Gastrin-stimulated gastric epithelial cell invasion: the role and mechanism of increased matrix metalloproteinase 9 expression

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The gastric hormone gastrin regulates the organization of the gastric epithelium, but the cellular control mechanisms are yet unknown. Epithelial remodelling typically involves extracellular proteolysis mediated by the matrix metalloproteinases (MMPs). Since a gene-array analysis of the gastric cancer cell line AGS-G_R suggested that gastrin increased MMP-9 expression, we examined the control of MMP-9 expression by gastrin. Gelatin zymography confirmed gastrin induction of MMP-9 in AGS-G_R cells, but showed a small inhibition of MMP-2. Immunocytochemical studies showed that MMP-9 was localized to vesicles that appeared to traffic along the processes that were extended in response to gastrin. Gastrin stimulated the invasion of AGS-G_R cells through artificial basement membrane, which was reduced by an inhibitor of MMP-2/-9. There was also an increase in MMP-9 in the stomach of patients with elevated plasma gastrin

and multiple-endocrine neoplasia type 1 (MEN-1) syndrome, suggesting *in vivo* regulation of MMP-9 expression by gastrin. Finally, we showed that the expression of 1.9 kb of human MMP-9 gene promoter coupled with luciferase (MMP-9-luc) was increased 7.65 ± 1.2 -fold by gastrin, via a pathway which includes stimulation of protein kinase C, and activation of Raf and the mitogen-activated protein (MAP) kinase pathway. The tumour suppressor menin (which is mutated in MEN-1 syndrome) inhibited the expression of MMP-9-luc by gastrin. These results suggest that gastrin increases MMP-9 expression, which is associated with increased invasion, and this is a putative mechanism regulating remodelling of the gastric epithelium.

Key words: enterochromaffin cells, menin, zymography.

INTRODUCTION

The pyloric antral hormone gastrin regulates gastric acid secretion acutely and over long periods, and influences the organization of the gastric epithelium [1]. Gastrin acts at gastrin–cholecystokinin B (CCK_B) receptors found normally on histamine-producing enterochromaffin-like (ECL) cells, acid-secreting parietal cells, central nervous system neurones, some tumour cells and some smooth-muscle cells [2,3]. It is generally thought that gastrin releases histamine from ECL cells, which then functions as a paracrine mediator acting directly on the parietal cells to increase acid secretion [4,5]. Several lines of evidence suggest that gastrin might also be involved in regulating the organization of gastric glands. Thus in mice, targeted deletion of the genes encoding either gastrin or gastrin–CCK_B receptor resulted in a decrease in the number of parietal and ECL cells and reduced gland length, but the number of mucus neck and surface epithelial cells was normal [6–8]. A common class of stem cell gives rise to all the epithelial cells of the gastric gland [9]; therefore gastrin probably regulates the proportion and distribution of different cell types during differentiation.

Elevated concentrations of plasma gastrin are associated with hyperplasia of ECL cells, and in extreme cases with ECL-cell dysplasia and a progression to ECL-cell carcinoid tumours [10,11]. The growth of ECL-cell carcinoid tumours is gastrin-dependent, and decreases in plasma gastrin concentrations

frequently cause their regression [12]. These tumours occur in approx. 30% of patients with elevated plasma gastrin concentrations due to a gastrin-secreting tumour in multiple-endocrine neoplasia type 1 (MEN-1) syndrome [13]. However, the responses to elevated circulating gastrin concentrations are complex; e.g. in the transgenic mice overexpressing gastrin, there is an age-dependent remodelling of the gastric epithelium associated with loss of parietal cells and a progression to foveolar hyperplasia, which is a preneoplastic condition [14,15].

Tissue remodelling depends on restructuring of the extracellular matrix (ECM), primarily through the release of ECM-degrading proteases and regulation of their activity [16]. These mechanisms are frequently disrupted in tumorigenesis, and to some extent accounting for the tendency of cancer cells to migrate, invade and colonize distant organs. Several interacting systems of extracellular proteases have been identified as significant in these processes. One significant group is the matrix metalloproteinases (MMPs), of which there are approx. 25 different members and at least four MMP inhibitors, known as the tissue inhibitors of MMPs [17].

