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Interaction of leptin with gastric myofibroblast transdifferentiation in Helicobacter pylori*-infected Mongolian gerbils: the effect of rebamipide*

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SUMMARY

Background: Our recent histochemical studies have revealed the marked increase of myofibroblasts in the *Helicobacter pylori*-infected Mongolian gerbil fundic mucosa, while the mediators, which facilitate the conversion of fibroblasts to the myofibroblasts have remained unknown.

Aim: The present study was undertaken to clarify the alteration of leptin in the control and *H. pylori*-infected Mongolian gerbil stomach. The effector sites of rebamipide were also investigated in relation to leptin.

Methods: The localization of leptin was investigated by the indirect immunofluorescence. Plasma leptin levels were determined by ELISA method. The localization of ^{3}H-rebamipide binding sites was investigated by autoradiography.

Results: Serum leptin content in *H. pylori*-infected Mongolian gerbils was significantly increased. The presence of leptin immunoreactivity was recognized in the endothelial cells of the microcirculatory network and very weakly in the glandular cells in the control group, while in the *H. pylori*-infected group leptin was markedly recognized in the mesenchymal cells. Rebamipide bound to the fibroblasts and surface mucous cells and decreased the leptin immunoreactivity in the gastric mucosa.

Conclusions: Leptin was mostly found in the mesenchymal cells. Rebamipide administration brought about the decrease of leptin in the gastric mucosaof the *H. pylori*-infected gerbils.

INTRODUCTION

Our recent histochemical studies have revealed the marked increase of the number and size of myofibroblasts in the *Helicobacter pylori*-infected human and Mongolian gerbil fundic mucosa.[1] As to the proliferation of this myofibroblast, transforming growth factor β, platelet-derived growth factor-BB, basic fibroblast growth factor (bFGF) and endothelin has been reported to play an important role,[2, 3] while the mediators which stimulate the conversion of undifferentiated mesenchymal cells or fibroblasts to the myofibroblasts have remained to be clarified. In the gastrointestinal tract, leptin has attracted attention as one of the luminal factors mostly secreted from the fundic glandular cells, but recently the hepatic fibroblast has been reported to have both leptin and leptin receptors[4] and its relation with the gastric mucosal myofibroblast attracts attention.

The present study was undertaken to clarify the alteration of content, immunoreactivity and receptors of leptin between the control, *H. pylori*-infected Mongolian gerbil fundic mucosa. In addition, the effector sites of rebamipide, one of the mucoprotective agents, and its effect on leptin content in the *H. pylori*-infected Mongolian gerbils were also investigated.

