Differential Expression of Transforming Growth Factors α and β in Rat Intestinal Epithelial Cells

Shin-ya Koyama and Daniel K. Podolsky
Departments of Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114

Abstract

Expression of transforming growth factor α (TGF α), and transforming growth factor β (TGF β) was assessed in isolated primary rat intestinal epithelial cells as well as a rat intestinal crypt cell–derived cell line (IEC-6). A gradient in TGF β was present, with high concentrations of a 2.5-kb transcript found in undifferentiated crypt cells and progressively lower amounts of the TGF β transcript in increasingly differentiated villus cell populations. In contrast, the concentration of 4.5-kb TGF α transcript was higher in differentiated villus cells than in mitotically active, undifferentiated populations of crypt epithelial cells. The concentrations of transforming growth factors α and β as determined by radioreceptor binding inhibition assay and direct assessment of transforming growth factor biological activity correlated with Northern blot analysis. Although gradients in the expression of the TGFs were present, equivalent binding was observed in the different intestinal cell populations when assessed with ^{125}I-TGF β and ^{125}I-EGF (TGF α). No EGF transcripts were detected in any intestinal cell population, suggesting that the true ligand of the EGF receptor was TGF α. IEC-6 cells expressed both TGF α and TGF β transcripts. In addition to the transcripts identified in the primary intestinal cells, this cell line contained an additional larger TGF α transcript (4.8 kb) and smaller TGF β transcripts (2.2 and 1.8 kb). TGF α and TGF β may play a significant role in the regulation of the balance between proliferative and differentiated cell compartments in the intestinal epithelium through both autocrine and paracrine mechanisms.

Introduction

The intestinal mucosa is composed of a highly dynamic epithelial cell population in which proliferating cells are spatially segregated in the crypt from a gradient of increasingly differentiated cells present along the longitudinal axis of the villus. The maintenance of mucosal integrity in the face of constant and rapid turnover of these cell populations suggests that exquisite mechanisms must exist to balance proliferative activity with both commitment to differentiation and loss of mature cells from the villus. A number of observations in both primary cells and established cell lines derived from the intestinal epithelium and human colon carcinomas indicate that soluble peptide growth factors may be essential in regulating proliferative activity in intestinal epithelial cells (1–5). A number of investigators have speculated that epidermal growth factor may play an important role in view of the detection of epidermal growth factor (EGF)[1] receptors on enterocytes (6–8).

Over the past several years, two additional classes of peptides initially identified through their ability to stimulate or augment anchorage independent growth of nontransformed fibroblasts and designated transforming growth factors α and β (TGF α and β) have been recognized to have potent effects on proliferation of many types of epithelial cells (9). TGF α, structurally homologous to EGF has been found to stimulate thymidine incorporation in many cell types (10–11). This action appears to be mediated through the same receptor that binds EGF indicating that the physiologically relevant ligand of some receptors initially detected through binding of EGF may not be obvious. In contrast, TGF β has been found to inhibit proliferative activity of many epithelial cell types (12–14). Although the mechanisms are incompletely understood, TGF β-mediated growth inhibition may be coupled to expression of features of differentiation in some cell systems (15).

A number of observations suggest that these more recently described factors may also participate in the modulation of proliferation and differentiation in the intestinal epithelium. Coffey and his co-workers have demonstrated the production of either TGF α and/or TGF β by some human colonic carcinoma–derived cell lines (3, 4). The associated presence of receptors for these ligands on some of the same cell lines suggests that these peptides may provide autocrine regulation of growth in these cells. Preliminary studies in this laboratory using the nontransformed IEC-6 line derived from the rat small intestine found that TGF β can be a potent growth inhibitor in this system and can promote expression of at least some features of the differentiated villus cell (2, 16). In this report, we demonstrate that TGF α and β are indeed present in intestinal epithelial cells but are differentially produced within the crypt-to-villus continuum.

