. After being washed extensively with 1× PBS-T, the immunocomplexes on the filters were reacted for 1 min with chemiluminescence reagent (NEL-100; DuPont-NEN). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film.

**Preparation of nuclear protein and EMSA.** Nuclear proteins were prepared by the procedure described previously (21, 53), and the protein contents in nuclear preparations were determined by the method described by Bradford (2). The double-stranded oligonucleotides used in these experiments were 5′-TGGAGAGCCAGACAAAAAGCCAGCATTAGCCGAGCAC-3′, which contains the consensus Smad-binding sites (underlined). These oligonucleotides were radioactively end-labeled with [γ-32P]ATP and T4 polynucleotide kinase (Promega). For EMSA, 0.035 pmol 32P-labeled oligonucleotides (~30,000 cpm) and 10 μg nuclear protein were incubated in a total volume of 25 μl in the presence of 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and 1 μg poly(dI-dC). The binding reactions were allowed to proceed at room temperature for 20 min. Thereafter, 2 μl of bromophenol blue (0.1% in water) were added, and protein-DNA complexes were resolved by electrophoresis on nondenaturing 5% polyacrylamide gels and were visualized by autoradiography. The specificity of binding interactions was assessed by competition with an excess of unlabeled double-stranded oligonucleotide of identical sequence.

**Immunohistochemical staining.** The immunofluorescence procedure was carried out according to the method of Violkind and Swierenga (47) with minor changes (7). Cells were incubated with the primary antibody against Smad3 or Smad4 at 4°C overnight and then incubated with secondary antibody conjugated with FITC for 2 h at room temperature. After the slides were rinsed three times, they were mounted and viewed through a Zeiss confocal microscope (model LSM410). Images were processed by using Photoshop software (Adobe, San Jose, CA).

**Luciferase assays and transfection.** Transient transfactions were performed by using the LipofectAMINE kit and were performed as recommended by the manufacturer (In-vitrogen, Carlsbad, CA). The cells were collected at 48 h after the transfection, and luciferase activities of reporters were assayed with a commercial kit (Promega). The luciferase activity from individual transfections was normalized by β-galactosidase activity from cotransfected plasmid pCMV-β-galactosidase. The experiments were done in triplicate and are reported as mean relative light units/β-galactosidase.

**Statistics.** All data are expressed as means ± SE from six dishes. Autoradiographic and immunohistochemical staining experiments were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined by using Duncan’s multiple-range tests (8).

**RESULTS**

**Effect of polyamine depletion on Smad3/Smad4 protein expression and cellular distribution.** Our previous studies demonstrated that exposure of IEC-6 cells to 5 mM DFMO for 4, 6, and 8 days almost completely depleted cellular polyamines and that decreased levels of polyamines resulted in a significant increase in expression of TGF-β and the TGF-β type I receptor (17, 49). Because the activated TGF-β receptors propagate the signal by increasing Smad activity in a variety of cell types (1, 54), the focus of the current study was to determine whether the altered TGF-β expression following polyamine depletion is associated with changes in Smad proteins in normal intestinal epithelial cells. Results presented in Fig. 1A show that depletion of cellular polyamines by treatment with DFMO increased TGF-β content in culture medium, which was completely prevented by exogenous spermidine. Consistently, basal levels of Smad3 and Smad4 proteins also increased significantly in DFMO-treated cells. The induction of protein levels for Smad3 and Smad4 occurred at day 4 and remained elevated 6 and 8 days after exposure to DFMO (Fig. 1, A and B). The levels of Smad3 in cells exposed to DFMO were ∼5.1, ∼5.3, and ∼5.5 times the normal values (without DFMO) at 4, 6, and 8 days after DFMO, respectively. Although basal expression of Smad4 protein was higher than that of Smad3 in IEC-6 cells, levels of Smad4 also increased significantly and were more than twice the normal values at 4, 6, and 8 days after DFMO treatment. Spermidine (5 μM) given together with DFMO completely prevented the increased levels of Smad3/Smad4 proteins. Putrescine (10 μM) had an effect equal to spermidine on Smad3/4 expression when it was added to cultures that contained DFMO (data not shown).

