Gene expression of activin, activin receptors, and follistatin in intestinal epithelial cells

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Gene expression of activin, activin receptors, and follistatin in intestinal epithelial cells. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G89–G97, 2000.—Gene expression of activin, activin receptors, and follistatin was investigated in vivo and in vitro using semiquantitative RT-PCR in intestinal epithelial cells. Rat jejunum and the intestinal epithelial cell line IEC-6 expressed mRNA encoding the βA-subunit of activin, α-subunit of inhibin, activin receptors IB and IIA, and follistatin. An epithelial cell isolation study focused along the crypt-villus axis revealed the expression of activin A in upper villus cells. The human intestinal cell line Caco-2 was used as a differentiation model of enterocytes. Four- to fivefold induction of βA mRNA was observed in postconfluent Caco-2 cells grown on filter but not in those cells grown on plastic. In contrast, follistatin mRNA was seen to be reduced after reaching confluence. Exogenous activin A dose-dependently suppressed the proliferation and stimulated the expression of apolipoprotein A-IV, a differentiation marker, in IEC-6 cells. These results suggest that the activin system is involved in the regulation of such cellular functions as proliferation and differentiation in intestinal epithelial cells.

IEC-6; Caco-2; RT-PCR; crypt-villus axis; differentiation

ACTIVINS, which are members of the transforming growth factor-β (TGF-β) superfamily, have been shown to regulate a number of different cell functions, including cell proliferation and differentiation (for review, see Refs. 16 and 20). These molecules are synthesized as either a single homo- or heterodimer of two highly related β-subunits (βA and βB), resulting in three different isoforms of activins: activin A (βAβA), activin B (βBβB), and activin AB (βAβB) (18, 33). Additional isoforms may arise from putative activin C-, D-, and E-chains, which have recently been cloned (7, 13, 24). In addition, the βA- and βB-subunits also form heterodimers with another dissimilar α-subunit, generating inhibin A (αβA) and inhibin B (αβB) (20). Activins and inhibins act as functional antagonists in some cell systems (18, 33). Activins bind to binary cell surface receptors, which are those receptors composed of two single membrane-spanning serine-threonine kinases designated type I and type II, called Act RI and Act RI1, respectively (for review, see Ref. 21). Still another type of activin-binding protein is known as follistatin (for review, see Ref. 22). Follistatin, a family of proteins generated by alternative splicing and proteolytic processing, has a specific and high affinity for activin, whose effect it neutralizes in a variety of systems. Thus the biological function of activins is regulated in concert with its receptors, antagonists (inhibins), and neutralizing binding proteins (follistatins).

Despite a number of studies investigating the biology of activins, studies concerning their expression and function in the intestine are limited. Kawamura et al. (15) demonstrated that human embryonic intestinal epithelial cell line HFs741 nt expressed mRNA encoding the βA-subunit of activin and released bioactive activin A. More recently, Hubner et al. (14) reported the increased expression of the βA-subunit of activin in surgical specimens from the intestine of patients with inflammatory bowel disease, suggesting an important role of activin in inflammatory processes of the intestine. The intestinal epithelium, which is functionally divided into a zone of proliferation confined to crypts and a zone of differentiation situated in the villi, undergoes continuous renewal throughout the lifespan of an animal (3, 11). The terminally differentiated cells of the epithelium are removed by a process of apoptosis occurring throughout the villus and by exfoliation at the tip of the villus (9, 11). Thus the renewal process of the intestinal epithelium occurs along the crypt-villus axis. This entire sequence of cell proliferation, differentiation, apoptosis, and exfoliation has been thought to be regulated by tissue-specific programmed gene expression and by such extrinsic factors as the presence of nutrients and growth factors (1, 2, 4, 17, 28). In view of the ability of activins to regulate such fundamental cellular functions as proliferation, differentiation, and apoptosis in diverse cell systems, it is believed that they play a role in the maintenance of homeostasis in the intestinal epithelium.

