Differentiated intestinal epithelial cells express high levels of TGF-β receptors and exhibit increased sensitivity to growth inhibition

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Abstract: Background: Intestinal epithelial cells (IECs) within crypts continuously divide and differentiate as they migrate up towards the luminal surface of the mucosa. With the onset of differentiation, IECs lose their proliferative potential, but the exact mechanism remains unknown. This current study examined the involvement of the TGF-β signaling pathway in this process. Methods: Studies were conducted in the IEC-6 cell line derived from rat small intestinal crypt cells. Cell differentiation was induced by forced expression of the Cdx2 gene, a transcription factor responsible for controlling intestinal epithelial cell differentiation. Results: Forced expression of the Cdx2 gene in stable Cdx2-transfected IEC-6 cells resulted in a differentiated phenotype as indicated by morphological features and increased expression of sucrase-isomaltase. Levels of TGF-β type I receptor (TGFβRI) and TGF-β type II receptor (TGFβRII) increased in these differentiated epithelial cells. The induced TGFβRI and TGFβRII expression in Cdx2-transfected IEC-6 cells was associated with increased sensitivity to TGF-β-induced growth inhibition. Depletion of cellular polyamines further increased TGF-β receptor expression and additionally enhanced the response to TGF-β-induced growth inhibition. Increased TGFβRI and RII in polyamine-deficient cells were also associated with an induction in JunD/AP-1 activity. Conclusions: These results indicate that the loss of the proliferative potential in differentiated IECs results partially from the increased expression of TGF-β receptors.

Keywords: Cdx2 gene, intestinal epithelium, TGF-β Receptors, AP-1 binding sites, cell growth, electrophoretic mobility shift assay, supershift assays
modified Eagle medium (DMEM) supplemented with 5% heat-inactivated FBS, 10 μg/ml insulin, and 50 μg/ml gentamicin sulfate. Flasks were incubated at 37 °C in a humidified atmosphere of 90% air-10% CO2. Stock cells were subcultured once a week at 1:20; medium was changed three times weekly. The cells were restarted from original frozen stock every 7 passages. Tests for mycoplasma were routinely negative and passages 15-20 were used in the experiments.

Our previous studies demonstrate that polyamine depletion induces TGF-β gene expression through posttranscriptional regulation and that elevation of TGF-β gene expression plays a critical role in the inhibition of undifferentiated intestinal epithelial cell proliferation [2, 6]. Polyamine-deficient cells also highly express TGF-βRI, which is associated with increased sensitivity to growth inhibition when exposed to exogenous TGF-β [6]. This current study further examines changes in TGF-βR expression during IEC differentiation and defined its role in the sensitivity to growth inhibition induced by exogenous TGF-β.

Materials and methods

Chemicals and supplies

Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture media, isopropyl-β-D-thiogalactopyranoside (IPTG), and dialyzed fetal bovine serum (dFBS) were obtained from Invitrogen (Carlsbad, CA), and biochemicals were from Sigma (St. Louis, MO). TGF-β was purchased from R&D Systems (Minneapolis, MN). Anti-TGFβRI and RII antibodies were from Cell Signaling Technology (Danvers, MA). DL-α-difluoromethylornithine (DFMO) was obtained from Genzyme (Cambridge, MA).

Cell culture and general experimental protocol

The IEC-6 cell line was purchased from the American Type Culture Collection (ATCC) at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al [17]. IEC-6 cells originated from intestinal crypt cells, as judged by morphological and immunological criteria. They are non-tumorigenic and retain the undifferentiated character of epithelial stem cells. Stock cells were maintained in T-150 flasks in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% heat-inactivated FBS, 10 μg/ml insulin, and 50 μg/ml gentamicin sulfate. Flasks were incubated at 37 °C in a humidified atmosphere of 90% air-10% CO2. Stock cells were subcultured once a week at 1:20; medium was changed three times weekly. The cells were restarted from original frozen stock every 7 passages. Tests for mycoplasma were routinely negative and passages 15-20 were used in the experiments.