To extend the finding of increased Smad expression following polyamine depletion, we further explored the cellular distribution of Smad3/Smad4 proteins in the cells grown in the presence or absence of DFMO for 6 days with the use of an immunohistochemical staining technique. In control cells, the immunostainings for Smad3 and Smad4 were observed in a perinuclear area (Fig. 2A, right). Consistent with our data from Western blot analysis, these immunostainings for Smad3/Smad4 were observed in a loosely defined perinuclear area (Fig. 2A, left) but the apparent immunoreactivities for Smad4 were observed in a more than twice the normal area at 4, 6, and 8 days after DFMO treatment. Spermidine (5 μM) had an effect equal to spermidine on Smad3/4 expression when it was added to cultures that contained DFMO (data not shown).
Cells treated with DFMO plus spermidine was indistinguishable from that in control cells (Fig. 2, A vs. C).

Changes in Smad sequence-specific DNA-binding activity in polyamine-deficient cells. Consistently, increased levels of Smad3/Smad4 proteins in cells exposed to DFMO were paralleled by a marked increase in Smad-binding activity as measured by EMSA (Fig. 3A). The Smad-binding activities were increased by approximately sixfold at 4, 6, and 8 days after DFMO treatment. Spermidine given together with DFMO completely prevented the increase in Smad-binding activity. To evaluate the specificity of the binding reaction in Fig. 3A, competitive inhibition experiments were carried out in control cells and in cells exposed to DFMO for 6 days. The Smad-binding activities in both control (Fig. 3Ba) and DFMO-treated cells (Fig. 3Bb) was dose-dependently inhibited when various concentrations of spermidine were added.

Fig. 2. Cellular distribution of Smad3 (left) and Smad4 (right) proteins in IEC-6 cells described in Fig. 1. The IEC-6 cells were plated in a 4-well chamber slide and grown in DMEM containing 5% dFBS in the presence or absence of DFMO (5 mM) or DFMO plus spermidine (5 μM) for 4, 6, and 8 days, monolayer of cells was washed once with D-PBS, and then 1 ml of fresh medium was added. The medium was collected following 12 h of further culture, and the content of TGF-β was measured. B: representative autoradiograms of Western blot from cells described in A. Whole cell lysates were harvested, applied to each lane (30 μg) equally, and subjected to electrophoresis on 10% acrylamide gel. Levels of Smad3 (~55 kDa) and Smad4 (~60 kDa) were identified by probing nitrocellulose with the specific antibodies. After the blot was stripped, actin (~42 kDa) immunoblotting was performed as an internal control for equal loading. C: quantitative analysis of Western blot by densitometry from cells described in B. a, Smad3; b, Smad4. Values are means ± SE of data from 3 separate experiments; relative levels of Smad3 and Smad4 were corrected for loading as measured by densitometry of actin. *P < 0.05 compared with control and DFMO plus SPD.