The aim of the present study was twofold. First, we attempted to demonstrate gene expression of activin subunits, activin receptors, and follistatin in intestinal epithelial cells while, secondly, trying to obtain information relevant to the association of activins with proliferation and/or differentiation of epithelial cells. In the present study, we demonstrated the expression of mRNA encoding the βA-subunit of activin, α-subunit of in- 

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hibin, Act RIB, Act RIIA, and follistatin in the rat jejunum, the intestinal epithelial cell line IEC-6, and the human colon adenocarcinoma cell line Caco-2. In addition, we observed that βA mRNA was expressed more abundantly in villus cells than in crypt cells and that activin A protein was expressed in upper villus cells, suggesting that expression of activin A is associated with a termination of proliferation, initiation, and/or a maintenance of differentiation of intestinal epithelial cells. It was also shown that gene expression of the βA-subunit of activin was coupled with cellular differentiation in Caco-2 intestinal cells, which have been known to differentiate after reaching confluence to form an epithelium that shares many characteristics of mature small intestinal mucosa in vivo (10, 24, 26, 32). In contrast, we observed that the follistatin gene was downregulated in differentiated Caco-2 cells. Furthermore, it was shown that the addition of activin A to the culture medium suppressed the proliferation and increased the expression of apolipoprotein A-IV (apo A-IV) gene, a differentiation marker of enterocytes, in IEC-6 cells. These results suggest that the activin system is involved in the maintenance of homeostasis in the intestinal epithelium.

**MATERIALS AND METHODS**

Animals. Male Wistar rats (Japan SLC, Hamamatsu, Japan) were housed in individual cages in a temperature-controlled (23 ± 2°C) room with a dark period from 1900 to 0500. They were allowed free access to water and to a purified diet consisting of (as wt/wt) 25% casein, 65% sucrose, 5% corn oil, 4% mineral mixture, and 1% vitamin mixture (30). This diet is used as a standard rat diet in our laboratory because we have found that it yields a maximal growth rate. Rats weighing between 250 and 300 g were anesthetized by an intraperitoneal injection of Nembutal (pentobarbital sodium 50 mg/kg body wt; Abbott Laboratories, North Chicago, IL). After laparotomy, a 10-cm portion of the jejunum at 3 cm distal to the ligament of Treitz was excised and the luminal contents were thoroughly washed with ice-cold saline. The mucosa was scraped with a glass slide and immediately plunged into liquid nitrogen. It was then stored at −80°C for RNA isolation. In a separate experiment, a 20-cm portion of the jejunum was excised and was subjected to isolation of the epithelial cells. Furthermore, a 5-cm segment of the jejunum was excised, embedded in optimum cutting temperature compound (Miles Scientific, Elk hart, IN), frozen in liquid nitrogen, and stored at −80°C for immunohistochemistry.

This study was approved by the Hokkaido University Animal Use Committee, and animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Intestinal cell isolation. Epithelial cells were differentially isolated from the jejunal segments using the distended intestinal sac method described by Traber et al. (31). Briefly, the jejunal segment was rinsed thoroughly with a washing solution composed of 0.15 M NaCl, 1 mM dithiothreitol (DTT), and 40 µg/ml phenylmethylsulfonyl fluoride (PMSF). The segment was then filled with buffer A, composed of 96 mM NaCl, 27 mM sodium citrate, 1.5 mM KCl, 8 mM KH2PO4, 5.6 mM Na2HPO4, and 40 µg/ml PMSF (pH 7.4), and the ends were clamped with hemostats. The filled segment was submerged in 0.15 M NaCl at 37°C for 15 min and then drained, and the solution was then discarded. Next, the segment was filled with buffer B, composed of 109 mM NaCl, 2.4 mM KCl, 1.5 mM KH2PO4, 4.3 mM Na2HPO4, 1.5 mM EDTA, 10 mM glucose, 5 mM l-glutamine, 0.5 mM DTT, and 40 µg/ml PMSF (pH 7.4), incubated in 0.15 M NaCl at 37°C for 4 min, and then drained. The drained solution was referred to as fraction 1. This step was repeated for another nine cycles at time intervals of 2, 2, 3, 4, 5, 7, 10, 10, and 10 min, generating fractions 2-10, respectively. Finally, these fractions were combined to yield six new fractions: fraction I from fractions 1 and 2, fraction II from fractions 3 and 4, fraction III from fractions 5 and 6, fraction IV from fractions 7 and 8, fraction V from fraction 9, and fraction VI from fraction 10. Aliquots of each fraction were subjected to determine the alkaline phosphatase (ALP) activity using p-nitrophenyl phosphate as a substrate (35). The protein concentration in each fraction was determined by the method of Lowry et al. (19). The cells from each fraction were collected by pelleting at 100 g for 5 min at 4°C, washed once with PBS, and then immediately subjected to isolation of RNA. All solutions used were preoxygenized with 95% O2-5% CO2 and warmed to 37°C.