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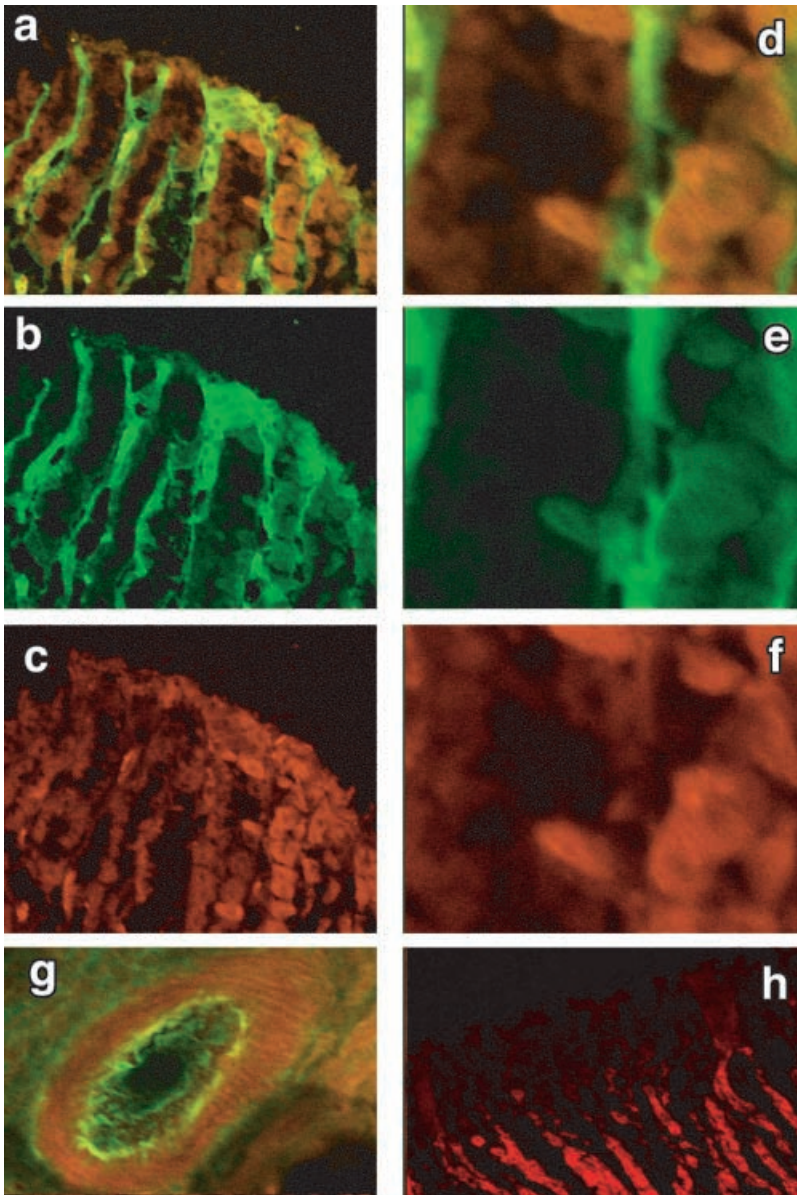

Figure 1. Leptin immunoreactivity in the control Mongolian gerbil fundic mucosa. A–C: In the tip portion of the fundic mucosa, the leptin immunoreactivity is mostly seen in the mesenchymal cells and weakly in the surface mucous cells. Magnification × 400. D–F: In higher magnification, leptin immunoreactivity is localized in the endothelial cells of the true capillaries. Magnification × 1600. A, D: overlaid view of lower two pictures. B, D: leptin immunoreactivity. C, F: alexa-phalloidin fluorescence showing the localization of f-actin.

MATERIALS AND METHODS

H. pylori infection was induced by the oral administration of CagA and VacA positive *H. pylori* strain (ATCC43504) 1, 2, 3 and 6 months before the experiments.

Procedures for immunohistochemistry

The stomach tissues were treated with Zamboni's fixative and the indirect immunofluorescence method using monoclonal antibodies against leptin (YC-040, Yanaihara Institute Inc., Shizuoka, Japan) were performed, counterstained with Alexa Fluor 594 phalloidin (Molecular Probes, Leiden, the Netherlands) and observed 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).

Procedures for autoradiography

The localization of ^{3}H-rebamipide binding sites was investigated by the *in vitro* autoradiography using

