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Expression of Amphiregulin, a Novel Gene of the Epidermal Growth Factor Family, in Human Gastric Carcinomas

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The expression of mRNA for amphiregulin (AR), a novel gene of the epidermal growth factor family, was examined in 8 human gastric carcinoma cell lines and 32 gastric carcinoma tissues as well as corresponding normal mucosa. Of the 8 gastric carcinoma cell lines, 7 expressed 1.4 kb AR mRNA at various levels. The expression of AR mRNA by TMK-1 and MKN-28 cells was increased by treatment with epidermal growth factor or transforming growth factor *α*. In surgical cases, all the gastric carcinoma tissues and their adjacent normal mucosa expressed AR mRNA. Interestingly, 20 (62.5%) out of 32 tumors expressed AR mRNA at higher levels than their corresponding normal mucosas (tumor/normal≧1.2). No obvious correlation was observed between the AR mRNA levels and the histological types or tumor staging of gastric carcinoma. Immunohistochemically, AR protein was localized to the cytoplasm and/or nucleus in tumor cells. These results suggest that AR produced by tumor cells may be involved in the pathogenesis and/or progression of human gastric carcinoma.

Key words: Amphiregulin — Gastric carcinoma — Growth factor

Human gastric carcinomas express a variety of growth factors and their receptors that regulate the growth of cancer cells.[1-3] We have previously reported that EGF[5] and TGF-*α* produced by tumor cells act as autocrine growth factors and synchronous expression of EGF, TGF-*α* and EGFR evidently contributed to the biological malignancy of gastric carcinoma.[4-6] Recently, several new members of the EGF family such as *cripto*,[7] AR[8] and heparin-binding EGF-like growth factor (HB-EGF),[9] have been identified. Except for *cripto*, all of these factors bind to EGFR and stimulate autophosphorylation.[9-12]

AR is a glycosylated, 84-amino-acid polypeptide originally purified from the conditioned medium of the human breast carcinoma cell line, MCF-7, after treatment with phorbol 12-myristate 13-acetate.[8] The mature AR protein is believed to be derived from the proteolytic cleavage of a 252-amino-acid transmembrane precursor.[13] AR shares significant structural and functional homology with EGF and TGF-*α*.[11] AR stimulates or inhibits the proliferation of cells depending on the concentration of AR and the nature of the target cell.[8,14] It has recently been reported that AR acts as an autocrine growth factor for a colon carcinoma cell line.[15] AR has been detected in a variety of human carcinoma cell lines such as breast, ovary, pancreas and colon carcinoma.[8,13,15]

In the present work, we have studied the expression of AR mRNA and protein in human gastric carcinoma cell lines and gastric carcinoma tissues by Northern blot analysis and immunohistochemistry.

MATERIALS AND METHODS

Cell cultures and tumor tissues Eight cell lines established from human gastric carcinomas were maintained in RPMI-1640 (Nissui Co., Ltd., Tokyo) with 10% fetal bovine serum (Whittaker M.A. Bioproducts Inc., Walkersville, MD). TMK-1 cell line (poorly differentiated adenocarcinoma) was established in our laboratory.[16] HSC-39 and KATO-III cell lines which were established from signet ring cell carcinoma were kindly provided by Dr. K. Yanagihara (Hiroshima University, Hiroshima)[17] and Dr. M. Sekiguchi (University of Tokyo, Tokyo), respectively. The other five gastric carcinoma cell lines (MKN-1, adenosquamous carcinoma; MKN-7, MKN-28 and MKN-74, well differentiated adenocarcinoma; MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima). A431 and HeLa cells were also used.

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[5] The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGF-*α*, transforming growth factor alpha; AR, amphiregulin; cDNA, complementary DNA; kb, kilobase; T/N ratio, tumor/normal ratio; poly(A)$^+$, polyadenylated; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

A total of 32 cases of gastric carcinoma were examined. Tumor tissues and their corresponding normal mucosa were surgically removed, frozen immediately thereafter in liquid nitrogen and stored at −80°C until use. We confirmed microscopically that the tumor tissue specimen consisted mainly of carcinoma tissue and that non-neoplastic mucosa did not exhibit any tumor cell invasion or show significant inflammatory involvement. The definition of stage grouping and the histological classification were made according to the criteria of the Japanese Research Society for Gastric Cancer.

