Regulation of Transforming Growth Factor Expression in Rat Intestinal Epithelial Cell Lines

S. Suemori, C. Ciacci, and D. K. Podolsky

Gastrointestinal Unit, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts 02114

Abstract

Autocrine and paracrine modulation of transforming growth factor expression was assessed in rat intestinal epithelial cell lines designated IEC-6 and IEC-17. Addition of the transforming growth factor α (TGFα) homologue epidermal growth factor (EGF) to media of subconfluent IEC-6 cells led to autocrine stimulation of TGFα expression as well as increased expression of the transforming growth factor β_1 (TGFβ_1). Increased expression of TGFα was maximal between 3 and 6 h after addition of EGF and subsequently declined coincident with increasing level of expression of TGFβ_1, which achieved maximal levels 6 h after addition of EGF and was sustained for more than 12 h. Addition of TGFβ_1 also led to autocrine induction of its own expression coincident with suppression of TGFα expression. Addition of TGFβ_1 was associated with increased expression of β-actin when standardized to a constitutive transcript (GAPDH). Similar responses to addition of EGF and TGFβ_1, were observed in another intestinal epithelial cell line, designated IEC-17. Modulation of expression of TGFs was attenuated when cells were grown on the complex extracellular matrix produced by the Engelbreth-Holm-Swarm tumor (Matrigel), reflecting the baseline induction of TGFβ_1 expression when compared to IEC-6 and IEC-17 cells maintained on plastic. These observations suggest that expression of TGFs is controlled by autocrine mechanisms in intestinal epithelial cell lines and proliferation stimulated by TGFα may be initially self-reinforcing but ultimately downregulated by induction of TGFβ_1. (*J. Clin. Invest.* 1991. 87:2216–2221.) Key words: autocrine • paracrine • epidermal growth factor • extracellular matrix

Introduction

Understanding of the elements regulating intestinal epithelial growth and proliferation is incomplete. Although a number of studies have suggested that constituents of the extracellular matrix and cell–cell interactions make important contributions to these processes, it is clear that a number of peptide growth factors are also essential (1–6). Indeed, it is likely that growth regulation reflects integration between the action of peptide growth factors and constituents of the extracellular matrix. There is little doubt that the full variety of the peptides which play a role in the control of intestinal epithelial cells has not

been defined. Nonetheless, there is increasing appreciation of the diversity of these factors generally and the importance of several specific peptides produced in the intestinal mucosa (1–8). A number of peptide growth factors may act through a classic endocrine mode but many of the more recently recognized factors are expressed by the epithelial cells themselves or other mucosal elements and act through paracrine and autocrine mechanisms.

Many observations have suggested that epidermal growth factor (EGF)[1] produced at remote sites may play a role in modulating fetal intestinal development and cellular proliferation in the mature animal (9–11). Some of these findings suggest still another mechanism of regulating growth of mucosal epithelial cells in which factors present in the intestinal lumen interact at the mucosal surface. However, extensive degradation of luminal peptide and the more recent visualization of EGF receptors restricted to nonluminal membrane domains make the relevance of this concept uncertain (10, 12). Furthermore, the production within the intestinal mucosa itself of transforming growth factor α (TGFα), a growth factor which binds the so-called EGF receptor suggest that EGF may not be the physiologically relevant ligand in the intestinal epithelium (13).

A number of observations in our laboratory suggest that TGFα may have an important role in the regulation of the proliferation of intestinal epithelial cells (2, 13). This protein initially identified and named for its ability to stimulate anchorage independent growth of nontransformed indicator fibroblasts has been shown to be a distinct member of the EGF-like family of growth factors with extensive homology to EGF as well as other peptides. All of its actions appear to be mediated through the same receptor recognized by EGF, though in a few select experimental settings the biological effects of these two factors can be partly dissociated (14).

We and others have demonstrated that TGFα is selectively expressed by villus epithelial cells with markedly less production in the proliferative crypt cell population (13, 15). This surprising and seemingly counterintuitive gradient of expression may reflect both the aggregate effect of several other growth factors modulating TGFα expression in vivo as well as its own autoregulation. In addition, constituents of the basement membrane and products of mesenchymal cells present in the lamina propria could also be important. Further, the distribution raises the important possibility of paracrine activity, a concept supported by the presence of cell surface TGFα, which in other cell systems has been demonstrated to lead to *trans*-cellular activation of EGF/TGFα receptors (16, 17). The importance of TGFα in the regulation of intestinal epithelial cell growth is supported by the demonstration of TGFα production by a number of colon cancer–derived cell lines and resulting

autocrine growth stimulation in several of these lines which also simultaneously express the relevant receptor (3–5, 18).