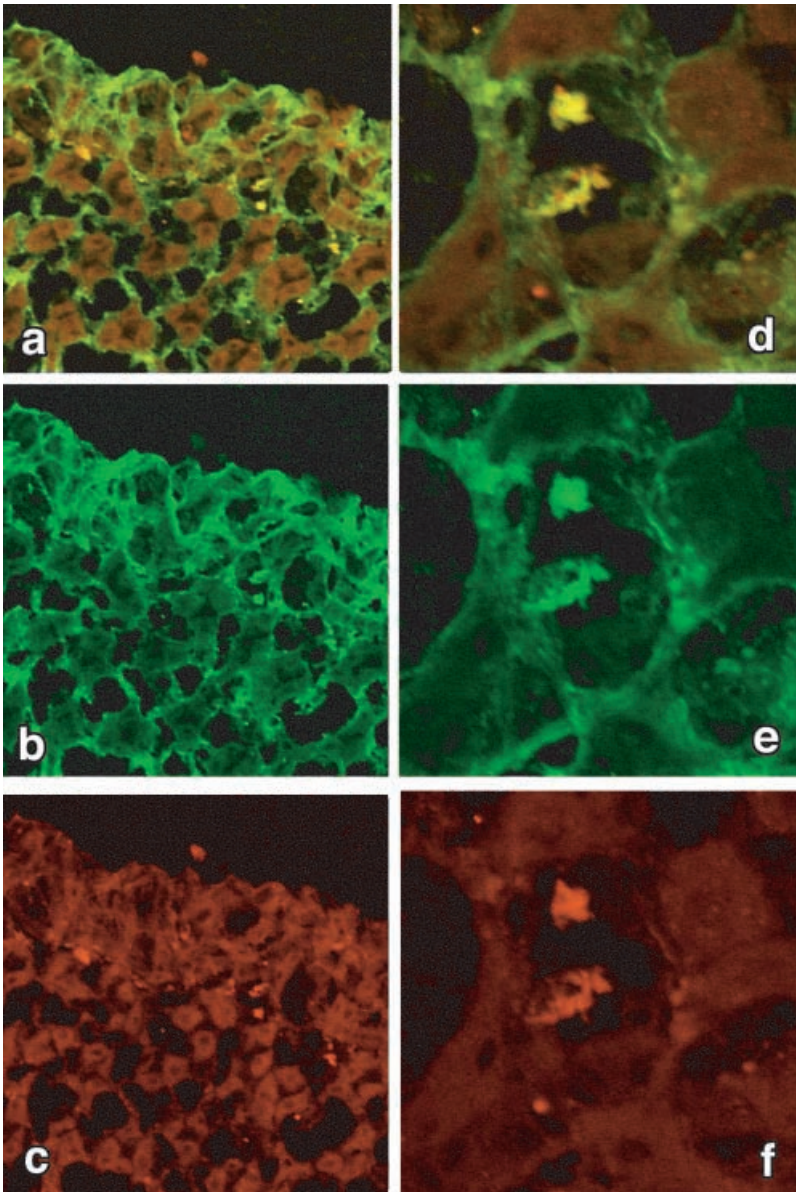

Figure 2. Leptin immunoreactivity in the *H. pylori*-infected Mongolian gerbil fundic mucosa for 3 months. A–C: In *H. pylori*-infected group, the leptin immunoreactivity is recognized both in the mesenchymal and surface epithelial cells. Magnification × 400. D–F: In the lamina propria mucosae, some mesenchymal cells show strong leptin fluorescence as well as f-actin localization, suggesting this cell coincide with myofibroblast. Magnification × 1600. A, D: overlaid view of lower two pictures. B, D: leptin immunoreactivity. C, F: alexa-phalloidin fluorescence showing the localization of f-actin.

unfixed cryostat sections[5] and autoradiography of soluble compounds.[6]

In the former method, 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-day exposure, the specimens were developed, fixed, counterstained with methylgreen and observed by confocal laser microscopy.

In the autoradiography for soluble compounds, aqueous solution of ^{3}H-rebamipide (37 MBq/100 g b.w.) with and without unlabelled rebamipide of 100 times higher concentration was administered through intra-aortic catheter, and the stomach tissues were freeze-dried and embedded in Epon under low pressure. The semithin and ultrathin sections were made using LKB ultramicrotome using nickel meshes, treated with autoradiographic emulsion film by the wire-loop method and exposed for 30 days. After development

