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Rebamipide binds to iNOS-positive cells in acetic acid-treated but not in ethanol-treated rat gastric mucosa

M. NAKAMURA*, Y. AKIBA†, H. MATSUI‡, K. TSUCHIMOTO§ & H. ISHII†
*Center for Basic Research, the Kitasato Institute; †Department of Internal Medicine, School of Medicine, Keio University; ‡Kitasato Institute for Life Sciences, Kitasato University; §Kitasato Institute Hospital, Tokyo, Japan

SUMMARY

Background: Rebamipide is a gastroprotective agent to stimulate prostaglandin generation in gastric mucosa and attenuate the activity of neutrophils, but direct evidence for the effector sites of this agent has remained to be clarified.

Aim: The present study was undertaken to show the effector sites of rebamipide in control and ulcer-provoked rats.

Methods: The rats were divided into control, acetic acid- and ethanol-treated rats. In the acetic acid-treated group, 100% acetic acid was attached to the serosal surface of the stomach for 30 s, 7 days before the experiments. In the ethanol-treated group, a dose of 0.5 mL/100 g body weight of 50% ethanol was administered through orogastric intubation 2 h before the experiments. Using the unfixed cryostat sections, aqueous solution of ^{3}H-rebamipide was applied and the localization of the binding sites of rebamipide was investigated by autoradiography.

Results: In the control rats, rebamipide was found to bind to the surface epithelial cells. In the ethanol-treated group, few binding sites were observed in the damaged gastric mucosa. In the acetic acid-treated group, the marked accumulation of the binding sites of ^{3}H-rebamipide was observed in the mesenchymal cells in the lamina propria mucosae between the regenerated gastric epithelial cells. Combination of autoradiography and immunohistochemistry has revealed that iNOS-immunoreactive cells had the strong binding of rebamipide in the acetic acid-treated group. Some of these cells were CD68-positive macrophages, while others were CD68-negative, corresponding to polymorphonuclear leucocytes. In the ethanol-treated acute gastric mucosal injury group, few binding sites were observed in the damaged gastric mucosa.

Conclusions: Autoradiography has made it clear that rebamipide binds to iNOS-positive cells in the gastric mucosa 7 days after acetic acid-treatment.

INTRODUCTION

Recently, chronic and recurrent gastric ulcers have been thought to be formed by inflammation, mainly by *Helicobacter pylori* infection. Thus, understanding this process is very important to bring about better healing and less recurrence. Rebamipide, a gastroprotective agent, has been shown to attenuate the activity of neutrophils and the production of inflammatory cytokines stimulated by NSAIDs and/or *H. pylori*, as well as increase prostaglandin generation in gastric mucosa.[1] The direct effector sites of this agent, however, remain to be clarified, because of the difficulty in identifying the binding sites of this kind of water soluble agent.

Recently the combination of autoradiography, immunohistochemistry and confocal laser microscopy has made it possible to observe the binding sites of

Correspondence to: Masahiko Nakamura, Center for Basic Research, the Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo, 108-8642, Japan. E-mail: nakamura-m@kitasato.or.jp

radiolabelled agents simultaneously with the localization of cell type-specific antigens such as receptors, cytokines and enzymes including iNOS.

AIM

The present study was undertaken to show the effector sites of rebamipide in control rats, acetic acid-treated rats as a chronic gastric ulcer model and ethanol-administered rats as a model of acute gastric mucosal damage, using a combination of autoradiography, immunohistochemistry and confocal laser microscopy.

MATERIALS AND METHODS

The rats were divided into control, acetic acid- and ethanol-treated rats. In the acetic acid-treated group, 100% acetic acid was attached to the serosal surface of the stomach for 30 s, 7 days before the experiments. In the ethanol-treated group, a dose of 0.5 mL/100 g body weight of 50% ethanol was administered through orogastric intubation 2 h before the experiments.

Procedures for autoradiography

The localization of ^{3}H-rebamipide binding sites was investigated by *in vitro* autoradiography using unfixed cryostat sections.[2, 3]

Aqueous solution of ^{3}H-rebamipide (3.7 MBq/mL) with or without 100 times higher concentration of unlabelled rebamipide was reacted with unfixed cryostat sections of the gastric tissues, treated with autoradiographic emulsion (Konica NR-M2). After 30 days exposure at 4 °C in the darkroom, the specimens were developed, fixed, counterstained with methylgreen and observed by confocal laser microscopy.