Because gastrin appears to play a role in determining the organization of gastric gland cells, we also considered whether it is capable of regulating the expression of genes associated with cell migration and invasion. In the present study, we identified MMP-9 (92 kDa gelatinase B/type IV collagenase) in a cancer gene array as a possible gastrin-regulated gene in a

Abbreviations used: AP-1, activator protein 1; CCK_B, cholecystokinin B; DN, dominant negative; ECL, enterochromaffin-like; ECM, extracellular matrix; EGF-R, epidermal growth factor receptor; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; MEN-1, multiple-endocrine neoplasia type 1; MMP, matrix metalloproteinase; MMP-9-luc, human MMP-9 gene promoter coupled to luciferase; PKC, protein kinase C; RGS, regulators of G-protein signalling; SFM, serum-free medium.

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gastric epithelial cell line AGS-G_R expressing the gastrin-CCK_B receptor. We have investigated the mechanisms by which gastrin stimulates MMP-9 expression in these cells, and sought evidence for up-regulation in patients with elevated plasma gastrin in MEN-1 syndrome [13,18].

EXPERIMENTAL

Cells, plasmids and drugs

AGS cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.). In our laboratory, wild-type AGS cells do not express the gastrin-CCK_B receptor as determined in radioligand-binding assays, Western-blot analysis and reverse transcriptase-PCR. Also, these cells do not respond to gastrin in assays of proliferation or migration. The present studies were made with AGS-G_R cells stably transfected with a vector encoding the gastrin-CCK_B receptor as described previously [19]. Cells were cultured in Ham's/F12 medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin, and subcultured with 1% Trypsin-versene (Life Technologies, Paisley, U.K.) as described previously [19]. Expression vectors for wild-type and dominant-negative (DN) *c-jun* plasmids were provided by Tim Wang (UMAS Medical Center, Worcester, MA, U.S.A.), and regulators of G-protein signalling 2 (RGS2) by Scott Heximer (Washington University School of Medicine, St. Louis, MO, U.S.A.) [20]. Plasmids for DN-Ras (N17), and Raf1 were gifts from Michael Höcker (Campus Vichow-Klinikum, Berlin, Germany). Settara Chandrasekharappa (National Institutes of Health, Bethesda, MD, U.S.A.) provided the plasmid for the full-length wild-type menin [21]. The human MMP-9 gene promoter-luciferase vector containing 1.9 kb fragment upstream of the initiation site coupled with luciferase (MMP-9-luc) was a gift from Yasuyuki Sasaguri (University for Occupational and Environmental Health, Kitakyushu, Japan) [22]. Heptadecapeptide amidated gastrin (G17) was obtained from Peninsula (St. Helens, Merseyside, U.K.), and the gastrin-CCK_B receptor antagonist L-740093 was from Merck (Harlow, U.K.). Ro-32-0432, PD-98059 and MMP-2/-9 inhibitor (2R)-2-[(4-biphenylsulphonyl)aminol]-3-phenylpropionic acid were purchased from Calbiochem (Nottingham, U.K.). PMA and actinomycin D were obtained from Sigma (Poole, Dorset, U.K.).

Detection of MMP-9 secretion by gelatin zymography

AGS-G_R cells (1×10^6) were cultured in a complete medium for 24 h, washed twice in serum-free medium (SFM) and cultured for a further 48 h with or without G17 (1–10 nM). The medium was concentrated using a Microcon[®]10 centrifugal filter device from Millipore and subjected to gelatin zymography in a 7.5% polyacrylamide gel containing 0.25% gelatin [23]. The gel was washed in 2.5% (v/v) Triton X-100 followed by repeated washes in distilled water, and then incubated overnight at 37 °C in a gelatin refolding buffer followed by staining with 2% Coomassie Blue. Proteolysis was detected as white zones in a dark field. Canine MMP-2 and MMP-9 were purified as described previously and used as reference standards [24,25].

