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Effects of rebamipide on bile acid-induced inhibition of gastric epithelial repair in a rabbit cell culture model

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SUMMARY

Background: Anti-ulcer agents exert various functional effects on gastric epithelial cells.
Aim: The effects of a novel gastro-cytoprotective agent (rebamipide) on epithelial restoration following bile acid damage were assessed using primary cultured rabbit gastric epithelial cells.
Methods: Rebamipide was added to complete confluent cell sheets with deoxycholic acid just after creating a cell-free wound (2 mm^2). The restoration was monitored and analysed by phase contrast microscopy and an image analyser for 48 h. The migration speed was measured during the initial 3 h after wounding. Cell proliferation was detected by staining for bromodeoxyuridine (BrdU) at 12-h intervals. The labelling index was calculated per unit area. The major cytoskeletal protein actin was detected by immunohistochemical staining.
Results: In the controls, restoration was completed 48 h following wounding. Deoxycholic acid retarded this process. The addition of rebamipide to deoxycholic acid abolished the bile acid-induced retardation. The migration speed was 26 μm/h in the controls, 15 μm/h in the deoxycholic acid group and 27 μm/h in the deoxycholic acid plus rebamipide group. In the controls, BrdU-positive cells, which were rarely detected in the initial 24 h, were maximal at 36 h (labelling index 1.7%). In the deoxycholic acid group, proliferation was inhibited (peak labeling index; 0.5% at 48 h). Actin-containing stress fibres were detected throughout the cells and the periphery of the lamellipodia in the controls, and were disrupted in the deoxycholic acid-treated group. Rebamipide prevented these effects.
Conclusions: Deoxycholic acid significantly retarded restoration by the inhibition of both cell migration and proliferation, potentially through an effect on the cytoskeleton. Rebamipide protected the mucosal cells from bile acid mediated injury.

INTRODUCTION

Histamine H_2-receptor antagonists and proton pump inhibitors (PPI) are the most popular and effective agents for the treatment of peptic ulcer disease. In addition to these agents, gastric cytoprotective agents including prostaglandins and sucralfate—which have no direct effects on acid secretion—have been used in the management of these patients.[1, 2]

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Bile acids regurgitated from the duodenum have been implicated in the aetiology of gastritis and peptic ulcer disease. The mechanisms by which bile acids injure the gastric mucosa have not yet been elucidated. One possible explanation is that the bile acids dissolve the lipid component of the cell membrane, thereby acting as detergents within the stomach lumen without entering the cells.[3] Another possibility is that the bile acids first enter the mucosal cells and cause some intracellular disturbance that results in altered mucosal function.[4]

Rebamipide is a novel quinolinone-derived gastro-cytoprotective agent used in Japan and Korea for the

treatment of peptic ulcer diseases.[5,6] In this study, the effects of rebamipide were assessed using a deoxycholic acid-induced model of mucosal damage in primary cultured gastric epithelial cells.[7] The cultured cells used in this model were predominantly mucous cells.[7] This model is therefore useful for the clarification of the effects of various agents on the restoration of mucous cells.

MATERIALS AND METHODS

Preparation and culture of gastric epithelial cells

Fasted male Japanese white rabbits weighing 2.0 kg were used. Gastric epithelial cells were isolated and inoculated according to previously described methods.[7] They were cultured in Coon's modified Ham's F-12 medium, supplemented with inactivated 10% foetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin.

Total gastric epithelial cell restoration

The effects of deoxycholic acid (Sigma) and rebamipide (2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4-yl] propionic acid) (Otsuka Pharmaceutical Co., Tokushima, Japan) on gastric epithelial cell restoration were assessed by previously reported methods.[7–9] Following formation of the complete monolayer cell sheet, a circular artificial wound (cell-free area 2 mm^2) was created in the centre of the dish. Restoration was assessed using cell migration and proliferation assays which were performed in serum-free media. Epithelial restoration was monitored using an inverted phase-contrast microscope equipped with a time-lapse laser videodisc recorder (LVR-3000N; Sony, Tokyo). Changes in the cell-free area during restoration were quantitatively analysed using an image analyser (IBAS II, Carl Zeiss Japan, Tokyo).[7]

The effects of deoxycholic acid were assessed by its addition to the medium just after wounding in concentrations of 10 μM, 50 μM and 150 μM. The effects of rebamipide (10^{-6} M, 10^{-5} M and 10^{-4} M) were assessed by its addition to the medium with or without deoxycholic acid (150 μM) just after wounding. In the additional experiments, the effects of hydrogen peroxide (3 μM) on epithelial restoration were assessed by addition to the medium with or without rebamipide (10^{-5} M and 10^{-4} M).