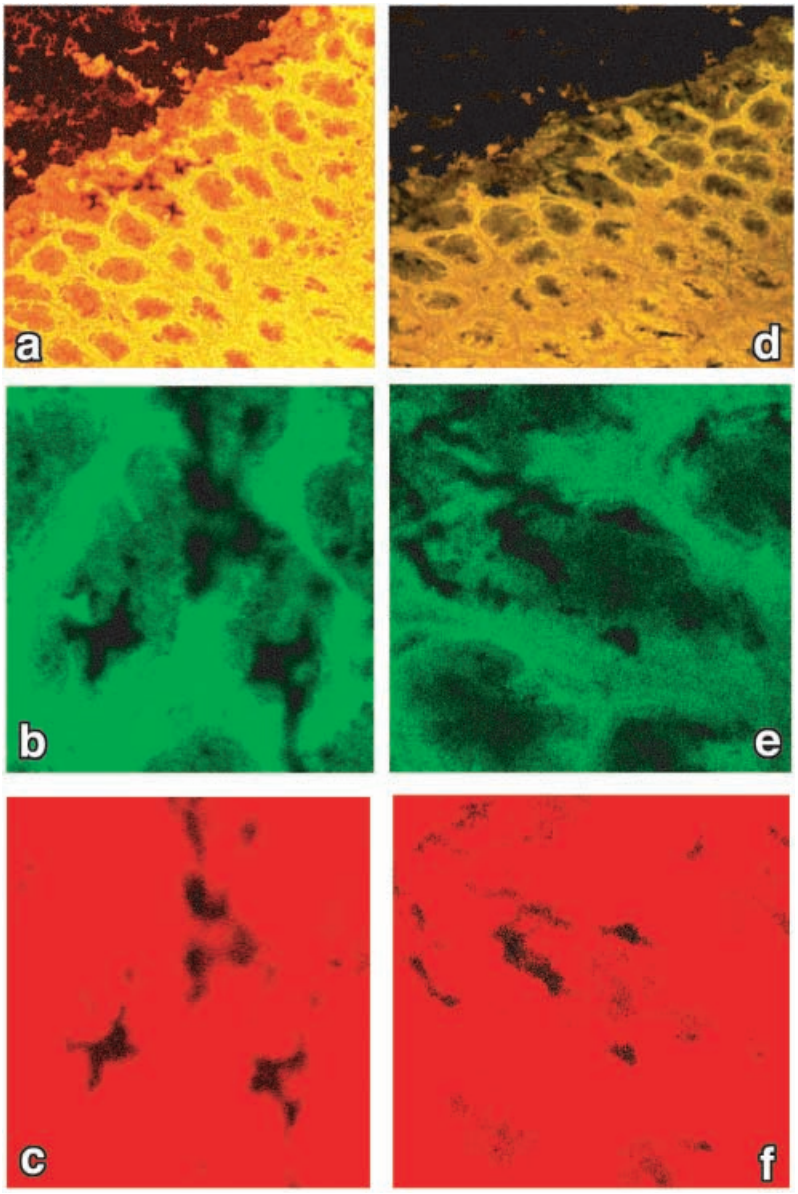

Figure 3. *In vitro* autoradiographs showing the binding sites of ^{3}H-rebamipide in the fundic mucosa using unfixed cryostat sections. A–C: In the control group, the silver grains showing the binding sites of ^{3}H-rebamipide are seen in the tip portion of the gastric mucosa (A). In higher magnification, the binding sites are mostly seen in the cytoplasm of the surface mucous cells (B, C). D–F: In the *H. pylori*-infected group, the silver grains are also seen near the surface mucous cells (D). The distribution of silver grains in higher magnification is rather scattered both on the surface mucous cells and the lamina propria mucosae (E, F). A, D: couterstained with methylgreen. Magnification × 200. B, E: counterstained with methylgreen. Magnification × 800. C, F: distribution of the silver grains by transmission mode of confocal laser microscopy. Magnification × 800.

and fixation, the specimens were observed by light and electron microscopy (JEOL JEM 1200EXII).

Estimation of serum leptin

Plasma leptin levels were determined by ELISA method (YK050, Yanaihara Institute Inc., Shizuoka, Japan) in the control, *H. pylori*-infected and *H. pylori*-infected and rebamipide-treated groups.