Patients

Endoscopic pinch biopsies of the gastric corpus were taken for Western blotting, and serum was obtained for assay of circulating gastrin from six patients (2 males, 4 females; 3 cases were from the same family; mean age 48.2 ± 3.0 years) with hypergastrinaemia secondary to gastrinoma found in association with MEN-1. Three MEN-1 patients had multiple ECL-cell carcinoid

nodules, identified at gastroscopy and confirmed by histology; routine histology also revealed linear or nodular ECL-cell hyperplasia, or microcarcinoids in these patients. Dyspeptic patients with normal endoscopy, negative *Helicobacter pylori* status by urease test of an antral biopsy and by histology, normal plasma gastrin concentrations and normal gastric histology were used as controls (6 males, 9 females; mean age 57.2 ± 2.7 years). The study was approved by the Ethics Committees of Royal and Broadgreen Liverpool Hospitals, NHS Trust and the South Sefton Ethics Committee of the Sefton Health Authority (Liverpool). All patients gave informed consent.

Western blotting and immunocytochemistry

Protein extracts were prepared in a lysis buffer and Western blotting was performed as described previously [19] by using a rabbit anti-MMP-9 antibody (Chemicon International, Temecula, CA, U.S.A.) and a goat anti-MMP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, MA, U.S.A.). The same MMP-9 antibody was used for immunocytochemistry by using a fluorescein-coupled anti-rabbit IgG (Vector Laboratories, Burlingame, CA, U.S.A.) as described previously [26].

Gastrin radioimmunoassay

The concentration of amidated gastrin in the plasma was determined by RIA using the L2 antibody specific for the C-terminal amide sequence of gastrin, as described previously [27].

Cellular migration and invasion assays

Transwell migration and invasion assays were performed in 24-well plates containing either 8 µm pore size Biocoat[®] control inserts (migration assays) or Matrigel-coated inserts (invasion assays), according to the manufacturer's instructions (Becton Dickinson, Bedford, MA, U.S.A.). Briefly, 2.5×10^4 AGS-G_R cells were diluted in 0.5 ml of SFM and placed in the insert immersed in 0.75 ml of SFM containing G17 (1 nM) with or without the MMP-2/-9 inhibitor for 15–22 h. After incubation, non-invading cells were removed from the upper surface of the membrane by scrubbing, and invading cells on the lower surface of the membrane were stained with Diff-Quik reagent (Dade Behring, Duding, Switzerland). The membranes were then removed, mounted in immersion oil and the invading cells were counted in five fields per membrane using a Zeiss 25 Axiovert microscope (Carl Zeiss Ltd., Welwyn Garden City, Herts., U.K.) and Intellicam software (Matrox, Stoke Poges, U.K.).

Transient transfection and luciferase assay

Cells (10^5) were plated in 6-well plates in full medium. The following day, the medium was removed and cells were transfected using TransFast (Promega, Chilworth, Southampton, U.K.) with 0.75–1 µg of total DNA in SFM for 1 h. Full medium (2 ml) was then added and the cells were incubated for 20 h. The media was replaced with 2 ml of SFM, and cells were incubated with G17 and other compounds as indicated for 6 h. In some experiments, AGS cells which lack the gastrin-CCK_B receptor were transfected with MMP-9-luc and then co-cultured with untransfected AGS-G_R cells, which express the receptor, and G17 was added as described above. Luciferase activity was measured with LucLiteTMPlus (Packard BioScience, Pangbourne, Berks, U.K.) using a LumiCount Platereader (Packard BioScience), according to the manufacturer's instructions. Results are presented as fold increase over unstimulated control, i.e. 1.0 signifies no change in luciferase activity. Transfection efficiency

was monitored by transfection of a plasmid encoding green fluorescent protein; under these conditions, 30–40 % of the cells were transfected. Protein concentration was determined using the Lowry protein assay kit (Sigma) to monitor the plating efficiency.

Statistics

Results are presented as means $\pm$ S.E.M.; comparisons were made using a *t* test and were considered significant at $P < 0.05$.

