Growth Stimulation by Coexpression of Transforming Growth Factor- α and Epidermal Growth Factor-Receptor in Normal and Adenomatous Human Colon Epithelium

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Abstract

Autocrine stimulation of the epidermal growth factor receptor (EGF-R), by coexpression of transforming growth factor- α (TGF- α), causes malignant transformation of some fibroblast cell lines. TGF- α and EGF-R are both known to be expressed in colon carcinoma tissue and have been shown coexpressed in colon carcinoma cell lines. TGF- α autocrine activation of EGF-R has been suggested as a potential mechanism contributing to abnormal growth control in colon cancer. We now report coexpression of TGF- α and EGF-R transcripts in morphologically normal colonic epithelium from five individuals, in colonic adenomas from three individuals, and in a nontumorigenic colon adenoma cell line, VACO-330. Functional studies demonstrate VACO-330 growth is stimulated by exogenous TGF- α and is completely abolished by a blocking anti-EGF-R antibody. Autocrine stimulation of EGF-R by TGF- α is therefore required for growth of the adenoma cell line. Autocrine stimulation of EGF-R by TGF- α does not cause malignant transformation of the colonic epithelial cell. In normal and adenomatous human colon TGF- α , via either an autocrine or paracrine mechanism, is likely an important physiologic stimulant of epithelial proliferation. (J. Clin. Invest. 1990. 86:356-362.) Key words: colon • autocrine • transforming growth factors

Introduction

Transforming growth factor- α (TGF- α)¹ is a secreted polypeptide that binds to the epidermal growth factor receptor (EGF-R) to produce a mitogenic stimulus (1). TGF- α production was initially detected in multiple transformed cell lines and in many naturally occurring human tumors (1-3). In many of these examples, the epidermal growth factor receptor was coexpressed along with TGF- α , leading to the suggestion that

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1. Abbreviations used in this paper: EGF, epidermal growth factor; EGF-R, EGF receptor; TGF- α , transforming growth factor- α .

J. Clin. Invest. © The American Society for Clinical Investigation, Inc. 0021-9738/90/07/0356/07 \$2.00 Volume 86, July 1990, 356–362 autocrine stimulation of the EGF-R by TGF- α could be one mechanism for tumors to escape normal growth controls (2–4). Indeed, using transfection to achieve coexpression of TGF- α and EGF-R results in transformation of several immortalized rodent fibroblast cell lines (5, 6). More recently TGF- α production has been demonstrated in skin, in activated macrophages, and in cultured normal breast epithelium (7–9). In nontransformed cultured normal breast epithelium the expressed TGF- α has been additionally shown to function as an autocrine growth factor that activates the coexpressed EGF-R (9). These observations suggest that autocrine growth stimulation by TGF- α need not be transforming, that TGF- α is expressed in nontransformed human tissues, and that paracrine or autocrine stimulation by TGF- α may be a physiologic stimulus to proliferation of some normal human tissues.

This study addresses the role of TGF- α in human colon epithelia. The human colonic epithelium is a rapidly proliferating organ that completely renews itself on a weekly basis (10). Neoplasia of the normal colonic epithelium proceeds through ordered stages first to adenomatous then to malignant change (11). EGF-R and TGF- α production have been previously individually detected in colon carcinomas and in numerous colon cancer cell lines (12-18). Coexpression of TGF- α and EGF-R and growth stimulation by TGF- α has been demonstrated in multiple colon cancer cell lines (16, 18), and TGF- α has been proposed as an autocrine growth factor in colon cancer (18). One of two recent investigations has now detected expression of TGF- α in normal colon (17, 19). In this study we provide evidence that TGF- α functions as a paracrine or autocrine growth factor in both adenomatous and nonneoplastic normal colon epithelium.

