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Regenerating gene expression in normal gastric mucosa and indomethacin-induced mucosal lesions of the rat

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Abstract: Regenerating (*reg*) gene expression was tested in rat gastrointestinal mucosa to investigate the role played by this gene in the healing of mucosal lesions. Expression of *reg* mRNA was higher in the stomach than in any other region of the gastrointestinal tract. The gastric cells that expressed *reg* mRNA were located in the deepest mucosal layer and were small in diameter. In an injured state following indomethacin treatment, *reg* gene expression was markedly augmented, accompanied by an increase of c-*fos* expression and healing of the mucosal lesions. These results suggest a role of the *reg* gene in the healing of gastrointestinal mucosal lesions.

Key words: stomach, gastric ulcer, mucosal healing, growth factor, c-*fos*

Introduction

Gastric mucosal cells are supplied continuously by the proliferation of progenitor cells, replacing exfoliating epithelial cells.[1–3] During the healing of acute gastric mucosal lesions, the proliferation of mucosal cells is accelerated to replace and re-epithelialize the damaged mucosa,[4] and it is speculated that, under such conditions, the proliferation of these progenitor cells may be controlled by various extracellular signals.[5,6] Several growth factors are reported to stimulate the proliferation of cultured gastric mucosal cells.[7–9] However, there is little information available concerning the changes in the gene expression of these growth factors during the healing of acute gastric mucosal lesions.

Regenerating (*reg*) gene (isolated from a cDNA library of rat regenerating pancreatic islets)[10] encodes a protein composed of 144 amino acids and was recently reported to stimulate the proliferation of β cells of rat pancreatic islets.[11,12] The transcription of the *reg* gene has been demonstrated in only a few organs, including the stomach,[13] but the role of this gene in the regeneration of gastric mucosal cells has not yet been investigated. The present study was designed to investigate alterations of *reg* gene expression during the healing of acute gastric mucosal lesions.

Materials and methods

Formation of indomethacin-induced gastric mucosal lesions

Six-week-old Sprague-Dawley male rats were used for the experiments. After 24-h fasting, the rats were injected subcutaneously with 0.5 ml/100 g body weight indomethacin solution (10 mmol/ml indomethacin suspended in saline with one drop of Tween-80).[14] They were sacrificed 1, 3, 6, 12, 24, and 48 h after the treatment ($n = 5$ each).

Dispersal of gastric mucosal cells

Gastric mucosal epithelial cells were dispersed from the gastric wall according to the method described by Brenna and Waldum,[15] with minor modifications. In brief, the contents of the glandular stomach were rinsed out with phosphate-buffered saline (PBS). The stomach was inverted to form an inside-out gastric bag, and was

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then filled with approximately 5 ml of 2.5 mg/ml pronase solution (Actinase E; Kaken Pharmacentical, Tokyo, Japan) in buffer A [0.5 mM NaH_2PO_4, 1.0 mM Na_2HPO_4, 70 mM NaCl, 5 mM KCl, 11 mM glucose, 2 mM ethylene diamine tetraacetic acid (EDTA), 50 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES, pH 7.2), 20 mM $NaHCO_3$ and 2% bovine serum albumin (BSA)]. The stomachs were incubated in pronase-free buffer A in an atmosphere of 95% O_2/ 5% CO_2 at 37°C for 30 min. The gastric bags were then transferred to buffer B (containing 1.0 mM $CaCl_2$ and 1.5 mM $MgCl_2$ instead of EDTA in buffer A) and agitated gently with a magnetic stirrer for 30 min. The epithelial cells dispersed in buffers A and B were collected as superficial epithelial cells. The gastric bags were further incubated in buffer B for another 30 min. The cells released into buffer B during this period were collected as epithelial cells from the middle layer. The epithelial cells still attached to the gastric walls were rinsed out in PBS; these were the epithelial cells from the deepest layers. The depth of the dispersed gastric mucosal cells was evaluated histologically.