RESULTS

Gastrin increases MMP-9 secretion and expression by AGS-G_R cells

We first sought to confirm, by gelatin zymography, the observation made using a gene array that gastrin increased MMP-9 transcription in AGS-G_R cells. Both MMP-2 and MMP-9 were identified by zymography in media from unstimulated AGS-G_R cells cultured in SFM. In cells stimulated with G17 (1–10 nM, 48 h) MMP-9 activity increased by 321 ± 60 % relative to control cells ($P < 0.05$). The specificity of the response was indicated by the fact that MMP-2 activity in the same samples exhibited either no change or only a slight decrease (86 ± 8 % of control) (Figure 1A). The increase in MMP-9 expression of AGS-G_R cells stimulated with G17 (1 nM, 6 h) was confirmed by Western-blot analysis; the effect of gastrin was inhibited by actinomycin D ($2 \mu\text{g/ml}$), suggesting control at the level of transcription (Figure 1B).

Gastrin induces redistribution of MMP-9 immunoreactivity in AGS-G_R cells

Immunohistochemical studies of MMP-9 in unstimulated AGS-G_R cells indicated localization to vesicles with a perinuclear distribution compatible with the endoplasmic reticulum and Golgi complex (Figure 2A). Treatment of these cells with gastrin stimulated the formation of lamellipodia and the extension of long processes with expanded terminal regions (Figure 2B). In gastrin-treated cells, MMP-9 immunoreactivity was localized to

Figure 1 Gastrin increases MMP-9 secretion and expression

(A) Gelatin zymography of samples of unstimulated AGS-G_R cells (0) and cells stimulated with G17 (1 and 10 nM, 48 h). Gastrin increases MMP-9 activity, but reduces MMP-2 activity. (B) Western-blot analysis of AGS-G_R cells stimulated with G17 (1 nM, 6 h) shows that gastrin-induced MMP-9 expression is inhibited by actinomycin D ($2 \mu\text{g/ml}$). Bands were identified as MMP-2 or MMP-9 by comparing with purified standards, as described previously [24,25]. Data are representative of three independent experiments.

Figure 2 Vesicular localization of MMP-9 in cellular processes and terminals after gastrin stimulation

Immunocytochemical localization of MMP-9 in unstimulated cells (A) and in AGS-G_R cells stimulated with G17 (1 nM, 6 h) (B). In unstimulated cells, MMP-9 exhibits a perinuclear vesicular localization, probably corresponding to the Golgi complex and the endoplasmic reticulum (short arrows). Gastrin-stimulated cells extend long processes with expanded terminal regions; MMP-9 is re-distributed along these processes and in the terminal regions (long arrows). Scale bars, 10 μM (A) and 20 μM (B).

vesicles that were distributed along the processes and in the terminal regions (Figure 2B). This phenotype is associated with increased migration (see below), suggesting delivery of MMP-9 to the advancing edge of motile cells.

Gastrin-stimulated MMP-9 expression and secretion increases cell invasion but not migration

Incubation of AGS-G_R cells with G17 (1 nM, 15 h) increased migration in a chemotaxis assay using Boyden-type chambers. The response was not influenced by $12.9 \mu\text{M}$ of (2R)-2-[(4-biphenylsulphonyl)aminol]-3-phenylpropionic acid, which is an inhibitor of MMP-2/-9 activity (Figure 3A). Gastrin (G17; 1 nM, 22 h) also stimulated invasion of AGS-G_R cells through $8 \mu\text{m}$ membranes coated with the artificial extracellular matrix Matrigel. By comparison, when unstimulated AGS-G_R cells were cultured on Matrigel, there was virtually no invasion (Figure 3B). Inhibition of MMP-2/-9 activity significantly decreased gastrin-stimulated invasion of AGS-G_R cells (Figure 3B); since gastrin decreased MMP-2 and increased MMP-9 activity in zymography, the results indicate that gastrin-induced invasion is at least partly attributable to the stimulation of MMP-9 activity (Figure 3B).

