Autocrine Action of Amphiregulin in a Colon Carcinoma Cell Line and Immunocytochemical Localization of Amphiregulin in Human Colon

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Abstract. Amphiregulin (AR) is a newly discovered glycosylated, 84-amino acid residue polypeptide growth regulator which has sequence homology to the EGF family of proteins. To obtain immunological reagents to study the biological role of AR, two synthetic peptides containing sequences corresponding to distinct regions of AR were used to generate polyclonal antibodies in rabbits. One preparation of antipeptide antibodies directed against residues 26-44 of AR (AR-Ab2) was most effective in the detection of native AR, whereas another preparation of antibodies against residues 8-26 (AR-Abl) was found to be most efficacious in the detection of AR in formalin-fixed and paraffin-embedded tissues.

The growth of a colon carcinoma cell line, Geo, which proliferates autonomously under serum-free conditions, was stimulated by the exogenous addition of AR or EGF. Half-maximal stimulation of this growth was observed at 40 and 200 pM of EGF and AR, respectively. A mAb to the extracellular domain of the EGF receptor blocked the stimulation of cell proliferation induced by the exogenous addition of AR, suggesting that this stimulation was mediated via the EGF receptor. Geo cells were found to constitutively express significant levels of the AR mRNA transcript as determined by analysis of the polymerase chain reaction–amplified cDNA product and AR protein was detected immunocytochemically using the AR-Abl antibodies in these cells. AR was immunoprecipitated specifically using the AR-Ab2 antibodies from the conditioned medium of Geo cells, which had been metabolically labeled with [^{35}S]cysteine. The secreted AR migrated as a broad band (18.5–22.5 kD) with a median molecular weight of ~20.7 kD in SDS-PAGE. Immunospecific removal of AR from serum-free medium conditioned by the Geo cells and readdition of the AR-depleted medium to Geo cells resulted in an ~40% inhibition of cell growth relative to controls. Furthermore, the growth of the Geo cells was also inhibited by ~50% by the addition of the anti-EGF receptor mAb alone. These results indicate that AR and the EGF receptor are involved in the autocrine growth of these cells and suggests that AR may act through the EGF receptor via an extracellular autocrine loop.

To study the expression of AR in human colon in vivo, AR was localized immunocytochemically in formalin-fixed, paraffin-embedded sections from normal and malignant human colon using the AR-Abl antibodies. In all normal colon specimens, AR was localized to the cytoplasm and nucleus of the terminally differentiated, non-proliferative surface columnar and secretory epithelial cells of the mucosa, but was not detectable in the proliferative epithelial cells of the crypts. In four out of five colonic carcinomas, AR was localized to the cytoplasm and/or nucleus of the cells. In conclusion, AR can act as an autocrine growth stimulator for colonic carcinoma cells in vitro and may perform a growth regulatory function in normal and malignant colon in vivo.

THERE is a rapidly growing number of EGF-like growth regulatory polypeptides such as transforming growth factor α (TGFα)[1] (Todaro and DeLarco, 1978), amphiregulin (AR) (Shoyab et al., 1988), cripto (Ciccodicola et al., 1989), and heparin-binding EGF-like growth factor (Higashiyama et al., 1991). With the exception of TGFα, little is known about the expression, mechanism of action and role that these growth factors may play in normal biological and pathological processes. AR is a glycosylated, 84-amino acid residue polypeptide originally purified from the conditioned medium of the human breast carcinoma cell line, MCF-7, after treatment with PMA (Shoyab et al., 1988). The

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1. *Abbreviations used in this paper*: AR, amphiregulin; PCR, polymerase chain reaction; TGFα, transforming growth factor α.

COOH-terminal 40-amino acid residue segment of AR (residues 44–84) has 38 and 32% sequence identity with EGF and TGFα, respectively (Shoyab et al., 1989). The very basic, hydrophilic, NH_2-terminal region of AR contains two putative nuclear targeting sequences positioned at residues 26–29 and 40–43 (Shoyab et al., 1989) and AR has been detected in the nucleus of both normal and malignant ovarian epithelial cells (Johnson et al., 1991). The mature AR protein is believed to be derived from the proteolytic cleavage of a 252-amino acid residue transmembrane precursor (Plowman et al., 1990).

AR can act to stimulate or inhibit the proliferation of cells depending on the concentration of AR and the nature of the target cell (Shoyab et al., 1988; Johnson et al., 1991). Unlike EGF or TGFα, AR does not promote the anchorage-independent growth of normal rat kidney (NRK) fibroblasts in the presence of transforming growth factor-β (Shoyab et al., 1989). Cultured human keratinocytes produce and respond to AR and the mitogenic activity of AR can be inhibited by heparin sulfate (Cook et al., 1991). AR can partially compete with ^{125}I-EGF for binding to the EGF receptor on A431 cells, but has a lower affinity for the receptor than does EGF (Shoyab et al., 1989). Many carcinoma cell lines and normal human tissues, including ovary, placenta, pancreas, cardiac muscle, testis, breast, lung, spleen, kidney, and colon, express the 1.4-kb AR mRNA transcript (Plowman et al., 1990). However, to date there is no available information regarding the localization of AR to specific cells of any normal tissue in vivo. Recently, AR mRNA has been detected in ~66% of primary or metastatic human colorectal carcinomas (Ciardiello et al., 1991*b*).

The colonic mucosa is a constantly self-renewing layer of epithelial cells which proliferate, mature, differentiate, and are eventually shed (reviewed in Wright, 1989; and Colony, 1989). Regrettably, the maintenance of normal colonic epithelial cells in long-term culture has not been possible and thus, studies probing the potential role that growth factors play in colonic epithelial cell growth relies heavily upon the use of colonic carcinoma cells in vitro (Colony, 1989; Mulder and Brattain, 1989). There is considerable evidence to suggest that EGF-like factors play a role in the growth of normal and malignant colonic epithelial cells. EGF has been shown to stimulate normal crypt cell proliferation in rats in vivo (Goodlad et al. 1987; Reeves et al., 1991) and Wright and co-workers (1990) have demonstrated that ulceration of the epithelium anywhere in the human gastrointestinal tract results in the induction of a novel EGF-secreting cell lineage that promotes ulcer healing. The EGF receptor has been detected in normal rat (Koyama and Podolsky, 1989) and human colonic epithelium (Markowitz et al., 1990). EGF receptors are expressed at a high frequency in colon carcinomas in vivo (Yasui et al., 1988; Moorghen et al., 1990) and in cell lines derived from these carcinomas (Wan et al., 1988; Murthy et al., 1989). Further, TGFα mRNA and/or protein has been detected in normal colonic epithelium (Markowitz et al., 1990; Cartlidge and Elder, 1989; Koyama and Podolsky, 1989) as well as in colon carcinomas (Malden et al., 1989; Liu et al., 1990).

In the work reported here, we have used a colon carcinoma cell line to study the mechanism of AR action and its potential role as an autocrine growth factor in vitro. We have also studied the expression and localization of AR in both normal and malignant human colon with the aim that the results will provide insight into the biological role that AR might play in vivo. The results demonstrate for the first time that AR can function as an autocrine growth factor for a carcinoma cell line in vitro and suggest that the mitogenesis induced by AR is mediated by the EGF receptor. We have also localized AR in vivo to the terminally differentiated, non-proliferative epithelial cells of normal colonic mucosa, but not to the proliferative cells of the crypts. Further, we demonstrate that AR can be detected immunocytochemically in the nucleus as well as the cytoplasm of normal and malignant colonic epithelial cells in vivo.