We have first investigated growth control of VACO-330, a nontransformed cell line established from a human colon adenoma (20). We find that VACO-330 expresses both TGF- α and EGF-R. VACO-330 growth can be accelerated by exogenous TGF- α and completely blocked by antibody against the EGF-R binding domain. In this cell culture model of adenomatous colon epithelium we thus establish growth depends on TGF- α autocrine stimulation. These investigations have been additionally extended to tissue samples obtained from nonmalignant human colon epithelium. In these samples coexpression of TGF- α and EGF-R transcripts in morphologically normal mucosa from five individuals and in adenoma tissue from three individuals was detected. We conclude that autocrine stimulation of EGF-R by TGF- α is characteristic of and required for growth of the adenoma cell line. Such autocrine stimulation is not itself transforming in colonic epithe-

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lium, rather, we conclude that TGF- α is likely important in stimulating proliferation, either by paracrine or autocrine mechanisms, in both normal and adenomatous colon epithelium. TGF- α antagonists may therefore be potentially useful pharmacologic agents in the treatment of colonic polyposis or other colonic diseases marked by disordered epithelial proliferation.

Methods

Cell lines. The VACO 330 cell line was maintained as described on rat tail collagen-coated plates in MEM supplemented with 2% fetal bovine serum, insulin, thyroxine, transferrin, selenium, and hydrocortisone (MEM2+ media) (20).

Tissue procurement. Adenomas were obtained from colectomy specimens and divided into sections for histopathologic analyses and RNA extraction using our previously described methods (21). Morphologically normal colonic epithelium was obtained from colectomy specimens by blunt dissection of the mucosa off the muscularis mucosae as previously described (21).

Nucleic acids. RNA was prepared from tissue and from the VACO-330 cell line by extraction with guanidine isothiocyanate as previously described (21–23). Total RNA was obtained by ultracentrifugation of the extract through a cesium cushion. Total RNA was resolved by electrophoresis on agarose-formaldehyde gels and judged to be undegraded based on the relative ethidium staining of 28S and 18S subunits (22, 23). Polyadenylated mRNA was selected by chromatography on oligo-dT cellulose (22, 23).

Northern analyses. 2 μ g of polyadenylated mRNA was resolved by electrophoresis on agarose-formaldehyde gels (23). RNA was transferred to Nytran nylon membranes (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. Double-stranded cDNA probes were labeled with ³²P by the random primer technique (24). Hybridization of probe to filters was at 42°C in 50% formamide, 5× SSPE, 2× Denhardt's solution, 0.1% SDS, 0.1 mg/ml salmon sperm DNA. Final washes were in 0.1× SSC, 0.1% SDS, 65°C. Blots were exposed on Kodak XAR film with an intensifying screen.

Probes. The human EGF-R probe, carried in plasmid pH 64.1, is a 1.89 kb Eco RI fragment of the EGF-R cDNA encoding the amino terminal portion of the molecule into the intracellular domain (25). The human TGF-α probe, carried in plasmid phTGF1-10-925, is a 925-bp complete cDNA (8). The human EGF probe, carried on plasmid hEGF15-1, is a complete 1.9-kb cDNA (26). The glyceraldehyde-3-phosphate dehydrogenase probe is a Pst I fragment of a chicken muscle (27). Blots hybridized to GAPDH underwent final washing at 56°C.

Growth curves. VACO 330 were plated on fresh collagen coated plates in MEM supplemented with hormones but without fetal bovine serum (MEM+ media). Cells were plated in 24 well plates at either 10,000 cells per well (low density) or 100,000 cells per well (high density). At 24 h additions of either, MEM+, EGF (Sigma Chemical Co., St. Louis, MO) or TGF- α (Gibco Laboratories, Grand Island, NY) were made. At defined time points total cell number was established by removing the cells from the plates with EDTA and counting in a hemacytometer.

Antibody studies. Anti-EGF-R monoclonals, Ab528 and Ab455 (28, 29), were purchased from Oncogene Science, Inc. (Manhasset, NY). Cells were plated in MEM+ media in 24 well plates at 100,000 cells per well (high density) and growth curves in the presence of antibody or antibody plus EGF performed as above.

Results

Coexpression of $TGF-\alpha$ and EGF-R by an adenoma cell line. We initially characterized VACO-330, a human cell line established from a small colonic tubular adenoma. VACO-330 exhibits typical epithelial features including tight junctions, apical brush boarder and basal nuclei (20). It neither clones in soft agarose nor forms tumors in nude mice (20). It has near diploid karyotype with the addition of a single chromosome 7. VACO-330 thus provides a cell culture model of the non-transformed colon adenoma cell.