In the rebamipide-treated group, 30 mg / kg b.w. of rebamipide in carboxymethylcellulose aqueous solution was administered through a gastric tube for 1 week before the experiments.

RESULTS

Immunoreactivity of leptin in control and H. pylori*-infected Mongolian gerbil fundic mucosa*

Leptin immunoreactivity was recognized in the mesenchymal cells in the lamina propria mucosa, i.e. endothelial cells of the microcirculatory network and fibroblasts showing weak alexa phalloidin reactivity. Some of the surface epithelial cells showed very weak leptin immunoreactivity (Figure 1). In the *H. pylori*-infected group leptin was markedly recognized in the mesenchymal cells including myofibroblast which were identified by strong alexa-phalloidin reactivity

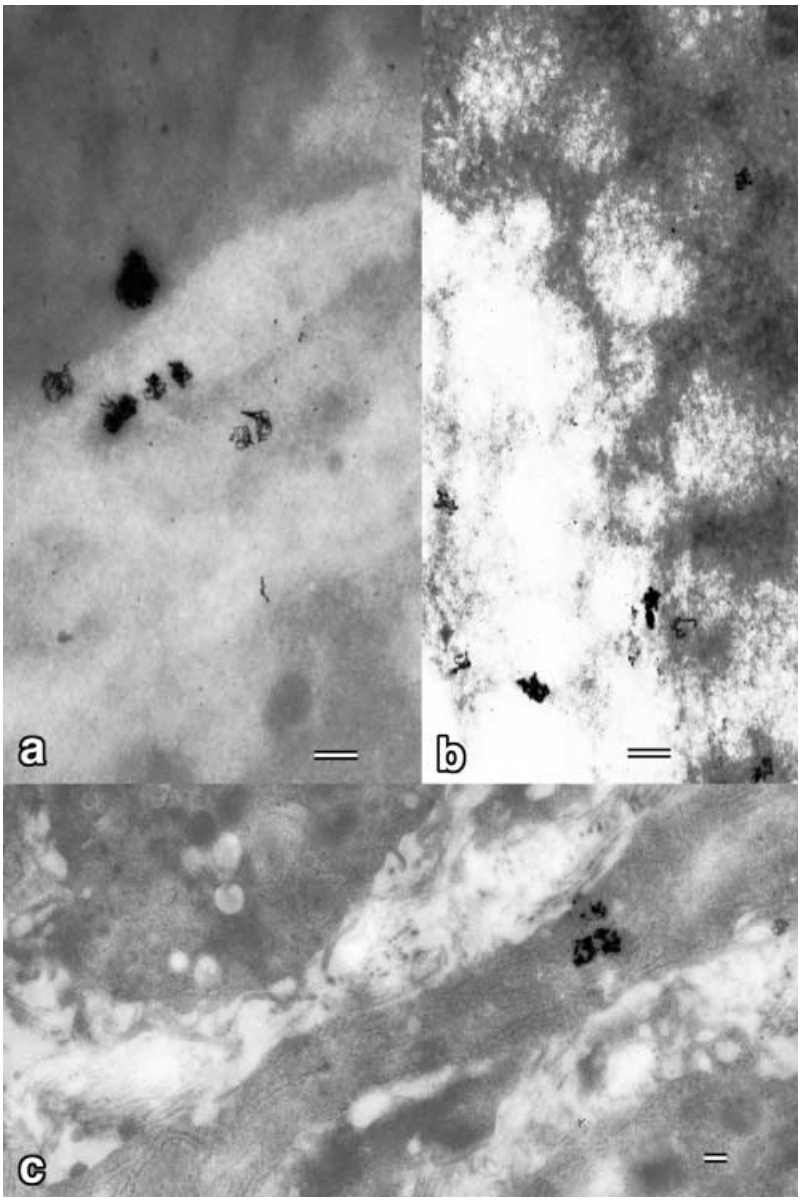

Figure 4. Electron microscopic autoradiographs of soluble compounds. A: In control group, the silver grains showing the binding sites of ^{3}H-rebamipide are seen near the basal plasma membrane of the surface mucous cells. Scale bar: 1 μm. B, C: In the *H. pylori*-infected group, the silver grains are seen in the cytoplasm of the surface mucous cell (B) and near the fibroblasts in the lamina propria mucosae (C). Scale bar: 1 μm.