Treatment with EGF and TGF-*α* TMK-1 and MKN-28 cells were used in this experiment. They were grown to subconfluence in the above-mentioned medium. After 24 h of serum starvation, 1 n*M* EGF or TGF-*α* was added. Human recombinant EGF and TGF-*α* were kindly provided by Wakunaga Pharm. Co., Hiroshima. The cells were harvested at 0 (control) time, 1 h, 3 h, 12 h and 24 h after the treatment and RNAs were isolated.

RNA preparation and Northern blot analysis RNAs were extracted by the standard guanidinium isothiocyanate-cesium chloride method.[18] Ten μg of poly(A)$^+$ selected RNA was electrophoresed on 1.0% agarose/formaldehyde gels and blotted onto nylon filter membranes. Filters were baked for 2 h at 80°C under vacuum. Hybridization, washing and autoradiography were performed as described previously.[19] The 1.1 kb *Eco*RI fragment of pAR9, which contains the human AR cDNA, was used as the AR probe (kindly provided by Dr. G. Plowman).[13] The β-actin DNA probe was purchased from Oncor Inc., Gaithersburg, MD.

Immunocytochemistry Cultured cells were plated into Lab-Tek 4 chamber slides (Nunc, Inc., Naperville, IL) at 2×10^4 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 and were treated with 0.5% Triton X-100 in PBS for 5 min at room temperature to enhance the staining of AR in the nucleus of cells.[14] After blocking of non-specific binding with 10% goat whole serum, the primary reaction was run with 0.5 ml of 10 μg/ml anti-AR antibody, AR-Abl IgG[15] or preimmune (control) IgG in 0.1% BSA/PBS. Immunospecificity of AR-Abl IgG was confirmed by preabsorption with the peptide antigen.[14, 15] After 3 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).

Deparaffinized sections (4 μm) of formalin-fixed tissues were immersed in methanol containing 0.03% hydrogen peroxide for 30 min to block the endogenous peroxidase activities and were incubated with non-immunized goat serum for 1 h to block the non-specific Fc-receptor in tissue. The slides were processed exactly as above. The sections were counter-stained with 3% methyl green. For negative controls, non-immune rabbit IgG was utilized in place of primary antibody. The control slides were invariably negative for immunostaining.

RESULTS

Amphiregulin mRNA levels in gastric carcinoma cell lines and surgical specimens The results of Northern blot analysis for AR in human gastric carcinoma cell lines are shown in Fig. 1. Of the 8 gastric carcinoma cell lines, 7 expressed a 1.4 kb AR mRNA at various levels. KATO-III, MKN-7, MKN-28 and MKN-74 cells expressed AR mRNA at extremely high levels, while TMK-1 cells expressed AR mRNA at low levels. AR mRNA was not detected in MKN-1.

We next examined the level of AR mRNA in human gastric carcinoma tissues and corresponding normal mucosa. The expression of AR and the clinicopathological findings of 32 human gastric carcinomas are summarized in Table I. Representative autoradiographs of Northern blot analyses for AR are shown in Fig. 2. The 1.4 kb AR transcript was detected not only in the tumor tissues but also in the corresponding normal mucosa. AR mRNA was expressed in the gastric carcinoma tissues at various levels. In 20 of the 32 cases (62.5%), the level of AR mRNA in the tumor tissue was higher than that in the corresponding normal mucosa (Table II). In 8 cases, the level of AR mRNA in carcinoma tissues was significantly higher than that in normal mucosas, the T/N ratio being more than 5.0. No relationship was found between the AR mRNA levels and the histological types or tumor staging of the gastric carcinomas.