Fig. 1. Changes in expression of transforming growth factor-β (TGF-β) and Smad3/Smad4 proteins in control IEC-6 intestinal epithelial cells and IEC-6 cells treated with either α-difluoromethylornithine (DFMO) alone or DFMO plus spermidine (SPD). A: levels of TGF-β. Cells were grown in DMEM containing 5% dialyzed FBS (dFBS) in the presence or absence of DFMO (5 mM) or DFMO plus spermidine (5 μM) for 4, 6, and 8 days, monolayer of cells was washed once with D-PBS, and then 1 ml of fresh medium was added. The medium was collected following 12 h of further culture, and the content of TGF-β was measured. B: representative autoradiograms of Western blot from cells described in A. Whole cell lysates were harvested, applied to each lane (30 μg) equally, and subjected to electrophoresis on 10% acrylamide gel. Levels of Smad3 (~55 kDa) and Smad4 (~60 kDa) were identified by probing nitrocellulose with the specific antibodies. After the blot was stripped, actin (~42 kDa) immunoblotting was performed as an internal control for equal loading. C: quantitative analysis of Western blot by densitometry from cells described in B. a, Smad3; b, Smad4. Values are means ± SE of data from 3 separate experiments; relative levels of Smad3 and Smad4 were corrected for loading as measured by densitometry of actin. *P < 0.05 compared with control and DFMO plus SPD.
Fig. 3. Changes in sequence-specific Smad DNA-binding activity in IEC-6 cells described in Fig. 1. A: Smad DNA-binding activity after different treatments. a, representative autoradiograms of EMSA for Smad-binding activity. Nuclear extracts were prepared from cells grown in the presence or absence of 5 mM DFMO with or without 5 μM SPD for 4, 6, and 8 days. EMSA was performed using 10 μg of nuclear protein and 0.035 pmol of 32P end-labeled oligonucleotides containing a consensus Smad-binding site. Positions of the specifically bound DNA-protein complex and freely migrating probes are indicated. b, quantitative analysis of EMSA by densitometry from cells described in Aa. Values are means ± SE of data from 3 separate experiments. *P < 0.05 compared with control groups. B: effects of unlabeled Smad oligonucleotide or the antibody against Smad3 or Smad4 added to the binding reaction mixture on Smad-binding activity. a, representative autoradiograms from control cells; b, representative autoradiograms from cells treated with DFMO for 6 days; c, effects of addition of the antibody against Smad3 or Smad4 on the Smad-binding activity. Nuclear extracts (10 μg) isolated from cells exposed to DFMO for 6 days were initially incubated with 2 μg of the indicated antibody for 40 min and then subjected to EMSA as described in A. IgG, lane with nuclear extracts and IgG. Three experiments were performed that showed consistent results.

The levels of mRNA by DFMO also significantly induced TGF-β mRNA expression and increased TGF-β content. The levels of transcription of the unlabeled Smad oligonucleotide were added to the binding reaction mixture. We also examined the effect of the unlabeled oligonucleotide containing a mutated Smad-binding site on Smad-binding activity and demonstrated that the Smad-mutated oligonucleotide did not inhibit Smad-binding activity in IEC-6 cells (data not shown). Results presented in Fig. 3Bc further show that preincubation of nuclear extracts with the specific antibody against Smad3 or Smad4 significantly inhibited the formation of Smad-binding complex. In contrast, preincubation with IgG had no inhibitory effect on Smad-binding activity. These results indicate that polyamine depletion activates Smad signaling in normal intestinal epithelial cells.

Effect of polyamine depletion on Smad-dependent transcriptional activity. To determine the possible transcription activation of induced Smad activity following polyamine depletion, the luciferase reporter activities of three Smad-dependent promoter constructs were measured in control cells and cells treated with DFMO alone or DFMO plus spermidine for 6 days. Results presented in Fig. 4 show that polyamine depletion-induced Smads were associated with significant increases in luciferase reporter activities of Smad-dependent promoters. Levels of pJun-Luc, pPAI1-Luc, and pSBE4-Luc luciferase reporter activity in cells exposed to DFMO for 6 days were ~7.8, ~3.1, and ~7.2 times the control values, respectively. On the other hand, spermidine at the concentration of 5 μM given together with DFMO completely prevented increases in luciferase reporter activity of Smad-dependent promoters.

To further define the relationship between induced Smads and the transcription activation following polyamine depletion, we examined the effect of inhibition of wild-type Smad4 by ectopic expression of a dominant negative mutant Smad4 (MSmad4) on transcriptional activation of Smad-dependent promoters in IEC-6 cells. Our previous studies have demonstrated that adenoviral vectors infect intestinal epithelial cells with nearly 100% efficiency within 48 h (39). In fact, >95% of IEC-6 cells were positive when they were infected with the adenoviral vector encoding green fluorescent protein that served as the marker for 24 h (data not shown). Results presented in Fig. 5 show that infection of polyamine-deficient cells with AdMSmad4 decreased luciferase reporter activity of Smad-dependent promoters. Levels of pJun-Luc and pSBE4-Luc luciferase reporter activity were decreased by ~40 and ~55%, respectively, when the adenovirus at the concentration of 20 plaque-forming units/cell was used. An adenovirus that lacked exogenous MSmad4 cDNA was used as the negative control and did not alter levels of Smad-dependent transcription activation in polyamine-deficient cells. These results clearly indicate that increased Smads have the transcription activation following polyamine depletion.