Materials and Methods

Cell Culture

Geo human colon carcinoma cell line (Brattain et al., 1981) was generously provided by Dr. M. Brattain (Baylor College of Medicine, Houston, TX) and the MDA-MB-453 and MCF-7 human breast cancer cell lines were acquired from the American Type Culture Collection (Rockville, MD). Cells were grown at 37°C in a 5% CO_2 atmosphere under serum-free conditions in McCoys 5A medium containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, 25 μg/ml gentamicin (GIBCO BRL, Gaithersburg, MD) and 2% (vol/vol) PC-1 serum-free supplement (Ventrex Laboratories, Portland, ME) (serum-free medium).

Purification and Characterization of Amphiregulin

AR was purified to homogeneity from the conditioned medium of MCF-7 cells which had been treated with PMA, and characterized as previously described (Shoyab et al., 1988, 1989). The purified AR was found to be homogeneous as determined by amino acid sequencing by Edman degradation, SDS-PAGE, gel exclusion HPLC and analytical reversed phase HPLC.

Generation and Characterization of Antipeptide Antibodies against Amphiregulin

Cys-AR^{8-26}-NH_2 and Cys-AR^{26-44}-NH_2 were synthesized and purified under contract by Multiple Peptide Systems (San Diego, CA). The numbering of the amino acid residues in AR is as described in Shoyab et al. (1989). The peptides were synthesized by the method of Houghten (1985) and purified by preparative reverse phase HPLC. 20 mg of each peptide was then conjugated through the side chain sulfhydryl to 16 mg of keyhole limpet hemocyanin using *m*-maleimidobenzoyl-N-hydroxysuccinimide ester and each conjugate was used to immunize two rabbits (Harlow and Lane, 1988). Before immunization, the rabbits were bled to generate control preimmune serum. Antisera were screened for a response to the peptide antigen starting 10 d after the first boost, as follows. 40 ng of the peptide in 50 μl of 0.1 M $NaHCO_3$, pH 9.1 was incubated in each well of an Immulon II ELISA plate (Dynatech Laboratories, Chantilly, VA) for 16 h at 4°C. The wells were then blocked with 3% BSA in PBS for 2 h at ambient temperature. 50 μl of various dilutions of preimmune and post-immune sera in 1% BSA/PBS was added to each well and incubated for 2 h at ambient temperature. Bound IgG was detected using biotinylated donkey anti-rabbit IgG, $F(ab')_2$ fragment followed by streptavidin-alkaline phosphatase (Amersham Corp., Arlington Heights, IL). Color development was performed using the phosphatase substrate system for ELISA (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Antisera were then screened for the ability to detect purified homogeneous AR in an ELISA format and to immunoprecipitate specifically [^{35}S]cysteine-labeled AR from medium conditioned by MCF-7 cells which had been treated with PMA. The ELISA for AR was performed as described above by adhering various quantities of purified AR to the bottom of an Immulon II ELISA plate and measuring the specific response generated using sera dilutions at 1:1,000. Antisera were tested for the ability to immunoprecipitate AR as described in the section titled "Metabolic Labeling with [^{35}S]Cysteine and Immunoprecipitation of Secreted Amphiregulin." The usefulness of these antibodies for the immunocytochemical detection of AR was determined using cell lines which are known to be positive and negative for AR mRNA. The following cell lines were used: MCF-7 before and after treatment with PMA (positive), SK-OV-3 (positive) (Plowman et al., 1990), LS 174T (positive), JVC

(negative), and WiDr (negative) (Ciardiello et al., 1991). The immunocytochemistry was performed as described in the "Immunocytochemistry of Cultured Cells" section.

Purification of Rabbit IgG

Rabbit IgG was purified from control serum and antiserum using Immunopure Immobilized Protein A/G gel (Pierce Chemical Co., Rockford, IL) exactly as described by the manufacturer. The purified IgG was dialyzed against PBS using tubing with a 12-14 kD cutoff (GIBCO BRL).

Mitogenesis Assay and Effect of an Anti-EGF Receptor mAb on the Amphiregulin-induced Mitogenesis

Mitogenesis assays were performed in serum-free medium (see "Cell Culture" section) in 48-well plates. Cells were initially plated at a density of 10,000 cells/well in 0.5 ml of serum-free medium and allowed to attach to the plate for 16 h. The medium was replaced with 0.4 ml of serum-free medium containing various concentrations of purified AR or EGF (GIBCO BRL). Each concentration of AR was assayed in triplicate and the control (no AR or EGF) was performed in six wells. After 4 d at 37°C, the cells were trypsinized in 100 μl and cells were counted using a hemocytometer. To ascertain the effect of an anti-EGF receptor mAb on the AR-induced mitogenesis, cells were plated in 12-well plates at a density of 30,000 cells/well and allowed to attach to the plate for 16 h. The next day the medium was changed to 0.98 ml of serum-free medium containing either 10 nM of an anti-EGF receptor IgG_1 mAb (No. 05-101; Upstate Biotechnology, Inc., Lake Placid, NY) or 10 nM of an isotype IgG_1 control mAb (No. 1170252, Boehringer Mannheim Biochemicals, Indianapolis, IN). After 30 min, 20 μl of 10 nM AR in serum-free medium was added to each well. 4 d later, cells were trypsinized, dispersed using a syringe with a 19-gauge needle and counted using a Coulter counter (Coulter Electronics Inc., Hialeah, FL).

Removal of Amphiregulin from Medium Conditioned by Geo Cells Using Anti-AR Antibodies

Cells were plated into 12-well plates at a density of 48,000 cells/well and allowed to attach for 12 h. The next day (day 2), the medium was replaced with 1 ml of fresh serum-free medium and the cells were allowed to grow for an additional day. On day 3, the conditioned medium from each well was collected and 34 μl of either 1 μg/μl purified AR-Ab2 IgG or preimmune IgG (control) in PBS was added to the conditioned medium. After a 45-min incubation at ambient temperature, 17 μl of Protein A agarose (GIBCO BRL) was added and the mixture rocked for 45 min. The processed conditioned medium was then separated from resin and sterilized using Spin-X centrifuge filter units (Costar, Cambridge, MA). This processed conditioned medium was added back to the appropriate wells of the 12-well plate and the cells were allowed to continue growing. On day 4, processing of the conditioned medium was repeated. On day 5, the cells were counted as described in the previous section.

Growth of Cells in the Presence of Anti-EGF Receptor mAb

Cells were plated into 12-well plates in 1 ml of serum-free medium at a density of 30,000 cells/well and allowed to attach to the plate overnite. The next day the medium was exchanged with serum-free medium containing either 10 nM of the anti-EGF receptor mAb, 10 nM of the isotype IgG_1 control mAb or no mAb. After 4 d, cells were counted.

