Amphiregulin Messenger RNA Is Elevated in Psoriatic Epidermis and Gastrointestinal Carcinomas

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Abstract

Amphiregulin (AR) is a heparin-regulated, epidermal growth factor-like growth factor capable of stimulating the proliferation of non-tumorigenic cells while inhibiting cell proliferation in some human tumor cell lines in vitro. In the present study, we have investigated AR mRNA expression in normal, hyperproliferative, and neoplastic human epithelium. Our results demonstrate that, compared with the adjacent uninvolved epithelium, AR mRNA expression is markedly elevated in epidermal biopsies derived from three human psoriatic lesions as well as in biopsies derived from five human colon carcinomas and three human stomach carcinomas. Moreover, analysis of a colon carcinoma by in situ hybridization revealed that AR mRNA is localized to the epithelium.

Introduction

AR (1-3) is a recently characterized member of the EGF family. This family includes EGF, vaccinia growth factor, TGF-α, AR (1-3), Cripto (4), and heparin-binding EGF-like growth factor (5). Studies are currently ongoing to delineate the involvement of the EGF family members in normal growth and development and in pathological conditions. The biological activity ascribed to AR includes the mitogenic stimulation of nontumorigenic cells as well as the inhibition of cell proliferation in some human tumor cell lines (1, 2). More recently, we have demonstrated that AR is a heparin-binding autocrine growth factor for human keratinocytes whose mitogenic activity is abrogated by its interaction with heparin (6) or other sulfated polysaccharides. We have also shown that both human keratinocyte and mammary epithelial cell cultures express AR mRNA, while dermal fibroblast and melanocyte cultures as well as certain mammary tumor cell lines do not express detectable levels of this transcript (6). It has been demonstrated that AR-specific mRNA is expressed in normal human breast, placenta, and ovary (3). Lower levels were detected in the normal human colon while no apparent expression of AR-specific mRNA was observed in normal human duodenum or epidermis (3). More recently, it has been shown that AR mRNA and protein are overexpressed in 60 to 70% of human primary and metastatic colorectal carcinomas (7). AR and TGF-α mRNA and protein are expressed by cultured human keratinocytes (6, 8), and TGF-α mRNA and protein are significantly elevated in psoriatic epidermis (9) and in several human carcinomas (10). Collectively, the results of these investigations suggest that AR and TGF-α may act as important regulators of growth for epithelial cells and that aberrant expression of these growth factors could facilitate the development of proliferative pathological conditions, such as psoriasis and colorectal carcinomas.

Materials and Methods

Processing of Tissue for RNA Isolation. Epidermal skin specimens from normal and psoriatic human skin were obtained by shave removal with a keratome (Castroviejo, 0.4-mm thickness). Normal tissue surrounding pathological specimens was trimmed away. Fresh specimens were immediately placed in chilled phosphate-buffered saline, transported to the laboratory and weighed. Gastrointestinal tumors (colon and stomach carcinomas) were surgically removed and subsequently processed as previously described (11); normal mucosa from tissue adjacent to the tumor was dissected away from the underlying submucosa. RNA was extracted from these tissues as described below. Utilization of human tissue was approved by institutional review boards of the Mayo Clinic and Vanderbilt University.

Isolation of RNA. Total cellular RNA was isolated from epidermal tissue by the RNAzol method (Cinna/Biotex Labs., Friendwood, TX) using a polytron homogenizer (Brinkmann). Total RNA and poly A+ RNA from gastrointestinal specimens were isolated as previously described (11). Poly A+ RNA from cultured normal human keratinocytes and human colon adenoma and tumor cell lines was isolated as previously described (6).

Northern Blot Hybridization Analysis. AR- and TGF-α-specific, [α-32P]UTP-labeled antisense cRNA was synthesized from linearized pTZ/AR-2 (6) and SP65/TGFα (8), respectively, and used as a probe to detect specific hybridization to the Northern blots, as previously described (6, 8). In some cases, an identical protocol was utilized to probe the same blot for the expression of the constitutively expressed cyclophilin (1B15) gene product (6, 12). In cases where expression of cyclophilin was not determined, equivalent amounts of intact RNA loaded onto gels were verified by staining of the gel with ethidium bromide and subsequent densitometry of 28S ribosomal RNA prior to RNA transfer.