Separation of dispersed gastric mucosal cells

The dispersed cells were separated using the method described by Soll,[16] with minor modifications. Briefly, the isolated cells were separated by counterflow elutriation (JE-6B; Beckman, Fullerton, Calif., USA). The dispersed cells were loaded into the separation chambers of the rotor at a flow rate of 8 ml/min. After $7.0 - 9.0 \times 10^8$ cells had been loaded, cell separation was carried out at 1800 rpm. Five different cell fractions (F1-F5) were obtained, at the following flow rates: F1, 20.0 ml/min; F2, 29.0 ml/min; F3, 37.0 ml/min; F4, 48.0 ml/min; and F5, 80.0 ml/min. The approximate diameters of the cells in each fraction were: F1, ≦ 11 μm; F2, ≦ 13 μm; F3, ≦ 15 μm; F4, ≦ 17 μm; and F5, ≦ 22 μm.

Extraction of RNA and Northern blot analysis

Total RNA was extracted from the excised stomachs with guanidine thiocyanate, followed by cesium chloride centrifugation.[17] The RNA was separated by electrophoresis on 0.66 M formaldehyde-1% agarose gel, transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), and fixed permanently to the membrane, using ultraviolet cross-linking. The probe used for Northern blot analysis was a 0.3-kb fragment of rat *reg* gene cDNA (open reading frame bases 187–491). This cDNA fragment was synthesized, according to the previously reported cDNA sequences,[10] in our laboratory by the reverse transcription polymerase chain reaction method. The sequence of this probe was confirmed by autosequencer. The c-*fos* probe was purchased from Takara Shuzo (Tokyo, Japan). Employing these probes labeled with ^{32}P, using a random prime labeling kit (Boehringer-Mannheim, Mannheim, Germany), hybridization was carried out at 42°C. Each hybridization was quantitated by scanning densitometry and compared after normalizing the expression of *reg* and c-*fos* to the expression of β-actin. These data were statistically analyzed by one-way analysis of variance.

Tissue preparation and in situ hybridization histochemistry

The rats were anesthetized with sodium pentobarbital (55 mg/kg) and perfused transcardinally with ice-cold Zamboni's solution (2 ml/g body weight). The stomachs were preserved in the same fixative for 6 h, and soaked in PBS with 30% (w/v) of sucrose at 4°C until they sank. The tissues were frozen quickly, cut into 7-μm sections, and floated onto poly-L-lysine-coated slides. The sections were rehydrated with PBS treated with proteinase K (10 μg/ml, Boehringer-Mannheim), postfixed in 4% paraformaldehyde, acetylated with 0.1 M triethanolamine with acetic anhydride (0.25% w/v), and dehydrated before hybridization.

In situ hybridization was performed by a procedure described previously,[18] with minor modifications. Hybridization was performed for 18 h at 47°C in a 200-μl hybridization mixture containing 1 × Denhardt's solution, 50% formamide, 10% dextran sulfate, 200 μg/ml salmon sperm DNA, 0.2% sarcosyl, 0.3 M NaCl, 20 mM Tris-HCl, 0.1 M Na_2HPO_4, and 0.2 μg digoxygenin-labeled complementary riboprobes (antisense or sense). The complementary riboprobes were prepared by ligating a 0.3-kb EcoR1 insert of rat *reg* gene cDNA into the pCR-II vector, using a TA cloning kit (Invitrogen, San Diego Calif., USA). The appropriate templates for *reg* antisense or sense RNA probes were generated by linearization with an appropriate restriction endonuclease (Xhol for the antisense probe; Bam HI for the sense probe). An RNA Labeling Kit (Boehringer-Mannheim) was employed to generate RNA probes by in vitro transcription from linearized cDNA templates in the nucleotide mixture (which included digoxygenin-labeled UTP), using the appropriate RNA polymerase (SP6 for the antisense; T7 for the sense). This antisense probe was complementary to the open reading frame of the *reg* gene (bases 187–491).

After hybridization, the tissue sections were treated with ribonuclease and 0.5 × SSC. The sections were then visualized with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitroblue tetrazolium chloride (NBT) under dark conditions, counterstained with methyl green.