Immunohistochemistry. To detect the activin A in rat jejunum, immunohistochemistry with catalyzed signal amplification was carried out using a TSA-Indirect kit (NEN Life Science Products, Boston, MA). Frozen sections (6 µm) were prepared with a cryostat, thawed onto glass slides, fixed with 4% (wt/vol) paraformaldehyde in PBS for 10 min, and then washed with TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) three times. Sections were incubated with 3% H2O2 in methanol for 10 min to inhibit endogenous peroxidase, washed with TNT buffer three times, and then blocked with TNB blocking buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.5% (wt/vol) blocking reagent (supplied in kit)) for 30 min. Samples were then incubated with primary antibody (anti-activin A monoclonal antibody, IgM class; a gift from Dr. Yuzuru Eto, Ajinomoto, Kawasaki, Japan) at 4°C for 24 h. After washing with TNT buffer three times, secondary antibody (peroxidase-conjugated goat anti-mouse IgM; Biosource, Camarillo, CA) was added for 30 min, followed by washing with TNT buffer three times. Sections were incubated with biotinyl tyramide solution (supplied in kit) for 10 min and then washed with TNT buffer. Samples were then incubated with peroxidase-conjugated streptavidin (supplied in kit) for 30 min and then visualized with diaminobenzidine solution (0.5 mg/ml) and 0.05% H2O2 for 5 min. The slides were rinsed with distilled water, counterstained with hematoxylin, dehydrated, air dried, and mounted. The negative control slides were treated with nonspecific mouse IgM.

Cell culture. IEC-6 and Caco-2 cells were obtained from the American Type Culture Collection at passages 13 and 18, respectively. Cells were maintained in Falcon 75-cm2 T-flasks (Nippon Becton Dickinson, Tokyo, Japan) in a standard culture medium at 37°C in a humidified atmosphere of 5% CO2-95% air. The standard culture medium for IEC-6 cells contained DMEM supplemented with 5% fetal bovine serum (FBS), 4 mM L-glutamine, 25 mM glucose, 1× nonessential amino acids (from 100× liquid; Gibco BRL, Tokyo, Japan), 5 mg/ml insulin, 100,000 U/ml penicillin, 100 mg/ml streptomycin, and 50 mg/ml gentamycin. The standard culture medium for Caco-2 cells was the same as that for IEC-6 cells except that the medium contained 20% FBS and no insulin. Media were replaced every two days or every day, depending on harvest times and degree of confluence.

For experiments in IEC-6 cells, the cells at 10 days postconfluence were harvested for isolation of RNA. In the case of Caco-2 cells, subconfluent cells were plated onto Falcon six-well plastic plates (Nippon Becton Dickinson) or Falcon 23.4-mm cell-culture inserts (pore size 0.45 µm;
Table 1. Oligonucleotide primers used for RT-PCR and inner oligonucleotide probes used for Southern hybridization of RT-PCR products

<table>
<thead>
<tr>
<th>Target Template</th>
<th>PCR Primers</th>
<th>Product Size, bp</th>
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<tr>
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<tr>
<td>β-A subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>5’-AGGAGGGCAATGTGAGGAGTGGAGGAGTGGAGGAGTGGAGGAG</td>
<td>106</td>
</tr>
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<td>Antisense</td>
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<td>106</td>
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<tr>
<td>α-subunit</td>
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<td></td>
</tr>
<tr>
<td>Sense</td>
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<td>106</td>
</tr>
<tr>
<td>Antisense</td>
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</tr>
<tr>
<td>Act RI</td>
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<tr>
<td>Sense</td>
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<td>106</td>
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<tr>
<td>Antisense</td>
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<tr>
<td>Antisense</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
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<td></td>
</tr>
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<td>β-A subunit</td>
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</tr>
<tr>
<td>Sense</td>
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<tr>
<td>Antisense</td>
<td>5’-GGAGGGCAATGTGAGGAGTGGAGGAGTGGAGGAGTGGAGGAG</td>
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Table 1.—Continued