(Figure 2). The surface mucous cells also showed the strong fluorescence.

Effector sites of rebamipide by autoradiography

In the control group, the binding sites of rebamipide were seen in the surface mucous cells (Figure 3). These binding were blocked by the concomitant application of cold rebamipide and found to be specific. In the *H. pylori*-treated group, the binding sites of rebamipide were accumulated on the mesenchymal cells including fibroblasts as well as surface epithelial cells. Electron microscopic autoradiographs also showed the similar distribution of the binding sites of ^{3}H-rebamipide (Figure 4).

Alteration of leptin concentration and effect of rebamipide

Serum leptin content in *H. pylori*-infected Mongolian gerbils was slightly increased as compared with the control group (Figure 5). Rebamipide treatment decreased the leptin immunoreactivity in the gastric mucosa, especially on the mesenchymal cells (Figure 6).

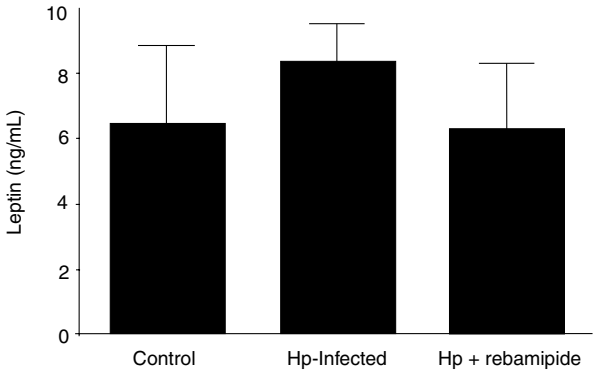

Figure 5. Leptin concentration and immunoreactivity by *H. pylori* infection and rebamipide. In the *H. pylori*-infected alone group, the serum concentration of leptin is slightly increased and in the *H. pylori* and rebamipide-treated group, that has decreased to the control level but they are not significantly different. Values are mean ± s.d. ($n = 5$).

DISCUSSION

Leptin, a protein product of obese gene expressed primarily by adipocytes, provides feedback information on the size of energy stores to central OB receptors controlling the food intake, energy expenditure and body weight.

Recently, this hormone has been reported be produced in the gastric mucosa of the rat and human.[7, 8] As to the role of leptin in the gastric leptin, the nutritional effect has been pointed out and leptin was suggested to be involved in early CCK-mediated effects activated by food intake.[9] Leptin has been also reported to accelerate ulcer healing and during this process up-regulation of TGFα and increased production of nitric oxide due to up-regulation of cNOS and iNOS in the ulcer area.[10] In most of these reports, leptin was shown to be localized in the chief cells. The present study has demonstrated the localization of leptin immunoreactivity in the microvascular endothelial cells. This discrepancy may result from the species difference. In addition, most of them have used the ordinary immunohistochemical procedures having moderate sensitivity. In the present study, confocal laser microscopic observation combined with fluorescent immunohistochemistry was employed and this method is advantageous to observe the localization of immunoreactivity in the small mesenchymal cells because of its high sensitivity and possibility to observe under higher magnification.

