IN ADDITION TO ITS WELL KNOWN roles in stimulating gastric acid secretion and gastric epithelial cell proliferation, the gastric hormone gastrin also regulates the expression of a variety of genes that appear to be involved in control of mucosal organization (7). These include both matrix metalloproteinases (MMPs) such as MMP-7 and MMP-9, and serine protease inhibitors including tissue inhibitors of matrix metalloproteinases (TIMPs) and plasminogen inhibitor (PAI)-2 (33, 34, 38, 39). The latter is part of the urokinase plasminogen activator (uPA) system that in many tissues is associated with thrombosis, fibrosis, tissue remodeling, and the progression to cancer. This system consists of the serine proteinase uPA, which converts plasminogen to plasmin, several plasminogen activator inhibitors (PAI-1, -2, and -3), and the uPA receptor (uPAR), which binds uPA at the advancing edge of migrating cells (30). In addition to activation of plasmin and consequent degradation of fibrin, uPA is associated with degradation of extracellular matrix, stimulation of angiogenesis, mitogenesis, cell migration and adhesion. The mechanisms controlling the expression of different members of the uPA system in gastric mucosa are still far from clear.

Recent studies have indicated that Helicobacter pylori infection is associated with increased expression of PAI-2 and other members of the uPA system in gastric mucosa (18, 19, 35). Members of the uPA system are also expressed in gastric cancer, and there is evidence that they are associated with poor clinical outcome in these tumors, as well as others (9, 11, 16, 23, 24). Together these observations suggest that the uPA system might have a role in controlling gastric mucosal organization and function.

Gastric neuroendocrine tumors may arise as a consequence of transformation of enterochromaffin-like (ECL) cells on a background of elevated plasma gastrin concentrations secondary to autoimmune atrophic gastritis leading to destruction of parietal cells, pernicious anemia (PA) and profound hypochlohydria, or as a consequence of gastrinoma particularly in the multiple endocrine neoplasia (MEN)-1 syndrome (4, 5, 10). Although the association of some gastric neuroendocrine tumors with hypergastrinemia is well recognized, the mechanisms by which gastrin determines tumor progression are not well understood. A previous study noted that gastrin treatment of AGS-G8 cells increased the abundance of PAI-1 detected in DNA arrays, and more recently uPA was shown to be upregulated in patients treated with the acid inhibitor esomeprazole who exhibited hypergastrinemia (25, 33). In the present study, we asked whether there was upregulation of members of the uPA system in hypergastrinemia and examined the consequences for cell migration and invasion in the case of PAI-1.

MATERIALS AND METHODS

Cells, plasmids, and reagents. AGS-G8 cells were maintained as previously published (36). A plasmid encoding 4.5 kb of the human PAI-1 promoter coupled to luciferase (PAI-1-luc) was generated as previously reported (19). Gastrin-17 was obtained from Bachem (St Helens, Merseyside, UK). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma (Poole, Dorset, UK); LY294002, PD98059, and U0126 were obtained from Promega (Southampton, UK). Human recombinant PAI-1 and Ro-32-0432 were obtained from Calbiochem (Nottingham, UK). Small interfering RNA (siRNA) for PAI-1 and control scrambled oligonucleotides were obtained from Santa Cruz.
Biotechnology (Santa Cruz, CA). Other chemicals were obtained from Sigma (Poole, Dorset, UK).

**Patients.** Endoscopic pinch biopsies were obtained from dyspeptic patients with normal endoscopy. Two antral biopsies were taken for urease test and two to four antral and two to four corpus biopsies were taken for histology; an additional six corpus biopsies were taken for RNA extraction. The present studies describe patients that were *H. pylori* negative by serology, antral urease test, and antral and corpus histology. Some patients were on proton pump inhibitors, and these were discontinued 1 wk before gastroscopy. On the basis of plasma gastrin concentrations, 16 subjects were selected with gastrin <30 pM (upper limit of the reference range) and 12 with gastrin >30 pM. In the former group 8 had been on proton pump inhibitors and in the latter 6. In addition biopsies were taken from ten patients with PA. Five patients with PA who exhibited macroscopic ECL cell neuroendocrine tumors were subjected to octreotide (25 μg/h) suppression test for 72 h and based on their response underwent antrectomy (13). Gastric biopsies were obtained at endoscopy before and immediately after octreotide, and before and 12 mo after antrectomy. In patients with macroscopic ECL cell nodules, the biopsies used for this study were taken from adjacent mucosa. The study was approved by the Ethics Committees of the Royal Liverpool and Broadgreen and Aintree University Hospitals NHS Trusts. All patients gave informed consent.