Another nearly ubiquitous family of peptide growth factors designated transforming growth factor β (TGFβ) may also play an especially important role in control of intestinal epithelial growth and differentiation. In preliminary studies, TGFβ_1 appeared to effect potent inhibition of proliferation of the intestinal epithelial cell line IEC-6 (2). In addition, exposure to TGFβ_1 was related to expression of at least some features of villus cell differentiation. Brattain and co-workers as well as others have demonstrated the ability of TGFβ_1 to promote features of differentiation in a number of colon cancer–derived cell lines consistent with the effect observed in the IEC-6 cells (4, 5, 19, 20). High levels of expression of both the mRNA transcript and the peptide has also been demonstrated in primary intestinal cells (13, 21).

Although these earlier findings indicate that both TGFα and TGFβ_1 are expressed by intestinal epithelial cells, their interactions in modulating proliferation and commitment to differentiation in these cells remain unknown. To define these processes, we have examined the modulation of coordinate expression of TGFα and TGFβ_1 in response to these same ligands and the further effect of a complex extracellular matrix on these interactions in established intestinal epithelial cell lines.

Methods

Receptor grade EGF and porcine platelet TGFβ_1 were obtained from R&D Systems (Minneapolis, MN).

IEC-6 cells, passage 15, and IEC-17 cells, first established in this laboratory by Dr. A. Quaroni (22), were grown in standard media using either untreated 60-mm plastic dishes or plates precoated with 100 μl Matrigel, a complex mixture of extracellular matrix produced by the Engelbreth-Holm-Swarm sarcoma line (23). Media of subconfluent cells was supplemented 2–4 d after seeding with either EGF (5 ng/ml) or TGFβ_1 (5 ng/ml) and cells harvested for preparation of mRNA at varying times. Previous studies have demonstrated that these concentrations effect maximal stimulation and inhibition of IEC-6 proliferation, respectively (2). Typically six dishes, seeded with 10^5 cells each were used for preparation of mRNA at each time point.

Northern blot analysis. Total cellular RNA was isolated from IEC-6 and IEC-17 cells by modification of the method of Chirgwin et al. as described (24); poly(A)$^+$/RNA was purified using an oligo (dT)-cellulose column ($\times$2), electrophoresed into 1.0% formaldehyde agarose gel, and blotted onto nylon membrane (Nytran, Schleicher & Schuell, Keene, NH) by standard methods; 3–7 μg of mRNA was applied to each lane. Assessment of specific transcripts was as follows: β-actin transcripts were assessed with a β-actin-specific oligonucleotide (39-mer) end labeled with T4 kinase (24). Blots were hybridized for 20 h at 45°C in 5$\times$ SSC, 1% SDS, 1 mM EDTA, 1$\times$ Denhart's plus 200 μg/ml salmon sperm (ss) DNA and washed with 0.2$\times$ SSC, 10 mM Na/P, pH 7.0, 10$\times$ Denhart's, 5% SDS, and 0.2$\times$ SSC in 1% SDS. TGFα transcripts were assessed with the pTGF-C1 riboprobe (25) kindly provided by Dr. R. J. Coffey, Jr. (Vanderbilt University, Nashville, TN) (26), which was linearized with Hind III before labeling with T7 polymerase. Hybridization with the riboprobe was carried out in the presence of 50% formamide, 5$\times$ SSC at 55°C for 20 h in 50 mM Na/P, pH 7.0, 1 mM EDTA, 2.5$\times$ Denhart's, 200 μg/ml ss DNA, and 0.1% SDS and washed three times in 0.1$\times$ SSC, 0.1% SDS at 65°C, before autoradiography. TGFβ_1 and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were prepared by random priming of a 1.08-kb Eco RI insert of a mouse TGFβ_1 cDNA clone designated pTGFβ as (27–29) containing the entire open reading frame generously provided by Dr. R. Derynck (Genentech Inc., South San Francisco, CA) and of a 550-bp, Hind III + Xba I digested fragment, from the GAPDH plasmid

pHcGAP (30) obtained from American Type Culture Collection (Rockville, MD), respectively. Hybridization was carried out at 42°C in 50% formamide, 5$\times$ SSC, 5$\times$ Denhart's, 10% dextran sulfate, and 20 mM Na/P, pH 7.0; blots were successively washed at 50°C in 2$\times$ SSC plus 0.1% SDS four times. Relative abundance of transcript was assessed by laser densitometer scanning, normalized to the density of the GAPDH transcript. Experiments were carried out a minimum of four times; SE $\leq$ 12% in all instances.