Fig. 1 demonstrates by northern analyses coexpression of both TGF- α and EGF-R transcripts in the VACO-330 cell line. Expression of both TGF- α and EGF-R transcripts is greater in the VACO-330 colon adenoma cell line than in the colon carcinoma cell line, SW480, previously demonstrated to coexpress these products (16). EGF-R expression in VACO-330 is clearly less than in line A431, in which the EGF-R gene is amplified, but far exceeds the SW620 line, in which EGF-R has previously been shown undetectable (16, 30). Southern analyses of VACO-330 genomic DNA showed no evidence of EGF-R gene amplification, but confirmed a 50% increase in EGF-R gene copy number, consistent with VACO-330's known trisomy 7 (data not shown).

Autocrine activation of VACO-330 EGF-R. We next addressed whether EGF-R transcript expression in VACO-330 results in expression of a functional receptor with growth promoting activity. Fig. 2 A shows supplementation of serum free VACO-330 growth medium with TGF- α or EGF (epidermal growth factor) increases VACO-330 growth four- to fivefold compared with unsupplemented controls. Both TGF- α and EGF attain maximal activity at 10 ng/ml. Stimulation of VACO-330 growth by either TGF- α or EGF was repeatedly demonstrated when VACO-330 were plated at 10,000 cell per 16-mm well (low density). However, exogenous TGF- α and EGF had no effect on growth of VACO-330 plated at 10-fold higher density of 100,000 cells/well (high density) (Fig. 2 B). This finding suggested that at high cell concentration endogenous TGF- α production might be sufficient to fully stimulate VACO-330 growth.



Figure 1. EGF-R and TGF- α coexpression in a colon adenoma cell line. RNA blot hybridization of polyadenylated RNA (2 μ g) extracted from colon adenoma cell line VACO-330 (V330), colon cancer cell lines SW480 and SW620, and reference cell line A431. Blots were hybridized with TGF- α (40) or EGF-R (8) cDNA probes.



Figure 2. Stimulation of VACO-330 growth by EGF and TGF- α . (A) VACO-330 cells were maintained as previously described in MEM media supplemented with hormones (insulin, thyroxine, hydrocortisone, and transferrin) and 2% fetal calf serum (25). Cells were replated at time 0 in MEM+ (MEM supplemented with hormones but without serum) at 10,000 cells per 16-mm well (low density). EGF or TGF- α was added at 24 hours and cell counts determined at 120 h. Shown is final cell number in growth factor-treated wells divided by the final cell number in control MEM+ only wells. (B) Cells were plated at 10,000 per well (low density) or at 100,000 per well (high density) in MEM+. At 24 h half the wells received 10 nM TGF- α . Shown are cell numbers attained at 120 h, divided by the initially plated cell number. In A and B mean values and standard errors are derived from triplicate experiments.

The dependence of growth of high density VACO-330 on endogenous TGF- α was tested by using antibodies to block the EGF-R ligand binding site. Ab528 is known to block ligand binding to and activation of the EGF-R without stimulating either receptor phosphorylation or cell division (28, 29). Ab455, directed against the carbohydrate moiety of the EGF-R, blocks neither ligand-receptor binding, ligand induced receptor phosphorylation, or ligand-induced mitogenesis (28,

29). Fig. 3 A shows addition of 10 nm EGF-R-blocking antibody, Ab528, totally abolishes growth of high density VACO-330. In contrast 20 nm nonblocking anti-EGF-R Ab455 has no effect on VACO-330 growth. Thus, only the anti-EGF-R antibody that inhibits EGF-R function will also inhibit VACO-330 growth. Ab528 has binding constant of 2.5 nM, and in other systems addition of exogenous EGF competes it from the EGF-R (31). To confirm Ab528 induced



Figure 3. Inhibition of VACO-330 growth by anti-EGF-R antibodies. (A) Growth curves of antibody treated VACO-330. VACO-330 cells were plated at time 0 in serum free MEM+ at 100,000 cells per 16-mm well (high density). At 24 h additions were made of 1-20 nM EGF-R blocking Ab528, or 20 nM nonblocking anti-EGF-R Ab455 (20, 28). Shown are daily average cell counts determined in duplicate wells followed through 120 h of growth. (B) TGF- α reversal of antibody mediated growth inhibition. Cells were plated as in A. At 24 h additions were made of: MEM+ media, MEM+ with 10 nM added TGF- α ; MEM+ with 10 nM added Ab528; and MEM+ with both 10 nM added Ab528 and 10 nM added TGF- α . Final cell numbers were determined at 120 h and are shown divided by the initially plated cell number. Means and standard errors are determined from triplicate experiments.