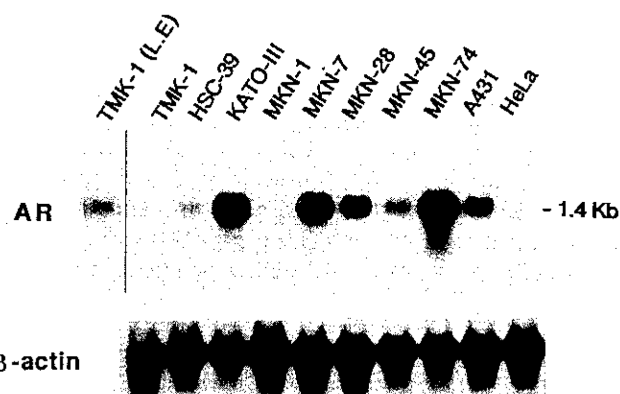

Fig. 1. Expression of mRNA for amphiregulin in gastric carcinoma cell lines. Ten μg of poly(A)$^+$-selected RNA was subjected to Northern blot analysis. A β-actin probe was applied as an internal control. L.E; long exposure.

Table I. Clinical Features and Amphiregulin mRNA Levels in 32 Primary Human Gastric Carcinomas

Case No.	Sex	Age	Histology[a]	Stage[a]	T/N ratio[b]	
					AR	β-actin
216	F	71	sci	III	5.6	1.0
218	M	63	sci	III	0.5	1.0
219	F	27	sci	IV	1.1	1.0
220	F	63	sci	III	0.4	1.1
226	M	59	well	I	0.7	0.9
243	M	79	poor	I	6.4	0.9
244	M	72	well	II	3.6	1.1
245	M	51	well	II	1.7	1.0
246	M	44	well	II	3.0	1.0
247	F	48	sci	III	2.0	1.0
248	M	73	well	II	6.0	0.9
249	F	45	poor	IV	0.5	1.0
289	F	79	poor	IV	0.8	1.1
291	M	62	poor	III	0.9	1.2
292	F	72	poor	II	0.6	1.1
294	F	37	sci	IV	5.4	0.9
326	M	74	sci	IV	2.2	0.9
327	M	50	well	III	5.0	1.0
332	M	75	poor	II	1.3	1.0
333	F	63	poor	II	0.8	1.0
334	F	71	sci	III	3.1	1.1
335	M	58	poor	II	1.5	0.8
339	M	62	well	III	0.8	1.0
340	F	73	poor	IV	11.7	1.1
501	M	60	poor	II	1.9	1.1
504	M	53	well	III	2.8	1.0
506	F	38	poor	I	10.6	1.1
507	F	62	poor	III	3.7	1.0
508	M	58	poor	IV	5.2	1.1
509	F	63	poor	II	1.2	1.0
510	M	57	sci	III	0.5	0.9
514	M	50	poor	IV	0.4	1.0

a) According to the criteria of the Japanese Research Society for Gastric Cancer (1985). Well, well differentiated adenocarcinoma including papillary and tubular adenocarcinoma; poor, poorly differentiated adenocarcinoma including signet ring cell carcinoma and mucinous adenocarcinoma; sci, scirrhous gastric carcinoma.
b) The ratio of densitometric measurements of autoradiographic signals from Northern hybridization in gastric carcinoma tissues (T) and corresponding normal tissues (N).

Table II. Relationship between Expression of Amphiregulin and Histological Type of 32 Gastric Carcinomas

Histological type[a]	Number of cases	Levels of mRNA expression[b]		
		T>N	T=N	T<N
Well	8	6 (75.0%)	0	2 (25.0%)
Poor	15	9 (60.0%)	1 (6.7%)	5 (33.3%)
Sci	9	5 (55.5%)	1 (11.1%)	3 (33.3%)
Total	32	20 (62.5%)	2 (6.3%)	10 (31.3%)

a) See footnote to Table I.
b) According to relative expression of AR gene (see Table I). T; gastric carcinoma tissues, N; corresponding normal tissues. T>N, more than 1.2; T<N, less than 0.8.

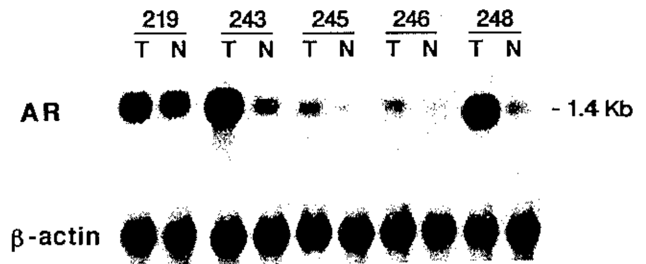

Fig. 2. Expression of mRNA for amphiregulin in human gastric carcinomas. Ten μg of poly(A)$^+$-selected RNA was analyzed as in Fig. 1. The signal intensity was measured by densitometric scanning. Numbers above the lanes are case numbers. T stands for tumor tissue and N, corresponding normal mucosa. A β-actin probe was applied as an internal control.