Cloning of AR cDNA into pSelect Vector. Oligonucleotide primers were synthesized corresponding to the sense, 5'-GCAC<sup>AAG-CTT</sup>CCCAAGAGCAGAGGTTGGCCCG-3', and antisense, 3'-TTCTCAGTTGAGCTGCATGTCGATTGC-5', strands of the published cDNA sequence of human AR (3). Underlined bases correspond to AR-specific cDNA sequences, nonunderlined sequences correspond to linkers (slash marks indicate restriction enzyme cleavage sites for HindIII), and the numbers correspond to the numbering of the previously published AR cDNA sequence (3). These primers were used to amplify the AR cDNA sequence using pTZ/AR-2 (6) as a template, using the previously published polymerase chain reaction protocol (6). Amplified cDNA was cloned into the pCR 1000 (Invitrogen, San Diego, CA) vector and sequenced. The AR cDNA
insert was isolated from the pCR 1000 vector by digestion with HindIII endonuclease and ligated into the HindIII site of linearized pSelect vector (USB, Cleveland, OH), which contains recognition sites for the SP6 and T7 RNA polymerases. The orientation of the construct placed the 3' end of the AR cDNA sequence adjacent to the T7 polymerase promoter. This construct was designated pSelect/AR.

**In Situ Hybridization Analysis.** Colon tumors and normal tissue adjacent to the tumor were sectioned and used for in situ hybridization analysis to detect the tissue-specific expression of AR mRNA utilizing a protocol similar to that previously described for TGF-α mRNA analysis to detect the tissue-specific expression of AR mRNA utilizing 15 units of SP6 or T7 RNA polymerase, respectively. Hybridization was carried out overnight at 55°C. Hybridized sections were washed twice for 10 min in a washing buffer (2xSSC; 10 mM β-mercaptoethanol; 1 mM EDTA), and subsequently incubated for 30 min in an RNase-containing solution (20 μg/ml of RNase A;500 mM NaCl;10 mM Tris buffer at pH 8.0). RNase-treated tissue sections were washed 2 times for 10 min each in washing buffer and then washed 3 additional times for a duration of 2 h (5, 20, and 95 min) in washing buffer containing 0.1x SSC at 55°C. Tissue sections were then subsequently washed 2 times for 10 min each in 0.5x SSC washing buffer. Tissue sections were dehydrated by incubation for 2 min each in a 0.3 M ammonium acetate solution containing 50%, 70%, and 90% ethanol. Dehydrated slides were air dried, dipped in Kodak NTB2 nuclear emulsion at 42°C, and stored dessicated at 4°C for 1 to 18 wk. Slides were developed using Kodak D19 developer and fixer. Developed slides were counterstained with toluidine blue.

**Results**

To examine AR and TGF-α expression in a nonmalignant hyperproliferative condition, shave biopsy samples from active psoriatic lesions and from normal uninvolved epidermis were obtained from three patients with widespread, untreated psoriasis vulgaris. Total RNA was extracted from these specimens and subjected to Northern blot analysis to detect expression of AR-specific mRNA. The results of this analysis (Fig. 1a) consistently demonstrated that AR mRNA (1.7 kilobases) expression is dramatically elevated in involved psoriatic epidermis (Lanes 2, 4, and 6) when compared with biopsies derived from uninvolved psoriatic epidermis (Lanes 1, 3, and 5) of the same individuals. All of these RNA samples were shown to be intact by detection of cyclophilin (1B15 expression). Because TGF-α mRNA previously has been shown to be increased in psoriatic lesions (9), we also analyzed RNA samples from two of the three patients for the expression of TGF-α mRNA. The results of this analysis demonstrated that, as expected, TGF-α mRNA (4.4 and 1.7 kilobases) was elevated in the RNA extracted from the active psoriatic lesions (Fig. 1b, Lanes 2 and 4), but to a lesser degree than AR (Fig. 1a, Lanes 2 and 4). We also have examined additional specimens from lesional and uninvolved epidermis of psoriatic skin (unmatched pairs), and in all cases AR mRNA expression was similarly elevated in the lesional samples. In other studies examining AR-specific mRNA expression in biopsy samples obtained from human basal cell and squamous cell carcinomas, we found that basal cell carcinomas did not express elevated levels of AR mRNA, while some squamous cell carcinomas did express elevated levels of AR-specific mRNA (data not shown).