TGF bioactivity. Subconfluent monolayers of IEC-6 cells were treated with TGFβ or EGF as above; at varying intervals, media was changed to remove the exogenously added ligand. After further culture for 4 h, bioactivity was determined by two approaches: the ability to stimulate anchorage independent growth in soft agar indicator NRK fibroblasts in the presence or absence of EGF as previously described (31, 32) and the effect on thymidine incorporation by untreated subconfluent IEC-6 cells (2). All assays were performed in triplicate; intradish variability was $\leq$ 15%.

Results

The expression of TGFα and TGFβ_1 were assessed by Northern blot analysis after exposure of IEC-6 cells to the TGFα homologue, EGF. Previous studies have demonstrated that EGF is not expressed by this cell line but promotes ^{3}[H]-thymidine incorporation at the concentrations added, mediating its effects through a receptor common to TGFα as in other cell types (11, 13). As demonstrated in Fig. 1, baseline expression of a 4.5-kb TGFα transcript was minimal in these subconfluent cells. Expression of TGFα was stimulated by addition of EGF, achieving highest levels 3 h after addition of this ligand. Subsequently, the expression of the TGFα transcript declined, returning to baseline concentration within 12 h. The temporal sequence of this stimulation and its decay despite the continuing presence of the ligand in the culture media are shown in Fig. 2. When normalized to a constitutive transcript (GAPDH), the relative concentration of TGFα was consistently two- to threefold elevated after addition of EGF. Although initial levels of TGFβ_1 in the growing IEC-6 cells were relatively lower than TGFα, EGF also led to increased expression of TGFβ_1 (Fig. 1). Highest expression was achieved by 3 h after addition of EGF. The relative concentration of TGFβ was fivefold elevated. Interestingly, elevated levels of TGFβ per-
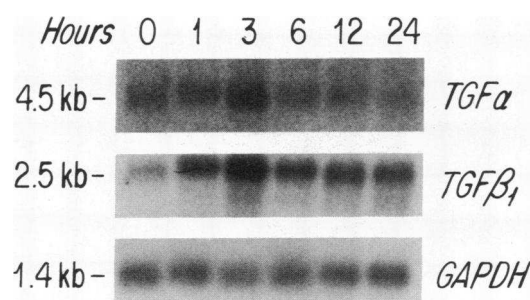

Figure 1. Northern blot analysis of the effect of EGF on expression of transforming growth factors in IEC-6 cells. Relative abundance of (*A*) TGFα and (*B*) TGFβ_1 transcripts and (*C*) GAPDH were assessed after electrophoresis of polyadenylated RNA prepared from subconfluent IEC-6 cells at varying times after addition of EGF (5 ng/ml): Northern blot were prepared by standard methods and sequentially hybridized with pTGF-C1 riboprobe, 1.08 kb Eco RI insert of pTGFβ as (labeled by random priming) and the 0.55 kb plasmid pHc GAPDH (labeled by random priming), using conditions specified in the text.
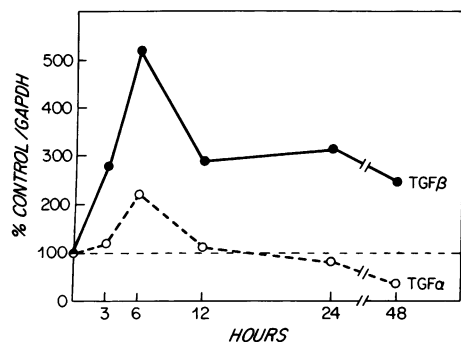
Figure 2. Effect of EGF on the relative abundance of TGFα and TGFβ₁ transcripts. The concentration of TGFα and TGFβ₁ transcripts at varying times after addition of EGF to subconfluent IEC-6 cells depicted in Fig. 1, were determined by densitometric scanning normalized to the content of the constitutive transcript GAPDH.

sisted 24–48 h after addition of the EGF ligand. It should be noted that cells remained subconfluent throughout the period of study, precluding a contribution of contact inhibition to modulation of TGF levels.