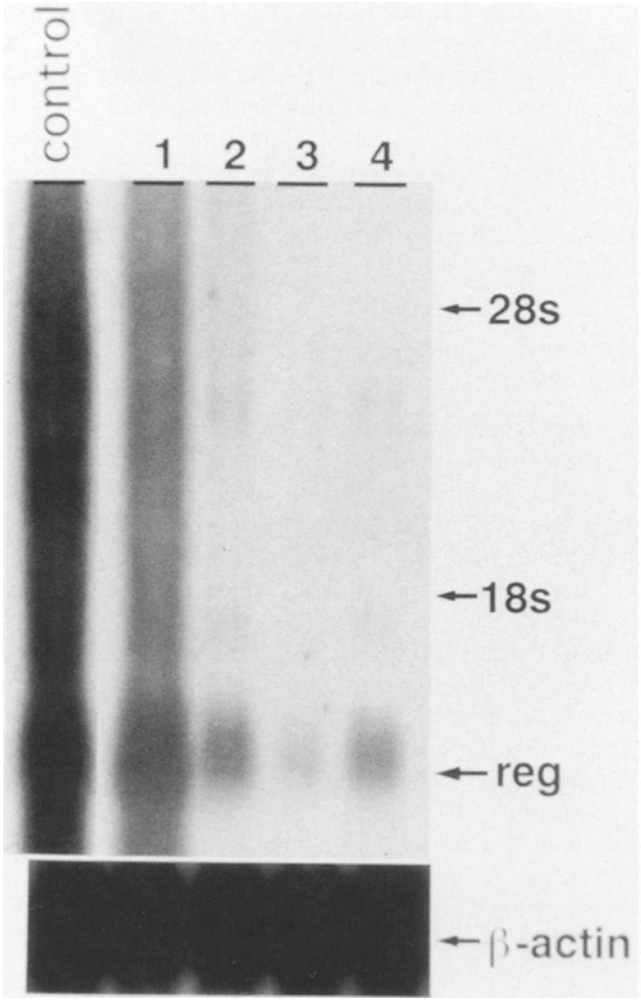

Fig. 1. Northern blot analysis of rat gastrointestinal tract, with rat *reg* cDNA as a probe. Each lane contains 20μg of total RNA. *Control*, Rat pancreas (since the control signal was very strong, the exposure time for this blot was shorter than that of the others.); *lane 1*, stomach; *lane 2*, jejunum; *lane 3*, ileum; *lane 4*, colon. The expression of β-actin mRNA is also shown