Increased MMP-9 in corpus and carcinoids of hypergastrinaemic MEN-1 patients

We then examined whether MMP-9 expression was changed in the tissues from hypergastrinaemic patients. In control subjects, with plasma gastrin in the normal range ($< 30 \text{ pM}$), Western-blot analysis of biopsies of the gastric corpus showed that MMP-9 was undetectable in 8/15 cases (Figure 4); in the remaining normal subjects, the abundance of MMP-9 in Western-blot analysis was low, but detectable. By contrast, in gastric biopsies from 4 out of 5 subjects with hypergastrinaemia due to gastrinoma in MEN-1 syndrome, there was a strong band of MMP-9 at 92 kDa (Figure 4). Moreover, in MEN-1 patients with gastric ECL-cell carcinoid tumours ($n = 3$), there was also a strong band of MMP-9 in the extracts of these tumours (Figure 4). The specificity of the increase in MMP-9 abundance was indicated by the observation that when membranes were stripped

Figure 3 Gastrin-stimulated MMP-9 expression and secretion are linked to invasion but not migration

(A) G17 (1 nM, 15 h; hatched bars; open bars are controls) stimulates migration of AGS- G_R cells in a Boyden-type chamber, and this is not influenced by an inhibitor (inh.) of MMP-2/-9 activity {(2*R*)-2-[(4-biphenylsulphonyl)amino]-3-phenylpropionic acid; 12.9 μ M}. (B) G17 (1 nM, 22 h) also stimulates invasion of AGS- G_R cells in Matrigel-coated chambers, and this is decreased by inhibition of MMP-2/-9. Data are means $\pm$ S.E.M. for $n \geq 3$.

Figure 4 Increased MMP-9 abundance in corpus and carcinoid tumours of hypergastrinaemic MEN-1 patients

(A) Representative Western-blot analysis of MMP-9 (top) and MMP-1 (bottom) in biopsies from the gastric corpus (lanes 1, 4 and 5) of MEN-1 patients, an ECL-cell carcinoid tumour from a MEN-1 patient (lane 2), and from the gastric corpus of two control subjects (lanes 3 and 6). (B) Plasma concentrations of gastrin measured by RIA in MEN-1 ($n = 6$, hatched bar) and control subjects ($n = 15$, open bar). (C) Abundance of MMP-9 (left) and MMP-1 (right) in biopsies of the gastric corpus (wide hatched bars, $n = 5$) and ECL-cell carcinoid tumours of MEN-1 patients (close hatched bars, $n = 3$), relative to the abundance in those control subjects, where MMP-9 was detectable (open bars, i.e. 7 out of 15 cases). Results are means $\pm$ S.E.M., $P < 0.05$, t test.

and probed again with an antibody raised against MMP-1, there was a 54 kDa band of similar intensity detected in all lanes. This result suggested that (1) there was no difference in the expression of MMP-1 in MEN-1 patients compared with control and (2) there was equal loading of protein in each lane.

Gastrin increases MMP-9-luc expression in AGS- G_R cells: role of protein kinase C (PKC)

To examine the cellular mechanisms by which gastrin might regulate MMP-9 expression, we studied the expression of a promoter-reporter vector consisting of 1.9 kb of the 5'-upstream sequence of MMP-9-luc. In six independent experiments, G17 (1 nM) increased MMP-9-luc expression by 7.65 ± 1.20 -fold. The response to G17 was dose-dependent over a concentration range