Procedures for immunohistochemistry

The stomach tissues were treated with Zamboni's fixative and the indirect immunofluorescence method using polyclonal antibody against iNOS (Zymed, San Francisco, CA, USA) diluted to one eight-hundredth of the original concentration, followed by incubation with anti-rabbit goat immunoglobulins conjugated with FITC and counterstained with Alexa Fluor 594 phalloidin (Molecular Probes, Leiden, the Netherlands). Some of the specimens were treated with 1 N NaOH to remove the autoradiographic films and then stained with monoclonal antibody against macrophage (CD68; Zymed) by the indirect immunoperoxidase method. Observations were made by confocal laser microscopy (Leica TCS NT). For the quantitative analysis of the immunoreactivity, the immunoreactive area was estimated by counting the pixels by Ultimage (Graftec, France).[4]

Some of the specimens were treated with periodic acid Schiff (PAS) stain to observe the localization of mucus in the surface epithelial cells. In addition, endogenous peroxidase activity was examined by diaminobenzidine reaction according to the method of Graham and Karnovsky[5] to observe the localization of leucocytes and macrophages.

RESULTS

Binding sites of rebamipide in control rat fundic mucosa

In the control rats, the tip portion of the fundic stomach is composed of the surface epithelial cells having PAS-positive mucus granules (Figure 1a,b). Rebamipide was found to bind to the surface epithelial cells and not to the fundic glandular cells (Figure 1c–f).

Binding sites of rebamipide in ethanol-treated rat fundic mucosa

In the ethanol-treated group, the upper half of the fundic mucosa showed hyaline degeneration (Figure 2a,b). Few binding sites were observed in the damaged gastric mucosa (Figure 2c). By the quantitative analysis, the silver grains were significantly decreased, compared with the control group (Figure 3).

Binding sites of rebamipide in acetic acid-treated rat fundic mucosa

In the rat gastric mucosa seven days after the acetic acid treatment, the regenerated gastric mucosa was formed surrounding the ulcerative lesion. These mucosa showed strong endogenous peroxidase activity (Figure 4a). The binding sites of 3H-rebamipide were observed in the mesenchymal cells in the lamina propria mucosae between the regenerated gastric epithelial cells (Figure 4b–e), while weak reaction was recognized on the surface mucous cells. Combination of autoradiography and immunohistochemistry has revealed that iNOS-immunoreactive cells had the strong binding of