Effect of polyamine depletion on TGF-β expression and cell proliferation. Depletion of cellular polyamines by DFMO also significantly induced TGF-β mRNA expression and increased TGF-β content. The levels of...
TGF-β in the cells treated with DFMO for 6 days were approximately two times the normal values (data not shown). Increased expression of TGF-β in the polyamine-deficient cells was associated with an inhibition of cell growth, in which cells were arrested in G1 phase. Cell numbers were decreased by 50% in DFMO-treated cells, and the population of G1-phase cells increased from 39% in control cells to 70% in cells exposed to DFMO for 6 days. TGF-β expression and cell growth were returned to normal when DFMO was given together with DFMO (data not shown). Similar results have been published previously (34).

Effects of inhibition of TGF-β on Smad activation in polyamine-deficient cells. To elucidate whether the increased Smad activity following polyamine depletion results from an increased expression of TGF-β, the following three logically linked studies were carried out. First, we examined the effect of exposure of normal IEC-6 cells (without DFMO) to exogenous TGF-β on Smad activity. Figure 6A clearly shows that treatment with TGF-β at the concentration of 5 ng/ml increased expression of Smad3 protein but had no effect on
Three experiments were performed that showed consistent results. B: sequence-specific Smad DNA-binding activity. a, Representative autoradiograms of EMSA for Smad-binding activity from cells described in A. Positions of the specifically bound DNA-protein complex and freely migrating probes are indicated. b, Quantitative analysis of EMSA by densitometry from cells described in Ba. Values are means ± SE of data from 3 separate experiments. *P < 0.05 compared with controls (0 h).

Smad4. Significant increases in Smad3 protein occurred at 1.5 h and peaked between 3 and 6 h after treatment with TGF-β. Maximum increases in Smad3 protein were over five times the pretreatment control level. Furthermore, increase in Smad3 protein was paralleled by that of Smad-binding activity (Fig. 6B). Levels of Smad-binding activity were ~2.3, ~7.2, and ~6.5 times the control levels at 1.5, 3, and 6 h after treatment with TGF-β, respectively.

Second, we examined whether dysfunction of TGF-β caused by using an immunoneutralizing anti-TGF-β antibody prevented the induced Smads in polyamine-deficient cells. Cells were grown in the presence of DFMO for 3 days, exposed to the medium containing DFMO and the antibody against TGF-β at different concentrations for 24 h, and then transfected with the pPAI1-Luc or pSBE4-Luc luciferase reporter construct. Levels of Smad-binding activity were measured 48 h after the transfection. As shown in Fig. 7, A and B, treatment with TGF-β antisense oligomers decreased levels of Smad3 protein but had no effect on Smad4 in polyamine-deficient cells. When the DFMO-treated cells were exposed to 4 or 6 μM TGF-β antisense oligomers for 72 h, the Smad3 protein levels were decreased by >80%. Treatment with TGF-β sense oligomers at the same concentrations had no effect on expression of both Smad3 and Smad4. Inhibition of Smad3 protein levels by TGF-β antisense oligomers also suppressed transcription activation of Smad-dependent promoters in polyamine-deficient cells (Fig. 7C). The luciferase activities of pJun-Luc and pPAI1-Luc reporter constructs were decreased by ~85% after exposure to 4 μM TGF-β antisense oligomers. In addition, there was no apparent loss of cell viability in cells treated with DFMO alone or DFMO-treated cells exposed to TGF-β antisense or sense oligomers (data not shown). Together, these findings strongly suggest that induced Smad activation following polyamine depletion is due to increased TGF-β in normal intestinal epithelial cells.