Isolation of Cellular RNA

Isolation of total cellular RNA from cells was accomplished using the method of Rappolee et al. (1988) with the following modifications. Approximately 70% confluent cell monolayers in 75 cm^2 flasks were trypsinized, immediately centrifuged at 600 *g* for 5 min and the resultant pellet lysed in 2.0 ml of 4 M guanidinium thiocyanate, 25 mM Tris/HCl, 0.01 M 2-mercaptoethanol, 0.5% (wt/vol) sodium sarcosine, pH 7.4. DNA was sheared by passing the lysates through a 19-gauge needle attached to a 10-cc syringe. Lysates were layered onto a 2.7-ml cesium trifluoroacetate gradient (1.51 g/ml) (Pharmacia Fine Chemicals, Piscataway, NJ) and resolved by ultracentrifugation at 125,000 *g* for 24 h at 15°C. The RNA pellet was dissolved in RNase-free H_2O, digested with RNase-free DNase I (1 U/μg RNA) (Promega, Madison, WI) for 30 min at 37°C and extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1). RNA was precipitated with 0.3 M sodium acetate and ethanol, vacuum dried, redissolved in RNase-free H_2O, and the concentration and purity of the RNA ($OD_{260\ nm/280\ nm}$) was determined.

Reverse Transcription and Polymerase Chain Reaction

cDNA was prepared using oligo(dT) primers with the RiboClone cDNA Synthesis System (Promega) according to the modified method of Gubler and Hoffman (1983) using avian myeloblastosis virus reverse transcriptase. The resulting cDNA samples (100 ng each) were co-amplified using 30 cycles of polymerase chain reaction (PCR) with sense and antisense primers as described by Saiki et al. (1988). Sense and antisense primers for AR correspond to positions 510-533 and 738-761 of the AR cDNA (Plowman et al. 1990), respectively, and correspond to the mature protein coding regions. The DNA thermal cycle profile consisted of 1-min denaturation step at 94°C, a 2-min annealing step at 55°C, and a 1-min primer extension step at 72°C. PCR products were electrophoresed at constant voltage (100 V) on a 2% Seakem GTG, 0.5% Seakem ME agarose gel (FMC BioProducts, Rockland, ME) containing Tris/acetate EDTA buffer (Quality Biologicals, Inc., Gaithersburg, MD).

Metabolic Labeling with [^{35}S]Cysteine and Immunoprecipitation of Secreted Amphiregulin

Cells were grown to ~70% confluence in a 25-cm^2 flask. The cells were washed three times with MEM Select-Amine medium (GIBCO) which lacked L-cystine. 5 ml of the Select-Amine medium was added to the cells and incubated at 37°C. After 30 min, 2.6 ml of the Select-Amine medium containing 0.8 mCi of [^{35}S]cysteine was added to the cells. After 22 h, the conditioned medium was collected and placed on ice. Proteinase inhibitors (300× stock solutions) were added to yield final concentrations of 4 mM EDTA, 1 μM pepstatin A, 0.2 mM PMSF, and 1 μM leupeptin. The pH of the solution was adjusted to pH 7.2 with 1 M NaOH and centrifuged at 800 *g*. The supernatant was collected, 15 μl of preimmune serum added and incubated at 4°C for 60 min. 75 μl of a protein A agarose resin (GIBCO) was added and the sample rocked for 60 min at 4°C. The sample was centrifuged at 800 *g*, supernatant removed, and split into three equal aliquots. Each aliquot received either 5 μl of preimmune serum,, 5 μl of postimmune serum or 5 μl of postimmune serum plus 20 μg of the peptide antigen. The samples were incubated at 4°C. After 60 min, 25 μl of the protein A agarose was added to each sample and rocked at 4°C for 60 min. The samples were then centrifuged in a microfuge (Microfuge 12; Beckman Instruments, Inc., Palo Alto, CA) at 10,000 rpm for 2 min. The supernatant was discarded and the resins washed three times with 1 ml of 50 mM Tris, pH 7.5, containing 0.05% NP-40. 50 μl of SDS-PAGE sample buffer containing 2.5% 2-mercaptoethanol was added to the resin and it was heated to 85°C for 10 min. The samples were then run in a 16% acrylamide SDS-PAGE gel, fixed for 30 min in 25% isopropanol/10% acetic acid, treated with Amplify fluorographic reagent (Amersham Corp.), and autoradiographed at −80°C.

Immunocytochemistry of Cultured Cells and Formalin-fixed, Paraffin-embedded Sections

Cultured cells were plated into Lab-Tek 4 chamber slides (Nunc, Inc., Naperville, IL) at 20,000 cells per chamber and grown to ~70% confluence. The cells were washed twice with PBS and fixed with 1.5% formaldehyde in PBS for 45 min at room temperature. The cells were then washed four times with PBS. 10% goat serum in PBS (0.5 ml) was then added to each chamber and incubated for 45 min at room temperature. This solution was aspirated and 0.5 ml of 10 μg/ml preimmune (control) IgG or AR-Abl IgG in 0.1% BSA/PBS was added. AR-Abl IgG that was preabsorbed with peptide (1 μg IgG per 1 μg peptide) for 2 h at 37°C was also used at this step to confirm specificity. After a 1-h incubation at room temperature the cells were washed twice with PBS and bound IgG was detected using the Vectastain ABC kit for rabbit IgG (Vector Laboratories, Burlingame, CA). Cells were counterstained with Mayer's hematoxylin (No. GHS-1-16, Sigma Chemical Co., St. Louis, MO) for 2 min and slides mounted. Paraffin blocks of formalin-fixed tissues were obtained from the Department of Surgical Pathology of the George Washington University Medical Center (Washington, D.C.) and from the University of Alabama Medical Center (Birmingham, AL). Formalin-fixed, paraffin-embedded sections (5 μm) were deparaffinized and hydrated with xylenes and a graded alcohol series. Sections were washed in distilled H_2O for 5 min and in PBS for 10 min. Sections were then incubated in 0.3% H_2O_2 in methanol for 30 min to inactivate any endogenous peroxidase activity. The sections were

washed three times with PBS and incubated in 10% goat serum in PBS for 45 min at room temperature. The slides were processed exactly as above except that the sections were exposed to primary antibody for 12 h.

Results

Characterization of Antipeptide Antibodies against Amphiregulin

Peptides corresponding to residues 8-26 (peptide 1) and residues 26-44 (peptide 2) of AR were synthesized and used to generate antipeptide antibodies. These regions of AR were chosen since they had no significant sequence homology to any known protein (Shoyab et al., 1989). The antisera generated from the rabbits were screened for the ability to detect the peptide antigen, native AR and detect AR in formalin-fixed cells or tissues as described in Materials and Methods. One antisera generated against peptide 2 and designated AR-Ab2 was found to be most effective in the detection of native AR. The AR-Ab2 antibodies specifically immunoprecipitated secreted AR from the culture medium of PMA-treated MCF-7 cells after the cells had been metabolically labeled with [^{35}S]cysteine and in an ELISA were capable of detecting as little as 1 ng of purified AR (data not shown). None of the antisera generated showed any cross reactivity with EGF or TGFα in an ELISA. The ability of antisera to detect cell-associated AR after fixation with formaldehyde was tested by performing immunocytochemistry on cell lines which are positive or negative for AR mRNA. These experiments revealed that one antibody preparation directed against peptide 1 and designated AR-Ab1 could detect AR in formalin-fixed specimens. The AR-Ab1 antibodies were able to detect ~5 ng of purified AR, and could easily detect as little as 40 pg of the peptide antigen in an ELISA (data not shown). In immunocytochemical staining of cultured cells, the AR-Ab1 antibodies were found to stain only cells which express the AR mRNA. We have observed that there is a corresponding increase in the level of expression of AR mRNA and the intensity of staining using the AR-Ab1 antibodies in human mammary epithelial cells following transformation with various oncogenes (N. Normanno, T. Saeki, N. Kim, G. R. Johnson and D. S. Salomon, unpublished work). The AR-Ab2 antibodies also were found to be useful for immunocytochemical applications, but reactivity under these conditions was less than the staining achieved with the AR-Ab1 antibodies.