Figure 5 Gastrin increases MMP-9-luc expression in AGS- G_R cells

In Figures 5 and 6, unless otherwise stated, hatched and cross-hatched bars represent responses of 1.9 kb of MMP-9-luc to G17 (1 nM) and PMA (100 nM) respectively. (A) Progressive increase in MMP-9-luc expression with increase in the concentration of G17 from 100 pM to 3 nM. (B) The expression of MMP-9-luc in response to G17 was inhibited by the gastrin- CCK_B receptor antagonist L-740093 (L740; 100 nM) and by co-transfection with a plasmid encoding RGS2. (C) Inhibition of G17 stimulation of MMP-9-luc by the PKC inhibitor Ro-32-0432 (Ro; 1 μ M), by the MEK inhibitor PD-98059 (PD; 20 μ M) and by co-transfection of plasmids encoding DN-Ras and DN-Raf. (D) Inhibition of PMA-stimulated MMP-9-luc expression by PD-98059 (20 μ M), by Ro-32-0432 (1 μ M) and by co-transfection of a plasmid encoding DN-Raf but not DN-Ras. For co-transfections, 0.4 μ g each of MMP-9-luc plasmid and co-transfected plasmid were added per well. Results were obtained after a 6 h stimulation; data are means $\pm$ S.E.M. for $n \geq 3$. * $P < 0.05$, t test.

of 100 pM–3 nM (Figure 5A). The increased expression was maximal after 6–8 h, and therefore subsequent studies were performed after 6 h stimulation.

The effect of G17 was virtually abolished by an antagonist of the gastrin- CCK_B receptor, namely L-740093 (Figure 5B). Co-transfection with a plasmid encoding RGS2, which inhibits signalling by $G_{\alpha_{11}}$ [20], also significantly inhibited the responses to gastrin (Figure 5B). In addition, the MMP-9-luc response to gastrin was decreased by approx. 50% by addition of the PKC inhibitor Ro-32-0432 (Figure 5C). Consistent with the idea of PKC stimulation of MMP-9-luc expression in these cells, activation of PKC by phorbol ester PMA also increased MMP-9-luc activity, and this was almost completely reversed by Ro-32-0432 (Figure 5D). The data therefore suggest that both PKC-dependent and -independent pathways mediate the effect of gastrin.

Mechanism of MMP-9 production by AGS- G_R cells

Many of the effects of gastrin are mediated by release of paracrine factors, including histamine and epidermal growth factor receptor (EGF-R) ligands [1]. Since the latter are reported to stimulate MMP-9 expression in some systems, we examined the possibility that the MMP-9-luc responses to gastrin involved a paracrine mediator. To this end, AGS cells which lack the gastrin- CCK_B receptor were transiently transfected with MMP-

Figure 6 Role of AP-1 and menin in MMP-9-luc responses to gastrin

(A) The induction of MMP-9-luc by G17 (1 nM, 6 h) was inhibited by co-transfection with DN *c-jun* and by wild-type menin. (B) Stimulation of MMP-9-luc by PMA was also inhibited by DN *c-jun* and wild-type menin. See the legend to Figure 5.

9-luc, and co-cultured with AGS- G_R cells, which express the receptor. In response to G17 (1 nM, 6–24 h), there was only minimal increase in luciferase activity (control, 1.0 ± 0.07 ; G17, 1.43 ± 0.05 ; $n = 3$). Moreover, the EGF-R tyrosine kinase inhibitor AG1478 (30 nM), which inhibits some paracrine responses to gastrin in AGS- G_R cells [28], had no effect on the response when MMP-9-luc was expressed in these cells (results not shown). In keeping with the idea that EGF-R stimulation is not of major importance for MMP-9 expression in AGS- G_R cells, we then found that three different EGF-R ligands produced very modest increases in MMP-9-luc expression in AGS- G_R cells (control, 1.00 ± 0.05 ; 100 ng/ml EGF, 1.70 ± 0.08 ; 100 ng/ml transforming growth factor α , 1.55 ± 0.19 ; 100 ng/ml heparin-binding ('HB')-EGF, 1.78 ± 0.6). Together these data indicate that gastrin mainly acts directly on AGS- G_R cells to increase MMP-9-luc expression, rather than by release of EGF-R ligands.