Migration assay

Migration velocities of the cells located on the wound edge at the time of wounding were measured. The speed of cell migration from 1 to 4 h following wounding was monitored by tracing cell movement using the equipment described above. The velocity of cell migration was calculated independent of cell proliferation.[7,9] The migration speed (μm/h) was reported in the controls, the deoxycholic acid (150 μM)-treated group, the rebamipide-treated group (10^{-4} M), and the deoxycholic acid (150 μM) plus rebamipide (10^{-4} M) group.

Proliferation assay

DNA synthesizing cells were sequentially detected during the process of re-epithelization every 12 h using indirect immunohistochemical methods with monoclonal anti-bromodeoxyuridine (BrdU, Sigma) antibody. The BrdU labelling index around the wound was calculated according to the methods of previous reports.[7,10]

Actin staining

Actin in the gastric epithelial cells was stained during restoration using the phalloidin–rhodamine method according to our previous report.[7]

Statistical analysis

All experiments were performed in triplicate from five separate cell preparations ($n = 5$). The results were expressed as the mean $\pm$ s.d. Statistical analysis was performed using analysis of variance and the non-paired Student's *t*-test.

RESULTS

Gastric epithelial restoration

In the controls, the cells on the edge of the wound began to form pseudopodia-like structures (lamellipodia) just after wounding. The lamellipodia started ruffling and moving toward the centre of the cell-free area (Figure 1a). This migration occurred as sheet migration. The cell-free area was repopulated in a time-dependent

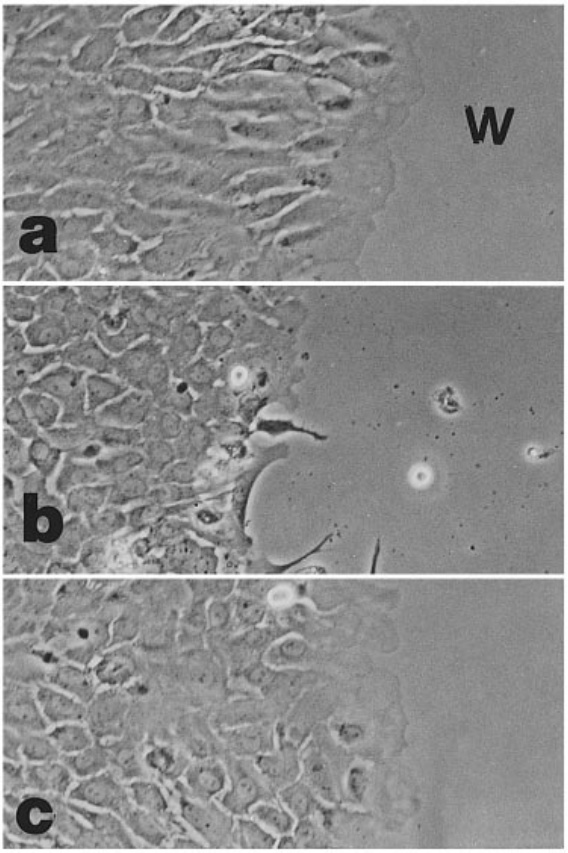

Figure 1. Phase contrast micrographs showing lamellipodia formation. (a) Lamellipodia were formed shortly after wounding in the controls. (b) Deoxycholic acid (150 μM) suppressed the formation of lamellipodia. (c) Addition of rebamipide (10^{-4} M) with deoxycholic acid (150 μM) resulted in full formation of lamellipodia. Original magnification $\times 100$.

manner and was eventually completely repaired. Active ruffling movement of the lamellipodia was characteristic for normal epithelial restoration. The lamellipodia disappeared following complete restoration.

