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Gastric restitution is inhibited by dexamethasone, which is reversed by hepatocyte growth factor and rebamipide

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SUMMARY

Background: Glucocorticoids have been shown to induce peptic ulcers, especially when co-administered with NSAIDs. Hepatocyte growth factor (HGF) plays a role in gastric ulcer repair, facilitating the restitution of gastric mucosal epithelial cells. HGF expression is induced by PGs in gastric fibroblasts. We hypothesized that dexamethasone (DEX) may inhibit PG production and HGF expression, thus inhibiting HGF-induced gastric epithelial restitution.

Aim: To investigate the effect of DEX on gastric restitution, using cultured gastric cells, the role of HGF in the restitution inhibited by DEX, and the effect of rebamipide on DEX- inhibited restitution.

Methods: Human gastric fibroblasts were prepared from human stomach obtained at surgery; PGE2 and HGF is determined by ELISA; Restitution was assessed by the round wound restitution model, using coculture of gastric fibroblasts and epithelial cells; COX-2 and HGF mRNA were quantified by TaqMan RT-PCR system.

Results: 1. DEX inhibited HGF mRNA and COX-2 mRNA. Accordingly, it inhibited PGE2 and HGF release. 2. DEX inhibited the restitution of gastric cells. 3. The inhibition of restitution was reversed by HGF and rebamipide to the same extent. 4. Rebamipide induced PGE2 and HGF.

Conclusion: DEX inhibits restitution via HGF depletion, and rebamipide reverses the inhibited restitution by HGF induction.

INTRODUCTION

Various kinds of steroids such as dexamethasone (DEX) are, in many cases, used for chronic inflammatory diseases such as rheumatic arthritis, even though they are known as inducers for peptic ulcers[1, 2] especially when patients take both steroids and NSAIDs.[3] Considering that patients with rheumatic diseases are likely to be taking both drugs simultaneously, steroid-induced ulcer is one of the important clinical problems in the field of gastroenterology which remains to be solved as a non-*Helicobacter pylori* gastric mucosal injury. We previously demonstrated that hepatocyte growth factor (HGF) strongly facilitated the proliferation and restitution of gastric mucosal epithelial cells.[4–7] HGF expression is induced by prostaglandins (PGs) more strongly than other factors in gastric fibroblasts.[8] Cyclooxygenase-2 (COX-2) is the key enzyme for production of PGs, which is also regarded as an important key factor for gastric ulcer healing.[9] Therefore, we hypothesized that COX-2 expression, PG production, and HGF expression are the series of processes that are the key for gastric ulcer healing. DEX is known to enhance the expression of IκB which inhibits the activation of NF-κB.[10–12] COX-2, an inducible isozyme of cyclooxygenase, is selectively expressed in response to various factors such as inflammatory cytokines and lipopolysaccharide and its expression is suppressed by the glucocorticoids such as DEX. NF-κB site is involved in both expression of the

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COX-2 gene and its suppression by DEX.[13–17] Phospholipase A2, the enzyme liberating the prostaglandin precursor arachidonic acid, was also inhibited by DEX.[18] As a result, prostaglandin production is inhibited by DEX in either event. Previously, we showed the possibility that NSAIDs-induced gastric mucosal impairment is attributed to its inhibition of HGF via PG.[19] Since the risk of ulcers synergistically increases when the patients take both corticosteroids and NSAIDs[3] it is of interest whether the mechanism of steroid ulcer can be explained in association with NSAID ulcer. In the present study, we assumed that DEX may inhibit the COX-2 expression and as a result inhibit PG production and HGF expression, or that DEX inhibits phospholipase A2 and as a result, prostaglandins and HGF. In either case, it may inhibit the HGF-induced gastric epithelial restitution. This may be one of the mechanisms of steroid ulcers. We also investigated the effect of rebamipide, a locally acting gastroprotective agent that is widely used clinically, on the PGE2 release and HGF induction by gastric cells, and accordingly on the restitution inhibited by dexamethasone.