**Real-time PCR.** Real-time PCR was performed using TaqMan chemistry with TaqMan Gene Expression 2× master mix (Applied Biosystems, Warrington, UK) and a 7500 real-time PCR system (Applied Biosystems) as previously reported (19). The following primer and probe sets were used: human *uPA* forward primer 5′-gctcctcctgttcgacagtca, reverse primer 5′-cggatcttcagcaaggcaat, probe 5′-ctgacacgcttgctcaccacaacga; and an 18S rRNA control kit (Eurogentec, Southampton, UK). uPA, uPAR, and PAI-1 mRNA abundances were determined relative to 18S and/or GAPDH in the same sample as appropriate.

**Gastrin radioimmunoassay.** Plasma samples were assayed for total amidated gastrin concentrations by radioimmunoassay using antibody, L2 which reacts at the COOH terminus of G17 and measures G17, G34, and minor components such as G14 with similar affinity (8).

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissue sections were processed for immunohistochemical detection of PAI-1 (American Diagnostica, Greenwich, CT), chromogranin A (Dako UK, Ely, UK) and synaptophysin (Leica Microsystems, Milton Keynes, UK) as previously described (19). In control sections, the primary antibody was omitted.

**Luciferase reporter assays.** Gastric glands were prepared from gastroscopic biopsies of control subjects as previously described (38) and transfected with PAI-1-luc according to the method used previously (19). AGS-GR cells (2×10^5) were plated in medium containing 10% bovine serum (full medium). The following day, medium was removed and cells were cotransfected with PAI-1-luc (0.25 μg/well) together with a constitutively active Renilla luciferase reporter (pRL-SV40, 0.5 ng/well, Promega) by use of TransFast (Promega) in serum-free medium for 1 h. After transfection, 2 ml of full medium was added and 20–24 h later cells were transfected to serum-free medium with G17 and other compounds as indicated in the text for a further 6 h. Luciferase activity was then measured by dual luciferase assay (Promega) according to the manufacturer’s instructions in a Lumat LB9507 luminometer (Berthold Technologies, Redbourne, Herts, UK). Results are presented as fold increase over unstimulated control, so the value of 1.0 signifies no change in luciferase activity.

**Migration and invasion assays.** Migration and invasion assays were performed in 24-well plates containing either 8-μm pore Biocoat control inserts (migration assay) or Matrigel-coated inserts (invasion assay)
according to the manufacturer’s instructions (Becton Dickinson, Bedford, MA). AGS-GR cells (10,000 cells/well for migration assays; 20,000 cells/well for invasion assays) in 0.5 ml serum-free medium were plated in the insert with or without addition of G17 for 18 h (migration) or 24 h (invasion). Cells invading the lower surface of the membrane were stained with Reastain Quick-Diff reagents (Reagena, Toivala, Finland). The total cells in five fields per membrane were counted, and the mean of three membranes per experiment was taken.

Adhesion assays. AGS-GR cells (1 × 10⁵) were plated with full media in 24-well plates and incubated with or without G17 for 45 min at 37°C. Media and nonadherent cells were then removed, cells were washed three times with PBS, and adherent cells were stained with 0.02% crystal violet. Adherent cells were then washed and solubilized with 2 mM Na₂HPO₄·50% ethanol, and absorbance was measured at 550 nm by use of a SpectraCount plate reader (Packard BioSience, Pangbourne, UK).

Knockdown of PAI-1 by siRNA. Transfection of siRNA for PAI-1, or control siRNA, was performed on cells in suspension by using an Amaxa Nucleofection Apparatus (Amaxa, Köln, Germany), solution V., program B023, according the manufacturer’s instruction. Knockdown was verified by quantitative PCR and Western blotting.