In the groups treated with deoxycholic acid, the formation and ruffling movement of the lamellipodia was deficient compared to the controls (Figures 1b and 1c). Epithelial restoration was much slower in the controls than in the deoxycholic acid groups (50 μM to 150 μM, Table 1). Treatment with deoxycholic acid significantly retarded the restoration process. Rebamipide itself did not accelerate mucosal restoration. However, rebamipide abolished the suppressive effects of deoxycholic acid on epithelial restoration (Table 2). In the additional experiments, hydrogen peroxide significantly suppressed the epithelial restoration but rebamipide abolished this effect (Table 3).

Cell migration

The velocity of cell migration was 26.1 ± 2.5 μm/h in the controls, 15.2 ± 1.1 μm/h in the deoxycholic acid (150 μM) group ($P < 0.01$), and 26.4 ± 2.8 μm/h in the rebamipide (10^{-4} M) group. The migration speed was 27.2 ± 2.8 μm/h in the deoxycholic acid plus rebamipide (10^{-4} M) group.

BrdU staining

BrdU-positive proliferating cells existed 80.0 ± 60.6 μm (mean $\pm$ s.d., $n = 150$) from the edge of the wound (Figure 2). Sequential training for BrdU following wounding in the controls revealed that BrdU-positive cells were rarely detected in the 12-h and 24-h groups. The number of BrdU-positive cells peaked in the 36-h group. They were rarely detected following complete repair of the artificial wound. The BrdU labelling index was almost undetectable in the 12-h and 24-h groups. It was 1.7% in the 36-h group, and 0.1% in the 48-h group. In the group treated with deoxycholic acid

Table 1. The size of cell-free area during epithelial restoration (mm²)

	0 h	12 h	24 h	36 h	48 h
Control	2.12 ± 0.11	1.00 ± 0.10	0.52 ± 0.14	0.21 ± 0.02	0
DCA (10 μM)	2.00 ± 0.08	0.98 ± 0.13	0.48 ± 0.12	0.20 ± 0.12	0
DCA (50 μM)	2.10 ± 0.09	1.09 ± 0.15	0.61 ± 0.21	0.42 ± 0.04†	0.28 ± 0.08*
DCA (150 μM)	2.04 ± 0.05	1.24 ± 0.03†	0.78 ± 0.09*	0.57 ± 0.11*	0.47 ± 0.05*

The size of the cell-free area was measured as described in Materials and Methods.
Data are expressed as the mean $\pm$ s.d. in five separate experiments ($n = 5$).
DCA = deoxycholic acid.
* $P < 0.01$ vs. control; † $P < 0.05$ vs. control.
h = hours after wounding.

Table 2. The effects of rebamipide on the size of the cell-free area during epithelial restoration (mm^2)

	0 h	12 h	24 h	36 h	48 h
Control	2.12 ± 0.11	1.00 ± 0.1	0.52 ± 0.14	0.21 ± 0.02	0
DCA (150 μM)	2.04 ± 0.05	1.24 ± 0.03*	0.78 ± 0.09†	0.57 ± 0.11†	0.47 ± 0.05*
Rebamipide (10^{-4} M)	2.04 ± 0.04	1.01 ± 0.12	0.50 ± 0.08	0.19 ± 0.08	0
DCA (150 μM)					
+ rebamipide (10^{-4} M)	2.02 ± 0.06	1.02 ± 0.12‡	0.48 ± 0.10§	0.20 ± 0.04§	0
+ rebamipide (10^{-5} M)	2.01 ± 0.10	1.19 ± 0.42	0.56 ± 0.12‡	0.28 ± 0.08§	0.12 ± 0.10*
+ rebamipide (10^{-6} M)	2.08 ± 0.04	1.22 ± 0.04	0.81 ± 0.32	0.54 ± 0.24	0.46 ± 0.12

Data are expressed as the mean ± s.d. in five separate experiments ($n = 5$).
DCA = deoxycholic acid; + rebamipide = DCA (150 μM) plus rebamipide treated group.
* $P < 0.05$ vs. control; † $P < 0.01$ vs. control; ‡ $P < 0.05$ vs. DCA group; § $P < 0.01$ vs. DCA group.
h = hours after wounding.