Amphiregulin Is a Mitogen for Geo Cells

The Geo cell line is derived from a human colon carcinoma (Brattain et al., 1981) and proliferates autonomously under serum-free conditions. To determine if AR is capable of stimulating the growth of Geo cells, the effect of AR on the anchorage-dependent growth of the Geo cell line was studied under serum-free conditions. The exogenous addition of either AR or EGF was found to stimulate the proliferation of the Geo cells (Fig. 1). Maximal stimulation of growth (~2.8-fold over controls) was observed at a concentration of 1-5 nM AR, whereas for EGF maximal stimulation of growth (~3.5-fold) was observed at 0.2 nM. The Geo cells were more sensitive to the exogenous addition of EGF as compared to AR, with half-maximal stimulations of growth at 40 and 200 pM for EGF and AR, respectively.

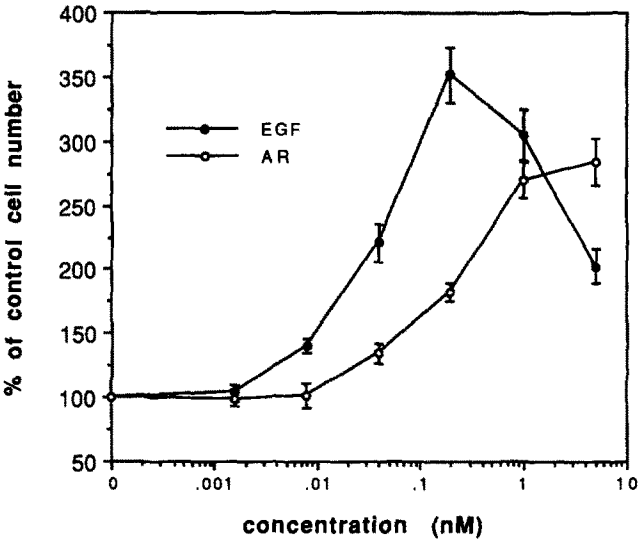


Figure 1. Stimulation of growth of Geo cell line by exogenous amphiregulin or EGF. Mitogenesis assays were performed in serum-free medium as described in Materials and Methods. Data points represent the mean ± SEM of experiments performed in triplicate and the effect of growth factor on growth is expressed relative to growth in the absence of growth factor (*control*). The cells were initially plated at a density of 10,000 cells/well and after 4 d control wells contained ~31,500 cells.

A mAb against the EGF Receptor Blocks Amphiregulin-induced Mitogenesis

If the AR-induced mitogenesis involves an interaction between AR and the EGF receptor, it should be possible to block this mitogenesis using a mAb directed against the extracellular domain of the EGF receptor. This commercially available mAb reacts with the extracellular domain of the EGF receptor on all human cells, competes for binding of EGF to the EGF receptor on human cells, blocks the biological effects induced by EGF, but does not activate the tyrosine kinase activity of the EGF receptor (Dr. J. Denry Sato, personal communication). Geo cells have been previously shown to express ~9,000 EGF receptors per cell (Wan et al., 1988; Mulder and Brattain, 1989). As shown in Fig. 2, 10 nM of the anti-EGF receptor mAb completely blocked the stimulation of growth of the Geo cell line induced by 200 pM of exogenous AR. An isotype control mAb was used to confirm that the inhibition induced by the anti-EGF receptor mAb was specific. The presence of the control mAb, by itself, had no significant effect on cell growth (Fig. 2).

Geo Cells Constitutively Express the Amphiregulin mRNA Transcript

To demonstrate that the Geo cells express AR mRNA under serum-free conditions, PCR amplification of cDNA was used since this technique is capable of detecting mRNA transcripts in small numbers of cells (Rappolee et al., 1989). Total cellular RNA was reverse transcribed using oligo(dT) primers to generate double stranded cDNA substrates and sense and antisense oligonucleotide primers corresponding to positions 510-533 and 738-761 of the AR cDNA (Plowman et al., 1990), respectively, were used to PCR amplify an ~250-bp region encoding the mature protein. The gener-

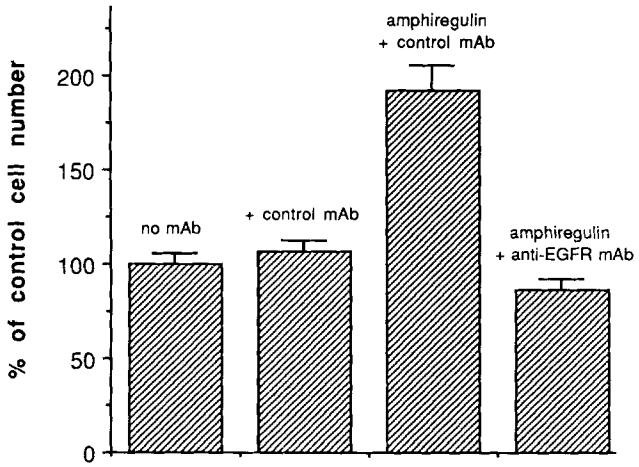


Figure 2. Effect of an anti-EGF receptor mAb on amphiregulin-induced mitogenesis. Geo cells were grown in serum-free medium in the presence of 0.2 nM AR containing either 10 nM of an anti-EGF receptor mAb or 10 nM of an isotype control mAb. After 4 d, cells were trypsinized and counted. Data points represent the mean ± SEM of experiments performed in triplicate. Results are expressed relative to the growth of the cells in the absence of AR and mAb (*control*). The cells were initially plated at a density of 30,000 cells/well and after 4 d control wells contained ~81,400 cells.