growth inhibition is mediated through EGF-R blockade, we used excess TGF- α to compete Ab528 from the VACO-330 EGF-R. Fig. 3 *B* shows that whereas addition of TGF- α has no effect on basal growth of high density VACO-330, addition of 10 nM TGF- α to Ab528-treated VACO-330 does, as predicted, reverse the Ab528-induced inhibition of VACO-330 growth. This result confirms that Ab528 inhibits VACO-330 growth specifically by antagonizing EGF-R activation. These findings demonstrate that growth of high density VACO-330 remains dependent on a functional EGF-R, and suggest that in high density VACO-330 EGF-R stimulation is fully provided by endogenous production of TGF- α . Thus in VACO-330 TGF- α is both expressed and, in an autocrine manner, stimulates VACO-330 growth.

In vivo coexpression of EGF-R and TGF- α . We next addressed whether coexpression in VACO-330 of TGF- α and EGF-R transcripts has been selected for in culture or is representative of colon adenoma growth in man. RNA was obtained and extracted from three human colonic adenomas. Fig. 4 A shows Northern analyses demonstrating that in each of the colon adenomas TGF- α and EGF-R transcripts are coexpressed at levels comparable to those seen in the VACO-330 adenoma cell line. We finally addressed whether coexpression of TGF- α and EGF-R occurs in morphologically normal colon epithelium or only in neoplastic adenomatous epithelium. Fig. 4 *B* shows Northern analyses demonstrating that in each of five different samples of morphologically normal colon epithelium TGF- α and EGF-R transcripts are coexpressed at levels comparable to those seen in the VACO-330 adenoma cell line.

While expression of EGF has been previously detected in the small bowels of patients with Crohn's disease and in one reported sample of human colon (19, 32); Northern analyses did not in our hands detect EGF expression in any of the RNAs we prepared from normal or adenomatous colon tissue or from the VACO-330 cell line (data not shown).

Discussion

This study demonstrates that in colon epithelium autocrine growth stimulation by TGF- α can occur independently of malignant transformation. This study further demonstrates that in the VACO-330 colon adenoma cell lines, growth is absolutely dependent on autocrine stimulation by TGF- α . And this study demonstrates that TGF- α and EGF-R are coexpressed in



Figure 4. (A) RNA blot hybridization of polyadenylated RNA extracted from adenomas of three patients, P1-3, compared with 2 μ g of VACO-330 polyadenylated RNA. Duplicate blots were first hybridized with TGF- α or EGF-R probes, then rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (29). (B) RNA blot hybridization of polyadenylated RNA extracted from morphologically normal colon epithelium of five patients, N1-5, compared with 2 μ g of VACO-330 polyadenylated RNA. Duplicate samples were first hybridized with 2 g of VACO-330 polyadenylated RNA. Duplicate samples were first hybridized with 2 g of VACO-330 polyadenylated RNA. Duplicate samples were first hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (27). Underlined samples were present together on the same nylon blot and are therefore directly comparable.



man both in morphologically normal and in adenomatous colonic epithelium. Together these observations suggest that TGF- α is likely in man an important stimulant of proliferation of normal and adenomatous colonic epithelium and that TGF- α stimulation in vivo may work by either paracrine or autocrine mechanisms.

We find that in the VACO-330 adenoma cell line TGF- α growth stimulation is through an autocrine mechanism. We cannot distinguish whether in adenoma tissue TGF- α and EGF-R are expressed on the same or on different cells. However, our hypothesis that in adenoma tissue TGF- α is an important growth factor is consistent with previous observations that (a) adenomas from patients with inherited polyposis coli produce factors supporting anchorage-independent growth of Rat-1 cells (33), and (b) that EGF stimulates proliferation of primary cultures established from colonic adenomas (34). Our findings that adenoma cell line growth is indeed dependent on autocrine activation of the EGF-R suggests that EGF-R antagonists may have potential clinical utility in the treatment or prevention of colonic adenoma in high risk individuals.