Addition of TGFβ₁ to growing IEC-6 cells also led to alterations in TGF expression. Significant inhibition of TGFα expression was observed within 6 h after addition of TGFβ₁ and persisted for 24 h (Fig. 3 and 4). Interestingly, the suppression of TGFα was attenuated with continued cell culture, returning to baseline levels 48 h after addition of TGFβ.

As demonstrated in Fig. 3, TGFβ₁ also led to stimulation of its own expression as previously reported (21). Relative levels two- to threefold greater than baseline normalized to GAPDH were observed within 6 h after TGFβ₁ addition. TGFβ₁ remained elevated for 24 h after addition of this ligand but declined subsequently to baseline level or below at 48 h. (Fig. 4). The decline in TGFβ transcript expression was coincident with reappearance of TGFα expression. The attenuation of TGFβ₁ expression and the late reemergence of TGFα could not be
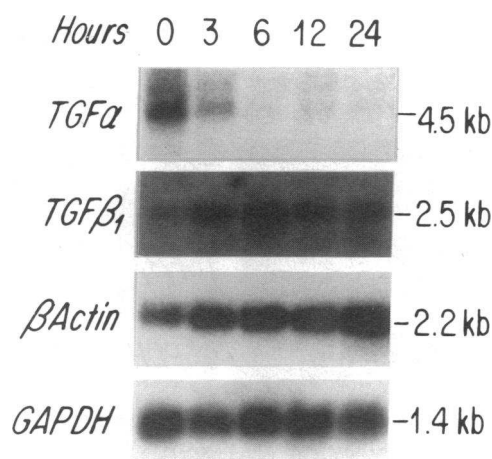

Figure 3. Northern blot analysis of effect of TGFβ₁ on expression of transforming growth factors in IEC-6 cells. Relative abundance of (*A*) TGFα, (*B*) TGFβ₁, (*C*) β-actin, and (*D*) GAPDH were assessed after addition of TGFβ₁ (5 ng/ml) to subconfluent IEC-6 cells. Northern blot analysis as detailed in legend to Fig. 1 and text. The β-actin was detected with an end-labeled specific 39-mer oligonucleotide (14).
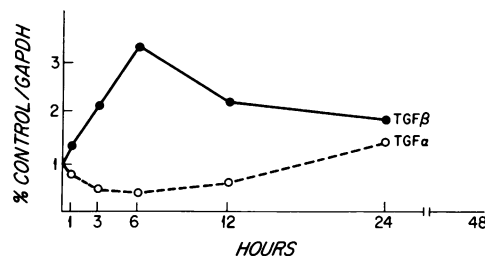

Figure 4. Effect of TGFβ₁ on the relative abundance of TGFα and TGFβ₁ transcripts. The concentration of TGFα and TGFβ₁ transcripts at varying times after addition of EGF to subconfluent IEC-6 cells depicted in Fig. 3, were determined by densitometric scanning normalized to the content of the constitutive transcript GAPDH.

correlated with disappearance of TGFβ₁ bioactivity from the media when assessed by ability of the supernatant to stimulate soft agar colony formation by indicator NRK fibroblasts or capacity to affect thymidine incorporation by previously untreated IEC-6 cells (Fig. 5). As demonstrated in Fig. 5, TGFα (defined by the ability to stimulate soft agar colony formation by the indicator line in the absence of supplemental EGF) and TGFβ (defined by the ability to augment soft agar colony formation in the presence of supplemental EGF) bioactivity corresponded to levels of expression of TGFα and TGFβ at the mRNA level observed after addition of TGFα/EGF or TGFβ to IEC cells (cf. Figs. 1 and 2 to Fig. 5 *A;* cf. Figs. 3 and 4 to Fig. 5 *B*). It should be noted that addition of TGFβ₁ to IEC-6 cells in these studies was correlated with increased expression of at least two markers of intestinal epithelial differentiation consistent with earlier findings, sucrase (not shown) and actin. Thus, as depicted in Fig. 3, the relative amount of an actin transcript steadily increased compared with the truly constitutive GAPDH transcript.