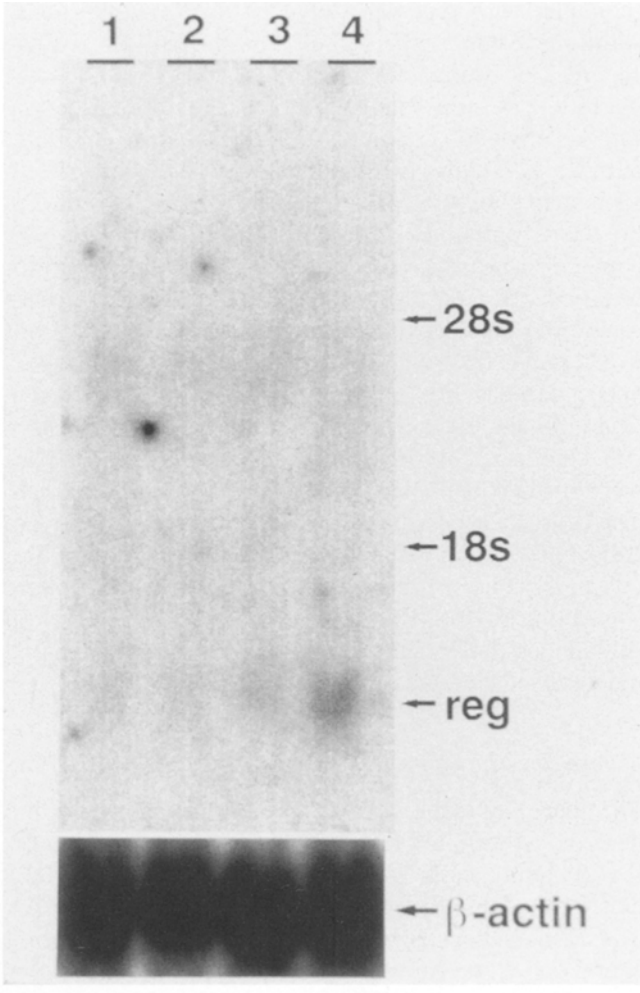

Fig. 2. Northern blot analysis demonstrating the expression of *reg* mRNA in the individual layers (superficial, middle, and deepest) from gastric mucosa of untreated rat. *reg* mRNA was demonstrated predominantly in the deepest layer. *Lane 1*, whole stomach; *lane 2*, superficial mucosal layer; *lane 3*, middle mucosal layer; *lane 4*, deepest mucosal layer. The expression of β-actin mRNA is also shown

Results

In addition to expression in the pancreas, *reg* mRNA was expressed throughout the entire gastrointestinal tract, but most prominently in the glandular stomach (Fig. 1). To identify the specific gastric cell layers that expressed the *reg* gene, RNA was extracted from the individual layers (superficial, middle, and deepest mucosal layers) of the gastric mucosa. The depth of the dispersed gastric mucosal cells was evaluated histologically. The superficial, the deepest, and the middle layers corresponded to the upper half, lower quarter, and the remander of the gastric mucosal layer, respectively (data not shown). *reg* mRNA was demonstrated predominantly in the deepest mucosal layer (Fig. 2). When *reg* gene expression was analyzed more precisely in elutriated cells, the gene was found to be expressed almost exclusively in cells that were less than 11μm in diameter (Fig. 3).

Three h after injection, indomethacin induced multiple linear erosions in the gastric mucosa. The severity of the mucosal lesions was maximal (in terms of the total length of linear erosions) between 6 and 12h after injection, and then they gradually healed. *reg* gene expression in the stomach was significantly stimulated 1h after the injection of indomethacin, and then gradually increased, reaching the maximal level 6h after indomethacin treatment (Figs. 4 and 5). The response of c-*fos* expression was slower than that of *reg* gene expression. c-*fos* expression had increased 3h after the