We also investigated the expression of AR- and TGF-α-specific transcripts in total and poly A+ RNA from biopsy samples of normal and tumor tissue isolated from human colon and stomach (Fig. 2d). Relative to the levels of AR mRNA expression observed in adjacent normal colonic epithelium (Fig. 2a, Lanes 1, 3, 5, 7, and 9), expression of AR mRNA (1.7 kilobases) was elevated in all of the colon tumors of the five patients examined (Fig. 2a, Lanes 2, 4, 6, 8, and 10). We also examined RNA isolated from carcinoma of the stomach (Fig. 2b, Lanes 2, 4, and 6) and RNA isolated from adjacent normal gastric epithelium (Fig. 2b, Lanes 1, 3, and 5) for expression of AR mRNA. In all three paired specimens examined, AR-specific mRNA (1.7 kilobases) was elevated in all tissue examined (Fig. 2c, Lanes 1, 3, 5, and 7). In comparison with AR mRNA expression, TGF-α mRNA levels were not uniformly elevated in the malignant tissue; in only three pairs (Fig. 2c, Lanes 1 to 4; Fig. 2d, Lanes 1 and 2) was TGF-α mRNA expression higher in the tumor tissue than in the normal epithelium. Examination of 6 human colon tumor cell lines (LIM 1215, SW 620, WIDR, Moser, HT29, and 1863) and a human colon adenoma cell line (VACO-
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Fig. 2. Expression of AR- and TGF-α-specific mRNA in normal human gastrointestinal epithelium and gastrointestinal carcinomas. Isolation of total and poly A* RNA from normal human gastrointestinal epithelium and adjacent tumors was performed as described in "Materials and Methods." Subsequent Northern blot analysis of AR- and TGF-α-specific mRNA was performed as described in "Materials and Methods." a, Northern blot analysis of AR-specific expression in total (Lanes 1 to 4, 20 μg/lane) and poly A* (Lanes 5 to 10, 2 μg/lane) RNA derived from biopsies of colon tumors (Lanes 2, 4, 6, 8, and 10) and adjacent normal colonic epithelium (Lanes 1, 3, 5, 7, and 9). b, Northern blot analysis of AR-specific expression in poly A* (2 μg/lane) RNA derived from biopsies of gastric tumors (Lanes 2, 4, and 6) and adjacent normal gastric epithelium (Lanes 1, 3, and 5). c, Northern blot analysis of TGF-α-specific expression in total (Lanes 3 to 4, 20 μg/lane) and poly A* (Lanes 5 to 10, 2 μg/lane) RNA derived from biopsies of colon tumors (Lanes 2, 4, 6, 8, and 10) and adjacent normal colonic epithelium (Lanes 1, 3, 5, 7, and 9). d, Northern blot analysis of TGF-α-specific expression in poly A* (2 μg/lane) RNA derived from biopsies of gastric tumors (Lanes 2, 4, and 6) and adjacent normal gastric epithelium (Lanes 1, 3, and 5). Equivalent loading of intact RNA onto gels was verified by staining gels with ethidium bromide and densitometry of 28S ribosomal RNA prior to RNA transfer (data not shown). Migration of the 18S and 28S ribosomal RNAs is indicated.

Fig. 3. Localization of AR RNA expression in histological sections of malignant colon tissue. A human colon tumor was prepared and subjected to in situ hybridization for the detection of AR-specific mRNA expression as described in "Materials and Methods." a, bright-field photomicrograph of thin section through human colon tumor. b, dark-field autoradiograph of serial thin section probed with radiolabeled cRNA corresponding to the antisense strand of human AR cDNA. Hybridization is indicated by exposed grains which appear as lightened areas on the photomicrograph. c, dark-field autoradiograph of serial thin section probed with radiolabeled cRNA corresponding to the sense strand of human AR cDNA. × 417.