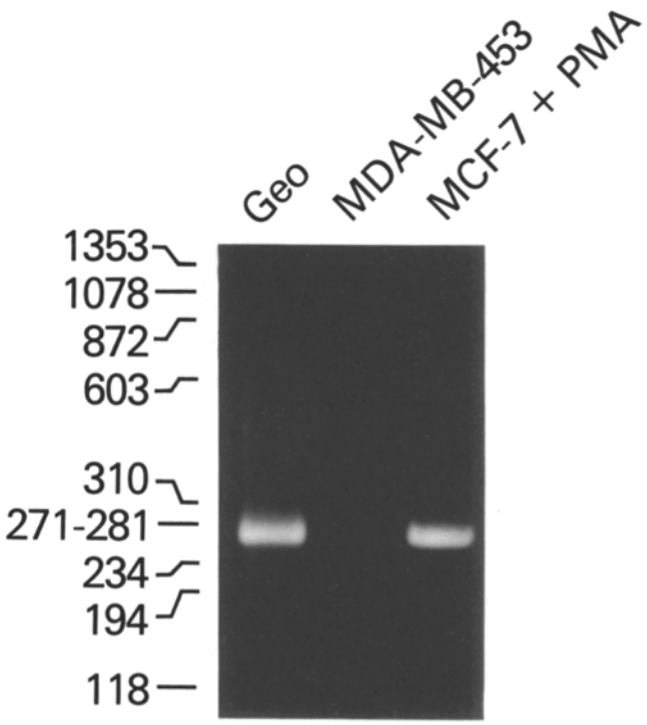


Figure 3. Analysis of AR mRNA expression by Geo cells. Poly(A)$^+$ mRNA was converted into cDNA as described in Materials and Methods section. PCR amplification (30 cycles) of 100 ng of cDNA derived from each cell line was performed, products fractionated in an ethidium bromide-containing Seakem agarose gel and bands visualized by UV illumination. The sense and antisense primers correspond to positions 510-533 and 738-761 of the AR cDNA (Plowman et al., 1990), respectively, and yield a predicted target sequence of ~250 bp upon PCR amplification. cDNA from Geo (*left lane*), MDA-MB-453 (*center lane*) and MCF-7 cells after 24 h treatment with 100 ng/ml PMA (*right lane*) were PCR amplified. The positions of a HaeIII digest of ϕX174 are shown to the left (in bp).

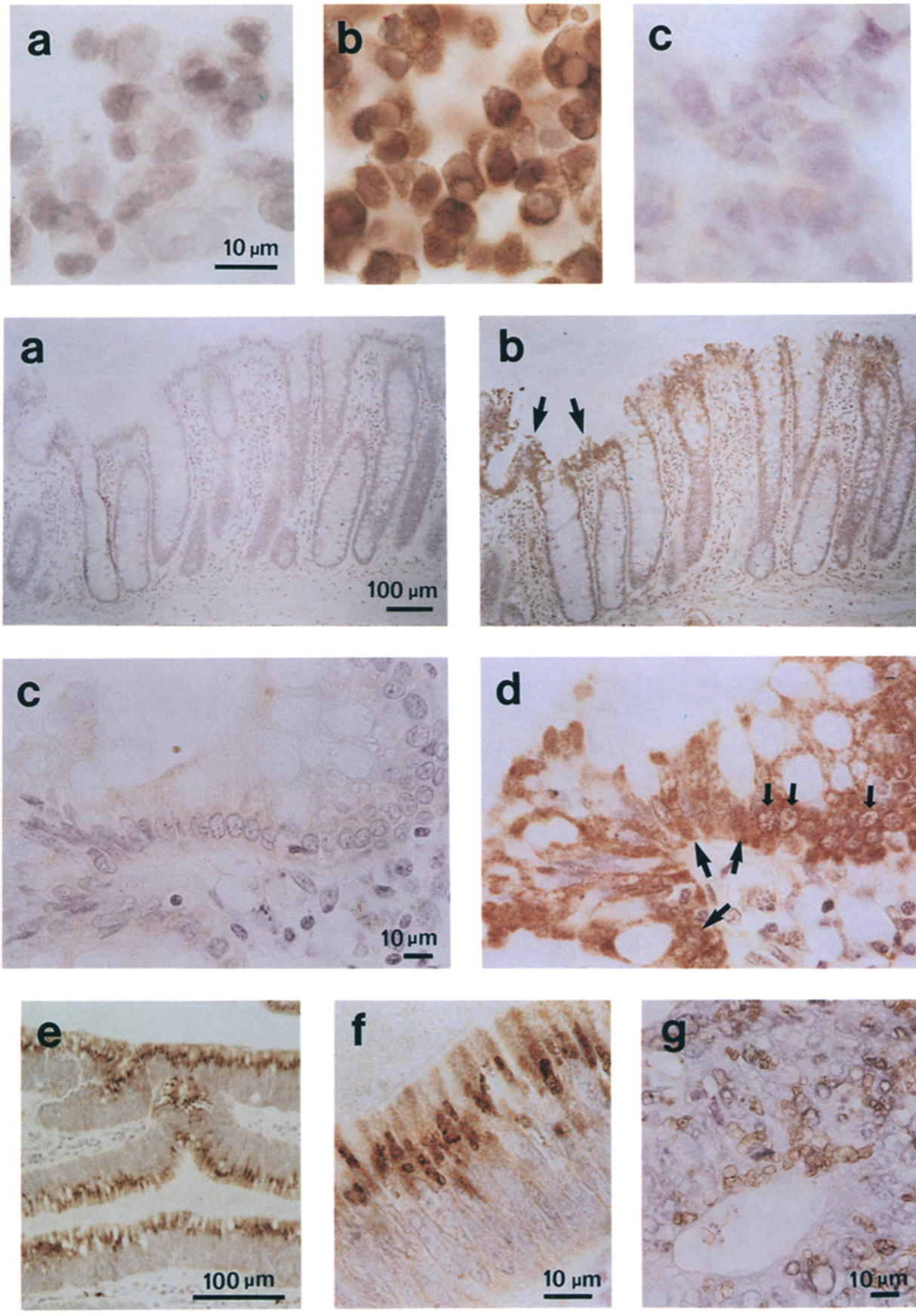
ated target sequence was then validated by size fractionation of the PCR products on an agarose gel. The MDA-MB-453 and PMA-treated MCF-7 cell lines which have been previously demonstrated to be negative and positive for expression of the 1.4-kb AR mRNA transcript (Plowman et al., 1990), respectively, were used to further validate the procedure. Fig. 3 shows that primer-directed amplification (30 cycles) of 100 ng of cDNA from Geo (Fig. 3, *left lane*) and MCF-7 cells treated with 100 ng/ml of PMA for 24 h (Fig. 3, *right lane*) yield the diagnostic ~250-bp band specific for AR, whereas there was no detectable band produced by PCR amplification of the MDA-MB-453 cDNA (Fig. 3, *center lane*). Further, digestion of the ~250-bp target sequence produced by PCR amplification (Geo and PMA-treated MCF-7 cells) with the BsmI restriction endonuclease results in the generation of two fragments of ~80- and ~170-bp size, exactly as predicted (data not shown). Thus, the results unequivocally demonstrate that Geo cells constitutively express the AR mRNA transcript under serum-free conditions.

Antibodies to Amphiregulin Detect the Protein in Geo Cells

Fig. 4 *b* demonstrates the intense brown immunospecific staining of Geo cells which resulted from use of the purified AR-Ab1 IgG. Controls using the IgG purified from the preimmune serum (Fig. 4 *a*) or the AR-Ab1 IgG which had been preabsorbed with the peptide antigen (Fig. 4 *c*) did not result in significant staining of the cells.

