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# Pepsinogen C Gene Product Is a Possible Growth Factor during Gastric Mucosal Healing[1]

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**We isolated, by the subtraction cloning method, a pepsinogen C (PGC) gene fragment (the sequence between the 968th and 1179th base pairs) from a rat gastric mucosal cDNA library as a cDNA clone encoding a substance that promotes growth of the normal rat gastric mucosal cell line RGM1. Northern blot analysis revealed that PGC gene expression was enhanced not only in acetic acid-induced chronic gastric ulcers but also in indomethacin-induced gastric mucosal lesions. PGC gene expression was also increased in the *Helicobacter felis*-infected stomachs. Thus, the PGC gene may play a role in gastric epithelial cell growth during gastric mucosal healing.** 

Pepsinogen, a precursor of pepsin, is mainly synthesized and secreted by the gastric mucosal cells. There are two major groups of pepsinogen, A (PGA) and C (PGC), which can be separated electrophoretically and immunochemically (1,2). Although pepsinogens are aspartic proteinases and play important roles in nutrient digestion in the stomach, whether PGA and PGC have other physiological roles is unknown. It is believed that a small percentage of pepsinogen synthesized is released into the circulation and that the serum pepsinogen concentration reflects the number of cells, mainly in the stomach, that produce pepsinogen (3,4). Recently, the serum pepsinogen concentrations of patients with *Helicobacter pylori*-induced gastritis were found to be elevated (5,6). Whether this elevation is due to leakage from damaged cells or enhancement of pepsinogen synthesis by mucosal cells of the inflamed tissues is unclear, but this finding indicates that pepsinogens may play a role in inflammatory processes in the gastric mucosa.

In this study, as part of our search for new growth factor genes in the stomach, we used the subtraction cloning method and isolated a PGC gene fragment from rat stomach with acetic acid-induced ulcers. The role of PGC as a possible novel gastric mucosal growth factor is discussed.

## MATERIALS AND METHODS

*Preparation of rat gastric ulcer and gastritis models.* Seven-week-old male Sprague-Dawley rats were used in this study. Chronic ulcers were induced on the anterior walls of the stomachs with acetic acid using the method described by Kinoshita *et al.* (7). Five days after ulcer induction, the rats were killed, their glandular stomachs were excised, and the RNA was extracted, for synthesis of a subtracted cDNA library and Northern blot analysis. Gastric mucosal lesions were induced with indomethacin, as described previously (7). Six hours after indomethacin injection, the rats were killed and their glandular stomachs were excised for measurement of the ulcer index (total length of the ulcers) and Northern blot analysis. A group of rats received omeprazole (50 mg/kg body wt orally daily) for 7 days before indomethacin treatment. All the control rats received equivalent volumes/kg of vehicle.

*Synthesis of subtracted cDNA library.* Each rat glandular stomach with acetic acid-induced ulcer was divided into two (anterior ulcerated and posterior non-ulcerated) segments, cDNA was synthesized from poly(A)$^+$RNA obtained from the ulcerated segments and hybridized with photobiotinylated poly(A)$^+$RNA obtained from the non-ulcerated segments. The resulting photobiotinylated mRNA-cDNA hybrids were complexed with streptavidin and removed from the hybridization mixture by phenol-chloroform extraction, using a Subtractor Kit (Invitrogen Co., San Diego, CA), leaving the unhybridized cDNAs behind. The subtracted cDNA library was synthesized from the subtracted cDNAs using a ZAP Express™ cDNA Synthesis Kit (Stratagene Cloning Systems, La Jolla, CA).

*Expression in COS-7 cells and [$^3$H]-thymidine incorporation by RGM1 cells.* The plasmid (pBK-CMV) library was constructed from the subtracted cDNA library by an *in vivo* excision technique using the ZAP Express cDNA Synthesis Kit. After amplification, the plasmid library was fractionated and transfected into COS-7 cells by the DEAE-dextran method as described previously (8). COS-7 cells were

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grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a 37°C, 10% CO2 humidified environment. After transfection, the COS-7 cells were cultured for 16 h under the above conditions. Then, the above culture medium was changed to that containing 0.5% fetal calf serum, the cells were incubated as above, and after 60 h the medium (conditioned medium) was collected. The conditioned medium was added to RGM1 normal rat gastric mucosal epithelial cells in culture and its growth-stimulating effect was determined by measuring [$^3$H]-thymidine incorporation (9). Transfected COS-7 cells, the conditioned media of which promoted DNA synthesis by RGM1 cells, were collected and the transfected plasmids were extracted using a slightly modified method of that described previously (10) and sequenced.