330) revealed that they all expressed mRNA corresponding to AR, while the human colon tumor cell line SW 480 did not express AR-specific mRNA (data not shown).

To confirm that the AR hybridization signal visualized in our Northern blot analysis was due to AR mRNA expressed in the carcinoma and not in the surrounding stromal tissue, we examined several malignant colon tumors for AR-specific RNA expression by in situ hybridization. Fig. 3 demonstrates the results obtained from a malignant human colon tumor (same as used for Fig. 2a, Lane 4). RNA coding for AR in the colon carcinoma was localized to the epithelium and was not expressed in the stromal tissue (Fig. 3C) when compared with the nonspecific (control) hybridization observed with AR sense RNA probe (Fig. 3B). These results are in agreement with a recent study demonstrating localization of AR protein in the epithelium of human colorectal tumors (7).

Discussion

The current study demonstrates that AR mRNA levels are elevated in both benign hyperproliferative (psoriasis) and malignant (stomach and colon carcinoma) human epithelium. In colon carcinoma, AR mRNA expression is predominantly localized to the malignant epithelium. Our results concerning AR mRNA expression in colon carcinoma are in agreement with a recent study demonstrating overexpression of AR and Cripto
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in a significant percentage of primary and metastatic human colorectal carcinomas (7). The fact that we have been able to detect AR mRNA expression in all samples (both normal and tumor) when compared with the previous study may reflect the use of high-specific-activity antisense RNA probes in this study instead of the DNA probes used in the previous study. Interestingly, unlike TGF-α, AR-specific mRNA expression was elevated in all gastric (three) and colon (five) carcinomas that we examined. Due to the unavailability of AR-specific antibodies we have yet to determine whether or not the increases in AR expression can be correlated with increased expression of AR protein. AR mRNA was also expressed in a human colon adenoma cell line and 6 of 7 of the colon carcinoma cell lines that we examined. We have previously demonstrated that AR mRNA and protein are expressed by cultured human keratinocytes but not cultured fibroblasts (6). Collectively, our observations suggest that expression of AR by epithelial cells in vivo may be involved in normal homeostasis in this tissue type and that overexpression of AR could contribute to the unregulated or aberrant proliferation observed in psoriasis and cancer. Moreover, our results and those of others (7) suggest that AR overexpression could be useful as a diagnostic marker in both malignant and nonmalignant proliferative disorders of human epithelia.

A variety of studies have demonstrated the antiproliferative effects of heparin (13–16). The inhibitory activity of this compound is thought to be dependent on both the size of the polysaccharide and the level of sulfation. Interestingly, heparin and heparin-like compounds in conjunction with corticosteroids or some heparin-like compounds alone have been demonstrated to be antiangiogenic (17–20) and in some cases have also been shown to impede tumor growth (18–20). TGF-α, a molecule which is structurally similar to AR, induces angiogenesis (10). Thus, it is tempting to speculate that AR produced by tumor cells could contribute to tumor angiogenesis, and that this activity could be blocked by heparin or other heparin-like compounds. Although in most cases the mechanism by which these sulfated molecules mediate growth inhibition is not understood, we have recently shown that heparin (6) and other compounds with similar structures block the autonomous growth (i.e., proliferation in the absence of exogenous polypeptide growth factors) of human keratinocyte cultures by abrogating the growth-stimulating activity of human keratinocyte-derived AR (6). Other studies from our laboratory have revealed that heparin blocks the ability of keratinocyte-derived AR to compete with EGF for cell surface binding (6). Thus, if AR overexpression is associated with the development or progression of diseases such as psoriasis and gastrointestinal cancer, it may be possible to favorably affect these diseases with the administration of sulfated polysaccharides or related compounds. Additional investigation will be necessary to determine the extent to which AR mRNA and protein overexpression contributes to the pathobiology of these and other proliferative diseases.

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References

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