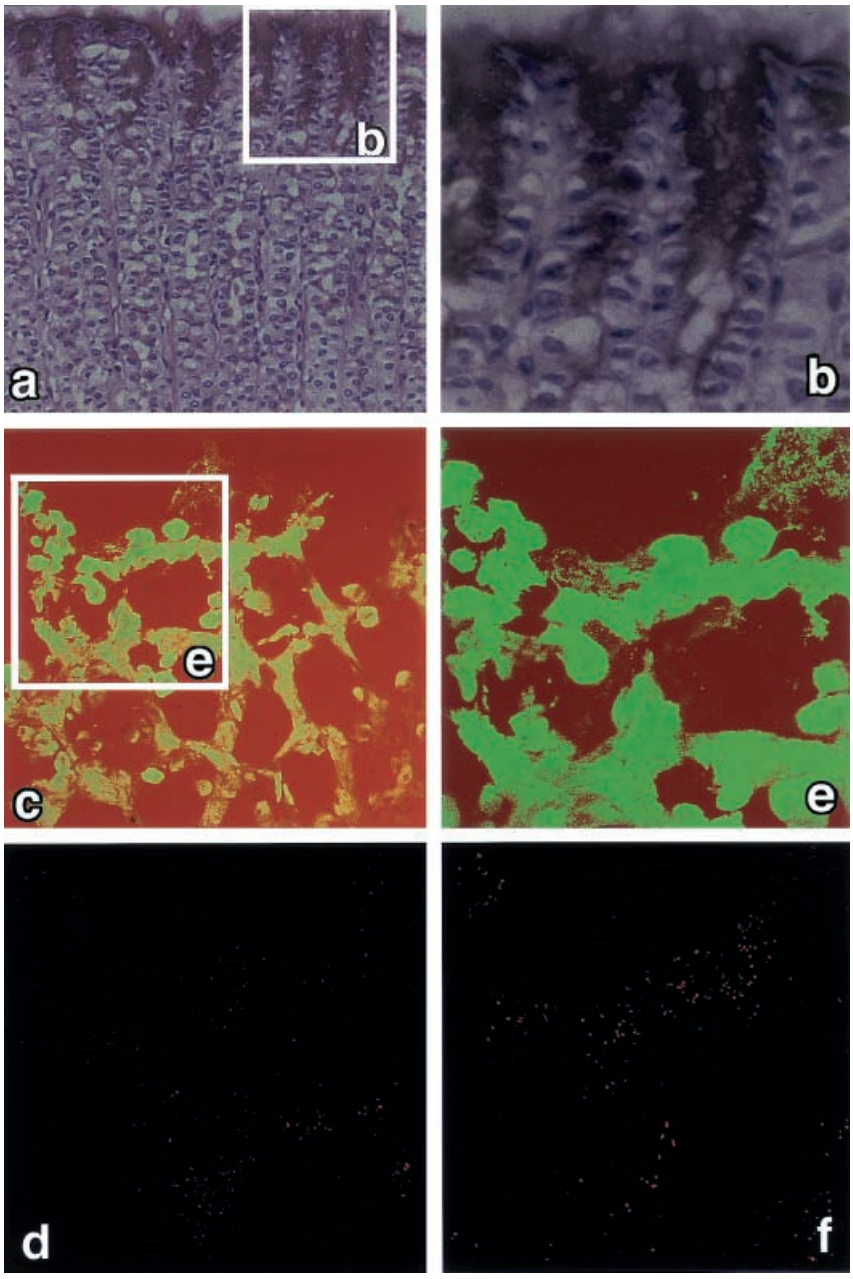

Figure 1. Light micrographs and autoradiographs showing the binding sites of ^{3}H-rebamipide in control rat fundic mucosa. (a, b) By the PAS staining, the secretory granules of the surface epithelial cells located in the foveolar portion of the fundic glands are well recognized. (b) Is the higher magnified view of the boxed area in (a). (a): ×200, (b): ×600. (c–f) In the upper half of the fundic mucosa, the silver grains showing the binding sites of ^{3}H-rebamipide are mostly seen on the surface epithelial cells. (c, e) Merged view of autoradiography and methylgreen autofluorescence. (d, f) Computer-assisted image showing the localization of silver grains. (c) and (d), (e) and (f) come from the same field. (e) is the higher magnified view of the boxed area in (c). (c, d): ×200, (e, f): ×400.

rebamipide in the acetic acid-treated group (Figure 4f–k). Some of these cells were CD68-positive macrophages, while others were CD68-negative, mostly corresponding to polymorphonuclear leucocytes.

DISCUSSION

In the present study, we have demonstrated that rebamipide has two binding sites, i.e. surface epithelial cells and iNOS-positive cells in the fundic mucosa of the control and acetic acid-treated rats.

As to the mucus secretion, rebamipide has been reported to promote gastric PGE2 production and mucus secretion through increasing EP4 gene expression in the antral mucosa[5]. The result of the present study coincides with the result of these pharmacological studies. The subcellular localization of rebamipide binding sites are rather dispersed in the cytoplasm and not near the plasma membrane, suggesting the effect of rebamipide is not receptor mediated. To clarify this point, electron microscopic autoradiography should be performed in the near future.