Our detection of EGF-R in morphologically normal human colon epithelium is consistent with previous observations demonstrating that in vivo intravenous EGF is a potent

mitogen for rat colonic epithelium (35). Our detection of TGF- α in five samples of morphologically normal colon is strongly confirmatory of one previous report of TGF- α expression in morphologically normal human colon mucosa (17). Our observations are at variance with one report detecting EGF but not TGF- α in one sample of human colon (19). It is possible that this particular sample reflected circumstances unique to the parent tissue donor. In this regard we note that induction of EGF has recently been reported in the small bowel of patients with Crohn's disease (32). Our study of morphologically normal colon epithelium contains a potential bias in that our tissue donors underwent colectomy due to coexistent colon carcinoma. Our morphologically normal mucosa was taken from the distal portion of the resection specimens, which were in all cases histologically free of disease and were well separated from the tumor mass. However, other investigators have demonstrated that the flat mucosa of individuals with colon carcinoma is in general hyperproliferative compared with that from normal individuals (36). While it may therefore be premature to extend our observations to the population at large, our data certainly suggest that TGF- α is likely an important colonic mitogen which in vivo acts to stimulate proliferation of the colonic epithelium. Our data support the

possibility that TGF- α antagonists may be useful pharmacologic agents in conditions marked by increase proliferation of colon epithelium.

This study adds to previous investigations that have established the utility of anti-EGF-R monoclonal antibodies, and particularly of Ab528, as useful reagents for demonstrating the biologic function of the activated EGF-R (9, 28, 29, 31, 37). Treatment of VACO-330 with Ab528 reveals an absolute dependence of VACO-330 growth on EGF-R activation. In this investigation we demonstrated growth inhibition of VACO-330 is due to Ab528 blockade of the EGF-R ligand binding site first by comparison with anti-EGF-R Ab455, which binds to the EGF-R without blocking receptor ligand interactions. Secondly, we demonstrated reversal of the Ab528-induced growth inhibition when Ab528 is completed from the EGF-R. Other investigators have observed similar reversal by ligand of the growth inhibitory effects of anti-EGF-R antibody. However, in high density VACO-330 we demonstrate such reversal under conditions in which the ligand, exogenous TGF- α , is in the absence of antibody without effect on cell growth. By separating the competition of Ab528 by ligand from any direct stimulatory properties of ligand, we further demonstrate that Ab528 growth inhibition depends on its causing EGF-R blockade. We anticipate future technical advances will likely allow the establishment of primary cultures of normal colonic epithelium. When such cultures become available, use of Ab528 will allow direct testing of our suggestion that growth of morphologically normal colonic epithelium is dependent on TGF- α stimulation.

This study adds to the emerging picture that TGF- α autocrine stimulation is not necessarily a transforming event, that occurrence in nature of TGF- α autocrine stimulation is not restricted to cancers, and that TGF- α autocrine stimulation may be a physiologic mechanism employed to increase proliferation of nontransformed epithelium. Previous investigations have shown that autocrine stimulation of the EGF-R, achieved by transfection of either TGF- α or EGF into the rodent Rat-1 and FR3T3 fibroblast cell lines, causes malignant transformation of those lines and confers on them the capacity for tumorigenic growth in nude mice (5, 31). Numerous previous studies have suggested a possible role for TGF- α as an autocrine growth factor in solid tumors in general and colon cancer in particular (2, 12-17, 38). However, this study demonstrates that in an immortalized colonic epithelial cell line, VACO-330, TGF- α autocrine stimulation of EGF-R is not transforming and induces neither anchorage independent growth in soft agar nor tumorigenic growth in the nude mouse. Our observations are consistent with the recent demonstration that TGF- α is an autocrine growth factor in nontransformed cultured normal breast epithelium (9). We concur with the authors of that study in the suggestion that normal and malignant epithelia may differ not in the existence of autocrine growth stimulation but in the response to such stimulation (9). Recent investigations have defined a series of mutations associated with the progression of colon neoplasia to colon cancer (9, 39). An important future application of the VACO-330 cell line will be as a nontransformed target for the introduction of these mutations. As our current observations show, such future experiments will allow direct determination of the ability of activated oncogenes to alter cellular responses to $TGF-\alpha$ autocrine stimulation.

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