Methods

Cells and cell culture. Primary rat intestinal epithelial cells were isolated from Sprague-Dawley rats by the method of Weiser (17). The recovery of a gradient of cells from crypt to villus was confirmed by

Address reprint requests to Dr. Daniel K. Podolsky, Gastrointestinal Unit, Massachusetts General Hospital, Boston, MA 02114.

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1. *Abbreviations used in this paper:* BHK, baby hamster kidney; EGF, epidermal growth factor; NRK, normal rat kidney; TGF, transforming growth factor.

measurement of sucrase activity (18), thymidine incorporation as described originally (17), and determination of actin expression in Northern blot analysis as described below. After initial confirmation of the recovery of a gradient from fully differentiated cells to crypt cells in nine successive fractions as originally described, successive pairs of fractions were pooled for subsequent studies, i.e., fractions 1 and 2, 3 and 5, 5 and 6, and 8 and 9. IEC-6 cells, passage 10–14, first established in this laboratory, were grown in standard conditions (16).

Northern blot analysis. Total cellular polyadenylated RNA was isolated from primary epithelial cells and IEC-6 cells as well as rat submaxillary gland and duodenum by modification of the method of Chirgwin et al. as described (19), electrophoresed into 1.0% formaldehyde-agarose gel, and blotted onto nitrocellulose paper by standard methods. Assessment of specific transcripts was as follows: γ-actin transcripts were assessed with a γ-actin-specific oligonucleotide (39-mer) generously supplied by Dr. Stephen Brand (Massachusetts General Hospital, Boston, MA) and end labeled with T4 kinase (20). Blots were hybridized for 20 h at 45°C in 5× SSC, 1% SDS, 1 mM EDTA, 1× Denhardt's plus 200 μg/ml salmon sperm (ss) DNA and washed with 0.2× SSC, 10 mM Na/P, pH 7.0, 10× Denhardt's, 5% SDS, and the 0.2× SSC in 1% SDS. Labeled TGF α cDNA probe was prepared by nick translation of the cDNA insert of plasmid pTGF-C1 (21) subcloned into the SP65 plasmid (22) as described and kindly provided by Dr. R. J. Coffey, Jr. (Vanderbilt University, Nashville, TN) (23). TGF β probe was prepared by nick translation of a 1.08-kb Eco R1 insert of a mouse TGF β cDNA clone designated pTGFBas (24) containing the entire open reading frame and generously provided by Dr. R. Derynck (Genentech, So. San Francisco, CA). Hybridization was carried out at 42°C in 40% formamide, 5× SSC, 5× Denhardt's, 10% dextran sulfate and 20 mM sodium phosphate, pH 7.0; blots were successively washed at 65°C in 2× SSC plus 0.1% SDS, 1× SSC plus 0.1% SDS, and 0.1× SSC plus 0.1% SDS. EGF-specific transcripts were assessed using an oligonucleotide probe specific for the carboxyl terminus of the coding region (bp 3,300–3,274), which had been endlabeled with T4 kinase (25). Blots were hybridized at 42°C for 24 h in 5× SSC before being washed sequentially in 0.5–1.0% SDS in 0.2× SSC. Cytophilin transcripts were assessed with the plB15 riboprobe (26) kindly provided by R. Coffey, Jr., which was linearized with Pst I before being labeled with T7 polymerase. Hybridization with the riboprobe was carried out in the presence of 50% formamide, 5× SSC at 55°C for 20 h in 50 mM P/Na (pH 7.0), 1 mM EDTA, 2.5× Denhardt's, 200 μg/ml ssDNA (salmon sperm) and 0.1% SDS and washed three times in 0.1× SSC, 0.1% SDS at 65°C, before autoradiography.

Detection of TGF activities. Primary intestinal epithelial cells freshly isolated as indicated above were suspended in 5 vol of 1% acetic acid and sonicated with a sonifier (model S-75; Branson Cleaning Equipment Co., Shelton, CT; 15-s bursts at maximum power setting no. 5) and the soluble extracts obtained as the supernatant after centrifugation (105,000 *g* for 60 min). Extracts were adjusted to a concentration of 1.0 mg/ml protein as determined by the method of Lowry et al. (27) and either used directly for soft agar colony stimulation assays or first lyophilized for competitive binding assays.