DISCUSSION

As discussed in the introduction, normal intestinal mucosal growth depends on an adequate supply of polyamines in the dividing cells within the crypts. Although few specific functions of polyamines at cellular and molecular levels are defined, there is little doubt that polyamines regulate intestinal epithelial cell proliferation by virtue of their ability to modulate expression of various growth-related genes (3, 19, 21, 22). Levels of Smad3/Smad4 proteins and their transcription activity were measured 48 h after the transfection in the presence of DFMO. Administration of the anti-TGF-β antibody not only significantly prevented the increase in Smad3 protein (Fig. 7, A and B) but also inhibited Smad-dependent transcription activity (Fig. 7C), although it had no effect on induced levels of Smad4 in DFMO-treated cells. In the cells treated with the antibody, levels of Smad3 protein were decreased by ~45% at 0.5 μg/ml and ~62% at 2 μg/ml, respectively. Consistently, luciferase activities of the pPAI1-Luc reporter construct were decreased by ~55% at 0.5 μg/ml and ~65% at 2 μg/ml the antibody (Fig. 7Ca), and levels of the pSBE4-Luc luciferase activity were decreased by ~33% at 0.5 μg/ml and ~45% at 2 μg/ml (Fig. 7Cb) in the DFMO-treated cells. The heat-inactivated anti-TGF-β antibody served as control in this experiment and showed no additional effect on Smad activity.

Third, we determined the effect of inhibition of TGF-β expression by using TGF-β antisense oligomers on Smads in polyamine-deficient cells. The protocol of this study was similar to the previous study involving the anti-TGF-β antibody. After polyamine-deficient cells were treated with TGF-β antisense or sense oligomers for 24 h, they were transfected with Smad-dependent luciferase reporter constructs. Levels of Smad3/Smad4 proteins and the luciferase reporter activity were examined 48 h after the transfection. As shown in Fig. 8, A and B, treatment with TGF-β antisense oligomers decreased levels of Smad3 protein but had no effect on Smad4 in polyamine-deficient cells. When the DFMO-treated cells were exposed to 4 or 6 μM TGF-β antisense oligomers for 72 h, the Smad3 protein levels were decreased by >80%. Treatment with TGF-β sense oligomers at the same concentrations had no effect on expression of both Smad3 and Smad4. Inhibition of Smad3 protein levels by TGF-β antisense oligomers also suppressed transcription activation of Smad-dependent promoters in polyamine-deficient cells (Fig. 8C). The luciferase activities of pJun-Luc and pPAI1-Luc reporter constructs were decreased by ~85% after exposure to 4 μM TGF-β antisense oligomers. In addition, there was no apparent loss of cell viability in cells treated with DFMO alone or DFMO-treated cells exposed to TGF-β antisense or sense oligomers (data not shown). Together, these findings strongly suggest that induced Smad activation following polyamine depletion is due to increased TGF-β in normal intestinal epithelial cells.
Increasing evidence indicates that polyamines positively regulate the transcription of growth-promoting genes such as c-fos and c-myc (3, 35, 50) and negatively control expression of growth-inhibiting genes. Polyamines may also modulate the transcriptional activity of critical extracellular matrix metalloproteinases in a Smad-dependent manner (34).
genes including TGF-β/TGF-β receptor (34, 40), p53 (19, 22), and junD (20, 36) at the posttranscriptional level in normal intestinal epithelial cells. It has been shown that increased polyamines not only stimulate expression of c-fos and c-myc genes by enhancing their mRNA synthesis but also inhibit expression of TGF-β/TGF-β receptor, p53, and junD genes by increasing their mRNA degradation, leading to stimulation of intestinal epithelial cell proliferation. In contrast, decreased levels of polyamines lead to reduction of levels of growth-promoting proteins through inhibition of their gene transcription and to increased levels of growth-inhibiting factors through stabilization of their mRNAs, which are associated with an increase in G1-phase growth arrest. However, the exact signaling pathways by which products of these growth-related genes mediate intestinal epithelial cell proliferation after increased or decreased cellular polyamines remain to be elucidated.