Modulation of expression of TGFα and TGFβ₁ after addition of either EGF or TGFβ₁ was also examined in a second rat intestinal epithelial cell line, designated IEC-17 to assess the relevance of initial studies using the IEC-6 line. The IEC-17 line was established from neonatal duodenal epithelium by the same methods used to establish the IEC-6 lines and has been noted to undergo complex morphologic differentiation as well as produce a complex basement membrane (33). Indeed, as shown in Fig. 6, rapid suppression of TGFα coincident with augmented TGFβ₁ expression after addition of TGFβ₁ is observed in the IEC-17 cells paralleling patterns observed in the IEC-6 line. Similarly, addition of EGF resulted in stimulation of TGFα expression with increased levels of TGFβ₁ transcript in a manner parallel to that observed in the IEC-6 cells.

Previous reports have suggested that features of the villus cell differentiated phenotype may be observed in IEC-6 after plating on a complex mixture of extracellular matrix produced by a sarcoma which contain laminin, entactin (nidogen), collagen, type IV, and fibronectin as well as other less well-defined constituents (1, 23). The impact of growth on this matrix on the responsiveness of these cells to exogenous growth factor was examined 2–4 d after initial seeding. As demonstrated in both panels of Fig. 7, baseline expression of TGFβ₁ was substantially greater in cells seeded on the Matrigel matrix when compared with cells seeded on uncoated plastic dishes. Thus, the content of TGFβ₁ at the zero time relative to the addition of either EGF or TGFβ₁ was significantly higher than that noted in cells

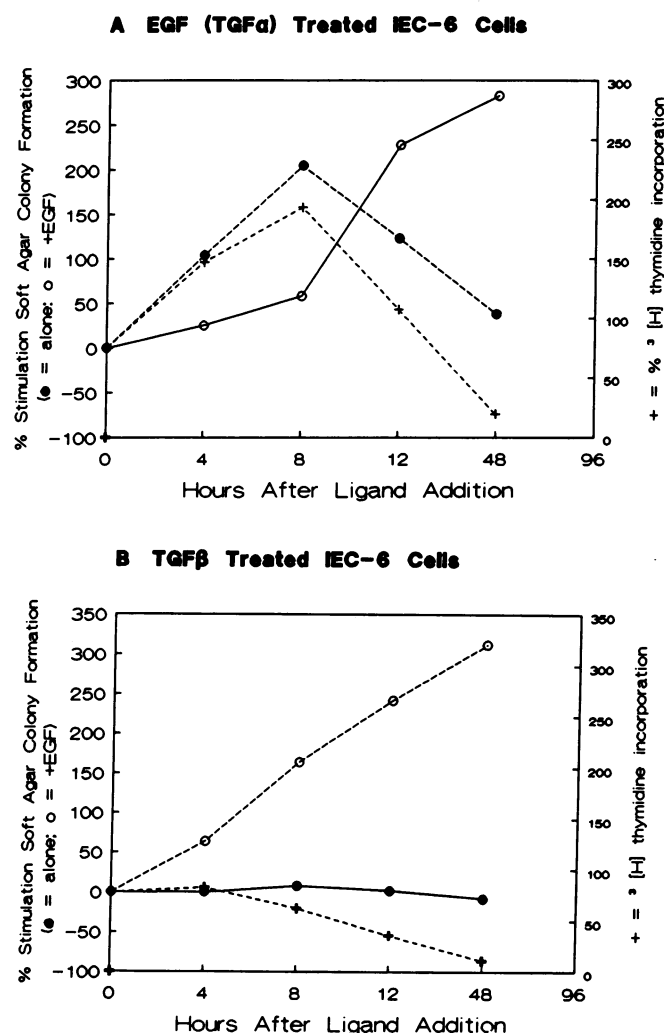## A EGF (TGFα) Treated IEC-6 Cells

B TGFβ Treated IEC-6 Cells

Figure 5. Effect of EGF and TGFβ treatment on TGFα and TGFβ bioactivity expressed by IEC-6 cells. EGF (5 ng/ml) or TGFβ₁ (5 ng/ml) was added to subconfluent IEC-6 cells for varying lengths of time; media was changed to remove the exogenous ligand. After 4 h subsequent incubation, the newly conditioned media was removed and bioactivity assessed by ability to stimulate colony formation of NRK fibroblasts in soft agar alone (TGFα bioactivity; ●) or in the presence of EGF (TGFβ bioactivity; ○) (14, 33, 34). Conditioned media was also assessed for its effect on thymidine incorporation in previously untreated subconfluent IEC-6 cells using previously described methods (2).