Table 3. The effects of rebamipide on the cell-free area during the restoration (mm^2)

	0 h	12 h	24 h	36 h	48 h
Control	2.12 ± 0.11	1.00 ± 0.1	0.52 ± 0.14	0.21 ± 0.02	0
H_2O_2 (3 mM)	2.05 ± 0.03	1.29 ± 0.04*	0.87 ± 0.03*	0.72 ± 0.05*	0.48 ± 0.05*
H_2O_2 (3 mM)					
+ rebamipide (10^{-4} M)	2.09 ± 0.03	0.93 ± 0.08†	0.52 ± 0.11†	0.25 ± 0.10†	0.08 ± 0.09†
+ rebamipide (10^{-5} M)	2.12 ± 0.05	1.17 ± 0.15	0.85 ± 0.09	0.61 ± 0.09	0.35 ± 0.05†

Data are expressed as the mean ± s.d. in five separate experiments ($n = 5$).
+ rebamipide = H_2O_2 (3 mM) plus rebamipide treated group.
* $P < 0.001$ vs. control; † $P < 0.001$ vs. H_2O_2 group.
h = hours after wounding.

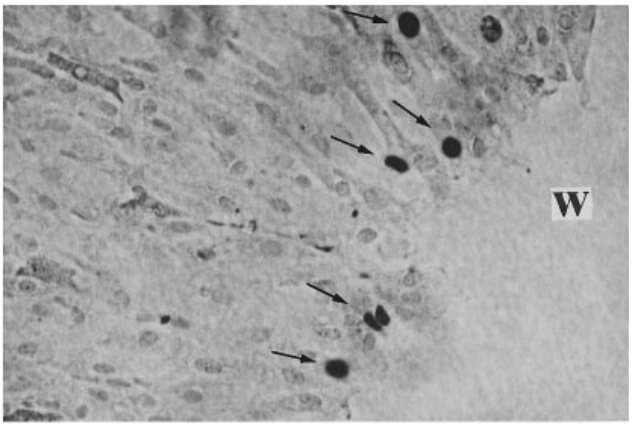

Figure 2. Detection of proliferating cells during the restoration in controls using BrdU staining. BrdU-positive cells (arrow heads) are detected only around the wound. These cells were detected in the greatest numbers 36 h after wounding. Original magnification × 100.

(150 μM), BrdU-positive cells were detected much later following wounding compared to the controls. Proliferating cells were negligible in the 12-h, 24-h and 36-h groups. A small number of BrdU-positive cells were detected in the 48-h group (0.4%, Table 4). The BrdU labelling index in the rebamipide group was similar to the controls at all time points (Table 4). In the deoxycholic acid plus rebamipide group, the labelling index returned to control levels (Table 4).

Actin staining

In the controls and the rebamipide group, specific fluorescence for actin could be detected on the stress fibres throughout the cytoplasm of the epithelial cells in the monolayer. Specific fluorescence was also detected in the submembrane area of the lamellipodia of the migrating cells. In the deoxycholic acid group, specific fluorescence intensity for actin was weak in the entire monolayer field. The actin-containing stress fibres were partly disrupted by the treatment. In the deoxycholic acid plus rebamipide group, the distribution of actin was similar to that in the controls (Figure 3).

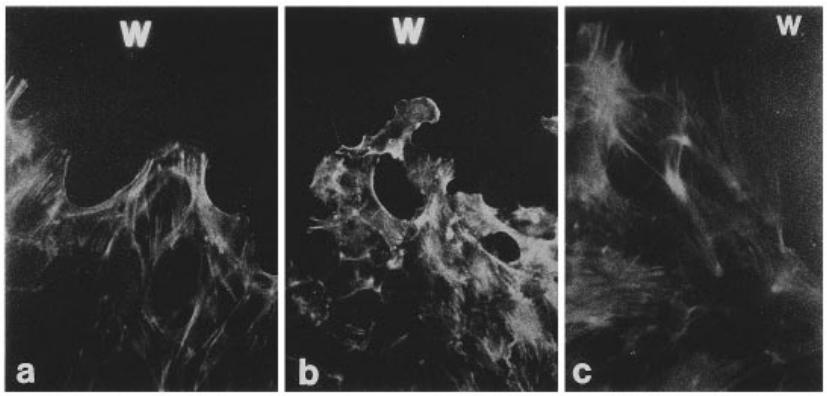

Figure 3. Actin staining. (a) Actin-containing stress fibres were detected throughout the cytoplasm of control cells. (b) Specific fluorescence on the actin fibres was partly disrupted in the deoxycholic acid (150 μM) group. (c) Rebamipide prevented the damaging effects of deoxycholic acid on the actin filaments. Original magnification $\times 250$.