Stimulation of colony formation in soft agar was assessed using the normal rat kidney (NRK) or baby hamster kidney (BHK) fibroblast indicator lines essentially as described (28, 29). Briefly 10^3 cells were seeded in 60-mm dish supplemented with 100 μl cell extract, genuine EGF (1 μg), genuine murine TGF β (5 ng) or 1% acetic acid in the presence or absence of EGF. Colony number and size were scored after 10 d.

Competitive radioreceptor binding assays for TGF α were carried out with the cell extract after lyophilization and resuspension in 0.1 vol of the appropriate binding buffer. TGF α was assessed through competitive inhibition of ^{125}I-EGF binding to A431 cell membranes as described previously (30).

EGF and TGF β binding to isolated intestinal epithelial cells. Specific EGF binding was determined by incubation of intestinal epithelial cells isolated as described above (10^6/100 μl binding buffer) with ^{125}I-EGF (10^6 cpm/1.6 μg/100 μl) at 24°C for 30 min using conditions previously described reference 27, after subtraction of nonspecific binding (defined as the radiolabel bound in the presence of 200-fold excess unlabeled ligand) and 0 time controls. Specific TGF β binding was determined by incubation of cells with ^{125}I-TGF β (10^6 cpm/1.1 μg/100 μl) at room temperature for 60 min using previously described conditions (31, 32), subtracting nonspecific binding and ligand bound at zero time.

Results

Primary intestinal epithelial cells were obtained as sequential populations ranging from fully differentiated villus cells that exhibited sucrase in high specific activity and the virtual absence of thymidine synthetase to crypt cells that lacked sucrase activity but demonstrated a high degree of incorporation of tritiated thymidine (Fig. 1). Microscopic examination of the isolated fractions revealed them to be devoid of any nonepithelial components. Cells remained viable after recovery, with >98% trypan blue exclusion in all fractions. The validity of the gradient of cells recovered by these procedures was further confirmed by Northern blot analysis of γ actin, which in the context of these populations is related to the development of microvilli and thus a marker of cell differentiation (see Fig. 1, *inset*).

The presence of mRNA transcripts encoding TGF α and TGF β was assessed by Northern blot analysis using oligodT selected RNA prepared from the intestinal epithelial cell populations. As demonstrated in Fig. 2 *A*, increasing concentrations of a single 4.5-kb TGF α transcript were observed in differentiated villus cell populations. This gradient did not reflect either differential RNA degradation or gel loading as demonstrated by rehybridization of the same blots with a cytophillin specific riboprobe. Although cytophillin has been reported to be constitutively expressed (26), a gradient of expression inverse to that observed with TGF α was found with highest concentrations in the crypt cell. These observations also preclude the possibility that the failure to detect specific TGF α transcript in crypt fractions simply reflected RNA degradation. Despite the lack of significant levels of TGF α transcript in primary intestinal crypt cells, paradoxically IEC-6 cells, a cell line thought to be derived from the rat crypt cell, expressed high levels of TGF α (Fig. 2 *A*). Interestingly, the major TGF α transcript in this cell line was slightly larger than that found in the primary cells (4.8 vs. 4.5 kb). In addition, a small amount of a still larger transcript was also observed in the IEC-6 cell line.

A gradient in the expression of a TGF β specific transcript was also observed (Fig. 2 *B*). However, in contrast to TGF α, highest levels of a 2.5-kb TGF β transcript were found in crypt cells, with diminishing amounts of the transcript in successive populations of villus cells. Although preliminary studies using a labeled linearized riboprobe for hybridization suggested the presence of a number of transcripts (not shown), only a single genuine transcript was found in primary epithelial cells when the nick translation labeled gel purified excised insert was used. An identical TGF β transcript was also observed in RNA prepared from IEC-6 cells (Fig. 2 *B*). In addition, two smaller transcripts, ~ 2.2 and 1.8 kb in size, not observed in the primary cells, were present in IEC-6 cells.