The stable Cdx2-transfected IEC-6 cell lines were developed and characterized by Suh and Traber [18] and were kind gifts from Dr. Peter G. Traber (University of Pennsylvania, Philadelphia, PA). The expression vector, the LacSwitch system (Stratagene, La Jolla, CA), was used for directing the conditional expression of Cdx2, and IPTG served as the inducer for gene expression. IEC-6 cells were transfected with pOPRSVCdx2 by electroporation technique, and clones resistant to selection medium containing 0.6 mg G418/ml and 0.3 mg hygromycin B/ml were isolated and screened for Cdx2 expression by Northern blot, RNase protection assays, and electrophoretic mobility shift assay (EMSA). Stock stable Cdx2-transfected IEC-6 (IEC-Cdx2L1) cells were grown in DMEM used as parental non-transfected IEC-6 cells. Before experiments, cells were grown in DMEM containing 4 mM IPTG for 16 days to induce cell differentiation.

The general protocol of the experiments and the methods used were similar to those described previously [19-21]. In brief, IEC-6 and IEC-Cdx2L1 cells were plated at 6.25 X 10^4 cells/cm^2 in DMEM plus 5% dFBS, 10 μg/ml insulin, 50 μg/ml gentamicin sulfate, and 4 mM IPTG. The cells were incubated in a humidified atmosphere at 37 °C in 90% air-10% CO2 (vol/vol) for 4 days, which was followed by a period of different experimental treatments.

Reverse transcription and PCR

Total cellular RNA was isolated by using the RNeasy Mini Kit (Qiagen, Valencia, CA). Ten micrograms of total RNA were reversely transcribed using a first-strand cDNA synthesis kit (Invitrogen) and random hexamers [pd(N)6 primer]. The reaction mixture was incubated for 1 h at 42 °C and then heated at 90 °C for 5 min to inactivate the reverse transcriptase. The specific sense and antisense primer for TGFβRI
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included 5'-TACAGTGTGTCGCCACCTCTGT-3' and 3'-ACACGTTGTAAGTTTGTGC-5'. The expected size of TGFβ-RI fragments was 177 bp, located at 128- to 305-bp in the coding region of the TGFβ-RI cDNA [22]. The specific sense and antisense primer for TGFβ-RI included 5'-CAGTCCCTTGGACAACC-3' and 3'-GGTGAAGTGGACCTCCTGCTGCC-5'. The expected size of TGFβ-RII fragments was 503 bp, located at 421- to 922-bp also within the coding region of the cDNA [15]. PCR was performed by a GeneAmp PCR system (Perkin-Elmer) using Taq polymerase. Two microliters of the first-strand cDNA reaction mixture was used in the PCR reaction. The cDNA samples were amplified in a thermal cycler under the following conditions: the mixture was annealed at 59°C (1 min), extended at 72°C (2 min), and denatured at 94°C (1 min) for 35 cycles. This was followed by a final extension at 72°C (10 min) to ensure complete product extension. The PCR products were electrophoresed through a 1% agarose gel, and amplified cDNA bands were visualized by ethidium bromide staining. To quantify the PCR products, cDNA bands were visualized by ethidium bromide staining. To quantify the PCR products, the amounts of mRNA of TGFβ-RI and TGFβ-RII, an invariant mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Immediately after each of the experiments, the optical density (OD) values for each band on the gel were measured by a gel documentation system (UVP, Upland, CA). The OD values in the TGFβ-RI and TGFβ-RII signals were normalized to the OD values in the GAPDH signals. The normalized values in the controls were expressed as 1 arbitrary unit for quantitative comparison [2, 6].

Western blot analysis

Cell samples, dissolved in ice-cold NP40×-buffer (25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1 mM EDTA, 205 mM sodium pyrophosphate, 10% glycerol, 1% Triton×-100, 10 mg/ml aprotinin), were sonicated and centrifuged at 14,000 rpm for 15 min at 4°C. The protein concentration of the supernatant was measured by the methods described by Bradford [23], and each lane was loaded with 50 µg of protein equivalent. The supernatant was boiled for 5 min and then subjected to electrophoresis on 10% acrylamide gels according to Laemmli [24]. Briefly, after the transfer of protein onto nitrocellulose filters, the filters were incubated for 1 h in 5% nonfat dry milk in 1x TBS-T buffer (Tris-buffered saline, pH 7.4, with 0.1% Tween 20). Immunologic evaluation was then performed overnight at 4°C in 5% nonfat dry milk/TBS-T buffer containing specific antibodies against TGFβ-RI and TGFβ-RII. The filters were subsequently washed with 1x TBS-T and incubated with the secondary antibodies conjugated with HRP for 1 h at room temperature. The immunocomplexes on the filters were reacted for 1 min with Chemiluminiscence Reagent (NEL-100 DuPont NEN).