seeded on plastic (cf. Figs. 1, 3, and 6). The augmented expression of TGFβ₁ appeared to be a dominant effect in the profile of TGF expression after addition of EGF or TGFα to these IEC-6 cells. There was no further increase in TGFβ₁ expression observed after addition of either of these ligands. Similarly, TGFα expression was lower in all cells maintained in the matrix and was not significantly stimulated by addition of EGF. Increased actin expression was observed in the cells maintained on Matrigel paralleling the increased expression observed after TGFβ treatment of cells grown on plastic.

Discussion

There is little doubt that numerous factors contribute to modulation of intestinal epithelial growth. Evaluation of these pro-
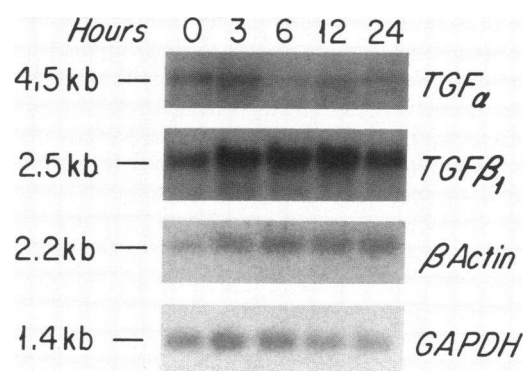

Figure 6. Effect of TGFβ on expression of TGFα, TGFβ₁, and β-actin expression in IEC-17 cells. Relative abundance of TGFα, TGFβ₁, β-actin, and GAPDH were assessed at varying times after addition of (*A*) EGF (5 ng/ml) or (*B*) TGFβ₁ (5 ng/ml), by Northern blot analysis as detailed in legend to Fig. 1 and text.

cesses in primary tissue is in many ways confounded by the complexities of cellular heterogeneity and the inability to dissociate the role of protein ligands and extracellular matrix as well as other contributing factors. To explore the modulation of transforming growth factors in these cells, we have examined their regulation in two nontransformed cell lines which retain many features of rat primary proliferation competent (crypt) intestinal epithelial cells.

These studies suggest that dynamic regulation of TGFα and TGFβ₁ expression may play an important role in these processes. It is clear that autocrine regulatory mechanisms are important insofar as both IEC-6 and IEC-17 cells were found to respond to the TGFs which are also produced as endogenous products by these cells. More particularly, it is notable that each peptide effected an autocrine stimulation of its own expression. Although these studies focus on exogenously added
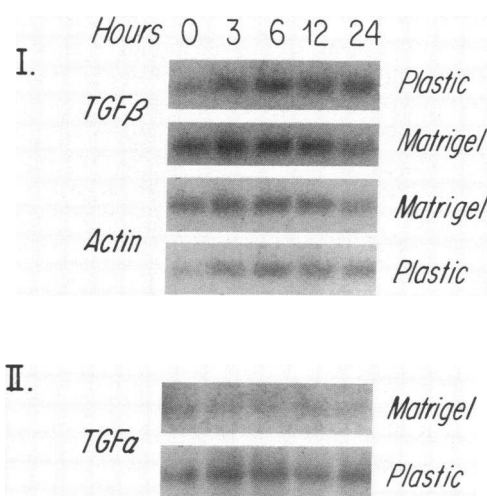

Figure 7. Effect of extracellular matrix on TGF and actin expression in IEC-6 cells. Northern blot analysis was performed at varying times after addition of either (*I*) TGFβ₁ or (*II*) EGF to subconfluent IEC-6 cells seeded on plastic or Matrigel-coated dishes. Northern blot analysis of the (*I*) TGFβ₁-treated IEC-6 for TGFβ₁ and actin, and (*II*) EGF-treated IEC-6 for TGFα accomplished by methods described in text and legend to Fig. 1. Equivalent loading verified by rehybridization with probe for the constituitive transcript GAPDH.

ligand, the observed bioactivity in the conditioned media suggests that the responses likely approximate in vivo responses. It should be noted that the conditioned media containing TGFβ bioactivity also caused the described increase in TGFβ and suppression of TGFα expression when added to previously untreated IEC-6 cells (data not shown). However, it is possible that the "autocrine" effects of exogenously added ligand may not be strictly comparable with the endogenously produced factor. Local concentrations at receptors might vary and the response to receptors on the surface could differ from possible intracellular ligand-receptor interaction.