Table 4. Cell proliferation during gastric epithelial cell repair

	BrdU labelling index (%) per unit area (0.109 mm²)			
	12 h	24 h	36 h	48 h
Control	0	0.3 ± 0.1	1.7 ± 0.3	0.1 ± 0.1
DCA (150 μM)	0	0*	0.2 ± 0.1*	0.4 ± 0.3
rebamipide (10^{-4} M)	0	0.2 ± 0.1	1.6 ± 0.4	0.2 ± 0.1
DCA + rebamipide	0	0.3 ± 0.1	1.6 ± 0.8	0.2 ± 0.1

Labelling index after the staining of BrdU was calculated according to the following formula:

Labelling index (%) = (BrdU-positive cells)/(total cells) $\times$ 100.

Data are expressed as the mean ± s.d. in five separate experiments.
* $P < 0.01$ vs. control.
h = hours after wounding; BrdU = bromodeoxyuridine; DCA = deoxycholic acid.

DISCUSSION

In this restoration model the ruffling movement of lamellipodia plays a key role in mucosal restoration by active migration. The basic event in this type of cell movement is an actin- and myosin-based phenomena which is controlled by the Ca^{2+}-calmodulin system.[7] In this model, cell migration partly resembles the *in vivo* restitution reported by Lacy & Ito.[12] Cell proliferation was not involved in their reported restitution process. However, in our model, cell proliferation was detected in the later stages of restoration.[7] Thus, in this study we assessed the effects of bile acid on total epithelial restoration, cell migration and cell proliferation. Using these measurements, deoxycholic acid was found to significantly decrease the total epithelial restoration rate, with a suppression of migration speed and cell proliferation around the wound.

The detailed cellular mechanism by which bile acids induce epithelial damage is still unknown. However, one possible hypothesis is through the detergent effects of bile acids on the cell membrane.[3,13] Another possible explanation is the role of intracellularly trapped bile acids. Changes in intracellular calcium regulates the activity of critical cellular enzyme systems. Recurrent elevations in intracellular calcium concentrations cause cellular damage.[14] Molloy *et al.*[15] have recently reported that deoxycholic acid—when added to cultured gastric mucosal cells—induces sustained elevations in intracellular calcium concentrations, consequently causing gastric mucosal cell damage. It is speculated that this type of hypercalcemia was present in our model, since the distribution of actin filaments was dramatically altered following treatment with deoxycholic acid.

Rebamipide has been reported to have cytoprotective effects in a bile acid-induced rat gastric mucosal injury model.[16] The mechanism of action of this drug is complicated, but several major effects have been documented. It is well known that rebamipide is a free radical scavenger.[17–19] In addition, it accelerates prostaglandin production and promotes mucus production in clinical peptic ulcer disease and experimental animal models, resulting in better quality ulcer healing.[20] In this study, we have shown the protective effects of rebamipide on the cytoskeletal protein actin, which may play an important role in epithelial restoration. The detailed mechanism of this protection of the cytoskeletal system is not fully understood, but may involve the Ca^{2+} signal transduction system which is altered by deoxycholic acid treatment. As shown in the additional experiments, rebamipide played a role as a radical scavenger in this experimental model. Further studies are necessary to

clarify whether these favourable effects of rebamipide are direct or indirect to cell organells, including the cytoskeletal system.

In conclusion, deoxycholic acid retarded the process of epithelial restoration in an *in vitro* cultured cell system. Rebamipide prevented the effects of the bile acid, possibly through the protection of the cytoskeletal system. Bile acid regurgitated from the duodenum to the stomach can cause gastric mucosal lesions. Rebamipide may be useful in the treatment of gastric ulcers and gastritis caused by bile reflex.

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