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<th>Inner Oligonucleotide Probes</th>
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<th>Human</th>
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<tr>
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<td>α-subunit</td>
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<td>5’-GGAGGGCAATGTGAGGAGTGGAGGAGTGGAGGAG</td>
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<tr>
<td>Act RI</td>
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<td></td>
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<tr>
<td>Sense</td>
<td>5’-CTTGTTTGTGAGGAGTGGAGGAGTGGAGGAGTGGAGGAG</td>
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<td>Antisense</td>
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<td>106</td>
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<tr>
<td>Antisense</td>
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Nippon Becton Dickinson in six-well plastic plates at initial densities of 0.5 × 10^6 cells/well. The cells were cultured in standard medium and harvested at preconfluence and at 1, 5, 10, 15, and 20 days postconfluence for isolation of RNA.

In separate experiments, preconfluent IEC-6 cells were cultured with the standard medium containing graded concentrations of recombinant human activin A (1, 10, 100, and 500 ng/ml; a gift from Dr. Yuzuru Eto) or 1 ng/ml of human TGF-β1 (Genzyme, Cambridge, MA) for 24 h, and then the cell proliferation was measured using cell proliferation ELISA (bromodeoxyuridine) kit (Boehringer Mannheim, Mannheim, Germany) as recommended by the manufacturer. In addition, postconfluent IEC-6 cells were incubated with serum-free medium containing activin A (10 and 100 ng/ml) or TGF-β1 (1 ng/ml) for 48 h and then harvested for isolation of RNA.

Isolation and analysis of RNA. Total RNA was isolated from jejunal mucosa, jejunal epithelial cells, and cell lines using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s protocol. Total RNA samples were treated with DNase RQ1 (Promega, Madison, WI) to remove any genomic DNA. Next, 5 µg of total RNA was annealed with 0.5 µg of oligo(dT)12–18 primer (GIBCO BRL) at 70°C for 10 min, followed by RNA digestion with RNase H (GIBCO BRL). The first-strand cDNA was then synthesized in 20 µl of first-strand buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 0.2 mM dNTP, and 1.25 units Moloney murine leukemia virus RTase (GIBCO BRL) at 37°C for 50 min, followed by RNA digestion with RNase A (GIBCO BRL). The first-strand cDNA sample (0.5 µl) was added to 50 µl of a PCR reaction mixture containing 0.5 µM gene-specific primers (Table 1), 2 mM MgCl2, 0.2 mM dNTP, and 1.25 units Ex-Taq polymerase (Takara, Otsu, Japan). Each cycle of PCR included 1 min of denaturation at 94°C, 1 min of primer annealing at different temperatures.
RESULTS

Each band was normalized by comparing it with the intensity of 100-bp ladder. βA, βA-subunit of activin; α, α-subunit of inhibin; RIB, activin RIB; RIIA, activin RIIA; Foll, follistatin.

For semi quantitative PCR, the kinetics of amplification was studied for each combination of primers in preliminary experiments, and PCR was performed at an exponential range. The PCR products, which were separated on 2% agarose gel electrophoresis, were transferred to a nylon membrane (Biodyne Plus, Pall, NY), and the blots were then hybridized with each inner oligonucleotide probe (Table 1) labeled with digoxigenin using a DIG oligonucleotide tailing kit (Boehringer Mannheim). Prehybridization, hybridization, and detection were all carried out with a DIG luminescence detector (Boehringer Mannheim). The hybridization was performed at 42°C overnight, and posthybridization washing was performed with 0.1× standard saline citrate-0.1% SDS at 60°C for 15 min 3 times. The bands developed on X-ray film with minimal contamination of cells from the lamina propria. ALP mRNA levels were higher in fractions I–III than in fractions IV–VI, suggesting the pretranslational regulation of ALP expression along the crypt-villus axis of small intestine. Furthermore, a histochemical analysis of intestinal remnant after isolation of the epithelial cells demonstrated that the lamina propria of the villus cores was left almost completely intact, whereas the majority of epithelial cells were eliminated (data not shown). These results suggest that epithelial cells were successfully isolated from along the crypt-villus axis with minimal contamination of cells from the lamina propria. Figure 2B shows that mRNA for the βA-subunit of activin was more abundant in the higher villus fractions (I–III) than in the lower villus-to-crypt fractions (IV–VI). In contrast, no marked changes were observed in mRNA for the α-subunit, Act RIIA, and follistatin (Fig. 2, B and C). Act RIB mRNA levels were higher in fractions IV–VI than in fractions I–III.