The mechanisms by which this self-reinforcing cycle of peptide growth factor expression is ultimately curbed is suggested by dynamic serial evaluation of transcript levels after addition of one of the ligands. Thus, the observed sequential temporal stimulation of TGFα with stimulation of TGFβ$_1$ expression after addition of EGF (TGFα) ligand suggest a mechanism for cellular self control of a proliferative stimulus. This control derives from the potent inhibition of proliferation which TGFβ$_1$ causes in this cell line in a manner similar to that observed in many epithelial cell types (2, 28, 30). It should be noted that it is not possible finally to determine whether the inhibition of proliferation by TGFβ$_1$ is a direct effect or a reflection of its inhibition of expression of proliferation promoting TGFα. The ability of TGFβ$_1$ to cause growth inhibition even in the presence of the exogenous TGFα homologue EGF indicates that TGFβ$_1$ inhibits proliferation in these cells directly and not through inhibition of TGFα production.

It has been suggested that TGFβ$_1$ may control proliferation in epithelial cells through modulation of the composition of extracellular matrix which in turn regulates proliferation (30). The present findings suggest that this is of less importance in these cells than direct inhibition for two reasons. First, the temporal disparity in the previously documented abrupt inhibition of thymidine incorporation by IEC-6 cells when TGFβ$_1$ is added to cells grown on plastic, compared to the delayed inhibition of proliferation when cells are initially seeded on matrix suggest that a direct effect of TGFβ$_1$ may be most important in these cells. Conversely, the curb of IEC proliferation by matrix appears temporally related to stimulation of endogenous TGFβ$_1$ expression (Fig. 6). These latter findings provide a possible mechanistic consistency in the observed expression of features of differentiated intestinal phenotype by IEC-6 cells after addition of exogenous TGFβ$_1$ and growth on the Matrigel complex reported by others (1). Although the latter has been noted to be devoid of TGFβ, it is apparent that it serves to stimulate endogenous expression of this growth factor by IEC-6 cells.

These findings suggest a self-limiting mechanism through which proliferation of intestinal epithelial cells may be intrinsically controlled. In this context, it is possible that the unexpected expression of TGFβ within primary crypt cells previously observed in this laboratory, is reflective of an important regulatory mechanism in the transition from undifferentiated mitotically active cell to nondividing enterocyte committed to terminal differentiation. Basement membrane constituents could lead to tonic levels of TGFβ$_1$ expression which acts through autocrine and possibly paracrine mechanisms to suppress cell proliferation.

These interrelationships suggest a mechanism which may be especially well suited to respond to mucosal injury. It is possible that injury with loss of epithelial cells and destruction of the basement membrane lead to reduced TGFβ$_1$ expression

and corresponding relief from the tonic inhibition of cell proliferation by surviving crypt epithelial cell elements. Overcompensation in cell proliferation may be regulated directly by coordinated though temporally delayed stimulation of TGFβ$_1$ expression. In addition, the reconstitution of the basement membrane may further reinforce TGFβ$_1$ expression.

Whereas these are necessarily abstractions in simplifying the number of factors which likely play a role in modulating intestinal epithelial cell proliferation, a number of observations suggest that the factors identified by these studies, TGFα, TGFβ$_1$, and the extracellular matrix, are indeed among the most important of these determinants. In particular, it should be noted that TGFβ$_1$ exerts a dominant inhibiting effect in the presence of known growth promoting peptide including EGF (2). It will be especially important to define the constituents within the extracellular matrix which contribute to this process and their relative importance. It will be just as important to explore the contribution of mesenchymal and immune cellular constituents to these processes which might be mediated in part indirectly through their influence on the constitution of the basement membrane. Understanding these processes should provide insight into both regulation of proliferation *per se* and a foundation to understand repair mechanisms after mucosal injury.

Acknowledgments

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