Geo Cells Secrete Amphiregulin

The AR-Ab2 antibodies which are directed against residues 26-44 of AR were used to ascertain whether the Geo cells secrete AR. The cells were metabolically labeled with [^{35}S]cysteine and after 22 h the conditioned medium was collected. Immunoprecipitations were performed and the resultant material was fractionated under reducing conditions in a 16% acrylamide SDS-PAGE gel. Radiolabeled AR was immunoprecipitated specifically from conditioned medium using AR-Ab2 antibodies (Fig. 5, *Geo, center lane*). The secreted AR migrated as a broad, diffuse band with a molecular weight that ranged from ~18.5 to 22.5 kD and had a median molecular weight of ~20.7 kD. Immunoprecipitations performed with the preimmune serum (Fig. 5, *Geo, left lane*) or with the AR-Ab2 serum in the presence of excess peptide antigen (Fig. 5, *Geo, right lane*) confirmed the specificity of the immunoprecipitation using the AR-Ab2 antibodies. Furthermore, immunoprecipitations performed using the MDA-MB-453 negative control cell line (Fig. 5, *MDA-MB-453*) also confirmed the specificity of the immunoprecipitation of AR from Geo-conditioned medium. AR which has been immunoprecipitated from medium conditioned by Geo cells and AR from medium conditioned by PMA-treated MCF-7 cells migrate in an almost identical fashion in SDS-PAGE (data not shown). The migration of the AR as a broad, diffuse band with median molecular weight of ~21 kD in SDS-PAGE is characteristic of AR and is most likely due to heterogeneous glycosylations and truncation of


a
10 μm
b
c
a
100 μm
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10 μm

Figure 4. Detection of amphiregulin in Geo cells by immunocytochemistry. Immunocytochemistry was performed using the purified AR-Ab1 IgG directed against residues 8-26 of AR as described in the Materials and Methods section. The cells were exposed to either 10 μg/ml of preimmune control IgG (*a*), 10 μg/ml of AR-Ab1 (*b*), or 10 μg/ml AR-Ab1 IgG that was preabsorbed with 20 μg/ml peptide antigen (*c*). All cells have been counterstained with Mayer's hematoxylin.

Figure 7. Immunohistochemical detection of amphiregulin in normal and malignant human colon using the AR-Ab1 antibodies. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections as described in the Materials and Methods section. Normal colonic mucosa (*a–d*) is shown after staining with preimmune IgG (*a* and *c*) or AR-Ab1 IgG (*b* and *d*). The arrows in *b* denote the intense staining observed at the luminal surface of the normal mucosa. The luminal surface epithelium of a normal colon is shown at high magnification in *c* and *d*. The large and small arrows in *d* denote examples of intense, uniform nuclear staining and punctate nuclear staining of AR, respectively. Staining of a well-differentiated (*e* and *f*) and poorly differentiated (*g*) adenocarcinoma of the colon is shown using the AR-Ab1 IgG. All sections have been counterstained with Mayer's hematoxylin.

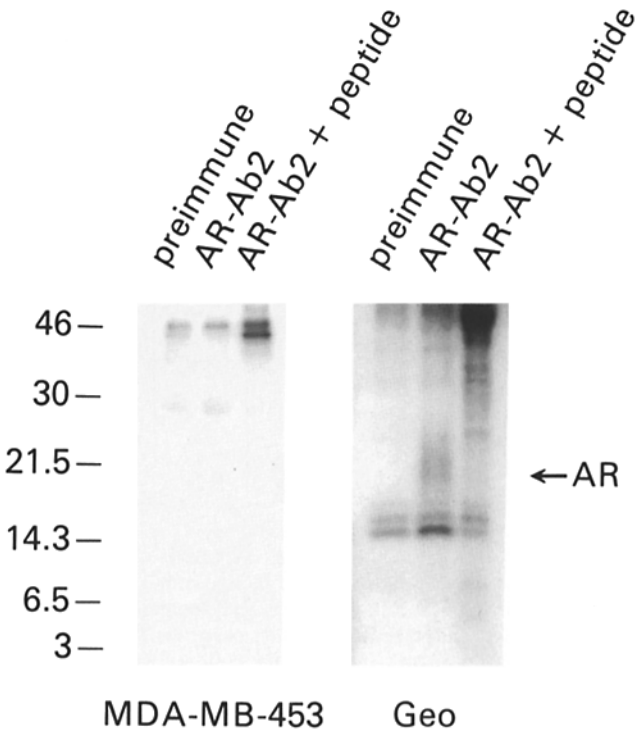

Figure 5. Immunoprecipitation of amphiregulin secreted by Geo cells. Geo and MDA-MB-453 cells were metabolically labeled with [^{35}S]cysteine and secreted AR was immunoprecipitated as described in the Materials and Methods section. Immunoprecipitations were performed using preimmune sera (*left lanes*), AR-Ab2 sera (*center lanes*) or AR-Ab2 sera in the presence of excess peptide antigen (*right lanes*). Samples were run under reducing conditions in a 16% acrylamide SDS-PAGE gel and autoradiographed at −80°C. The arrow on the right denotes the position of AR in the gel. The positions and mol wt of marker proteins in kilodaltons are shown at the left.

the six NH_2-terminal amino acid residues of the AR molecule (Shoyab et al., 1988). To confirm that the Geo cells secrete a biologically active EGF-like factor, serum-free medium was conditioned by the Geo cells and then tested for the ability to support the proliferation of the MCF-10A cell line, a mammary epithelial cell line which has an obligatory requirement for the exogenous addition of EGF for growth (Soule et al., 1990). Geo conditioned medium (diluted 2–20-fold with fresh medium) and purified AR were both found to support the growth of the MCF-10A cells and thus were able to supplant the growth requirement of these cells for EGF (data not shown).

Proliferation of Geo Cells Is Inhibited by Immunospecific Depletion of Amphiregulin from the Extracellular Medium and by an Anti-EGF Receptor mAb

The inclusion of the AR-Ab1 or AR-Ab2 antibodies was found to have no significant effect upon the anchorage-dependent growth of the Geo cells in serum-free medium, indicating that these antibodies are incapable of neutralizing the biological activity of secreted AR under these conditions. These antibodies were also incapable of inhibiting the action of exogenously added AR on Geo cells (data not shown). Therefore, to test the hypothesis that AR is an autocrine growth factor for the Geo cells a different strategy was used. AR was immunospecifically removed from serum-free medium conditioned by the cells using the purified AR-Ab2 IgG and then the AR-depleted medium was added back to the cells. The cells were allowed to continue growing for another day and then the culture medium was again depleted of AR. It is important to note, as demonstrated in Fig. 5, that this procedure specifically removes AR from the Geo-conditioned medium. The consequence of this depletion of AR from the medium was a 39.9% inhibition of Geo cell growth relative to cells which were treated in an identical manner using the purified preimmune IgG as the control (Fig. 6 *A*). The processing of the conditioned medium using the control preimmune IgG and readdition of the medium to the cells had only a minor growth inhibitory effect (~20%) when compared to cells in which the conditioned medium was not processed at all, demonstrating that the processing alone did not have a dramatic effect on mitogenic activities in the conditioned medium. These results indicate that the AR secreted by Geo cells is biologically active and stimulates the growth of these cells via an autocrine mechanism.

To ascertain if the EGF receptor is involved in the autocrine growth of Geo cells, the cells were grown in the presence of the anti-EGF receptor mAb under serum-free conditions. The anti-EGF receptor mAb also inhibited the growth of the Geo cells by 50.5% relative to their growth in the absence of any mAb (Fig. 6 *B*). The presence of an isotype control mAb had no significant effect on the growth of the cells. These results indicate that both secreted AR and the EGF receptor are involved in the autocrine growth of these cells.