To determine whether TGF α- and β-specific transcripts were associated with actual growth factor production, extracts of primary intestinal epithelial cell populations were assessed for TGF activities and specific protein by soft agar colony

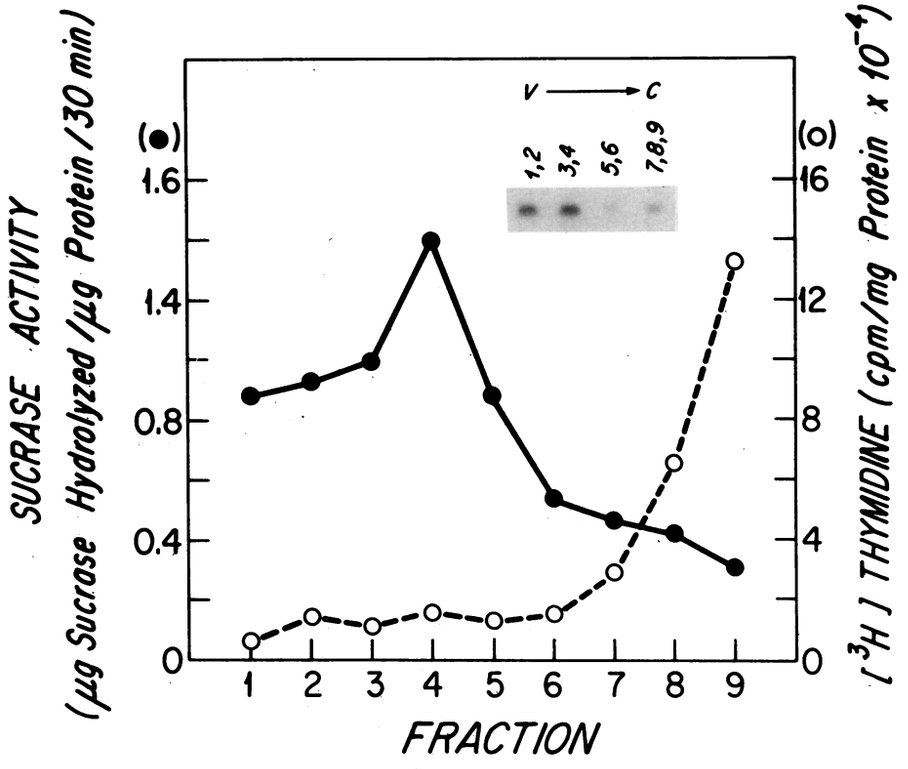

Figure 1. Separation of intestinal epithelial cell populations. Intestinal epithelial cells were isolated by the method of Weiser (17). Individual fractions were assayed for sucrase (18) and thymidine uptake and incorporation (17). Gradient of differentiated villus cells to undifferentiated, proliferative crypt cells runs from fractions 1 to 9. (*Inset*) Northern blot analysis for γ-actin-polyadenylated RNA was prepared from pooled combined adjacent fractions (19) and electrophoresed in 1.0% formaldehyde-agarose before being transferred to nitrocellulose. Blots were probed with an γ-actin-specific oligonucleotide (39-mer) end labeled with T4 kinase (20) as detailed in the text.

stimulation and competitive inhibition of binding by genuine labeled factors respectively. Indeed, TGF α biological activity, as defined by stimulation of colony formation by nontransformed fibroblasts in soft agar, was present in villus cell extracts. Thus, the extract of the villus cells stimulated the number of colonies formed in soft agar by NRK fibroblasts when added alone. These data are consistent with the presence of TGF α material, although they do not exclude the possibility of the presence of other growth factors that might effect formation of soft agar colonies. Peak biological activity was observed in the extracts of villus cell fractions 3 and 4, which exhibited the highest concentrations of the TGF α transcripts in Northern blots. This distribution correlates with cells exhibiting the highest concentration of sucrase activity. Extracts of the villus cell populations were found to have the biological equivalent of 1.3–1.5 pmol EGF equivalent/mg protein, in contrast to <0.5 pmol EGF equivalent in crypt cell extracts.