Western blots. Protein extracts of AGS-GR cells were prepared and Western blotting was performed as previously described (22). Samples were probed with antibodies to PAI-1 (GeneTex, Irvine, CA) followed by horseradish peroxidase-conjugated secondary antibody and detection by incubation with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and HyperFilm (Amersham) as previously described (22). Samples were reprobed for GAPDH (Biodesign, Saco, ME).

Statistics. Results are presented as means ± SE; comparisons were made by Student’s t-test or ANOVA as appropriate and were considered significant at P < 0.05.

**RESULTS**

**Increased gastric PAI-1 in hypergastrinemia.** In subjects with plasma gastrin concentrations that exceeded the reference range (>30 pM), PAI-1 mRNA abundance was increased approximately twofold compared with subjects with plasma concentrations within the reference range (Fig. 1, A and B). These subjects were H. pylori negative and so the increase was not attributable to *Helicobacter* infection, which has previously been associated with increased expression of members of the uPA system (19). Because PAI-1 interacts with both uPA and uPAR, we also analyzed these in the same samples, and in both cases there were increases, suggesting that the system as a whole was gastrin regulated (Fig. 1B).

To further explore the idea that increased gastrin might control expression of this system, we then examined mRNA abundance of PAI-1 in patients with hypergastrinemia due to chronic atrophic gastritis. Circulating gastrin in these patients was typically 10- to 100-fold elevated compared with normal subjects, and PAI-1 mRNA abundance was elevated ~10-fold (Fig. 1, C and D). Again, there were also significant increases in uPA and uPAR mRNA (Fig. 1D).

**Decreased gastric PAI-1 mRNA after octreotide or antrectomy.** In PA patients with ECL cell neuroendocrine nodules, 3-day infusion of octreotide was employed to establish suitability for subsequent antrectomy as previously described (13). After octreotide, there was decreased plasma gastrin. In biopsies taken from corpus mucosa that did not include ECL cell nodules, there was also decreased histidine decarboxylase expression.

![Fig. 2. Decreased PAI-1, uPA, and uPAR mRNA after octreotide (Oct) or antrectomy (Ant) in the gastric corpus of PA patients. PA patients received octreotide (25 μg/h, 3 days), plasma, and endoscopic biopsies from corpus mucosa unaffected by macroscopic nodules were taken before and at the end of the infusion period. Subsequently, the patients underwent antrectomy for treatment of gastric neuroendocrine tumours and plasma and gastric biopsies were taken 12 mo postoperatively. After both treatments there were decreases in plasma gastrin (A), histidine decarboxylase (HDC; B), uPA (C), and PAI-1 (D) [⁎P < 0.05, vs. the relevant control (Cont), n = 5].](http://ajpgi.physiology.org/)}
(HDC) mRNA abundance used as a marker for ECL cell function (Fig. 2, A and B). In addition, octreotide was associated with a decrease of ~70% in PAI-1 mRNA in these biopsies and with a significant but slightly smaller decrease in uPA mRNA, compatible with acute endocrine regulation of these transcripts (Fig. 2, C and D). Moreover, 12 mo after antrectomy, plasma gastrin was reduced to concentrations within or close to the reference range and HDC mRNA was also significantly reduced (Fig. 2, A and B). In these circumstances there was a 70% reduction in PAI-1 mRNA abundance (Fig. 2D). There was also decreased abundance of uPA mRNA which although statistically significant was less than that seen for PAI-1 and HDC mRNAs (Fig. 2C).

**Cellular localization of PAI-1.** In immunohistochemistry, PAI-1 was found to be localized to parietal cells in hypergastrinemic patients without chronic atrophy confirming the localization reported previously (Fig. 3A) (19). In PA patients with micronodular ECL cell tumors, there was clear localization of PAI-1 to ECL cell micronodules (Fig. 3B), characterized by chromogranin A and synaptophysin staining (Fig. 3, C and D). In addition, weak staining for PAI-1 was observed in the stroma (Fig. 3B), but staining in control sections was negative (Fig. 3E).