METHODS

Cultured cells

The primary culture of human gastric fibroblasts was prepared from a human stomach obtained at surgery, after enzyme digestion, according to previous methods.[7] RGM-1 was used as the gastric epithelial cells for restitution assay.

Measurement of PGE2 and HGF

PGE2 and HGF in conditioned medium was determined by enzyme-linked immunosorbent assay (ELISA), using PGE2 EIA kit (Cayman Chemical, AnnArbor, MI, USA) and HGF EIA kit (Institute of Immunology, Tokyo, Japan), respectively.

Restitution assay

Restitution was assessed using the round wound restitution model, using coculture of gastric fibroblasts and epithelial cells (RGM-1), according to the previously described method.[5, 6] In brief, the effect of dexamethasone, HGF, and rebamipide on restitution was studied using an *in vitro* gastric mucosal model which consisted of gastric epithelial cells (RGM-1) and human gastric fibroblasts. Confluent coculture of gastric epithelial cells and fibroblasts in 24-well culture plates were wounded with a custom-made scraper that produced a round wound with a diameter of about 1.5 mm in each well. Then the monolayers were washed with fresh serum-free medium, and were further cultured in fresh serum-free medium with various agents. The restitution was assessed by determining the denuded areas in a time course. Restitution of the coculture was assessed in a blind fashion to avoid observer bias. Accordingly, determination of the uncovered area was performed by a person who was unaware of the details of the experiment. Photomicrographs of the wounds were obtained at a 40-fold magnification using a Nikon microscope and camera. Then prints were made and the wound area was cut out from each print and weighed. The weight was precisely related to the area, since the thickness of the prints was constant. We used paper which weighed 220 g/m^2. Experiments were performed four times and the results were expressed as the mean ± S.E.

Assessment of COX-2 mRNA expression

The expression of COX-2 mRNA was assessed using a TaqMan™ quantitative RT-PCR system according to the manufacturer's instruction (Perkin-Elmer, Applied Biosystems, Roche Molecular Systems, Inc., Branchburg, NJ, USA).

The design of the TaqMan probes, combined with the 5′–3′ nuclease activity of rTth DNA polymerase, allowed for the direct detection of PCR products by the release of a fluorescent reporter during PCR on the ABI PRISM 7700 Sequence Detector.[20, 21] When the probe is intact, the proximity of reporter dye to the quencher dye results in suppression of reporter fluorescence, primarily by Forster-type energy transfer.[22] During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The nucleolytic activity of the rTth DNA polymerase enzyme cleaves the probe between the reporter and the quencher only if this region hybridizes to the target. rTth DNA polymerase did not digest the free probe. After degradation of the hybridized probe, the shortened probe dissociates from the target and polymerization of the strand continues. This process occurs during every cycle and does not interfere with the exponential accumulation of the product. The degradation of the oligonucleotide between the reporter and quencher dyes resulted in

increased fluorescence of the reporter proportional to the amount of the product accumulated.

Oligonucleotide primers and TaqMan probes for COX-2 and HGF were designed using Primer Express, version 1.0 (Perkin-Elmer Applied Biosystems Inc.). (Sense primer for COX-2: TTT GCA TTC TTT GCC CAG C; antisense primer COX-2: GGG AGG ATA CAT CTC TCC ATC AAT; TaqMan probe for COX-2: ACT CTG GCT AGA CAG CGT AAA CTG CGC; sense primer for HGF: ATG CAT GAC CTG CAA TGG G; antisense primer for HGF: GAG TAT AGC ACC ATG GCC TCG; TaqMan probe for HGF: TCA TCA GAC ACC ACA CCG GCA CAA.) The TaqMan probe consisted of an oligonucleotide with a 5′-reporter dye and a downstream, 3′ quencher dye. The RT-PCR reaction was carried out in 96 sample tubes per assay (25 μL per tube) in a reaction buffer containing 1 × TaqMan EZ buffer, 3 mM $Mn(OAc)_2$, 300 μM dA/dC/dG/dUTP, 2.5 unit rTth DNA polymerase, 200 nM primers (forward and reverse), 100 nM TaqMan probe, 100 ng total RNA. PCR conditions were 95 °C for 15 s, 58 °C for 1.5 min for 40 cycles on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems). COX-2 (HGF) mRNA was comparatively quantified using GAPDH mRNA as a control. COX-2 (HGF) mRNA was compared with GAPDH mRNA which is determined simultaneously by the same PCR reactions. COX-2 (HGF)/GAPDH was calculated using the following formula:

$$\text{COX-2 (HGF) mRNA/GAPDH mRNA} = 2y/2x = 2y - x$$

The formula was derived from the equation:

$$\text{The specific amount of product accumulated} = \text{COX-2 (HGF) mRNA} \times 2x = \text{GAPDH mRNA} \times 2y$$

(x cycles for COX-2 (HGF) mRNA and y cycles for GAPDH mRNA to reach a certain amount of accumulated product.)

Statistical analysis

Data are presented as mean ± S.E. for *n* determinations. Comparisons between two groups were made by a Student's *t*-test for grouped or paired data when appropriate; comparisons among several groups were made by analysis of variance, followed by Dunnett's test, when appropriate. In all analysises, statistical significance was attributed at a 95% or greater confidence level.

RESULTS

Effect of DEX on PGE2 release and HGF expression by gastric fibroblasts

Gastric fibroblasts were incubated with DEX at concentrations of 10^{-8}–10^{-4} M. After incubation for 6, 12, and 18 h, the conditioned medium was obtained and PGE2 and HGF in the medium was determined. Dexamethasone inhibited the expression of PGE2 and HGF in a time (data not shown) and dose-dependent manner (Figure 1).

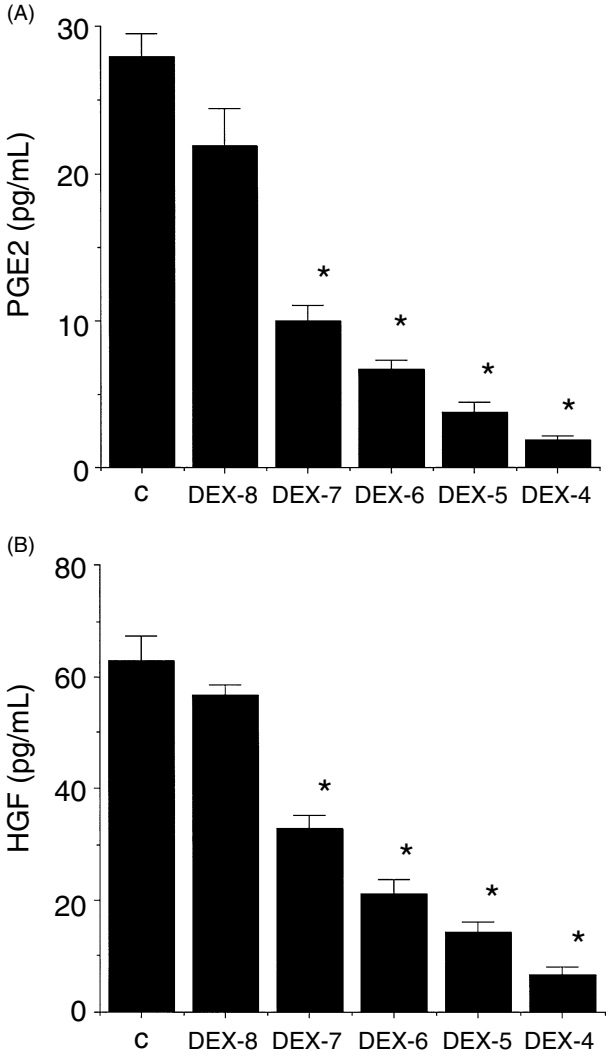

Figure 1. Effect of dexamethasone on PGE2 and HGF release by human gastric fibroblasts. (A) Dexamethasone significantly and dose-dependently inhibited PGE2 release by gastric fibroblasts, as assessed by ELISA; (B) Dexamethasone significantly and dose-dependently inhibited HGF release by gastric fibroblasts, as assessed by ELISA. (mean + S.E., $^*P < 0.01$ vs. control.) (DEX-4: dexamethasone 10^{-8} M, etc.)