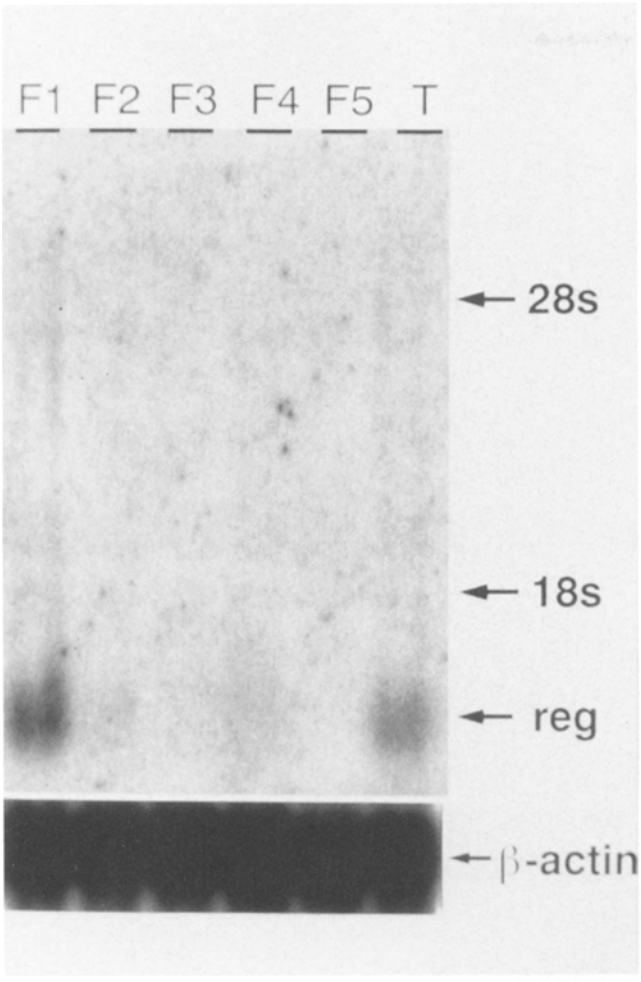

Fig. 3. Distribution of *reg* mRNA expression in elutriated gastric mucosal cell fractions. Each lane contains 20μg of total RNA. The fractions (*F1–F5*) were separated according to cell size, F1 being the smallest and F5 the largest cell fraction. The diameter of cells in each fraction is described in *Materials and Methods*. *T* represents total non-fractionated gastric mucosal cells. The expression of β-actin mRNA is also shown

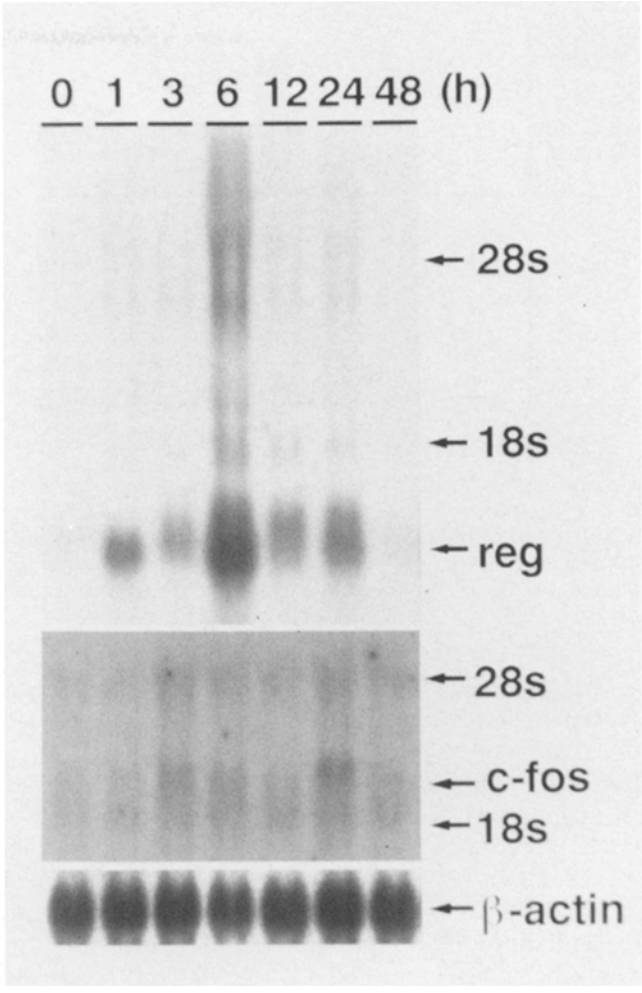

Fig. 4. Effects of indomethacin treatment on expression of *reg* (*upper panel*) and c-*fos* (*lower panel*) mRNAs in rat stomach. Samples were taken 0, 1, 3, 6, 12, 24, and 48h after indomethacin administration. The expression of β-actin mRNA is also shown

treatment and reached maximal level 24h after injection (Figs. 4 and 5).

As shown in Fig. 2, in untreated rat stomach, *reg* mRNA was expressed almost exclusively in the deepest zone of the mucosal layer, and this distribution pattern was not changed after injection of vehicle (Fig. 6a). In contrast, after injection of indomethacin, in addition to showing increased expression in the deepest layer, *reg* mRNA expression was detected in all mucosal layers (Fig. 6b). These findings were confirmed by in situ hybridization histochemistry (Figs. 7 and 8). In stomachs of untreated animals, *reg* mRNA was detected in a few small cells at the bottom of the crypt (Fig. 7). In contrast, in the injured stomach, many cells in all mucosal layers showed strong *reg* mRNA signals (Fig. 8).