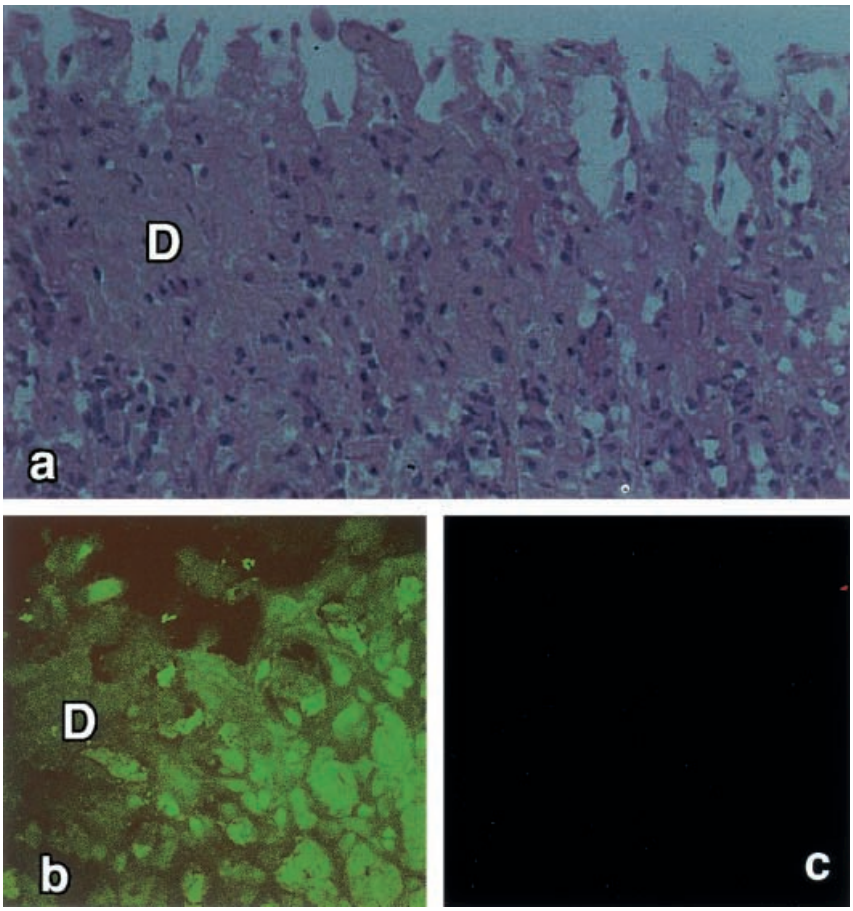

Figure 2. Light micrographs and autoradiographs showing the binding sites of ^{3}H-rebamipide in ethanol-treated rat fundic mucosa. (a) By the ethanol treatment, PAS-positive substances are lost and the epithelial cells showed hyaline degeneration (D). PAS staining. (b, c) Few silver grains showing the binding sites were observed in the degenerated area (D). (b) Merged view of autoradiography and methylgreen autofluorescence. (c) Computer-assisted image showing the localization of silver grains ×400.

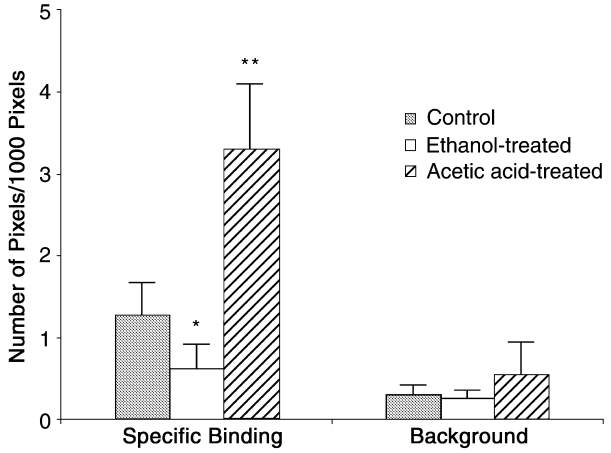

Figure 3. Comparison of ^{3}H-rebamipide binding in control, ethanol-treated and acetic acid-treated rat fundic mucosa. Values are mean ± s.d. ($n = 5$). *$0.001 < P < 0.01$, **$P < 0.001$, compared with the control.

In our previous study, iNOS expression was found to be present in some epithelial cells and vascular smooth muscle as well as in the inflammatory cells in the regenerating mucosa after acetic acid-induced gastric ulcer formation.[6] This means that iNOS alone is not sufficient for the identification of the inflammatory cells. As to the identification of the iNOS positive inflammatory cells, CD68 could be a good marker of macrophages[7] as shown in the present study. In the present study, rebamipide was found to bind to the CD68-positive macrophages and CD68-negative cells in the gastric mucosa in the healing process from acetic acid treatment. The latter cell was thought to be polymorphonuclear leucocytes from the lobulated nucleus as shown in the present study. This result corresponds to the report that rebamipide offers a potential for protection against reactive oxygen- and activated neutrophil-associated gastric mucosal injury by scavenging hydroxyl radical and inhibiting neutrophil activation.[8, 9]

Few binding sites were observed in the ethanol-induced gastric lesions in the present study. This suggests that inflammatory cells do not take part in the formation of ethanol-induced gastric mucosal

