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Gastrointestinal hormones stimulate growth of Foregut Neuroendocrine Tumors by transactivating the EGF receptor☆

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ABSTRACT

Foregut neuroendocrine tumors [NETs] usually pursuit a benign course, but some show aggressive behavior. The treatment of patients with advanced NETs is marginally effective and new approaches are needed. In other tumors, transactivation of the EGF receptor (EGFR) by growth factors, gastrointestinal (GI) hormones and lipids can stimulate growth, which has led to new treatments. Recent studies show a direct correlation between NET malignancy and EGFR expression, EGFR inhibition decreases basal NET growth and an autocrine growth effect exerted by GI hormones, for some NETs. To determine if GI hormones can stimulate NET growth by inducing transactivation of EGFR, we examined the ability of EGF, TGFα and various GI hormones to stimulate growth of the human foregut carcinoid,BON, the somatostatinoma QGP-1 and the rat islet tumor, Rin-14B-cell lines. The EGFR tyrosine-kinase inhibitor, AG1478 strongly inhibited EGF and the GI hormones stimulated cell growth, both in BON and QGP-1 cells. In all the three neuroendocrine cell lines studied, we found EGF, TGFα and the other growth-stimulating GI hormones increased Tyr^{1068} EGFR phosphorylation. In BON cells, both the GI hormones neurotensin and a bombesin analogue caused a time- and dose-dependent increase in EGFR phosphorylation, which was strongly inhibited by AG1478. Moreover, we found this stimulated phosphorylation was dependent on Src kinases, PKCs, matrix metalloproteinase activation and the generation of reactive oxygen species. These results raise the possibility that disruption of this signaling cascade by either EGFR inhibition alone or combined with receptor antagonists may be a novel therapeutic approach for treatment of foregut NETs/PETs.

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1. Introduction

Foregut neuroendocrine tumors (NETs)/pancreatic endocrine tumors (PETs) originate from the neuroendocrine cells of the diffuse endocrine system of the stomach, lung, first part of duodenum and pancreas [1,2]. Although these tumors have been considered rare, their incidence is increasing [2]. They are classified either on a clinical basis, depending on the presence of hormone hypersecretion (functioning vs. non-functioning), their grade of differentiation (well vs. poorly differentiated) or location of the primary lesion [2]. Overall, foregut NETs/PETs display a common secretory system and physiological response (production and/or secretion of neural/hormonal regulators), with a heterogeneous clinical presentation at diagnosis [3,4]. Although in some cases they present with a hypersecretion syndrome and show a relative slow growth rate, a subset show more aggressive growth. At present 60–80% of cases are diagnosed at advanced stages, with metastases [1,3]. The only effective approach is surgical resection, but it is possible in only 10–20% [3,4]. Other treatments for patients with advanced foregut NETs/PETs include the use of kinase inhibitors, peptide-radioligand therapies, biotherapy (interferon,somatostatin and chemotherapy) [1,2,5,6], but in most cases the survival rates remain low [1]. For this reason there is a need for new therapeutic approaches [5–7].

Growth factors and their receptors play an important role in controlling cell growth, differentiation and migration and this represents a key component in tumor development and growth [7,8]. Epidermal growth factor (EGF) and its receptor (EGFR) are frequently overexpressed by tumors [8]; including foregut NETs/PETs [9–12], especially in the most aggressive forms. *In vitro* studies on carcinoids and PET cell lines demonstrate that activation of the EGFR receptor can stimulate stimulate growth and that its inhibition can inhibit basal growth [11,13,14].

Abbreviations: Bn, bombesin; EGFR, epidermal growth factor receptor; GI, gastrointestinal; GPCR, G protein coupled receptor; HNSCC, head and neck squamous cell cancers; MMP, matrix metalloproteinases; NETs, neuroendocrine tumors; NT, neurotensin; PETs, pancreatic endocrine tumors; PACAP, pituitary adenylate cyclase activating peptide; PKC, protein kinase C; ROS, reactive oxygen species; TGFα, Transforming growth factor alpha; VIP, vasoactive intestinal peptide

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A frequent mechanism in tumors in controlling their growth is transactivation of EGFR by the activation of G protein-coupled receptors (GPCRs), generally by gastrointestinal (GI) hormones/neurotransmitters or other biologically active substances, such as prostaglandins and lipids [15–17]. The abnormal secretion, either autocrine or paracrine of these substances in tumors, leads to the activation of GPCRs, which, in turn, transactivate EGFR [15,16,18]. This occurs in a number of cancers [head/neck squamous cell carcinoma (HNSCC) [16], lung, prostate, breast, colon] as well various endocrine cancers and those of other tissues [15,17–21]. Among GI hormones/neurotransmitters, neurotensin, bombesin-related peptides, bradykinin, gastrin and PACAP are known to play an important role in controlling tumor growth by transactivation of EGFR in a number of cancers [17–23].

It is reported that a number of GI hormones/neurotransmitters can affect the growth of neuroendocrine tumors [7,24]. Even though it has been shown that activaton of EGFR can alter the basal growth of NETs [11,13,14], it is unknown whether EGFR-transactivation is involved in the stimulation by GI hormones/neurotransmitters of their growth or if so, the cellular mechanisms involved.

Therefore the aim of this study was to address these issues by investigating the ability of GI hormones/neurotransmitters to stimulate the growth of various foregut neuroendocrine tumor cell lines and determining whether transactivation of EGFR was important in mediating this growth effect and if so, define the celluar signaling cascades involved.