The TGF-β family consists of a group of closely related genes that are widely distributed in a variety of human and animal tissues, including the gastrointestinal mucosa (5, 6, 10, 29). TGF-β is an important physiological regulator for normal intestinal mucosal growth, and administration of TGF-β inhibits proliferative activity and promotes the development of differentiated function in intestinal epithelial cells (12, 28, 32, 54). We have recently demonstrated that polyamine depletion by DFMO dramatically stabilizes TGF-β mRNA and inhibits the mRNA degradation, leading to an increase in TGF-β expression (34). Addition of immunoneutralizing anti-TGF-β antibody to the culture medium significantly prevents growth inhibition in DFMO-treated cells. Polyamine depletion also increases expression of the TGF-β type I receptor in IEC-6 cells (40). Inhibition of TGF-β type I receptor expression decreases TGF-β-mediated growth inhibition in normal cells and prevents the increased sensitivity to exogenous TGF-β in polyamine-deficient cells. These findings strongly suggest that increased TGF-β/TGF-β receptor expression is one mechanism by which decreased levels of polyamines inhibit cell renewal in the intestinal mucosa.

The data from the current studies provide new evidence that Smad proteins are involved in the processing functions of cellular polyamines in intestinal epithelial cells. Exposure of IEC-6 cells to DFMO for 4, 6, and 8 days significantly increased levels of both Smad3 and Smad4 proteins and induced their nuclear translocation. Smad3 is shown to be highly expressed in intestinal epithelial cells, and activation of this R-Smad is ligand specific (10, 17, 29, 31). It is not surprising that polyamine depletion by DFMO increases Smad3 protein and enhances its nuclear translocation, because treatment with DFMO is known to stimulate expression of TGF-β/TGF-β receptor in IEC-6 cells (34, 40). It is likely that activated Smad3 results from the increased expression of TGF-β/TGF-β receptors in polyamine-deficient cells. This contention is strongly supported by data presented in Figs. 7 and 8 that show that inhibition of TGF-β by treatment with either immunoneutralizing anti-TGF-β antibody or TGF-β antisense oligomers prevented the increased Smad activation in the absence of cellular polyamines.

The current studies also demonstrated that polyamine depletion induced Smad4 nuclear translocation in IEC-6 cells. Smad4 functions as a common mediator for all R-Smads and forms heteromeric complexes with Smad3 after ligand activation (12, 31, 54). The observed change in Smad4 in the current studies seems to be a secondary response to the activation of Smad3 in DFMO-treated cells. In support of this possibility, treatment with exogenous TGF-β did not alter levels of Smad4 protein in normal IEC-6 cells (without DFMO), although it significantly increased Smad3 expression (Fig. 6). Furthermore, exposure to immunoneutralizing anti-TGF-β antibody or TGF-β antisense oligomers did not prevent the increased levels of Smad4 protein in polyamine-deficient cells. The other possibility also exists that polyamine depletion induces Smad4 expres-

![Fig. 9. Schematic diagram depicting the proposed role of TGF-β-Smad signaling pathway in the inhibition of normal intestinal epithelial cell proliferation following polyamine depletion. In this model, polyamines are the negative regulators for expression of the TGF-β gene, whereas Smad proteins are the downstream intracellular effectors of activated TGF-β receptors. Decreased cellular polyamines, by either inhibition of their synthesis, stimulation of the catabolism, or suppression of polyamine uptake, increase expression of TGF-β through stabilization of TGF-β mRNA, enhance the release of TGF-β, and subsequently phosphorylate (P) the TGF-β type II receptor (R-II). The phosphorylated R-II activates TGF-β type I receptor (R-I), induces the formation of Smad3/Smad4 heteromeric complexes, and stimulates their nuclear translocation. The activated Smads in the nucleus bind to the specific DNA site and cooperate with Smad DNA-binding partners (DBP) such as some activating protein-1 proteins to activate or repress transcription of specific target genes, thus leading to the inhibition of normal intestinal epithelial cell proliferation following polyamine depletion.](http://ajpgi.org)
sion through a mechanism independent from the activated Smad3 in intestinal epithelial cells. In addition, this increased Smad expression and nuclear translocation in DFMO-treated cells are specifically related to polyamine depletion rather than to a nonspecific effect of DFMO, because the stimulatory effect of this compound on Smads was completely prevented by the addition of exogenous spermidine.