TGF β CSA was also detected in the extracts of intestinal cells. Thus, when added in the presence of EGF, intestinal cell extracts augmented both the number of colonies formed by the BHK fibroblasts and the size of the individual colonies as observed when genuine TGF β was added with EGF in control experiments. These observations are consistent with TGF β activity but do not preclude the possible presence of other important growth factors. Again, highest levels of the biological activity were correlated with the distribution of the transcript, with highest concentration in the crypt cell–derived extract.

TGF α activity detected in intestinal cell fraction extracts in soft agar colony formation assays correlated with the results of competitive radioreceptor binding assays. Concentrations of TGF α/EGF were calculated from inhibition of specific ^{125}I-EGF binding to A431 cell membranes relative to standard assays constructed using genuine unlabeled EGF. As summarized in Table I, inhibition of specific EGF binding consistent with the presence of either TGF α or EGF was present in villus cell extracts as a protein equivalence of ~ 3.6 ng EGF/mg cell protein, or roughly the same as that detected in the biological assay.

To complement the soft agar colony formation and competitive binding inhibition assays, as well as Northern blot analysis, which established intestinal cell production of TGFs, specific binding of EGF/TGF α and TGF β by the separated intestinal epithelial cell populations was evaluated. Interestingly, all populations appeared to specifically bind both ligands (Table II). Essentially, equivalent concentrations of EGF/TGF α receptor were present on crypt cells and sequentially isolated populations of villus cells. Specific TGF β binding was also observed in both crypt and villus cells.

The presence of specific EGF binding sites in intestinal epithelial cells is consistent with a capacity to respond in some fashion to either EGF and/or TGF α, because both appear to bind to the same receptor in other systems (12–14). As described above, Northern blot analysis with the TGF α-specific probe demonstrated the intrinsic production of this peptide by villus cells. Northern analysis was also carried out with EGF-specific oligonucleotide probes (Fig. 3). EGF-specific transcripts were not found in any intestinal epithelial cell population with an oligonucleotide probe that hybridized strongly to mRNA prepared from submaxillary gland and the full-thickness duodenum (including the Brunner's glands) included as positive controls.

Discussion

The intestinal mucosa contains a dynamic population of epithelial cells in which the mitotically active components are