*Expression in RGM1 cells.* The plasmid library was transfected directly into RGM1 cells by the liposome-mediated transfection method using Lipofectoamine reagent (Gibco BRL, New York, NY). RGM1 cells were grown in Dulbecco's modified Eagle's medium and HAM F12 supplemented with 20% fetal calf serum in a 37°C, 5% CO2 humidified environment. After transfection, the RGM1 cells were cultured for 24 h under the above conditions, after which, the above culture medium was changed to that containing 10% fetal calf serum and 200 $\mu$g/ml G418, an antibiotic. After incubation (as above) for 72 h, the attached RGM1 cells were collected and transferred to a 96-well culture plate by the limiting dilution method (11). After incubation (as above) for at least 72 h, RGM1 cells which grew in the low-serum medium containing G418 were collected and their genomic DNA was extracted. The inserted fragments in the genomic DNAs were amplified by the PCR (polymerase chain reaction) method using a pair of primers specific to the vector (pBK-CMV), the amplified fragments were subcloned into pCRII (TA Cloning Kit; Invitrogen Co., San Diego, CA) and sequenced.

*Inoculation of mice with Helicobacter felis. Helicobacter felis* (strain CS1 obtained from ATCC) (12) cells ($1\times10^9$) were suspended in 1 ml Brucella broth medium supplemented with 2.5% fetal bovine serum and administered orally to 6-week-old BALB/cA Jcl mice as described previously (13). Twelve weeks later, the mice were sacrificed and their glandular stomachs were excised for Northern blot analysis. Infection was confirmed using a rapid urease test kit (PML MICROBIOLOGICALS, Tualatin, OR). The control mice received equivalent volumes/kg of vehicle.

*Northern blot analysis.* The total RNA was isolated from each mouse stomach, separated by electrophoresis on a 1.0% agarose gel, transferred to nitrocellulose membranes (Schleicher & Schuell Inc., Keene, NH) and hybridized with a $^{32}$P-labeled 0.4-kb cDNA fragment of the PGC gene or the complete PGC cDNA which was obtained from rat gastric tissue mRNA by RT-PCR amplification method. Hybridization was carried out at 42°C, followed by washing the filters twice for 20 min each at 55°C with 0.1 × SSC/0.1% SDS, as described previously (9), and the radiolabeled DNA probes were detected by a bioimaging analyzer BAS 2000 (FUJIX, Tokyo).

## RESULTS AND DISCUSSION

In order to identify genes specifically expressed in injured gastric mucosa, cDNAs were obtained from the rat gastric wall with acetic acid-induced ulcers by subtracting those from the non-ulcerated gastric wall and were transfected into COS-7 and RGM1 cells. We selected the transfected COS-7 cells with conditioned media showing potent growth stimulating effects on the normal rat gastric epithelial cell line RGM1 and successfully isolated several cDNA clones. DNA sequencing of these isolated clones revealed that the DNA sequence of one was identical to that of a part of exons

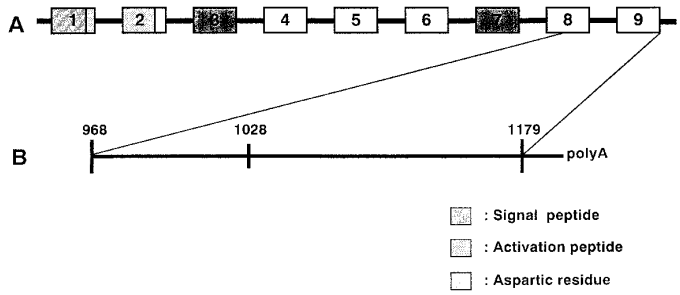


**FIG. 1.** Gene structure of pepsinogen C. **A,** The structure of nine exons is indicated. Exon 1 encodes the signal sequence and first few residues of the activation peptide and exon 2 encodes the remainder of the activation peptide and a few residues of the enzyme. The enzymatic active site, which includes aspartic acid residues, is present from exons 3 to 7. **B,** The structure of the clone obtained is indicated. It is identical to the sequence between the 968th and 1179th base pairs of PGC cDNA and its 3' non-coding region.

8 and 9 of the rat PGC gene (**Fig. 1**). Furthermore, in another experiment, we selected the directly transfected RGM1 cells that grew in the low-serum medium containing the antibiotic G418 and isolated several cDNA clones, one of which encodes the same part of the rat PGC gene obtained from the COS-7 cells (between the 968th and 1179th base pairs of the PGC gene). To clarify the specificity of cells, the conditioned medium of the COS-7 cells transfected with the rat PGC gene fragment as mentioned above was added to NIH/3T3 normal fibroblasts, but it did not show any growth stimulating effects (data not shown). These data suggest that PGC gene expression is enhanced in damaged gastric tissue and this gene is involved in the gastric mucosal cell growth in damaged gastric mucosa.