To reveal the expression of activin A protein in rat small intestine, immunohistochemistry was carried out using anti-activin A monoclonal antibody. Sakai et al. (27) examined the specificity of the antibody by preabsorption study and demonstrated that the immunostaining of activin A in rat tibia was completely absorbed by adding an excess concentration of recombinant human activin A, suggesting the specificity of the antibody used here. In the present study, as no positive signal was detected by the conventional immunoperoxidase staining (data not shown), we attempted to amplify the signal by using biotinyl tyramide. Figure 3 showed the representative results of immunohistochemistry with the catalyzed signal amplification. The signal was clearly seen in the epithelial cells in the uppermost part of the villus (Fig. 3, A and B). In contrast, negative control processed with nonspecific mouse IgM yielded no signal (Fig. 3C).

To more fully understand the relationship between the activin system and differentiation in the human intestinal cell line Caco-2, which has been used as a model for differentiation of intestinal epithelial cells in vivo, we investigated the time course for the expression...
of each gene. Figure 4 shows representative Southern blots of semiquantitative RT-PCR products in Caco-2 cells grown on filter or plastic. Each individual band was detected by agarose gel electrophoresis of RT-PCR products, and the size of each product was consistent with the predicted size (data not shown). Apo A-IV mRNA was detected as a differentiation marker, and the apo A-IV mRNA levels increased in both plastic- and filter-grown cells after reaching confluence (Fig. 4A). In addition, the apo A-IV mRNA was detected earlier in cells grown on filter than those grown on plastic. The βA-subunit mRNA in the cells on plastic showed no changes in the very low levels observed during the culture period (Fig. 4B). In marked contrast, the βA-subunit mRNA levels in the cells grown on the filter were drastically increased after reaching confluence and higher than in those grown on plastic. The α-subunit mRNA levels were also increased after confluence, and the levels tended to be higher in cells on filter than in those on plastic throughout the culture period (Fig. 4C). In contrast, the follistatin mRNA levels were drastically decreased after reaching confluence in both filter- and plastic-grown cells (Fig. 4F). The levels of mRNA for Act RIB and Act RIIA were also decreased after reaching confluence (Fig. 4, D and E).

Furthermore, we investigated whether exogenous activin could influence the proliferation and differentiation of intestinal epithelial cells. Addition of recombinant human activin A in the medium suppressed the proliferation (bromodeoxyuridine uptake) of IEC-6 cells in a dose-dependent manner (Fig. 5A). In addition, the apo A-IV mRNA levels in IEC-6 cells were significantly increased by incubating with exogenous activin A (Fig. 5B). However, these effects of activin A were markedly weaker than those of TGF-β1.

DISCUSSION

Although the mechanism underlying the renewal process in the intestinal epithelium, which includes cell proliferation, differentiation, and elimination, has not yet been fully elucidated, the entire sequence is believed to be regulated by tissue-specific programmed gene expression and by such extrinsic factors as the presence of nutrients and growth factors (1, 2, 4, 17, 28). In terms of growth factors, it has been reported that both epidermal growth factor and TGF-α promote cell proliferation (2). In contrast, TGF-β has been demonstrated to block mitotic activity (1) and to induce cell differentiation (17). As activins, which are members of the TGF-β superfamily, have been shown to regulate various cell functions, including cell proliferation, differ-

Fig. 2. Gene expression of βA-subunit of activin (B), α-subunit of inhibin (B), activin (Act) RIB (C), Act RIIA (C), and follistatin (C) in intestinal epithelial cells along the crypt-villus axis. Cells were differentially isolated from rat jejunum, and mRNA levels were determined by semiquantitative RT-PCR followed by Southern blot hybridization as described in MATERIALS AND METHODS. Representative Southern blots of PCR products are shown above each graph. Alkaline phosphatase (ALP) activity, mRNA levels for ALP, apolipoprotein (Apo) A-IV, and polymeric immunoglobulin receptor (pIgR) were determined as markers to validate differential isolation of cells along the crypt-villus axis (A). Signal intensity of Southern blots was normalized by comparing with each value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was detected as an internal control for PCR. Results are means ± SE of 3 rats. Results not sharing a common letter in each gene are significantly different (P < 0.05).
entiation, and apoptosis (16, 20), we may conclude that these molecules modulate the cell renewal process in the intestinal epithelium. However, few reports to date have demonstrated the expression and function of activins in the intestine.