Discussion

The role played by Reg protein in the regeneration and proliferation of pancreatic β cells has been shown in previous studies. Increased expression of the *reg* gene was demonstrated in regenerating islet cells after 90% of the pancreas was removed,[10] and this increase was correlated with β cell proliferative activity.[11,19] Further, recombinant rat Reg protein,[20] administered both in vivo and in vitro, enhanced the proliferation of pancreatic β cells and cultured islets.[12]

reg mRNA is present in the gastric wall.[13] However, its functional role in the stomach has not yet been clarified. In the present study, we demonstrated that in rat gastrointestinal tract, the expression of *reg* mRNA was most prominent in the stomach, confirming a previous report.[13] We showed, for the first time, that *reg* gene

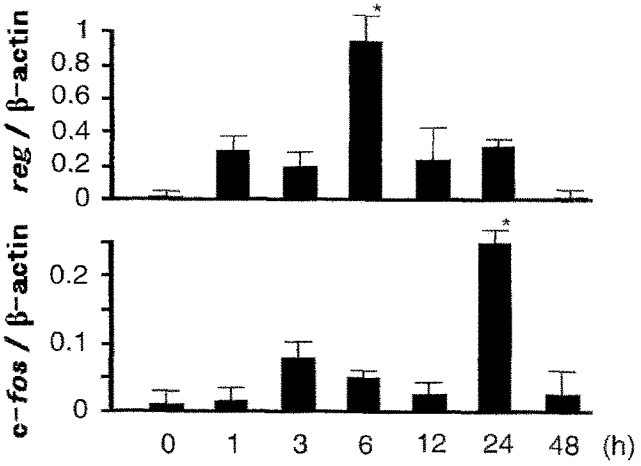

Fig. 5. Expression of *reg* mRNA, quantitated by normalization of hybridization, relative to the expression of β-actin mRNA, determined by scanning Northern blot analysis (Fig. 4), using densitometry. Values are means ± SE from five animals. $*P < 0.001$; significantly different from control (0 h)

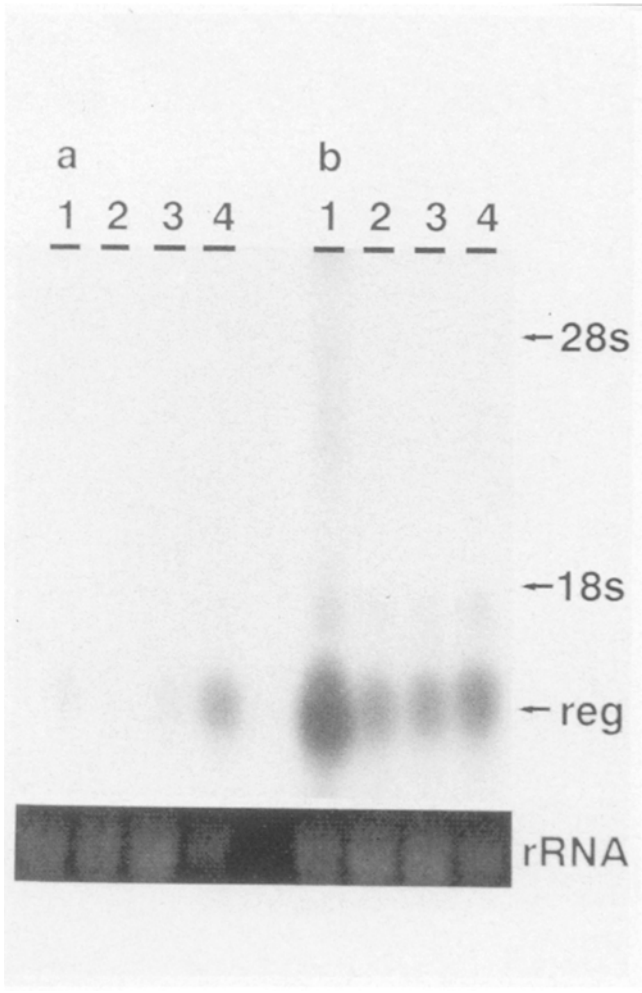

Fig. 6a,b. Effects of indomethacin treatment on distribution of *reg* mRNA in gastric mucosal cells. **a** Distribution of *reg* mRNA 6 h after vehicle administration; **b** Distribution 6 h after indomethacin treatment. *Lane 1*, whole stomach; *lane 2*, superficial mucosal layer; *lane 3*, middle mucosal layer; *lane 4*, deepest mucosal layer