Gastrin increases MMP-9-luc expression in AGS- G_R cells: role of the mitogen-activated protein (MAP) kinase pathway

Gastrin increases the expression of histidine decarboxylase gene via PKC stimulation of the MAP kinase pathway [29]. We found that PD-98059, which inhibits activation of the MAP kinase kinase, MAP kinase/ERK kinase (MEK), also significantly decreased the stimulation by G17 of MMP-9-luc expression (Figure 5C). Since PD-98059 inhibited the effect of PMA as well, it seems that PKC was upstream of MEK for induction of MMP-9 in AGS- G_R cells (Figure 5D). To further analyse the cellular mechanisms, we studied MMP-9-luc responses to gastrin and PMA during expression of a DN-Ras construct (N17-Ras). The latter had no significant effect on PMA-induced MMP-9, although it reduced the effect of gastrin. We then examined the responses in the presence of a co-expressed DN-Raf. The latter significantly inhibited the responses to both G17 and PMA (Figures 5C and 5D). The data suggest, therefore, that gastrin increases MMP-9 expression, in part, by activation of PKC, Raf and MEK. In addition, G17 may also activate a second pathway involving Ras, which is independent of PKC.

Role of activator protein 1 (AP-1) and menin in MMP-9-luc responses to gastrin

To examine the role of AP-1, we co-expressed DN *c-jun* and MMP-9-luc. There was a substantial inhibition of G17-stimulated luciferase expression by DN-Jun (Figure 6A). In view of the increased expression of MMP-9 in hypergastrinaemic MEN-1 patients, we then examined whether the tumour suppressor

menin (which is mutated in the MEN-1 syndrome) might modulate responses to gastrin. Co-expression of wild-type menin and MMP-9-luc significantly decreased the MMP-9-luc response to G17 (Figure 6A). In contrast, co-transfection of MMP-9-luc and the appropriate empty vector for menin did not modify the response to gastrin (empty vector control, 1.0 ± 0.03 ; empty vector + 1 nM G17, 6.70 ± 0.27 ; menin, 1.00 ± 0.05 ; menin + gastrin, 2.50 ± 0.28 ; $n = 6$). Finally, since gastrin acts at least in part via PKC, we examined the effect of DN-Jun and menin on the responses to PMA. The effect of the latter was virtually abolished by co-expression of both DN-Jun and menin (Figure 6B).

DISCUSSION

The present study indicates that gastrin induces expression of MMP-9 (92 kDa gelatinase B/type IV collagenase) via activation of the gastrin- CCK_B receptor and pathways involving PKC, Raf, MEK and AP-1. The data suggest that induction of MMP-9 enhances invasion of ECM by a gastric cancer cell line AGS- G_R . There is increased expression of MMP-9 in the stomach and ECL-cell carcinoid tumours of patients with high plasma gastrin concentrations and MEN-1 syndrome. Significantly, gastrin stimulation of MMP-9 expression was inhibited by the tumour suppressor menin, which is mutated in MEN-1 syndrome. The data provide a basis for understanding gastrin-stimulated mechanisms of cell migration and invasion in the gastric epithelium.

Cell migration and remodelling of the extracellular matrix are the features in several different aspects of normal and abnormal gastric epithelial function. These include the migration of epithelial cells into the gastric gland after leaving the cell cycle, and wound healing after epithelial damage. In addition, remodelling is a feature of the premalignant condition of atrophic gastritis, and cell migration and invasion are features of the progression of gastric cancer. Previous studies have shown expression of MMP-9 in normal parietal cells [30], and its increased expression in experimental ulcer models [31,32] and in gastric cancer [33–35], compatible with the view that MMP-9 plays a role in several different aspects of gastric epithelial restructuring. However, the mechanisms that might control MMP-9 expression and activation in the gastric epithelium have received relatively little attention, even though gastric cancer and peptic ulcer are common conditions. Our data indicate that gastrin may be one of the controlling factors.

Many of the physiological responses to gastrin are secondary to the release of paracrine mediators, including histamine and members of the EGF family [1]. As previous studies have shown that MMP-9 expression is induced by EGF-R stimulation as well as by cytokines and other growth factors such as tumour necrosis factor α and transforming growth factor β [36–39], we considered the possibility that gastrin might regulate MMP-9 expression in AGS- G_R cells by an indirect mechanism. However, using experimental methods that reveal paracrine control of proliferation in response to gastrin [28], we found evidence that the effect of gastrin on MMP-9 expression was mainly direct.