Preparation of nuclear proteins and EMSA

Nuclear extracts were prepared as previously described [2, 25]. Briefly, cells were harvested in ice-cold D-PBS with a cell scraper and centrifuged at 500 g at 4°C for 5 min. The resulting cell pellets were resuspended in 4 ml of ice-cold STM buffer containing 20 mM Tris-HCl (pH 7.85), 250 mM sucrose, 1.1 mM MgCl2, and 0.2% Triton X-100 and were incubated on ice for 5 min. The cell pellets were then washed once with STM buffer containing 0.2% Triton X-100 and once with STM buffer without Triton X-100. The isolated nuclei were then resuspended in STM buffer (without Triton x-100) that contained 0.4 M KCl and 5 mM β-mercaptoethanol and incubated on ice for 10 min to extract nuclear proteins. After centrifugation at 2,000 g at 4°C for 10 min, the supernatant was collected, aliquoted, and frozen at -80°C before use. The protein content of nuclear extracts was determined by the method described by Bradford [23].

The double-stranded oligonucleotides used in these experiments included 5’-CGCTTGATGAC TCA GCCGGAA-3’, which contains a consensus AP-1 binding site that is underlined (Santa Cruz Biotechnology, Santa Cruz, CA). These oligonucleotides were radioactively end-labeled with [γ-32P] ATP (3,000 Ci/mmol; Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (Promega, Madison, WI). For mobility shift assays, 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 2 mM Tris-HCl (pH 7.5), 8 mM NaCl, 0.2 mM EDTA, 0.2 mM β-mercaptoethanol, 0.8% glycerol, and 1 µg poly (dl-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. Gel
supershift assays were accomplished by adding 1 μg (in 1 μl) of TransCruz supershift JunD antibody (Santa Cruz Biotechnology) to the reaction mixture and incubating for an additional 30 min at room temperature.

Statistics

All data are expressed as means ± SE from six dishes. Autoradiographic results were repeated three times. PCR results were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined using Duncan’s multiple range test [26].

Results

TGF-β receptor gene expression in IEC-Cdx2L1 Cells

Consistent with our previous studies [6, 19], nontransfected parental IEC-6 cells revealed a simple monolayer of epithelial cells with sparse microvilli and no evidence of cellular differentiation. In contrast, differentiated IEC-Cdx2L1 cells exhibited multiple morphological and molecular characteristics of intestinal epithelial differentiation, as indicated by polarization, development of lateral membrane interdigitations, and microvilli at the apical pole (data not shown). The differentiated phenotype of stable Cdx2L1 cells was also shown by an induction in expression of enterocyte-specific marker, sucrase-isomaltase gene. As shown in Figure 1 stable IEC-Cdx2L1 cells grown for 12 and 16 days in the presence of IPTG resulted in a significant increase in TGFβ-RI & RII expression, compared to parental IEC-6 cells. The increase in TGFβ-RI mRNA and protein levels were noted on day 12 and remained elevated until day 16 after exposure to IPTG. The relative protein levels of TGFβ-RI in cells exposed to IPTG for 12 and 16 days were ~3.0 times higher in comparison to undifferentiated IEC-6 cells (Figure 1Ca). Similarly, TGFβ-RII mRNA and protein levels were also significantly increased in differentiated IEC-Cdx2L1 cells when cultures were grown in IPTG-supplemented media, compared to parental IEC-6 cells. The relative protein levels of TGFβ-RII were ~1.75 times higher than the values of normal IEC-6 cells (Figure 1Cb).