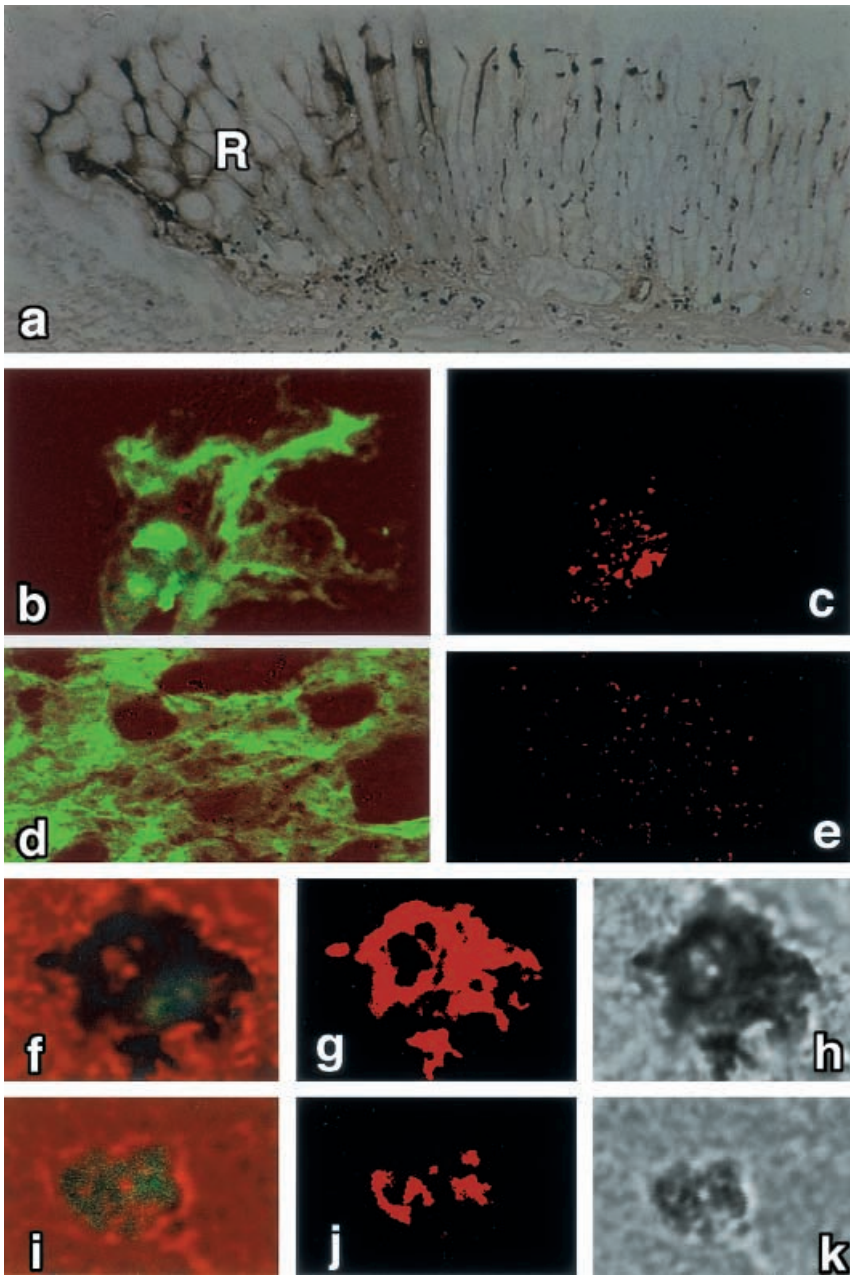

Figure 4. Light micrographs and autoradiographs showing the binding sites of ^{3}H-rebamipide in acetic acid -treated rat fundic mucosa. (a) Seven days after the acetic acid treatment, the regenerated mucosa (R) is seen in area surrounding the ulcer. The endogenous peroxidase-positive cells, i.e. leucocytes and macrophages, are richly distributed in this area. Graham and Karnovsky's staining ×200. (b–e) The binding sites of rebamipide are accumulated in the mesenchymal cells in the lamina propria mucosae. (b, d) Merged view of autoradiography and methylgreen autofluorescence. (d, e) Computer-assisted image showing the localization of silver grains. (b) and (d), (c) and (e) are taken from the same field ×300. (f–k) Light microscopic autoradiographs and histochemical observation of iNOS and CD68. All of the mesenchymal cells having the binding sites of rebamipide show iNOS immunoreactivity, but only large size cells are CD68-reactive (h) and small size cells having lobulated nucleus were not (k). (f, i) Merged view of autoradiography and fluorescence of iNOS immunoreactivity. (g, j) Computer-assisted image showing the localization of silver grains. (h, k) Immunoreactivity of CD68 by indirect immunohistochemical method. (f, g) and (h), (i), (j) and (k) are taken from the same field ×1000.

injury. As to this point, Davies *et al.*[10] reported that ethanol treatment did not affect COX-2 mRNA or protein expression, while aspirin up-regulated them. In the human alcoholics, the inflammation was recognized only in the antrum and not in the fundus.[11] From these observations, ethanol-induced gastric mucosal damage was not shown to be due to the inflammation, but rather to the direct chemical effect, coinciding well with our data. Although Chow *et al.*[12] reported the interaction of neutrophil in the formation of gastric lesion by ethanol and cigarette smoking, ethanol alone could not induce inflammation in the gastric mucosa.

CONCLUSIONS

Autoradiographic studies revealed that rebamipide binds to iNOS-positive cells in the gastric mucosa after acetic acid-treatment, while in control rat gastric mucosa, the binding sites were mostly seen in the surface mucous cells.

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