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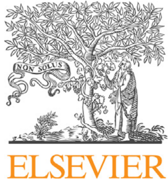

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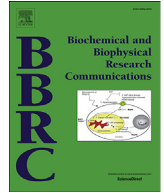

Connective tissue growth factor is activated by gastrin and involved in gastrin-induced migration and invasion

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A B S T R A C T

Connective tissue growth factor (CTGF) has been reported in gastric adenocarcinoma and in carcinoid tumors. The aim of this study was to explore a possible link between CTGF and gastrin in gastric epithelial cells and to study the role of CTGF in gastrin induced migration and invasion of AGS-G_R cells. The effects of gastrin were studied using RT-qPCR, Western blot and assays for migration and invasion. We report an association between serum gastrin concentrations and CTGF abundancy in the gastric corpus mucosa of hypergastrinemic subjects and mice. We found a higher expression of CTGF in gastric mucosa tissue adjacent to tumor compared to normal control tissue. We showed that gastrin induced expression of *CTGF* in gastric epithelial AGS-G_R cells via MEK, PKC and PKB/AKT pathways. CTGF inhibited gastrin induced migration and invasion of AGS-G_R cells. We conclude that CTGF expression is stimulated by gastrin and involved in remodeling of the gastric epithelium.

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1. Introduction

The CCN proteins are a family of matricellular proteins involved in many physiological and pathological processes, including carcinogenesis. The connective tissue growth factor (CTGF), also known as CCN2, is believed to interact with a wide range of molecules by virtue of its multi-domain structure. Such interactions enable CTGF to modulate and integrate different biological cues into a cognate biological response. CTGF is a prominent mediator of transforming growth factor β (TGFβ) signalling [1]. It interacts with components of extracellular matrix (ECM) and various cell surface receptors regulating cell and cell-matrix signalling [1]. CTGF is involved in tuning of cell adhesion, migration, extracellular matrix production, survival and tumorigenesis [2]. Furthermore, CTGF promotes migration of many mesenchymal cell types and has been identified in a variety of tumors, including gastric cancer [2–4].

The gastric hormone gastrin is well known for its role in stimulating gastric acid secretion and organization of the gastric mucosa by promoting epithelial cell proliferation, migration and invasion [5]. Gastrin is identified to regulate expression of molecules that exert important effects on extracellular matrix remodelling, including plasminogen activator inhibitors (PAIs) [6] and matrix metalloproteinases (MMPs) [7].

CTGF has been suggested to be involved in the neoplastic transformation of gastrointestinal neuroendocrine cells and has been reported in carcinoids [8,9]. Here we have assessed the hypothesis that gastrin regulates gastric CTGF expression and explored a potential role of CTGF in gastrin induced migration and invasion of gastric epithelial cells.

2. Materials and methods

Cells, plasmids and reagents. AGS-$G_R$cells were maintained as previously described [10]. CTGF expression vector was obtained from Open Biosystems (Thermo Scientific, Rockford, IL, USA) and empty pCMV vector from Addgene (Cambridge, UK). siRNA for CTGF, STAT3 and control siRNA were respectively obtained from Invitrogen (Carlsbad, CA, USA), Qiagen (Germantown, MD, USA) and Ambion (Austin, TX, USA). Human recombinant CTGF was

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obtained from Peprotech (London, UK). Gastrin (G-17) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The inhibitors Ro 31-8220 (3-[1-[3-(Amidinothio) propyl]-1H-indol-3-yl] -3-(1-methyl-1H-indol-3-yl) maleimide, Bisindolylmaleimide IX, Methanesulfonate) and AKT inhibitor XVIII, SC 66 ((2E, 6E)-2, 6-bis (4-Pyridylmethylene) cyclohexanone) were obtained from Millipore (Merck KGaA, Darmstadt, Germany). BIRB 796 (Doramapimod), PD184352 (C17H14ClF2IN2O2) and SB203580 (C21H16FN3OS) were obtained from Axon Medchem (Groningen, NL).

Patients and mice. Four subjects with normal (24.3 ± 2.2 pM) and four patients with high (67 ± 12.7 pM) plasma gastrin concentrations were selected from a cohort, aged 18 and over, who had clinical indications for undergoing upper gastrointestinal endoscopy. The study was approved by the Liverpool Local Research Ethics Committee and by the Royal Liverpool and Broadgreen University Hospitals NHS Trust and all patients gave written, informed consent. Corpus biopsies were collected in RIPA buffer and extracts were prepared as previously described [11]. In addition, four INS-Gas mice with elevated plasma gastrin (348.8 ± 184.1 pM) and FYB/N wild type controls with normal plasma gastrin (73.5 ± 12.8 pM) concentrations were maintained in an appropriately controlled environment with a 12-12 h light/dark cycle and were fed a commercial pellet diet with water *ad libitum* as previously described [7] [12]. Animals were killed by increasing CO_2 concentration. Gastric corpus extracts were prepared from unfasted animals in RIPA buffer as previously described [11]. All animal experiments were approved by the University of Liverpool Animal Welfare Committee, and were conducted in compliance with Home Office requirements and the UK Animals (Scientific Procedures) Act 1986.

The human materials used for microarray gene expression analysis were biopsies from gastric adenocarcinoma (n = 61) and adjacent (non-tumor) mucosa (n = 21) from patients, and age-sex matched normal mucosa form healthy individuals (n = 16), collected at St. Olavs Hospital, Trondheim, Norway. The patients gave written informed consent and the study was approved by the Regional Medical Research Ethics Committee of Central Norway (Approval No. 018-02). Samples were kept frozen at −80 °C until further processing.