Effect of DEX on COX-2 and HGF mRNA expression by gastric fibroblasts

Gastric fibroblasts in the 6-well plate were also incubated with DEX at concentration of 10^{-6} M for 4 h. Total RNA was extracted from the fibroblasts and COX-2 mRNA was quantitatively evaluated by TaqMan RT-PCR. Mean and standard error values were calculated from six independent determinations. COX-2 mRNA expression was significantly inhibited by DEX, indicating that PGE2 release inhibition by DEX was via COX-2 depletion (Figure 2A).

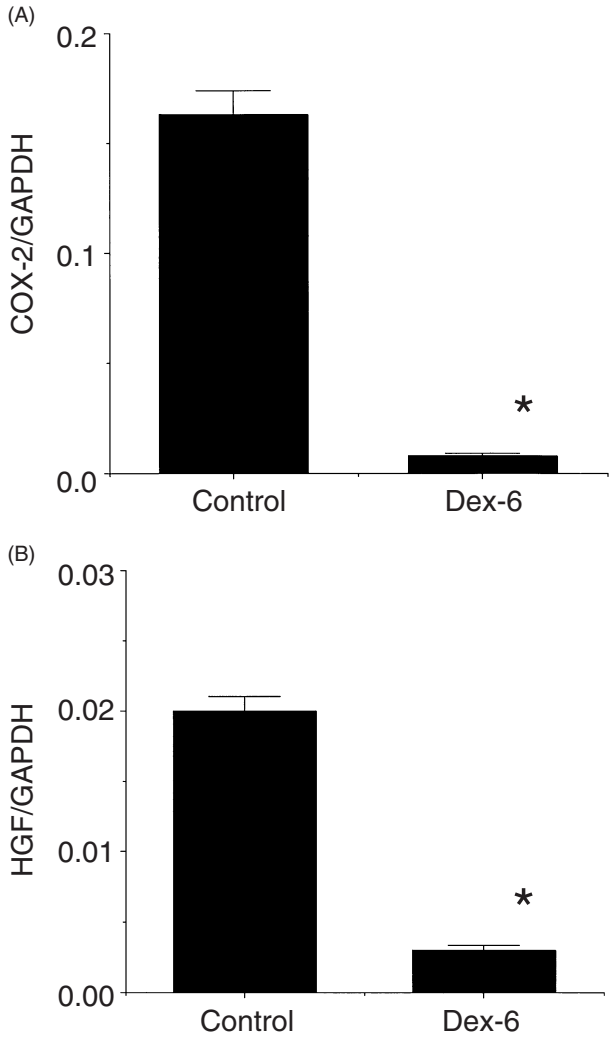

Figure 2. Effect of dexamethasone on COX-2 and HGF mRNA expression by human gastric fibroblasts. (A) COX-2 mRNA expression in gastric fibroblasts was significantly inhibited by dexamethasone, as assessed by TaqMan RT-PCR; (B) HGF mRNA expression in gastric fibroblasts was significantly inhibited by dexamethasone, as assessed by TaqMan RT-PCR. (mean + S.E. of six experiments, *$P < 0.01$ vs. control.) (Dex-6: dexamethasone 10^{-6} M).

HGF was mRNA was also significantly inhibited by DEX (Figure 2B).

Effect of DEX on gastric cell restitution

Coculture of gastric fibroblasts and epithelial cells in 24-well culture dishes were denuded with a scraper and incubated with culture medium in the presence of dexamethasone. Round denuded areas of about 1.5 mm in diameter were gradually covered by gastric cells as they migrated towards the centre of the denuded area over time, which was defined as restitution. DEX inhibited the restitution of the coculture of gastric epithelial cells and fibroblasts in a dose-dependent manner (Figure 3).