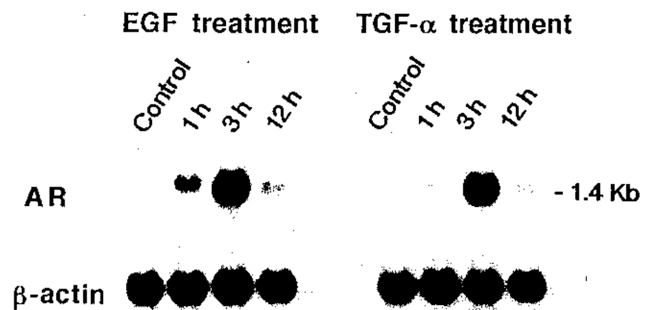

Fig. 3. Induction of amphiregulin mRNA expression in TMK-1 cells by EGF and TGF-α. Poly(A)$^+$ RNA was prepared from the cells after treatment with 1 nM EGF or 1 nM TGF-α for the indicated periods and analyzed as in Fig. 1. A β-actin probe was applied as an internal control.

Southern blot analysis was performed to detect potential alterations in the AR gene. Neither gene amplification nor rearrangement was observed in the gastric carcinoma cell lines or the surgical specimens examined (data not shown).

Induction of amphiregulin mRNA by EGF and TGF-α EGF and TGF-α induce the expression of various growth factor/receptor genes by gastric carcinoma cells.[20,21] Therefore, we studied the effect of EGF and TGF-α on the expression of AR mRNA. The time course of AR induction was determined by Northern blot analysis of AR mRNA derived from TMK-1 and MKN-28 cells. Both EGF and TGF-α induced the expression of AR mRNA by TMK-1 cells and maximal levels of AR mRNA were detected 3 h after the treatment (Fig. 3). The same result was observed in MKN-28 cells (data not shown).

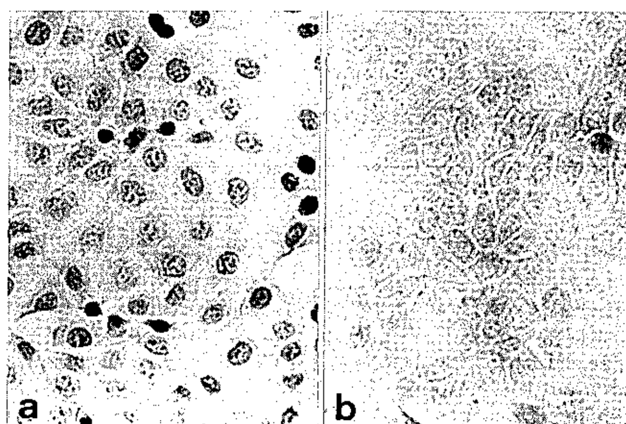

Fig. 4. Amphiregulin immunoreactivity in gastric carcinoma cells. Amphiregulin was detected in the nucleus of MKN-28 cells. The cells were exposed to either 10 μg/ml of AR-Ab1 (a) or preimmune control IgG (b). ×280.

Fig. 5. Amphiregulin immunoreactivity in human gastric carcinoma tissues. Amphiregulin was detected in the cytoplasm and nucleus of many tumor cells. Staining of a well differentiated adenocarcinoma of stomach (Case 246) is shown using the AR-Ab1 IgG. ×280.

Immunolocalization of amphiregulin in gastric carcinoma cell lines and tissues In order to confirm the expression of AR protein in cancer cells, immunocytochemistry was performed on gastric carcinoma cell lines and gastric carcinoma tissues. AR protein was detected in the nucleus of MKN-28 cells, which express AR mRNA at high levels (Fig. 4). However, no immunoreactivity was found in MKN-1 cells, which do not express AR mRNA (data not shown). In gastric carcinoma tissues, cytoplasmic and nuclear immunoreactivities were observed in variable numbers of cancer cells in all 4 cases tested (Case No. 219, 243, 245, 246) (Fig. 5). AR was also localized in normal epithelial cells.