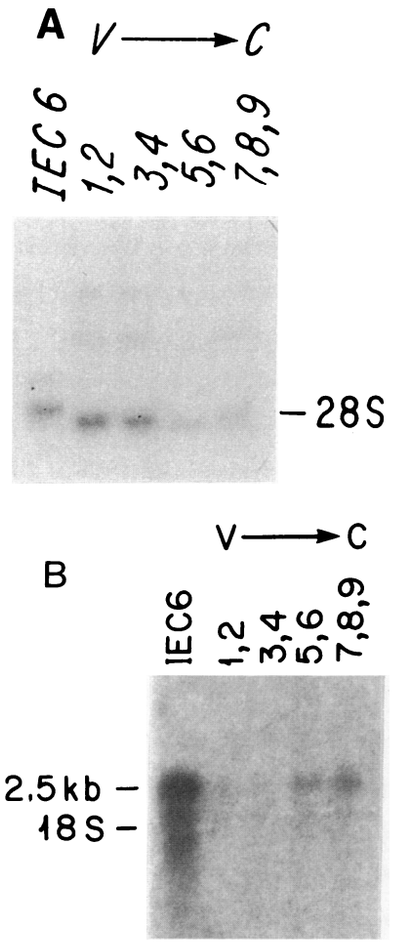

Figure 2. Northern blot analysis of TGFα and TGFβ in intestinal epithelial cells. Relative abundance of (A) TGFα and (B) TGFβ transcription, were assessed after electrophoresis of polyadenylated RNA (2 and 20 μg/lane, respectively) isolated from separated villus, mid villus and crypt cell populations (population fractions as defined in Fig. 1), as well as semiconfluent IEC-6 cells, a line derived from rat intestinal crypt cells (16). Northern blots of polyadenylated RNA were prepared by standard methods (19, 20) and probed with nick translation–labeled insert of the plasmid pTGF-C1 (21–23) or 1.08-kb Eco R1 insert of a mouse TGFβ cDNA clone designated pTGFβ as detailed in the text.

spatially segregated from differentiated cells. Undoubtedly a variety of substances may contribute to the regulation of proliferation and turnover in these cells. The studies described in this report suggest that the intestinal epithelial cells themselves produce at least two factors, TGF α and β, which are recognized to have profound effects on proliferation and differentiation in a wide variety of cells (13–19). In epithelial cells, TGF α and TGF β had often been found to have opposite effects, with TGF α promoting and TGF β inhibiting proliferative activity while promoting expression of a differentiated phenotype in some circumstances. It is therefore possible that the relative concentrations of TGF α and TGF β from crypt to villus serve to regulate intestinal epithelial cell turnover.

The detection of specific receptors for TGF α and TGF β on intestinal epithelial cells supports the assumption that these factors modulate some processes in these cells. However the true physiological ligand of the EGF receptor can only be spec-

Table I. Inhibition of EGF Binding by Extract of Rat Intestinal Epithelial Cells

Intestinal epithelial cells*	Ligand concentration
	ng/0.1 mg protein
1, 2 Villus	3.6
3, 4	3.3
5, 6	2.3
7–9 Crypt	0.4

Protein extracts were prepared from intestinal epithelial cells as described in the legend to Fig. 3. TGFα content was defined by extract (100 μg/100 μl) inhibition of ^{125}I-EGF (10^6 cpm/1.4 ng) binding to A431 membranes (10 μg/100 μl) coated plates at room temperature for 60 min and inhibition of ^{125}I-EGF binding to intestinal epithelial cells (fractions 3 and 4). Concentrations are calculated from concentration standard curves constructed by incubation with unlabeled genuine ligand. TGFα/EGF is expressed relative to EGF.
* Rat intestinal epithelial cell population as defined by Weiser (17) (Fig. 1).

Table II. EGF and TGFβ Binding to Isolated Rat Intestinal Epithelial Cells

Intestinal epithelial cell fractions*	^{125}I-EGF bound‡	^{125}I-TGFβ bound§
	cpm/10^6 cells	
1, 2 Villus	1,610	12,740
3, 4	1,370	14,600
5, 6	1,750	17,860
7–9 Crypt	1,710	19,340

* Intestinal epithelial cells were isolated and defined according to methods of Weiser (17).
‡ Specific EGF binding determined by incubation of 10^6 cells/100 μl binding buffer with ^{125}I-EGF (10^6 cpm/1.6–3.1 ng in the 100 μl) at 24°C for 30 min using conditions previously described (8). Nonspecific binding defined as radiolabel bound in the presence of 200-fold excess unlabeled ligand was subtracted. Mean of four experiments.
§ Specific TGFβ determined by incubation of 10^6 cells with ^{125}I-TGFβ (10^6 cpm/1.1–3.4 μg/100 μl) at room temperature for 60 min using conditions described previously (31, 32). Radiolabel bound in presence of 200-fold excess unlabeled ligand was subtracted. Mean of four experiments.