Results presented here further showed that polyamine depletion-induced Smad activity was associated with a significant increase in transcription activation of Smad-driven promoters in IEC-6 cells. Using the EMSA method and luciferase reporter assays, we demonstrated that polyamine depletion significantly increased Smad sequence-specific DNA binding and induced luciferase reporter activity of Smad-dependent promoters. Our results are consistent with others that indicated that induced nuclear Smad proteins mediate transcription of Smad target genes in a variety of cell types (10, 29, 52). The current studies also show that increased transcriptional activation following polyamine depletion is primarily due to the function of Smad3-Smad4 heteromeric complexes because ectopic expression of a dominant-negative mutant Smad4 prevented the increased Smad transcription activation in DFMO-treated cells. Recently, Smad DNA-binding sites have been identified in various gene promoters including PAI-1, junB, c-jun, and p21 by different approaches (15, 23, 33, 44, 55). In general, these sequences reported by different groups are essentially identical; regardless of whether one defines a Smad-binding site as the palindrome AGACGTCT, as the CAGA box, or as repeats of GACA, all identified sites contain the Smad box, 5′-GTCT-3′, or its reverse complement, 5′-AGAC-3′. Our results clearly indicate that induced Smad3-Smad4 heteromeric complexes in polyamine-deficient cells are able to bind to this specific DNA site, suggesting that Smads mediate transcriptional activation, probably through a direct interaction with SBE sites in target promoters.

The exact mechanism by which activated Smads induced by TGF-β following polyamine depletion mediate target gene transcription is obscure. Although initial studies show that Smads can bind directly to DNA and activate transcription of target genes, increasing evidence reveals that Smads primarily function as comodulators of transcriptional activity and that interaction between Smads and their DNA-binding partners are essential for Smads to function in regulating specific transcriptional response (1, 18, 42). Recently, Smad3/activator protein-1 (AP-1) interactions have been shown to control transcriptional responses to TGF-β in a promoter-specific manner (11, 23, 46, 51, 55). We have recently demonstrated that polyamine depletion significantly increases JunD/AP-1 activity in IEC-6 cells and that increased JunD/AP-1 plays an important role in growth inhibition of polyamine-deficient cells (20, 36). It is not clear at present whether Smads interact with JunD/AP-1 and if observed Smad/JunD complexes cooperatively regulate transcription of specific target genes and are involved in the G1-phase growth arrest following polyamine depletion. Further studies are needed to define the exact relationship between Smads and JunD/AP-1 in polyamine-deficient IEC-6 cells.

Combining the current findings with our previous studies (34, 40), we propose a model delineating the role of the TGF-β-Smad signaling pathway in the inhibition of intestinal epithelial cell proliferation following polyamine depletion (Fig. 9). In this model, polyamines negatively regulate posttranscription of the TGF-β gene, whereas Smads are the downstream intracellular effectors of activated TGF-β receptors. Decreased cellular polyamines stabilize TGF-β mRNAs, increase TGF-β synthesis, and cause the activation of TGF-β receptors. The resultant activation of TGF-β receptors induces Smad3/Smad4 levels and stimulates Smad nuclear translocation, resulting in the accumulation of Smad3-Smad4 complexes. In the nucleus, Smads bind to the specific DNA site and cooperate with their DNA-binding partners such as JunD/AP-1 to activate or repress transcription of specific target genes, thus leading to the inhibition of intestinal epithelial cell proliferation. In contrast, increased polyamines downregulate TGF-β-Smad signaling and enhance intestinal epithelial cell proliferation. These findings suggest that Smads are involved in the negative control of intestinal mucosal growth and that polyamines stimulate intestinal epithelial cell renewal in vivo at least partially through inhibition of the TGF-β-Smad signaling pathway under physiological conditions.

DISCLOSURES

This work was supported by Merit Review Grant from the Department of Veterans Affairs (to J.-Y. Wang) and by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-57819 and DK-61972 (to J.-Y. Wang).

J.-Y. Wang is a Research Career Scientist, Medical Research Service, United States Department of Veterans Affairs.

REFERENCES