DISCUSSION

Most gastric carcinomas overexpress EGF, TGF-α and EGFR and their expression is well correlated with biological malignancy.[4-6] Expression of *cripto* correlates with the degree of intestinal metaplasia of the stomach, and most well differentiated adenocarcinomas express *cripto* at higher levels than normal mucosa.[22] Therefore, EGF-related peptides appear to be major regulators of the growth and differentiation of gastric carcinomas.

In the present study, expression of AR, a new member of the EGF family, was examined in gastric carcinomas. AR mRNA was detected in 7 of the 8 gastric carcinoma cell lines at various levels. In the gastric carcinoma tissues, 62.5% of tumors exhibited higher levels of AR mRNA than the corresponding normal mucosa. Immunocytochemically, AR protein was localized in the nucleus and/or cytoplasm in tumor cells. Expression of AR mRNA has previously been detected in approximately 50% of primary human colorectal tumors but in only 15% of adjacent noninvolved colon mucosa.[23] Furthermore, AR protein has been detected by immunohistochemistry in approximately 50% of human colon carcinomas, whereas only 15% of adjacent noninvolved mucosa expressed AR.[24] It has been also reported that AR mRNA expression was markedly elevated in biopsy specimens derived from five colon carcinomas and three gastric carcinomas compared with the adjacent noninvolved mucosa.[25] Therefore, AR may participate in the development of gastric as well as colonic carcinomas. To elucidate the role of AR in the progression of gastric carcinoma, the expression of AR should be examined in a larger number of cases at various tumor stages.

AR immunoreactivity was localized in not only tumor cells but also normal epithelial cells of stomach. AR mRNA was detected in the normal gastric mucosa at various levels (Fig. 2). Although we can not explain the variation of AR mRNA levels in normal gastric mucosa at the present time, it might be related to the degree of hyperplasia and regeneration of gastric mucosa.

AR acts by binding to the extracellular domain of the EGFR and activating the EGFR tyrosine kinase.[12] In addition, the interaction of AR with the EGFR can result in the activation of the p185^{erbB2} receptor tyrosine kinase.[12] A recent study has shown that AR acts as an autocrine growth factor through the EGFR in a colon carcinoma cell line.[15] Namely, Geo colon carcinoma cells, which are derived from a well differentiated adenocarcinoma, secrete AR and the cell growth was stimulated by the addition of exogenous AR and in-

hibited by treatment with an anti-AR antibody or an anti-EGFR antibody. Since most gastric carcinoma cell lines express both AR and EGFR mRNA,[4] it is possible that AR may act as an autocrine growth factor for gastric carcinoma cell lines, as do EGF and TGF-α.

While AR acts through the EGFR at the surface of epithelial cells, the present study and previous reports[14, 15] demonstrated that AR can be detected in the nucleus of epithelial cells *in vitro* and *in vivo*. This staining pattern is similar to that of basic fibroblast growth factor,[26, 27] which has nuclear sites of action.[28] AR precursor contains both a signal peptide[13] and two putative nuclear targetting sequences positioned at residues 26–29 and 40–43.[11] It is not known whether AR is targeted directly to the nucleus after biosynthesis or is secreted and endocytosed via EGFRs. It would be of great interest to elucidate the biological function of AR in the nucleus of cells.

Although no gross alteration of the AR gene was detected by Southern blot analysis (data not shown), 62.5% of the gastric carcinoma tissues overexpress AR mRNA compared with their corresponding normal mucosa. The present study demonstrated that exogenous EGF and TGF-α increased AR expression by TMK-1 and MKN-28 cells. Since gastric carcinoma cells express EGF and TGF-α,[4-6] the endogenous production of these growth factors might result in the constitutive overexpression of AR in tumor cells via an autocrine mechanism. Aberrant transcriptional regulation of the AR gene may also contribute to the overexpression of AR in gastric carcinomas. The promoter region of the AR gene contains an Spl consensus sequence and a cAMP responsive element (CRE).[13] Recently, we have demonstrated that the level of the transcription factor, Spl, in carcinoma tissues is higher than that in normal mucosa of the stomach.[29] Therefore, Spl might be responsible for the differential expression of AR in carcinomas and normal mucosa.

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