In the present study using Southern blot hybridization of RT-PCR products, we demonstrated the mRNA expression of the \( \beta \)-subunit of activin, the \( \alpha \)-subunit of inhibin, Act RIB, Act RIIA, and follistatin in rat jejunum and the rat intestinal epithelial cell line IEC-6 (Fig. 1). From these results, we speculated that activins may modulate the function of intestinal epithelial cells in concert with antagonists (inhibins), receptors, and neutralizing binding proteins (follistatins). Initially, undifferentiated proliferative cells in the small intestine located in the region of the crypts give rise to differentiated nonproliferative cells that migrate along the length of the villus, at which point the terminally differentiated cells are removed by apoptosis and exfoliation. The present study demonstrated using a differential cell isolation technique in which the \( \beta \)-subunit mRNA levels were overtly higher in the epithelial cells located at the upper part of the villus than those at the crypt region in rat jejunum (Fig. 2). The \( \beta \)-subunit constitutes not only activins (A and AB) but also inhibin A. As we also showed that the mRNA for the \( \alpha \)-subunit of inhibin was expressed in rat jejunum and IEC-6 cells, it is possible that inhibin A, in addition to activin A, is synthesized in intestinal epithelial cells. However, unlike the \( \beta \)-subunit, no changes were observed in the \( \alpha \)-subunit mRNA levels in the isolated cells along the crypt-villus axis. Thus it is likely that synthesis of activin A but not inhibin A is progressively increased as the cells migrate from the crypt to the villus. In fact, we confirmed in the present study that the immunoreactive activin A protein was expressed in the epithelial cells on the uppermost part of the villus (Fig. 3).

Absence of an activin A signal on the midvillus despite the significant expression of \( \beta \)-subunit mRNA may be due to low sensitivity of the immunohistochemistry or pretranslational regulation of activin A synthesis. These results suggest that activin A is associated with a termination of proliferation, initiation, and/or maintenance of differentiation in intestinal epithelial cells.

As cells in the human colon carcinoma cell line Caco-2 spontaneously differentiate after reaching confluence to form an epithelium with many characteristics of mature small intestinal mucosa in vivo, this cell line has been used as an experimental model of cell differentiation in the small intestine (10, 12, 26, 32). In addition, the culture substratum has been reported to influence differentiation of Caco-2 cells. Wagner et al. (34) demonstrated that apoA-IV protein was detectable in the culture media earlier with filter-grown cells than it was with plastic-grown cells, despite similar apoA-IV mRNA levels. In the present study, the apoA-IV mRNA levels increased after reaching confluence in both filter- and plastic-grown cells, but the increment was earlier in filter-grown cells than in plastic-grown cells (Fig. 4A), a finding that is in contrast to those of Wagner et al. (34). Nevertheless, these results suggest that Caco-2 differentiation may be facilitated when the cells are grown on a filter. Under these conditions, we observed that the levels of \( \beta \)-subunit mRNA in filter-grown cells increased drastically after reaching confluence, whereas the levels in plastic-grown cells were extremely low throughout the culture period (Fig. 3). Thus our findings indicated that gene expression in the \( \beta \)-subunit was associated with a differentiation of cells, a finding that is consistent with the results for the expression pattern of \( \beta \)-subunit mRNA and activin A protein along the crypt-villus axis of rat jejunum. However, it remains to be shown why \( \beta \)-subunit mRNA in plastic-grown cells was not induced postconfluence despite the fact that apo A-IV mRNA as a differentiation marker was increased. In contrast to the
Fig. 4. Changes in time course of gene expression of βA-subunit of activin (B), α-subunit of inhibin (C), Act RIB (D), Act RIIA (E), and follistatin (F) in Caco-2 cells cultured on filter insert or plastic plate. Cells were harvested at preconfluence (-2) and 1, 5, 10, 15, and 20 days postconfluence. Blots above each graph illustrate representative Southern blots of semiquantitative RT-PCR products. Apo A-IV was detected as a marker for cellular differentiation (A). Signal intensity of Southern blots was normalized by comparing with each value of GAPDH, which was detected as an internal control for PCR. Results are means ± SE of 3 experiments. Open and closed circles represent results on plastic and filter, respectively. *P < 0.05 compared with preconfluent cells. †P < 0.05 compared with cells grown on plastic.
A-subunit, the follistatin mRNA levels decreased after reaching confluence in both plastic- and filter-grown Caco-2 cells. The reverse pattern of expression for the βA-subunit and follistatin suggests that higher levels of follistatin in undifferentiated proliferative cells may suppress the paracrine action of activin A secreted from differentiated nonproliferative cells. In the present study, however, we did not reveal the secretion of bioactive activin A and follistatin in the Caco-2 cells. Therefore, further studies will be required to establish the role of activin and follistatin in the proliferation and differentiation of Caco-2 cells.