To extend the findings that TGFβ-RI expression in IEC-Cdx2L1 cells increased after exposure to IPTG, we investigated whether treatment with IPTG for a short time could alter the expression of TGFβ-RI. Results presented in Figure 2 shows

Figure 1. Expression of the TGFβ type I receptor (TGFβ-RI) and type II receptor (TGFβ-RII) mRNA and proteins in parental IEC-6 and IEC-Cdx2L1 cells treated with 4 mM IPTG for 12 and 16 days. A: representative PCR-amplified products displayed in agarose gels for TGFβ-RI (177 bp) and TGFβ-RII (503 bp). Total cellular RNA was harvested at various times after IPTG treatment and the mRNA levels for TGFβ-RI and TGFβ-RII were determined by semi-quantitative RT-PCR analysis. B: representative autoradiograms of Western blots from cells described in A. Whole cell lysates were harvested, applied to each lane (50 μg) equally, and subjected to electrophoresis on 10% acrylamide gel. Levels of TGFβ-RI (~52 kDa) and TGFβ-RII (~75 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 blots by densitometry from cells described in B. a, TGFβ-RI; b, TGFβ-RII. Values are means ± SE of data from 3 separate experiments; relative levels of TGFβ-RI and TGFβ-RII were corrected for loading as measured by densitometry of actin. *P < 0.05 compared with cells treated without IPTG.
that TGFβ-RI gene expression failed to increase after exposing differentiated IEC-Cdx2L1 cells to IPTG for 24 and 48 hours.

Effect of exogenous TGF-β on cell growth in IEC-Cdx2 Cells

Our earlier studies have shown that the exposure of normal IEC-6 cells to exogenous TGF-β inhibited cell growth [6, 8]. In this study, we analyzed whether Cdx2-induced cellular differentiation affects the sensitivity of exogenous TGF-β on cell growth. Differentiated IEC-Cdx2L1 cells exhibited increased sensitivity to TGF-β-induced growth inhibition compared with those observed in parental IEC-6 cells (Figure 3). When various doses of TGF-β were tested, cell growth was inhibited linearly with increasing concentrations of TGF-β ranging from 1 to 10 ng/ml. Significant decreases in cell number occurred starting at 10 ng/ml and were ~40% of normal values. However, in differentiated IEC-Cdx2L1 cells, 2.5 ng/ml TGF-β showed decreases in cell number and continued to exhibit significant changes when a dose of 5 to 10 ng/ml was given; cell counts were ~50% of control values and decreased by ~75% (Figure 3B). These results indicate that differentiated IEC-Cdx2L1 cells are more sensitive to TGF-β-induced growth inhibition.
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Effect of inhibition of polyamine biosynthesis on TGF-β-Receptor mRNA expression and cell growth in response to exogenous TGF-β

Our previous studies demonstrated that the exposure of IEC-Cdx2L1 cells to 5 mM DFMO (specific inhibitor of polyamine synthesis) for 4 and 6 days almost completely depleted cellular polyamines [19]. Depletion of cellular polyamines by DFMO resulted in a significant increase in TGFβ-RI expression in differentiated IEC-Cdx2L1 cells (Figure 4). The increase in relative mRNA levels for TGFβ-RI was noted on day 4 and remained elevated on day 6 after exposure to DFMO. The levels of TGFβ-RI mRNA in cells exposed to DFMO for 4 and 6 days were ~3 times the normal values. Exogenous addition of spermidine at a dose of 5 µM combined with DFMO completed prevented the previously observed increase in TGFβ-RI gene expression. The levels of TGFβ-RI mRNA in cells treated with DFMO plus spermidine were indistinguishable from those in cells grown in control cultures. In contrast to TGFβ-Ri, polyamine depletion did not induce expression of the TGFβ-Rii gene in IEC-Cdx2L1 cells. No significant changes were detected in the relative levels of TGFβ-Rii mRNAs between control cells and cells exposed to DFMO with or without spermidine (Figure 4B).

When varying doses of TGF-β were tested, cell growth was inhibited with increasing concentrations of TGF-β ranging from 1 to 10 ng/ml. Significant decreases in cell number were noticed first at 5 and 10 ng/ml. In DFMO-treated cells, however, 1 ng/ml TGF-β significantly decreased cell count (Figure 5). When TGF-β at doses ranging from 1 to 10 ng/ml were given, cell numbers were decreased by > 50%. Consistent with the effect on TGFβ-RI expression, spermidine given together with DFMO prevented the increased sensitivity of polyamine-deficient cells to growth inhibition caused by exogenous TGF-β (Figure 5C). These results indicate that polyamine depletion increases TGFβ-RI expression and enhances its sensitivity to TGFβ-induced cell growth.