expression was markedly augmented during the regeneration of gastric mucosal cells after chemically induced mucosal damage. The response of *reg* gene expression was very rapid, being evident as early as 1 h after the induction of gastric damage. This early response of *reg* mRNA preceded the response of c-*fos* mRNA, which was evident 3 h after indomethacin injection. c-*fos*, one of the early response genes, is known to be related to the initiation of cell proliferation.[21] The expression of this gene is also reported to be enhanced when the proliferation of gastric mucosal cells is stimulated.[22] As noted above, Reg protein enhanced the proliferation of pancreatic β cells.[12] Therefore, it is tempting to speculate that the *reg* gene product may be involved in the proliferation and regeneration of gastric cells in the damaged mucosa through enhancement of c-*fos* expression.

Undifferentiated proliferating cells (hypothetical stem cells) of the gastric mucosa are present in the neck of gastric glands located in the middle layer of the gastric mucosa.[1,2] The cells present in this neck zone supply epithelial cells to the other layers of the gastric wall.[1,2] Accordingly, to clarify the topographical relationship between proliferating cells and the layers that express the *reg* gene, we investigated for the presence of *reg* mRNA in each layer of the gastric mucosa. Interestingly, *reg* mRNA was found only in the deepest mucosal layer and was not present in the middle layer. The deepest gastric mucosal layer consists predominatly of two kinds of cells, chief cells and endocrine cells,[1-3] chief cells being larger than endocrine cells.[23] Therefore, to clarity the type of cell, we separated cells from the deepest mucosal layer according to size, by the counterflow elutriation technique. Using this method, we have previously shown that endocrine cells are concentrated in fraction 1, consisting of the smallest cells, and that chief cells are present in fraction 4, consisting of larger cells.[23] In the present study, Northern blot analysis clearly revealed that *reg* mRNA was present only in fraction 1. Moreover, in situ hybridization histochemistry demonstrated *reg* mRNA signals in small cells at the bottom of the crypt. Taken together, these findings strongly suggest that the *reg* gene is expressed in gastric mucosal endocrine cells. This observation coincides with reports that regenerating pancreatic endocrine cells show the strongest expression of *reg* mRNA.[10,19,24]

However, it is of interest that, during the healing of gastric mucosal damage, the expression of *reg* mRNA not only increased in the deepest layer of the mucosa but was also seen in both the superficial and middle layers on Northern blot analysis and on in situ hybrid-