To investigate the effect of activin A on the cell proliferation and differentiation of intestinal epithelium, we used in the present study the IEC-6 cells, a model for proliferative undifferentiated intestinal epithelial cells. As TGF-β1 has reportedly blocked the mitotic activity (1) and induced the differentiation (17) of intestinal epithelial cells, this growth factor was used as a positive control in the present study. In fact, addition of 1 ng/ml of TGF-β1 to the culture medium suppressed the proliferation and stimulated the expression of apo A-IV mRNA in the IEC-6 cells (Fig. 5). Although exogenous activin A also suppressed the proliferation and induced the apo A-IV mRNA in a dose-dependent manner, higher doses were required compared with the case of TGF-β1. Thus cell proliferation and differentiation in the intestinal epithelium may be controlled more dominantly by TGF-β1 than by activin. Further investigations will be necessary to make clear how TGF-β1 and activin share biological functions in the intestine.

Cell cycle regulatory proteins such as cyclin-dependent kinase inhibitors (CdkIs) have been implicated in the proliferation and differentiation of intestinal epithelial cells. p21WAF1/CIP1 is one of the CdkIs, and its mRNA and protein product have been demonstrated to be expressed in the epithelial cells of the uppermost region of the crypt and the villus of small intestine (5, 8, 25). These observations suggest that p21WAF1/CIP1 expression is inversely related to proliferation and directly correlates to differentiation of intestinal epithelial cells. This speculation has been supported by cultured-cell studies demonstrating that p21WAF1/CIP1 mRNA and protein were increased in Caco-2 cells after reaching confluence (6, 8). In addition, it has been demonstrated that activin A inhibited the proliferation of Hep G2 hepatoma cells through upregulation of p21WAF1/CIP1 expression (37). Furthermore, Moustakas and Kardasis (23) reported that Smad proteins, which play an important role in the transduction of extracellular signals such as TGF-β and activin, transactivate the p21 WAF1/CIP1 promoter in Hep G2 cells. Thus it is likely that the p21 WAF1/CIP1 could mediate the regulation of proliferation and/or differentiation of intestinal epithelial cells by activin.

In summary, the present study demonstrated that the βA-subunit of activin, the α-subunit of inhibin, Act R1, Act RII, and follistatin genes were expressed in the intestinal epithelial cells and that activin A expression could be associated with cell differentiation. It was also shown that follistatin mRNA was expressed more abundantly in undifferentiated proliferative Caco-2 cells than in differentiated nonproliferative cells. The present results suggest that the activin system is involved in the maintenance of homeostasis in the intestinal epithelium. Further research is underway to determine the effects of blockade of activin signal transduction on the proliferation and differentiation of intestinal epithelial cells.

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**Fig. 5.** Effect of exogenous activin A on proliferation and differentiation of IEC-6 cells. A: bromodeoxyuridine incorporation in IEC-6 cells cultured with graded doses of recombinant human activin A or 1 ng/ml of human TGF-β1. Results are means ± SE of 3 wells. Results not sharing a common letter are significantly different (P < 0.05). B: gene expression of apoA-I/IV in IEC-6 cells cultured with recombinant human activin A or TGF-β1. Blots at top illustrate representative Southern blots of semiquantitative RT-PCR products. Signal intensity of apo A-IV was normalized by comparing with each value of GAPDH, which was detected as an internal control for PCR. Results are means ± SE of 3 experiments. Results not sharing a common letter are significantly different (P < 0.05).
REFERENCES