Effect of TGF-β on AP-1 DNA binding activity

Previous studies [25] show that activation of JunD/AP-1 represses IEC proliferation, the current study determines whether TGFβ-induced cell growth inhibition was associated with changes in JunD/AP-1 binding activity in differentiated IEC-Cdx2L1 cells. Cultures were initially grown for 4 days and then incubated with varying concentrations of TGF-β for an additional 6 h. Nuclear proteins were subjected to EMSA analysis to determine the AP-1 binding activity. Figure 6A showed that treatment with TGF-β at a concentration of 5 and 10 ng/ml significantly increased AP-1 DNA binding activity. Since AP-1 complexes consists of different components, gel supershift assays were performed using a specific JunD antibody. As can be seen in Figure 6B, the anti-JunD antibody, when added to the binding reaction mixture, dramatically super-
shifted the AP-1 complexes present in TGF-β (10 ng/ml)-induced IEC-Cdx2L1 cells. These results indicate that TGF-β increases JunD/AP-1 activity in intestinal epithelial cells.

Discussion

The TGF-β signaling pathway is involved in the regulation of a wide variety of biological processes including cell proliferation and apoptosis [27-30]. In normal development, TGF-β inhibits cell proliferation and induces cell differentiation, whereas during tumorigenesis, TGF-β is secreted from tumor cells causing them to lose their inhibitory function [31, 32]. In the intestinal epithelium, TGF-β plays a pivotal role in the regulation of normal mucosal growth [1, 8] and it inhibits cell proliferative activity and promotes the onset of differentiation [33]. Our earlier studies have demonstrated that cellular polyamines negatively regulates the expression of growth-inhibiting genes, including TGF-β and TGF-β receptors, p53, Smad, and JunD, at the posttranscriptional level in normal undifferentiated intestinal epithelial cells [2, 6, 8, 25, 34, 35]. This current study provides new evidence showing that differentiation induces TGFβ-R expression and increases the sensitivity of IECs to TGF-β-induced growth inhibition.
In intestinal epithelial cells, resistance and sensitivity to growth inhibition by TGF-β are mainly regulated by changes in TGF-β receptor expression. Current findings clearly show that differentiated IEC-Cdx2L1 cells are associated with increased TGFβRI expression. The IEC-6, IPEC (porcine jejunal enterocytes), and RIE-1 (rat intestinal epithelial cells) cell lines, all of which exhibit undifferentiated characteristics, have their growth inhibited by TGF-β and express the TGF-β receptors [6, 8, 36]. In many other cell types, TGF-β resistance has been associated with a decrease or absence of TGFβRI and RII expression [37, 38]. The results reported here indicate that the expression of TGFβRI is also implicated in the process by which polyamine depletion increases the sensitivity to TGF-β-mediated growth inhibition in differentiated intestinal epithelial cells. As shown in Figure 4, exposure to DFMO for 4 and 6 days increased the relative levels of TGF-βRI mRNA expression. In contrast, expression of the TGFβRII gene was not affected after exposure to DFMO in the presence or absence of exogenous spermidine. These expected results are not surprising because TGFβRII is a known constitutive, active kinase [39]. Polyamines may regulate TGFβRII function through a different mechanism rather than through mRNA synthesis. It is interesting and of important biological significance that the increased TGFβRI expression in differentiated IECs was associated with an increase in sensitivity to growth inhibition induced by exogenous TGF-β. These observations have great potential biological significance, since TGFβ-Rs and their downstream signals are highly expressed in gut epithelium and regulated under physiological conditions.

To further determine the mechanism by which activated TGFβ-R pathway induces sensitivity to TGF-β-mediated growth inhibition, AP-1 binding activity was determined by EMSA after administration of exogenous TGF-β. Administration of TGF-β increased JunD/AP-1 binding activity, suggesting the involvement of this negative AP-1 binding in this process. These findings were consistent with our earlier observations that JunD/AP-1 binding activity is increased following polyamine depletion and contributes to growth inhibition in gut mucosa in vivo as well as in vitro systems [25, 40].

In summary, these results indicate that differentiated intestinal epithelial cells induced by forced expression of the Cdx2 gene express high levels of TGFβRI and TGFβRII. The increased TGF-β receptor expression plays an important role in the process through which differentiated intestinal epithelial cells are more sensitive to growth inhibition induced by exogenous TGF-β. This signaling pathway is regulated by cellular polyamines. These findings suggest that the loss of proliferative potential in undifferentiated intestinal epithelial cells is due, at least in part, to the increased expression of TGF-β receptors.

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