Effect of HGF and rebamipide on the restitution inhibited by DEX

The culture medium was also supplemented with HGF in addition to DEX, in the restitution assay described above. The restitution inhibition by DEX was reversed by HGF in a dose-dependent manner (Figures 4A,C), indicating that HGF depletion may be the mechanism of restitution inhibition by DEX. Rebamipide also reversed the restitution inhibited by DEX in a dose-dependent manner, to similar extent as HGF (Figures 4B,C). This data suggests that rebamipide may be a good candidate

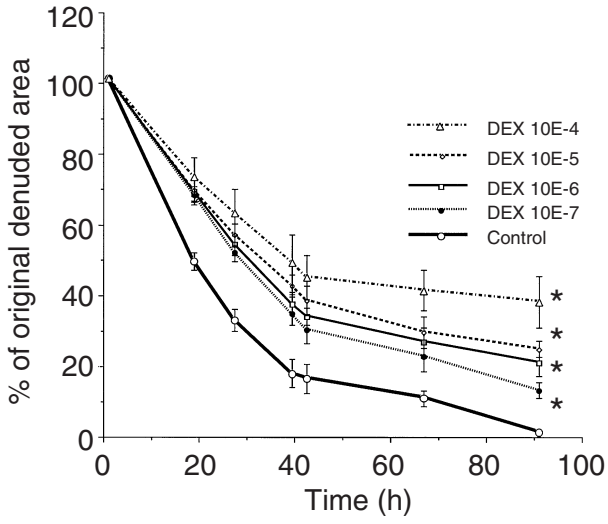

Figure 3. Effect of dexamethasone on restitution of gastric cells. Dexamethasone dose-dependently and significantly inhibited the restitution of coculture of gastric epithelial cells and fibroblasts, as assessed by round wound restitution model. (mean ± S.E., *$P < 0.01$ vs. control.)