**Gastrin increases PAI-1-luc expression via protein kinase C and mitogen-activated protein kinase pathways.** In glands cultured from gastroscopic biopsies and transfectected with PAI-1-luc, there is known to be expression in parietal and ECL cells cultured from gastroscopic biopsies and transfected with PAI-1-luc, gastrin induced a concentration-dependent increase in luciferase expression over the range 0.3 to 10 nM that was maximal 6 h after stimulation (Fig. 4, A and B). The response to 1 nM gastrin was inhibited by Ro-32-0432, which is an inhibitor of PKC; moreover, stimulation of PKC with PMA produced a robust increase in PAI-1-luc expression that was inhibited by Ro-32-0432 (Fig. 4, C and D). In addition, the response to both gastrin and PMA was partially reversed by U0126, which inhibits the activation of MAP kinase kinase (MEK)-1 and -2 (Fig. 4, B and D), indicating that the activation of MAP kinase was downstream of PKC. No significant effect was seen with an inhibitor of MEK1 (PD98059) pointing to the involvement of MEK2 in the AGS-GR cell response. The phosphatidylinositol-3 kinase inhibitor LY204002 had no effect on PAI-1-Luc expression (Fig. 4C).

**PAI-1 inhibits AGS-GR cell migration and invasion.** Since the uPA system is implicated in control of cell migration and invasion we examined the role of the system on G17-stimulated migration and invasion of AGS-GR cells in Boyden chambers. There was stimulation of AGS-GR cell migration and invasion in response to G17 as previously noted (35); in the presence of PAI-1 (40 nM) the migration and invasion responses to G17 were 56.6 ± 11.3 and 34.3 ± 1.7%, respectively, of those to G17 alone (P < 0.05 in both cases). We

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![Fig. 3. Immunohistochemical localization of PAI-1 to parietal cells and micronodular hyperplastic enterochromaffin-like (ECL) cells. A: in a hypergastrinemic patient. PAI-1 is localized to parietal cells (arrow) in a gastric biopsy from a region not exhibiting atrophy. B: in a hypergastrinemic patient with micronodular ECL cell hyperplasia. PAI-1 is localized to the nodules, and there is also light staining of surrounding stroma (arrows). C and D: in the same patient there is localization of chromogranin A and synaptophysin, respectively, to nodules (note, B–D are not consecutive sections). E: control section without primary antibody. Magnification ×20.](http://ajpgi.physiology.org/)

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considered the possibility that induction of PAI-1 might also influence gastrin-stimulated cell adhesion (26); however, although acute treatment of AGS-GR cells with 1 nM G17 increased adhesion in a dose-dependent manner, in conditions that increased PAI-1 and approximated to those used for migration and invasion assays (i.e., pretreatment with G17, 1 nM, 18 h) there was no change in the adhesion response compared with acute stimulation, suggesting that gastrin does not stimulate migratory and invasion responses as a consequence of long-term changes in cell adhesion (Fig. 5).

To address the significance of gastrin-stimulated PAI-1 expression in AGS-GR cell motility, we used siRNA to depress PAI-1 expression. After treatment with siRNA, gastrin-stimulated increases in PAI-1 mRNA abundance were virtually abolished (Fig. 6A). There was also substantial reduction in PAI-1 detected by Western blot, but no change in GAPDH abundance indicating the specificity of the knockdown (Fig. 6B). In cells with PAI-1 knockdown, there were no differences in basal rates of migration and invasion. However, there was significantly increased migration and invasion in response to an optimal concentration of G17 compared with cells treated with control siRNA (Fig. 7, A and B).

**DISCUSSION**

The present study shows that increased plasma gastrin concentrations are associated with increased expression of PAI-1 in the stomach. There are also changes in expression of other members of the uPA system in gastric mucosa, suggesting that the system as a whole is upregulated. The cellular control mechanisms involved in PAI-1 expression include the PKC and MAP kinase pathways. There is increased cell migration and invasion in CCK2-expressing AGS cells in response to gastrin, and this is increased by siRNA knockdown of PAI-1 expression, indicating that induction of PAI-1 plays a role in restraining cell migration and invasion and may therefore be considered to have a protective role in hypergastrinemia.

There are multiple different serpin inhibitors of plasminogen activators (PAI-1, PAI-2, PAI-3, and protease nexin). Previous work has shown gastric PAI-2 to be increased by gastrin and *H. pylori* infection (33, 35); however, PAI-2 possesses a relatively weak signal sequence and at least some effects, e.g., inhibition of apoptosis, appear to be mediated intracellularly. Although we previously found PAI-3 in stomach (19), we found no evidence of regulated expression. In contrast, PAI-1 mRNA abundance increases with raised plasma gastrin in *H. pylori* negative subjects and in PA subjects with profound hypergastrinemia and is decreased in the latter after treatments that lower plasma gastrin (octreotide infusion and antrectomy). Together with the results of promoter-reporter experiments, these observations indicate that gastrin regulates PAI-1 expression in the stomach.