Immunolocalization of Amphiregulin in Normal and Malignant Human Colon

The AR-Ab1 antibodies were used to detect AR in the normal

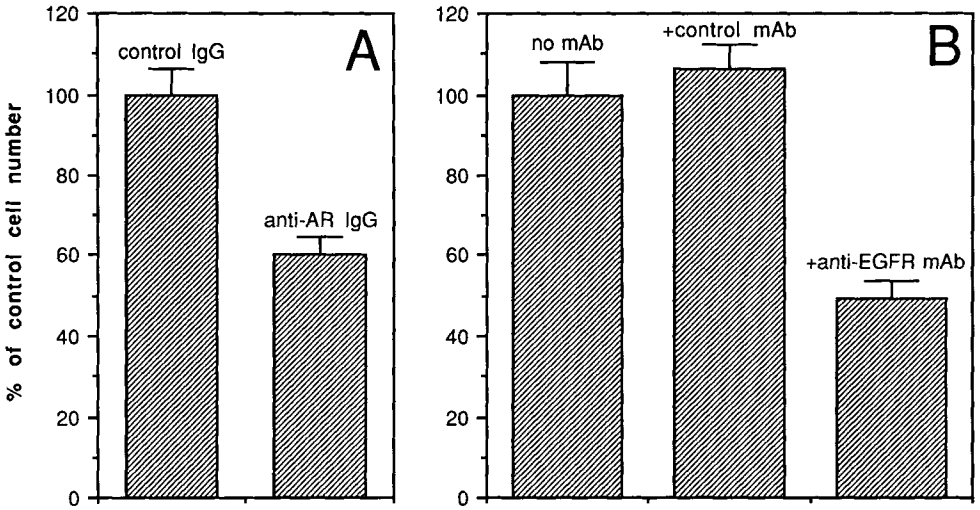

Figure 6. Inhibition of Geo cell growth by immunospecific depletion of AR from extracellular medium and by an anti-EGF receptor mAb. (*A*) Inhibition of cell proliferation by immunospecific removal of AR from conditioned medium. 2 d after initially plating of the Geo cells under serum-free conditions, the conditioned medium was collected and AR was specifically immunoprecipitated using purified AR-Ab2 IgG as described in the Materials and Methods section. The AR-depleted medium was then added back to the cells and after 24 h, the conditioned medium was processed again. After 24 h, the cells were trypsinized and counted. The results represent the mean ± SEM of experiments performed in triplicate using either the preimmune IgG (*control*) or the AR-Ab2 IgG. The cells were initially plated at a density of 48,000 cells/well and at the end of the experiment control wells contained ~100,500 cells. (*B*) Inhibition of cell proliferation by an anti-EGF receptor mAb. Geo cells were plated into 12-well plates and grown in serum-free medium containing 10 nM anti-EGF receptor mAb, 10 nM of an isotype control mAb or no mAb. After 4 d, the cells were trypsinized and counted. Results are expressed relative to the growth of the cells in the absence of any mAb and represent the mean ± SEM of experiments performed in triplicate. The cells were initially plated at a density of 30,000 cells/well and after 4 d control wells contained ~82,200 cells.

colon from five individuals who were free of any colon disease. Immunohistochemistry was performed on paraffin-embedded, formalin-fixed sections and the samples were examined at various degrees of magnification. In all normal specimens, AR was localized only in epithelial cells of the colonic mucosa. The brown immunoperoxidase staining revealed that AR was localized to the surface columnar and secretory (goblet) epithelial cells of the luminal surface of the mucosa (Fig. 7 *b*), and was not detectable in the less differentiated, proliferative cells of the crypts. Fig. 7 *a* shows the lack of staining using the control preimmune antibodies in a companion section from the identical tissue region. There was a gradient of increasing immunoperoxidase staining of AR towards the lumen, with the most intense staining observed at the luminal surface. In some of the specimens extracellular AR was occasionally detected within the lumen of the colon. At higher magnification, it was clear that the AR was localized to both the cytoplasm and the nucleus of the surface columnar and secretory cells of the mucosa (Fig. 7 *d*). Fig. 7 *c* shows the staining achieved using the control preimmune antibodies in a companion section from the identical tissue region. The larger arrows in Fig. 7 *d* denote the intense uniform staining of AR in the nucleus of some cells, whereas the smaller arrows denote punctate brown nuclear staining of AR that was also observed. In some instances, AR appeared to be associated with the plasma membrane of the cells. The large oval transparent cytoplasmic inclusions seen near the luminal surface in Fig. 7 *d* are mucin-rich secretory granules characteristic of secretory (goblet) cells (Colony, 1989). AR was not detectable within these secretory granules. Immunohistochemical analysis of the same sections using the AR-Ab2 antibodies which recognize a distinct region of AR relative to the AR-Ab1 antibodies yielded equivalent results, although with less intense staining (data not shown).

The AR-Ab1 antibodies were then used to detect and localize AR in five specimens derived from adenocarcinomas of the colon. AR was found in two well, one of two poorly and one undifferentiated carcinoma. The AR was localized to the carcinoma cells of the tumors. Figure 7 *e* shows the intense immunostaining of a well-differentiated adenocarcinoma using this anti-AR antibody. The subcellular localization of AR in the carcinoma cells of the tumors was somewhat heterogeneous within and among specimens. The AR was detected in either cytoplasm or nucleus of the cells, or in both of these subcellular locations. The cytoplasmic localization of AR in the cells of a well differentiated carcinoma is demonstrated in Fig. 7 *f*. The AR appears to be concentrated within a cytoplasmic organelle and it is possible that this subcellular organelle is the Golgi apparatus. Fig. 7 *g* demonstrates the nuclear localization of AR in the vacuolated nuclei of cells in a poorly differentiated colonic carcinoma. In some instances, extracellular AR was detectable in the lumen of the glands of the carcinomas. Controls performed using preimmune antibodies confirmed the specificity of the immunostaining observed in these specimens (data not shown).

Discussion

Evidence That the EGF Receptor Mediates the Mitogenesis Elicited by Amphiregulin

Geo cells are classified as a differentiated colonic carcinoma cell line relative to other colon carcinoma cell lines based upon a number of criteria (Wan et al., 1988; Mulder and Brattain, 1989). In general, the more differentiated cell lines express ~8–20 × 10^3 EGF receptors per cell and are growth stimulated by the exogenous addition of EGF, whereas poorly differentiated colon carcinoma cell lines express <3 × 10^3 receptors per cell and do not respond to the exogenous addition of EGF (Wan et al., 1988; Mulder and

Brattain, 1989). Our results indicate that while exogenous AR stimulates the growth of Geo cells, it is not as potent as EGF. AR is known to compete for binding of ^{125}I-EGF to EGF receptors on human A431 cells, but is not as effective as EGF (Shoyab et al., 1989). Conversely, AR was as effective as EGF at competing for binding of ^{125}I-EGF to EGF receptors on mouse AKR-2B cells (Cook et al., 1991). Nevertheless, there is no direct evidence to date to indicate that the EGF receptor is the receptor through which the biological action of AR is mediated. Our observation that a mAb to the extracellular domain of the EGF receptor completely blocks the mitogenesis induced by AR, taken together with the fact that AR can compete for binding of ^{125}I-EGF to the EGF receptor provides strong evidence that the EGF receptor is the cell surface molecule through which AR acts in Geo cells. Further, the observations that the addition of exogenous AR to A431 cells leads to phosphorylation of the EGF receptor (M. Shoyab, unpublished work) and that AR can support the growth of MCF-10A cells, an EGF-dependent cell line, provide additional evidence that AR can act through the EGF receptor. The fact that AR-induced mitogenesis can be completely blocked by a mAb to the EGF receptor suggests that there is not an additional cell surface receptor on Geo cells which can transduce a biological signal in response to AR.