To establish whether gastrin induction of MMP-9 occurs *in vivo* in functionally important circumstances, we studied a group of patients with high plasma gastrin concentrations, some of whom had ECL-cell gastric carcinoid tumours [11]. It is already known that gastrin regulates the expression of many genes expressed normally in ECL cells, and that elevated plasma gastrin is associated with increased ECL-cell numbers and function [1,10,40]. In addition, ECL-cell carcinoid tumours are known to be gastrin-sensitive [11,12]. The data clearly show that

MMP-9 was up-regulated in the stomach of the majority of patients (80%) with elevated plasma gastrin, and also in ECL-cell carcinoid tumours. The latter occur in about 30% of the patients with elevated plasma gastrin and mutation of the *menin* tumour suppressor gene [13]. We have shown that the expression of *menin* inhibited the induction of MMP-9 by gastrin, suggesting that loss of *menin* exacerbates gastrin-induced MMP-9 expression and so promotes cellular invasion.

The gastric ECL-cell carcinoid tumours studied by us are relatively rare, but they are important in that they provide a clear example of a gastrin-regulated neoplastic lesion. Further study is required to determine the extent to which control of MMP-9 expression by gastrin plays a role in gastric adenocarcinoma. It should, however, be noted in this context that there are conflicting reports on the expression of gastrin- CCK_B receptors in gastric adenocarcinoma. Studies using radioligand binding and reverse transcriptase-PCR suggest that <10% of gastric adenocarcinomas express the gastrin- CCK_B receptor [41,42]. In contrast, all of the 15 gastric adenocarcinomas were reported to be positive for the presence of the receptor, as revealed by immunohistochemistry of paraffin sections [43].

The gastrin- CCK_B receptor has high affinity for the C-terminal amidated forms of gastrin, such as G17. During biosynthesis, these peptides are generated from peptides with a C-terminal glycine, which have low affinity at the gastrin- CCK_B receptor [1]. The C-terminal glycine-extended gastrins may, however, exert their own biological activity by a distinct (but yet uncharacterized) receptor. Interestingly, there are reports that C-terminal glycine-extended gastrins increase both the migration of gastric epithelial cells and the production of MMP-1, MMP-2 and MMP-9 by colonic LoVo cells [44,45]. Further studies are needed to determine whether these products of *gastrin* gene expression increase MMP-9 expression in the normal stomach, or gastric cancer. Either way, both the profile of responses and the probable receptor mechanisms underlying the action of the C-terminal glycine-extended gastrins are likely to be distinct from those reported in the present study. The mechanism by which gastrin induces MMP-9 expression in AGS- G_R cells includes mediation by PKC, Ras, Raf and MEK. However, as with induction of histidine decarboxylase gene expression by gastrin [46], PKC appears to stimulate Raf independently of Ras. Interestingly, Ras stimulation of MMP-9 expression in OVCAR-3 cells was not mediated via Raf and MEK [36]. Activation of AP-1 appears to be important for MMP-9 expression in several systems [36,39]. As *menin* binds and inhibits the AP-1 family member Jun-D [21], the loss of *menin* may contribute to increased MMP-9 expression because of enhanced stimulation by pathways stimulating expression at AP-1 sites.

It is now clear that gastrin regulates many aspects of gastric epithelial function, including acute control of acid secretion and longer-term regulation of gene expression and epithelial organization [3]. Studies in genetically modified mice and in patients with elevated plasma gastrin indicate that these mechanisms are important both physiologically and in disease [1]. Multiple mechanisms are involved, and the present study identifies gastrin-induced expression of MMP-9 as contributing to increased cell invasion. The idea that gastrin might act through modulation of cell-matrix interactions by release of MMPs has not received attention previously. It is probable that other MMPs and inhibitors are involved in these events, and further study is needed to determine their identity and role. The characterization of these mechanisms is important since the epithelial remodelling that characterizes gastric atrophy is a common premalignant condition, which is associated with increased plasma gastrin concentrations. Moreover, the mediators of these processes may

prove to be important targets for therapeutic intervention in gastric pathology.

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