As to the lepton localization in the microcirculation, relation to angiogenesis has recently been pointed out,

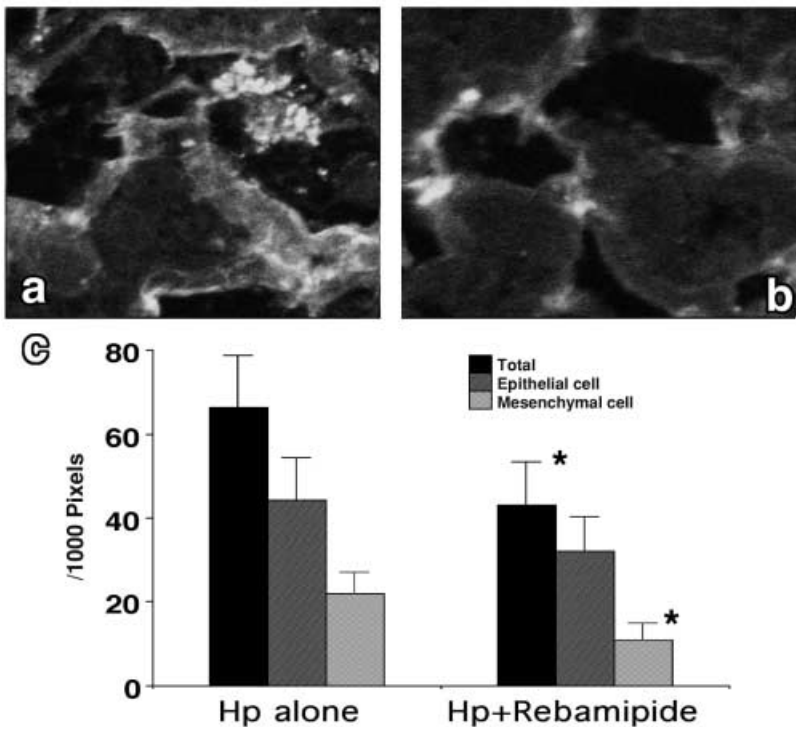

Figure 6. Comparison of leptin immunoreactivity in the *H. pylori*-infected and *H. pylori* and rebamipide-treated Mongolian gerbil fundic mucosa. A, B: Comparison of immunoreactivity of *H. pylori*-infected (A) and *H. pylori* and rebamipide-treated group (B). The intensity of the fluorescence has markedly decreased in the rebamipide-treated group. C: by the computer-assisted image analysis, fluorescence of leptin has significantly decreased in the mesenchymal cells and not in the epithelial cells. Values are mean ± s.d. ($n = 5$). *$P < 0.01$.

because leptin administration has been reported to bring about new fenestrated blood vessels.[11] Leptin has also been shown to synergistically stimulate angiogenesis with bFGF and vascular endothelial growth factor, the two most potent and commonly expressed angiogenic factors. These findings demonstrate that leptin has another new function-the increase of vascular permeability.[12] In addition, relation of leptin with angiopoietin-2 has also been pointed out in adipose tissue without a concomitant increase in VEGF.[13] The endothelia cell network of the microcirculatory system has been thought to be one of the largest endocrine organ in the body. Leptin could be one of the secreted substances from the endothelial cells. Taken together, the role of leptin in the angiogenesis and ulcer healing is plausible and the present study also shows its possibility.

Rebamipide, one of the mucoprotective agents used in Japan and other countries, has been reported to inhibit lipid peroxidation[14] as well as stimulation of mucous secretion.

In the present study, rebamipide has shown to decrease the leptin immunoreactivity in the fibroblasts and myofibroblasts. The relation between lipid peroxidation and myofibroblast is very interesting, because myofibroblast is closely related to fat-storing cell or Ito cell and the conversion from Ito cell to myofibroblast has been demonstrated in the chronic liver dysfunction. Further study is needed to clarify this point.

CONCLUSIONS

In the Mongolian gerbil fundic mucosa, leptin was to be mostly found in the microcirculatory system and myofibroblast as well as mucous cells. Rebamipide administration brought about the decrease of leptin immunoreactivity in the gastric mucosa especially in the mesenchymal cells, which suggests that rebamipide should act at least partly through the modulation of myofibroblast function by leptin.

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