Amphiregulin As an Autocrine Growth Stimulator

The Geo colon carcinoma cells constitutively express the AR mRNA, secrete AR protein into the extracellular medium, are growth stimulated by the exogenous addition of AR and are partially growth inhibited by depletion of AR from its extracellular medium and by an anti-EGF receptor mAb. This clearly demonstrates that AR is an autocrine growth stimulator for Geo cells. Taken together with the observation that an anti-EGF receptor mAb blocks the mitogenic stimulation invoked by AR, the findings suggest that AR can function through an extracellular autocrine loop that involves the EGF receptor. Since we were only able to partially inhibit the growth of the Geo cells, other secreted growth factors may stimulate the proliferation of these cells via autocrine mechanisms. It has been suggested that autocrine growth factors may play a role in malignant transformation and growth of cancer cells (Sporn and Todaro, 1980; Sporn and Roberts, 1985; Lang and Burgess, 1990). There is considerable evidence which indicates that TGFα may function as an autocrine growth stimulator for many types of cancer cells (reviewed in Salomon et al., 1990) including colon carcinoma cells (Wan et al., 1988; Murthy et al., 1989; Mulder and Brattain, 1989). Recently, Sizeland and Burgess (1991) have demonstrated that TGFα is one of two autocrine growth factors required for the autonomous growth of the LIM 1215 colon carcinoma cell line. With the recent discovery of other EGF-related molecules such as AR (Shoyab et al., 1988), cripto (Ciccodicola et al., 1989), and heparin-binding EGF-like growth factor (Higashiyama et al., 1991), it is becoming clear that the EGF receptor could potentially function as a common receptor for multiple autocrine growth factors. Ciardiello and co-workers (1991*b*) found that a majority of primary and metastatic colorectal carcinomas express the mRNA for AR and cripto, suggesting that these molecules may be involved in the pathogenesis and/or progression of this disease. While there is no evidence that the cripto protein is secreted from cells or that it binds to the EGF receptor, expression of the cripto cDNA has been shown to transform cells (Ciccodicola et al., 1989; Ciardiello et al., 1991*a*). Our work demonstrates that AR is another EGF-like growth regulator which can function in an autocrine mode.

Immunocytochemical Localization of Amphiregulin in Normal and Malignant Colon

Normal colonic epithelial cells arise, proliferate and mature within the crypts, and migrate to the mucosal surface where they are eventually shed (Wright, 1989; Colony, 1989). Colonic epithelial cell turnover time is estimated to be 4–8 d in humans (Lipkin and Deschner, 1976). The surface columnar and secretory (goblet) cells of the mucosal surface are terminally differentiated, non-proliferative cells (Wright, 1989; Colony, 1989). Our finding that AR was exclusively localized to these cells and not detectable in the proliferative cells of the crypts, suggests that AR is not an autocrine growth stimulator for normal colonic epithelial cells in vivo. The EGF receptor has been detected in normal human (Markowitz et al., 1990) and rat colonic epithelium (Koyama and Podolsky, 1989). In fact, normal rat crypt cell proliferation is stimulated by EGF in vivo (Goodlad et al., 1987; Reeves et al., 1991). Further, the number of EGF receptors is constant throughout the crypt–villus continuum in rat intestine (Koyama and Podolsky, 1989). In some instances, we were able to detect extracellular AR, indicating that the epithelial cells of the normal colonic mucosa are capable of secreting AR. Therefore, if one assumes that the biological role of AR in the normal colon is one of growth regulation, it is possible that AR is secreted by cells of the luminal colonic surface and acts in a paracrine fashion to stimulate or control the growth of proliferative crypt cells. As cells leave the crypt and are no longer proliferative, AR may be expressed and act to stimulate the growth of cells in the crypt, thus generating a tightly orchestrated process of mucosal renewal. This mechanism of epithelial cell turnover may be preferable to having crypt cells control their own growth through autocrine mechanisms, since autonomous growth could increase the likelihood of aberrant or uncontrolled crypt cell growth. Interestingly, the amount of the TGFα mRNA transcript was higher in differentiated villus cells than in mitotically active crypt epithelial cells of rat intestine, whereas TGFβ mRNA levels were higher in crypt cells than in villus cells (Koyama and Podolsky, 1989). These authors suggest that these TGFs may be involved in the balance between proliferation and differentiation of these cells through autocrine and paracrine mechanisms. Unlike normal colon, one can speculate that in colonic adenocarcinomas, AR might function principally as an autocrine growth factor in which the malignant cells have the capacity to secrete, as well as respond to AR.

AR contains two Arg-Lys-Lys-Lys sequences positioned at residues 26-29 and 40-43 (Shoyab et al., 1989). These sequences suggest that AR may be targeted to and translocated into the nucleus of cells (Roberts, 1989). We have previously found that endogenous AR is predominantly localized to the nucleus of both normal ovarian surface epithelial and ovarian carcinoma cells in vitro, suggesting that AR plays some biological role in the nucleus of cells (Johnson et al., 1991). Our finding that AR can be detected in the nucleus of colonic epithelial cells in vivo using antipeptide antibodies directed against residues 8-26 of AR substantiates this possibility.

This region of AR (residues 8-26) has no significant sequence homology to any known protein (Shoyab et al., 1988). We consistently detected AR in the nucleus and cytoplasm of the terminally differentiated cells of the normal mucosa. These cells are not mitotically active and it is not clear what biological role AR plays in the nucleus of these cells. This observation suggests that AR could play a role in the differentiation of the cells through a direct effect in the nucleus, although there is currently no evidence that AR plays any direct role in differentiation. In the colonic carcinomas, AR was also often detected in the nucleus of the cells. In some cells of the carcinomas it was exclusively localized in the cytoplasm of the cells, whereas in some instances it was localized just in the nucleus or in both subcellular locations. It is difficult to place any significance upon this observation without knowing what function AR serves in the nucleus of cells. There is evidence that other growth factors have nuclear sites of action (Burwen and Jones, 1987). The addition of exogenous basic fibroblast growth factor to endothelial cells leads to the accumulation of the growth factor in the nucleolus and transcription of ribosomal genes during the $G_0 \rightarrow G_1$ transition (Bouche et al., 1987). Renko et al. (1990) demonstrated that in cells which produce basic fibroblast growth factor the high molecular weight forms were localized in the nucleus, whereas the low molecular weight (18 kD) form was found primarily in the cytoplasm. A fibroblast growth factor-related protein, the *int*-2 oncoprotein, has been detected in the nucleus of cells (Acland et al., 1990). Attachment of a nuclear-targeting sequence from yeast histone 2B to a truncated defective heparin-binding growth factor-1 (acidic fibroblast growth factor) mutant results in the recovery of its mitogenic activity (Imamura et al., 1990). It is important to note that heparin binding-EGF-like growth factor contains one of the putative nuclear targeting sequences found in AR (Higashiyama et al., 1991), suggesting that AR may not be the only EGF-related growth regulator which is targeted to the nucleus. In summary, the finding that AR can be localized to the nucleus of cells in vivo strongly suggests that AR performs some biological function in the nucleus of cells. Thus, it will be important to determine what this function is to more fully understand the role that AR plays in normal and pathological biological processes.

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