Gastrin radioimmunoassay. Mouse plasma and human serum samples were assayed for total amidated gastrin concentrations by radioimmunoassay using antibody L2 (which reacts with G-17 and G-34 but not progastrin or Gly-gastrins) and ^{125}I-G-17 as previously described [13].

Immunohistochemistry. Tissue sections were deparaffinised, rehydrated, quenched for endogenous peroxidase before antigen retrieval in boiling citrate buffer. The sections were incubated with mouse anti-CgA (M0869, Dako, Glostrup, Denmark) (1:1500) or goat anti-CTGF (sc-14939, Santa Cruz Biotechnology, Dallas, TX, USA) (1:200) overnight at 4 °C. Reactions were visualized using room temperature incubation with the rabbit/mouse EnVision-HRP/DAB + kit (K5007, Dako), or biotinylated anti-goat (BA-5000, Vector Laboratories, Birlingame, CA, USA) (1:150) for 1 h, ABC reagent (PK-6100, Vectastain Elite ABC Kit (Standard), Vector Laboratories) for 30 min, and DAB chromogen (K5007, Dako). Hematoxylin was used as counterstain. Serial staining was done on the shared surface of neighbouring sections.

RNA extraction. AGS-G_R cells (2×10^5) were plated in 6-well plates in complete medium. After 48 h, cells were treated with G-17 (5 nM, 1 h) in serumfree medium. In the experiments with kinase inhibitors, cells were pretreated with inhibitors for 1 h prior to G-17 treatment. RNeasy Mini kit (Qiagen, Germantown, MD, USA) was used for total RNA extraction, following instructions provided by the manufacturer. Assessment of RNA integrity, quality and quantity were performed with a Nanodrop spectrophotometer (Nanodrop Technologies, Rockland, DE, USA).

Total RNA from the frozen stomach samples was isolated and purified using an Ultra-Turrax rotating-knife homogenizer and the mirVana miRNA Isolation Kit (Ambion, Thermo Fisher Scientific Inc., DE, USA) according to the manufacturer's instructions.

cDNA synthesis and quantitative real-time PCR (RT-qPCR). Aliquots of 1 μg of total RNA were reverse transcribed using Transcriptor First strand cDNA synthesis Kit (Roche Applied Science, Mannheim, Germany). cDNAs were amplified with the FastStart Universal SYBR Green Master (Rox) (Roche). The thermal cycling program used was: 5 min at 95 °C, then 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C followed by melting curve analysis. Relative *CTGF* mRNA abundance was calculated by $2^{-\Delta\Delta C_T}$ method [14]. All PCR reactions were performed in triplicates on StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and normalized by comparison to β-actin. Primer and probe sequences are shown in Suppl. Table 1.

Microarray gene expression analysis. cRNA was prepared with Ambion's Illumina® TotalPrep™-96 RNA Amplification Kit, using 300 ng total as input material. For each sample, the biotin-labelled cRNA concentrations were checked (NanoDrop, Thermo Fisher Scientific) and 750 ng hybridized to HumanHT-12 Expression BeadChips (Illumina, CA, USA). Only probes abiding to the Illumina detection P value of 0.01 in at least one sample were included in further analyses, using the limma (v. 3.12.1) Bioconductor package [15] for paired t-tests. A false discovery rate adjusted P value < 0.05 was taken as significant. The microarray data are available from ArrayExpress (accession no. E-MTAB-1338).

Western blot. For the patient samples, western blot analysis was performed as described in Ref. [7] using antibodies to CTGF (Santa Cruz Biotechnology). AGS-G_R cells (2×10^5 or 3×10^5/well) were plated in 6-well plates and grown for 48 h. After G-17 (5 nM, 1 h) stimulation, the cells were harvested in 200 μl RIPA (Pierce, Thermo Scientific). Proteins were resolved using NuPAGE Novex 10% Bis-Tris Gels (Invitrogen), transferred to polyvinylidene difluride (PVDF) membrane (Millipore, Merck KGaA, Darmstadt, Germany) and probed with anti-CTGF (1:400) (Santa Cruz Biotechnology) followed by HRP-conjugated secondary antibody, detected by Super-Signal® West Femto Maximum Sensitivity Substrate (Pierce, Thermo Scientific) and visualized with Odyssey FC Dual Mode imaging system (LI-COR, Lincoln, NE, USA). The blots were quantified with Image studio version 3.1 (LI-COR, Lincoln). Blots were reprobed for β-actin to normalise for protein loading.

Transient transfection. AGS-G_R cells (3.5×10^5/well) were plated in 6-well plates and transfected after 24 h with 2.5 μg of plasmid (pCMV-CTGF or control pCMV) or siRNA and 12.5 μl Metafectene PRO transfection reagent (Biontex Laboratories GmbH, Martinsried, Germany) per well.

Migration and invasion assays. Biocoat Cell Culture Inserts (migration assays) or Matrigel-coated inserts (invasion assays) (BD Biosciences, Bedford, MA, USA) were performed according to the manufacturer's instructions and as described in Ref. [6].

Statistical analysis. Results are presented as means ± SEM. Comparisons between data were made using paired Students *t*-test and were considered significant at P < 0.05.

3. Results

3.1. Gastric CTGF is increased in hypergastrinemia and in gastric adenocarcinomas

To investigate if gastric mucosal CTGF is associated with elevated plasma gastrin, gastric corpus biopsies selected from individuals with either low or high gastrin were processed for detection of CTGF by Western blot. In both cases, there was a major

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