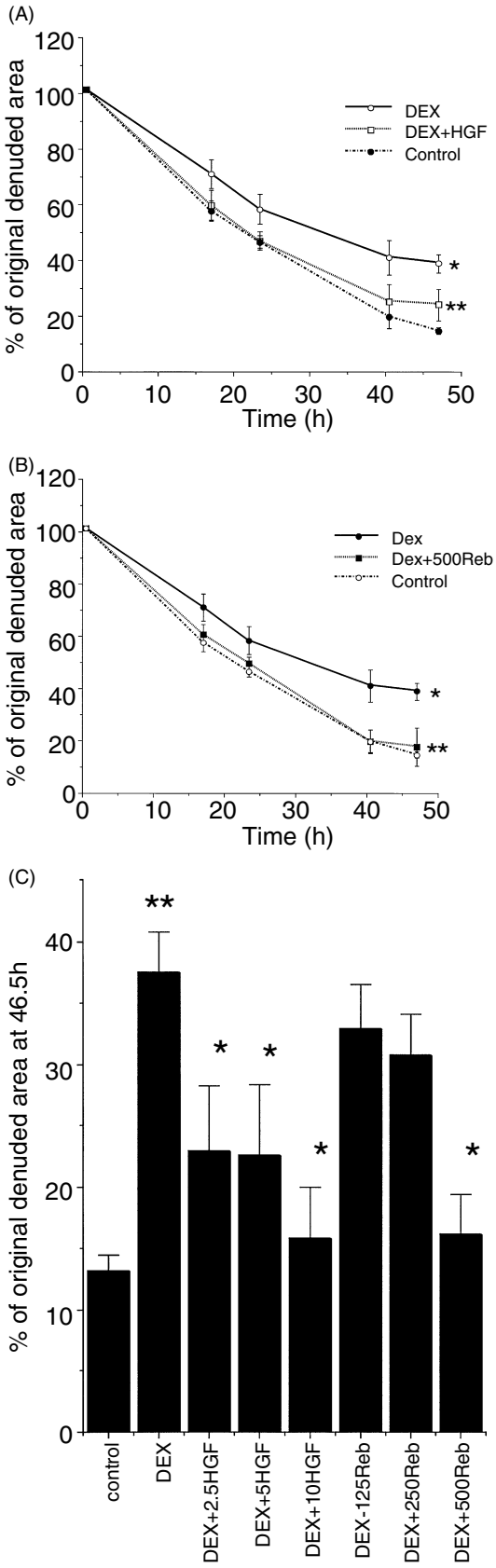

Figure 4. Effect of HGF and rebamipide on inhibited restitution by dexamethasone. (A) The restitution inhibited by dexamethasone (10^{-6} M) was reversed by HGF (10 ng/mL); (B) Rebamipide (500 μM) also reversed the inhibited restitution by dexamethasone. (mean ± S.E., $^{*}P < 0.01$ vs. control. $^{**}P < 0.01$ vs. dexamethasone alone); (C) Comparison of the effects of HGF and Rebamipide on the inhibited restitution by dexamethasone. Both HGF and rebamipide dose-dependently and significantly reversed the restitution inhibited by dexamethasone, to the same extent. The comparison was performed at 64.5 h after the restitution started. (mean + S.E., $^{*}P < 0.01$ vs. dexamethasone alone. $^{**}P < 0.01$ vs. control) (DEX: dexamethasone 10^{-6} M) (2.5HGF: HGF 2.5 ng/mL) (125Reb: rebamipide 125 μM).

for the treatment and/or prevention of steroid-induced gastric injuries.

Effect of rebamipide on PGE2 release by gastric fibroblasts

After incubation for 12 h with rebamipide at concentrations from 3 μM to 300 μM, the conditioned media of gastric fibroblasts were obtained and PGE2 was determined by ELISA. PGE2 release by gastric fibroblasts was increased by rebamipide dose-dependently (Figure 5).

Effect of rebamipide on HGF release by gastric fibroblasts

HGF concentration of the conditioned medium was also determined by ELISA. HGF release by gastric fibroblasts

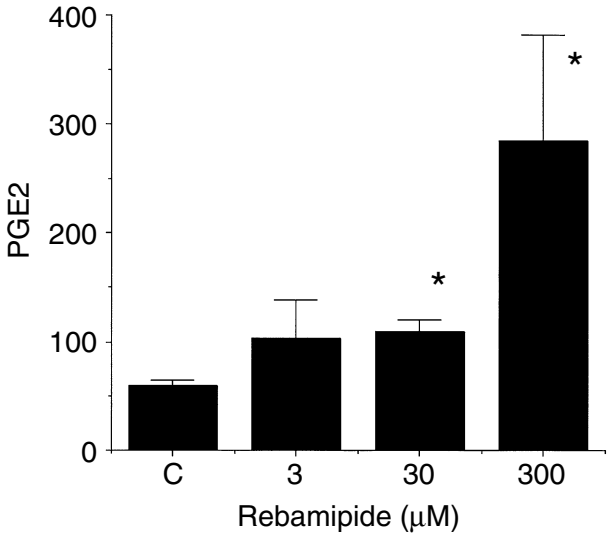

Figure 5. Effect of rebamipide on PGE2 by human gastric fibroblasts, as assessed by ELISA. Rebamipide significantly induced PGE2 release by gastric fibroblasts. (mean + S.E., $^{*}P < 0.01$ vs. control.)

was increased dose-dependently by rebamipide probably via PG release (Figure 6).

Effect of rebamipide on PGE2 release in the presence of H. pylori

PGE2 release by gastric fibroblasts was increased by rebamipide more significantly in the presence of *H. pylori* presence than without *H. pylori* (Figure 7).

DISCUSSION

Firstly, we demonstrated that DEX inhibited gastric restitution in the cultured restitution model. This is equivalent to steroid-induced gastric mucosal impairment in clinical observations.[1, 2] The mechanism of the steroid ulcers is not yet clear. Therefore, we tried to investigate the mechanism, using this *in vitro* culture system. As stated in the introduction, we proposed a hypothesis that ulcer repair involves a series of processes, namely, inflammation, COX-2, prostaglandins, HGF, and restitution. DEX inhibits COX-2 and PGs, which was confirmed in the gastric cells in the present study. Therefore, if the hypothesis is correct, dexamethasone inhibits restitution via HGF depletion, which is the effector for restitution.[6] The second new finding in the present study was that inhibition by dexamethasone was reversed by HGF, which supports this hypothesis.