It is well recognized that members of the uPA system are expressed in a wide variety of cell types, which in the case of...
hollow organs can include both epithelial and stromal cells. In the stomach, the CCK2-receptor is expressed by both parietal and ECL cells. The localization of PAI-1 and uPA to parietal cells is compatible with a direct role for gastrin in stimulating their expression. In the case of ECL cells, PAI-1 expression was clearest in micronodular ECL cell neuroendocrine tumors, again compatible with a direct effect of gastrin (albeit in the high concentrations characteristic of patients with these tumors). Previous studies have shown that gastrin stimulates the expression of several genes in gastric epithelial cells including HDC (14, 28), PAI-2 (33) VMAT2 (37), chromogranin A (6, 15), and trefoil factor-1 (20). In all these cases there is evidence for the involvement of PKC and MAP kinase pathways; the present data suggest that activation of similar pathways leads to PAI-1 expression in gastric epithelial cells, and this is also compatible with studies of PAI-1 expression in a variety of other cell types including neurons (27) and vascular smooth muscle cells (32).

However, in addition to expression in gastric epithelial cells, there is also PAI-1 expression in gastric stromal cells. These are not thought to express the CCK2 receptor under physiological conditions (2). Instead, it seems possible that gastrin exerts indirect effects on these cells. Studies of gastrin-activated paracrine mechanisms in AGS cell populations have identified both IL-8 and prostaglandins as mediating expression of PAI-2 (1, 35). In addition, there is evidence that gastrin activates epithelial-mesenchymal signaling pathways. Thus gastrin stimulates gastric epithelial MMP-7 expression and release that in turn cleaves insulin-like growth factor binding protein-5 produced by myofibroblasts to liberate IGF, which acts as an autocrine stimulant of myofibroblasts or as a paracrine stimulant of epithelial cells (12, 22). In support of this suggestion it should be noted that IGF-I has also been shown to stimulate PAI-1 expression in HepG2 cells (29) and IGF-II stimulated PAI-1-luc expression in gastric glands.

The expression of PAI-1 in cancer, including gastric cancer, is associated with poor prognosis (3, 9, 16, 17). This is sometimes referred to as paradoxical given that uPA is also associated with poor prognosis and it might be expected that inhibition of uPA by PAI-1 would be beneficial. However, it is recognized that PAI-1 has both uPA-dependent and -independent effects and that it can exert different actions with both beneficial and adverse consequences (21). Thus, in addition to inhibition of uPA, PAI-1 also binds vitronectin and disrupts the binding of vitronectin to integrins, e.g., α5β1 (31, 40). These actions lead to decreased cell adhesion and inhibition of migration. Although gastrin acutely stimulates cell adhesion, we did not find evidence for changes in gastrin-stimulated adhesion over a time course compatible with induction of PAI-1. In contrast, PAI-1 inhibited gastrin-stimulated cell migration and invasion, and suppression of PAI-1 synthesis by siRNA treatment led to increases in gastrin-stimulated cell migration and invasion, suggesting that induction of PAI-1 by gastrin normally restrains its actions on cell motility.

The progression to ECL cell neuroendocrine tumors in the stomach is driven by gastrin and enhanced by mutations of the MEN-1 gene, or mucosal inflammation. In general these
tumors are not thought to be particularly aggressive, and, for example, metastasis is a feature only of advanced stages of the disease. The factors governing progression remain poorly understood. However, the present study raises the possibility that some effects of gastrin may delay tumor progression. In particular, induction of PAI-1 may in this case limit cell migration and invasion and so serve a protective function. We suggest that this system provides a useful model for further studies of the role of PAI-1 in cancer progression.

ACKNOWLEDGMENTS
We thank David Berry for assistance with immunohistochemistry and members of the Mersy Neuroendocrine Tumour Multidisciplinary team and Gastroenterology Unit at Royal Liverpool University Hospital for clinical assistance.

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