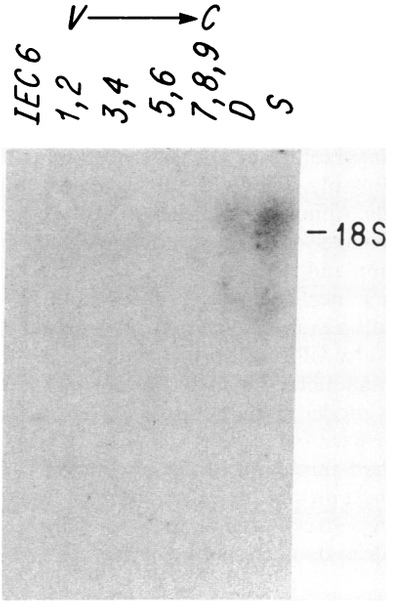

Figure 3. Northern blot analysis of EGF expression in intestinal epithelial cells. Presence of EGF-specific transcripts was assessed in polyadenylated RNA (20 μg) from primary intestinal epithelial cells ranging from villus (1 and 2) to crypt (7 and 9) as defined in Fig. 1, and the IEC-6 cell line as well as positive controls (1 μg) submaxillary glands (S) and duodenum (D). Blots prepared as described in legend to Fig. 2 and text were probed with an EGF-specific oligonucleotide probe for the carboxyl terminus of the coding region (bp 3,300–3,274) end labeled with T4 kinase. Blots were hybridized at 42°C for 24 h in 5× SSC before sequential washing in 0.5–1.0% SDS in 0.2× SSC.

ulated upon at this time. Previous reports documenting the presence of EGF binding sites on enterocytes have speculated that luminal EGF may control proliferation (6–10). However, it is possible that TGF α is the true ligand for these receptors at least in part insofar as these cells actually produce this homologous factor and apparently not EGF. Furthermore, the nature of the response of the villus cell to EGF/TGF α receptor occupancy regardless of the ligand remains unclear but cannot be proliferation in these presumably terminally differentiated cells. Although it is possible that the EGF/TGF α binding observed in villus cell populations reflects mere persistence of nonfunctional receptor after differentiation, it is also possible that additional processes other than mitotic activity may be mediated through this receptor.

The functional significance of TGF β expression and binding among the intestinal epithelial cells also remains to be determined. However, previous studies using the IEC-6 line derived from rat intestinal crypt cells demonstrated potent inhibition of proliferation and promotion of at least some attributes of villus cell differentiation when this peptide was added in vitro (2). This effect was dominant when TGF β was added in the presence of EGF and other growth factors that individually stimulated IEC-6 proliferation. Alternatively, TGF β could modulate the extracellular matrix of the intestinal epithelium including the lamina propria. Modulation of the extracellular matrix might in turn play an important role in regulation of mitotic activity and expression of a differentiated phenotype. Assessment of these biological activities in primary cells is precluded by their limited survival after isolation, so that caution is needed in extrapolating findings obtained with established cell lines.

The seemingly uniform distribution of TGF receptors among intestinal epithelial cell populations in the face of the differential distribution of intrinsic ligand production, suggests that these growth factors may operate through both autocrine and paracrine mechanisms. As noted above, maximal expression of TGF α was found in villus cells, with lesser concentrations of transcripts and peptide in the crypt cells while an opposite distribution was found for TGF β. The latter compares with findings noted in a preliminary report (33). Nonetheless, the paradoxical nature of the distribution of TGF α and TGF β in intestinal epithelial cells should be emphasized. Production of TGF α, which promotes proliferative activity, was greatest in differentiated cells, whereas the growth-inhibiting TGF β was predominantly found in undifferentiated mitotically active crypt cells. Although simplistic, these observations suggest that the balance of crypt proliferative activity with villus cell maturation and senescence may be controlled by a paradoxical feedback mechanism, i.e., crypt cell production of TGF β, which puts a brake on their own proliferation, may be counterbalanced by villus cell production of TGF α. Although other factors undoubtedly also play a role in modulating this balance, this model presupposes that TGF β acts through an autocrine or local paracrine mechanism, whereas TGF α acts in this regard through a longer range paracrine mode. Clarification of these processes may provide insight into the exquisite balance between cell proliferation, functional capacity, and cell loss in these dynamic populations.

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