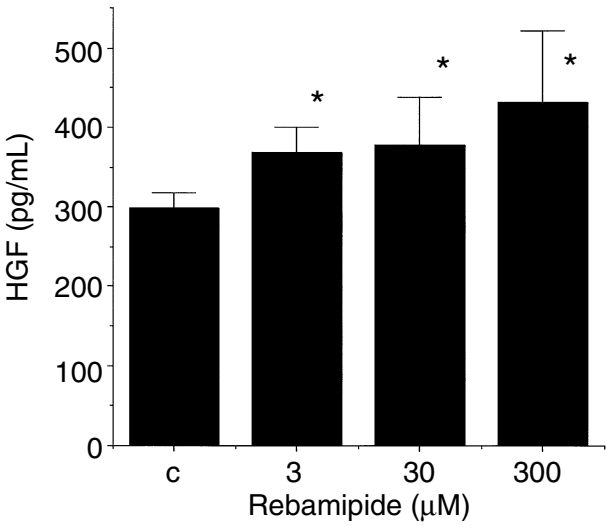

Figure 6. Effect of rebamipide on HGF release by gastric fibroblasts, as assessed by ELISA. Rebamipide dose-dependently induced HGF release by gastric fibroblasts. (mean + S.E., $^*P < 0.01$ vs. control.)

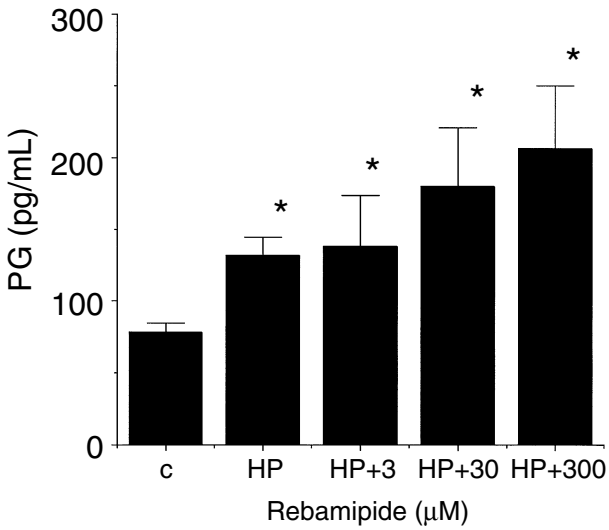

Figure 7. Effect of rebamipide on PGE2 release by gastric fibroblasts in *Helicobacter pylori* presence. Rebamipide induced PGE2 release by gastric fibroblasts more significantly in *Helicobacter pylori* presence. (HP: *Helicobacter pylori*) (mean + S.E., $^*P < 0.01$ vs. control.)

Previously, we have shown that NSAID-induced ulcer may be attributed to its HGF inhibition via COX-2 inhibition.[19] This is also based on the hypothesis described above. Therefore, the mechanism of NSAID-induced ulcer and corticosteroid-induced ulcer can be explained on the same basis. This is also in accordance with a case-controlled study based on a Tennessee Medicaid records[3] in a sense that both classes of drugs may synergistically cause peptic ulcers. We should evaluate the effect of rebamipide on NSAID-inhibited restitution in the same cultured system in the future.

Clinically, HGF may not be useful because of some reasons such as its deactivation by acid or its possible effect on other organs in the formation of neoplasms. Instead, locally acting agents which increase HGF may be useful for prevention or healing of steroid-induced gastric mucosal impairment. We took rebamipide as an example, since rebamipide is known to induce PGs and HGF, which was also confirmed in the culture system (unpublished data). We investigated its effect on the restitution in the same *in vitro* culture system. It reversed dexamethasone-inhibited gastric cell restitution. Therefore, it may be a good candidate for this purpose. This was our third new finding in the present study.

In conclusion, we demonstrated that DEX inhibited gastric cell restitution, which was reversed by HGF and

rebamipide. This may be explained by the hypothesis that repair includes a series of processes, namely, inflammation, COX-2, prostaglandins, HGF, and repair.

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