2. Materials and methods

2.1. Materials

Dulbecco's minimum essential medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS), were from Invitrogen (Carlsbad, CA); Thymidine, [6-^{3}H], >97%, 1 mCi (37 MBq) from Perkin Elmer (Boston, MA); Tris buffered saline (TBS) from Cellgro® (Mediatech Inc. Manassas, Va); Dimethyl sulfoxide (DMSO), trichloroacetic Acid (TCA), MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DL-dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), Tiron and N-Acetyl-cysteine were from Sigma–Aldrich (St. Louis, MO); Human EGF (Recombinant, E. coli), PACAP27, the tyrosine kinase inhibitor AG178, the Src family kinases inhibitor PP2 and PP3, GF 109203X and GM 6001 were from Calbiochem (San Diego, Ca). TGFα BioVision (Mountain View, Ca), neurotensin and bradykinin were from Bachem (Torrence, CA); the bombesin analogue, [DTyr^6, βAla^{11},Phe^{13},Nle^{14}]Bn [(6–14)] obtained from Dr. David H. Coy, Tulane University (New Orleans, La); protease inhibitor tablets were from Roche (Basel, Switzerland); α-tubulin and phospho-EGF receptor (Tyr^{1068}) antibodies and non-fat dry milk were from Cell Signaling Technology, Inc. (Beverly, MA) and horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit) and Supersignal Western Pico/Dura were from Thermo Scientific (Rockford, IL).

2.2. Methods

2.2.1. Cell culture

The metastatic human pancreatic carcinoid cell line, BON was obtained from Cancer Research UK Cell Services and grown in DMEM/F12K (1:1), with 10% FBS and supplemented with penicillin/streptomycin. The human somatostatinoma cell line, QGP-1, was obtained from Cancer Research UK Cell Services and the rat islet cell tumor, Rin-14B was obtained from ATCC (Manassas, VA). Both cell lines were grown in RPMI with 10% FBS and supplemented with penicillin/streptomycin. The cells were mycoplasma free and incubated at 37 °C in 5% CO_2/95% air.

2.2.2. Western blotting

Briefly, after adding lysis buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium azide, 1 mM EGTA, 0.4 mM EDTA, 1 mM DTT, 0.4 mM sodium orthovanadate, 1 mM PMSF, and one protease inhibitor tablet per 10 ml], lysates were sonicated, centrifuged at 13,000 rpm for 20 min at 4 °C and protein concentration was measured using the Bio-Rad protein assay reagent (Hercules, CA). Equal amounts of protein from lysates were loaded on to SDS-PAGE using 4–20% Tris-Glycine gels Invitrogen (Carlsbad, Ca). After electrophoresis, proteins were transferred to nitrocellulose membranes overnight. Membranes were washed twice in washing buffer (TBS plus 0.1% Tween®20), at room temperature for 1 h, and then incubated with primary antibody at 1:1000 dilution in washing buffer + 5% BSA [Phospho-EGFR (Tyr^{1068}) or tubulin, as loading control] overnight at 4 °C. Then, membranes were washed twice in blocking buffer (TBS, 0.1% Tween® 20, 5% non-fat dry milk) for 4 min and then incubated with HRP-conjugated secondary antibody (anti-rabbit) for 1 h at room temperature. Membranes were washed again twice in blocking buffer for 4 min, and then twice in washing buffer for 4 min followed by incubation with chemiluminescence detection reagents for 4 min and finally were exposed to Kodak Biomax film (MR, MS). The intensity of the protein bands was measured using Kodak ID Image Analysis.

2.2.3. [^{3}H]-Thymidine uptake

BON (1.5×10^5 cells/well) and QGP-1 (4×10^4 cells/well) cells were plated in 24-well plates in DMEM/F12K with 10% FBS or RPMI with 10% FBS, respectively. After 24 h cells were washed twice with PBS and starved in DMEM/F12K or RPMI without FBS, for additional 24 h. Then, peptides and hormones were added for 24 h. Six hours before the end of the incubation 1 μCi/ml of [^{3}H]-Thymidine was added to each well. Then cells were washed 3 times with 1 ml iced cold PBS and incubated with 1 ml of ice cold 5% TCA at 4 °C for 30 min. After that, TCA was aspirated and cells were washed twice with iced cold PBS. Finally, cells were resuspended in 0.5 ml of 0.5 N NaOH/0.5% SDS and placed in a scintillation vial and counted in a scintillation counter.

2.2.4. MTT assay

5×10^3 BON cells were seeded in 96-well plates and incubated for 24 h in 100 ml of DMEM/F12K with 10% FBS. Then, cells were washed twice in PBS and the starvation media (DMEM/F12K) with peptides and hormones were added for 24 h. Three hours before the end of the treatments 10 ml of MTT (1.2 mM, final concentration) was added to each well; after 3 h, 50 ml of 0.04 N HCl in isopropanol was added to each well and incubated at room temperature for 5 min. The plate was read at 595 nm using a microplate reader.

2.2.5. Statistical analysis

All results are expressed as mean ± SEM from at least 3 experiments, and results were considered significant if, in a paired t-Test and in one-way ANOVA (Dunnett's multiple tests, as a post test), p was <0.05. PRISM GraphPad software (GraphPad Software Inc., La Jolla, CA) was used for all statistical analysis.

3. Results

3.1. Ability of EGF, TGFα or various GI hormones to stimulate growth of BON and QGP-1 cells and effect of an EGFR inhibitor, AG1478

To assess the effect of GI hormones on BON cell line growth, we performed two different assays, the MTT assay (Fig. 1A) and an assessment of [^{3}H]-Thymidine uptake (Fig. 1B). EGF and TGFα stimulated BON cells growth by 30% and 40% in the MTT assay, respectively. With [^{3}H]-Thymidine assessment, growth stimulated by EGF or TGFα was 32% and 42%, and was 180% with the addition of 10% fetal bovine serum. With both experimental approaches, each GI hormone/neurotransmitter [a bombesin analogue (Bn-analogue),