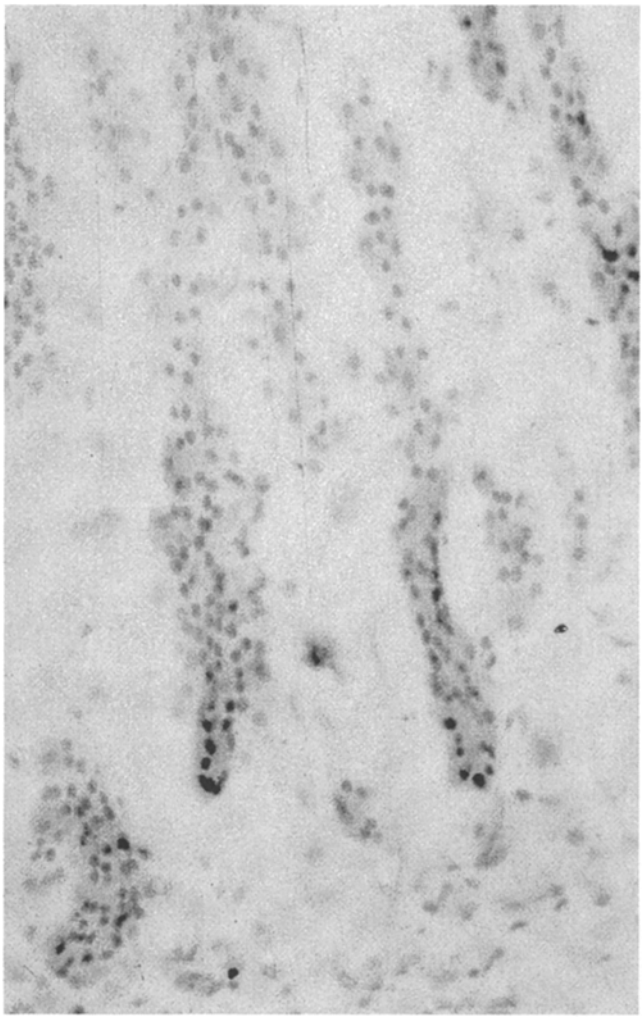

Fig. 7. Localization of *reg* mRNA in gastric mucosa of untreated rats, determined by in situ hybridization. The *reg* antisense riboprobe hybridized in a few small cells at the bottom of crypts. The *reg* sense riboprobe did not hybridize in the section (data not shown). ×200

ization histochemistry. Therefore, it is reasonable to speculate that, in addition to gastric endocrine cells, proliferating immature cells located in the middle layer of the mucosa begin to express the *reg* gene during the process of gastric mucosal regeneration.

In summary, we have demonstrated for the first time that *reg* gene expression in the rat gastric mucosa is augmented during the healing of mucosal damage. This increased expression of *reg* mRNA precedes the expression of c-*fos* mRNA and the proliferation of gastric mucosal cells. In a normal state, *reg* mRNA appears to be present only in gastric mucosal endocrine cells. During the healing of gastric mucosal damage, on the other hand, other cells may participate in the production of *reg* protein.

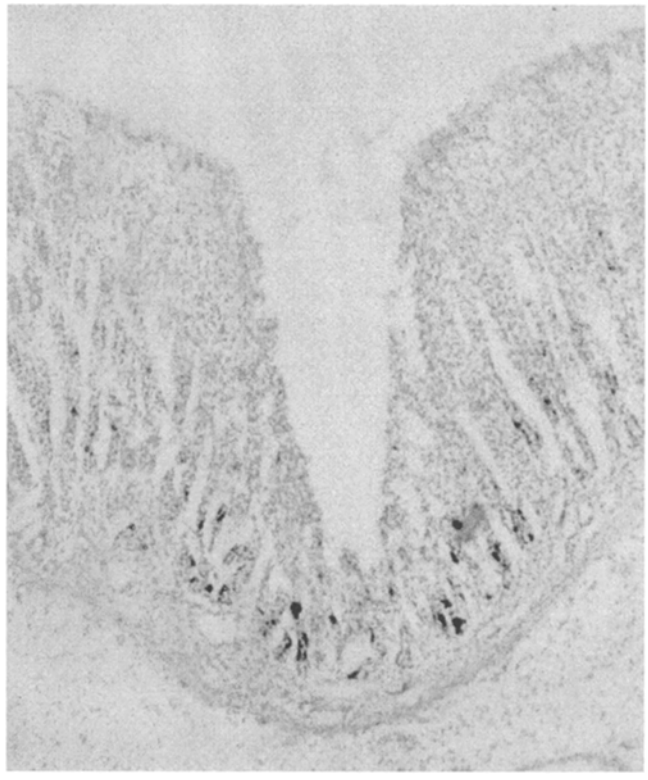

Fig. 8. Localization of *reg* mRNA in gastric mucosa of rats treated with indomethacin, determined by in situ hybridization. Increased expression of *reg* mRNA was detected in throughout all mucosal layers by the *reg* antisense riboprobe. The *reg* sense riboprobe did not hybridize in the section (data not shown). ×40

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