In order to ensure mRNA expression was augmented in gastric ulcer tissue, Northern blot analysis was performed using the cloned PGC cDNA as a probe. The amounts of authentic PGC gene, according to the molecular weight, in gastric tissues with acetic acid-induced ulcers were increased markedly in comparison with those in non-ulcerated gastric tissues (**Fig. 2**). Northern blot analysis was also performed using the complete PGC cDNA as a probe and the signals exhibited the same molecular size as those observed by using the cloned PGC cDNA (data not shown). Furthermore, PGC gene expression was enhanced in the indomethacin-induced gastric mucosal lesions compared with control tissues (**Fig. 3**). In this model, enhanced PGC gene expression was evident as early as 3 h after indomethacin administration and reached its maximal level 6 h after mucosal lesion induction, when the mucosal lesions were most severe (data not shown). When rats were pretreated with omeprazole, a proton pump inhibitor, attenuation of gastric mucosal lesion was associated with a considerable reduction of the enhanced PGC gene expression (**Fig. 3**). These results support

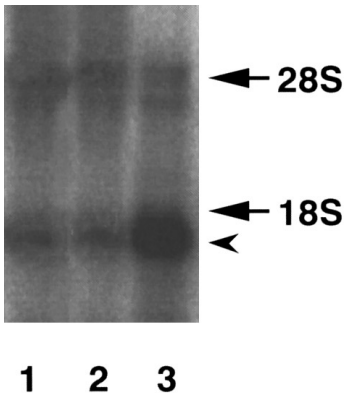


**FIG. 2.** Northern blot analysis of pepsinogen C gene transcripts in the rat stomach with acetic acid-induced chronic ulcers. RNA samples (10 $\mu$g/lane) from the following were analyzed: Lane: 1, whole normal stomach; 2, non-ulcerated posterior segment; 3, ulcerated anterior segment. The positions of the 28S and 18S rRNAs are indicated and the positions of PGC gene transcripts are indicated by arrowheads. The results are representative of three independent experiments.

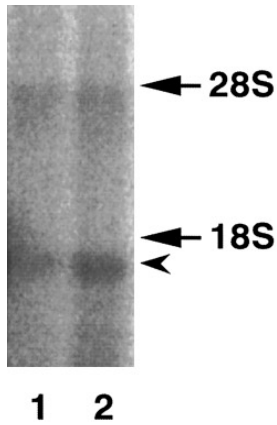


**FIG. 4.** Northern blot analysis of pepsinogen C gene transcripts in the *Helicobacter felis*-infected mouse stomach. RNA samples (10 $\mu$g/lane) from the following were analyzed: Lane: 1, whole non-infected stomach; 2, *Helicobacter felis*-infected stomach. The positions of the 28S and 18S rRNAs are indicated and the positions of PGC gene transcripts are indicated by arrowheads. The results are representative of three independent experiments.

the hypothesis that PGC gene expression is enhanced by gastric mucosal damage.

Recent studies have shown that the serum PGC concentrations of patients with *Helicobacter pylori* infection are elevated (5,6). In our study, we found that PGC gene expression in murine stomachs infected with *Helicobacter felis* was enhanced (**Fig. 4**). Therefore, the elevated serum PGC levels in *Helicobacter pylori*-infected patients are probably due to enhanced PGC gene expression, suggesting this gene plays a pathophysiological role in the development of *Helicobacter pylori*-induced gastritis.

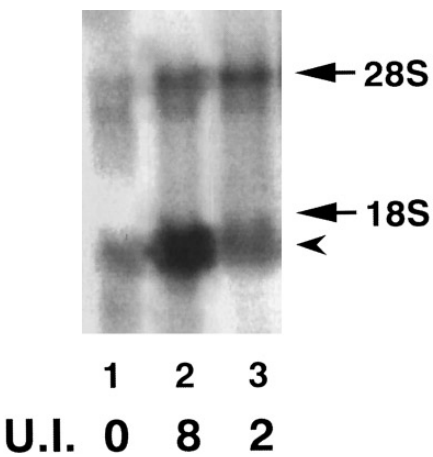


**FIG. 3.** Northern blot analysis of pepsinogen C gene transcripts in the rat stomach with indomethacin-induced gastric mucosal lesions. RNA samples (10 $\mu$g/lane) from the following were analyzed: Lane: 1, control rat stomach; 2, indomethacin-treated rat stomach; 3, omeprazole pre-treated indomethacin-treated rat stomach. The ulcer index (U.I.) is shown under each lane, the positions of the 28S and 18S rRNAs are indicated and the positions of PGC gene transcripts are indicated by arrowheads. The results are representative of three independent experiments.

In this study, we demonstrated clearly that the conditioned medium of COS-7 cells transfected with a c-terminal PGC gene fragment stimulated proliferation of gastric epithelial cells but not NIH/3T3 normal fibroblasts, suggesting that PGC itself or its c-terminal fragment, exerts a growth promoting effect on gastric epithelial cells. As PGC gene expression is enhanced in stomachs with mucosal lesions, including those due to *Helicobacter pylori* infection, PGC itself, or its c-terminal fragment, may play a role in gastric mucosal inflammatory or healing processes. Whether the c-terminal fragment of PGC is actually present in gastric tissue should be elucidated in future studies.

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