Expression of leptin in two-layered culture of gastric mucous cells and fibroblasts: effect of Helicobacter pylori attachment

M. NAKAMURA*, T. TAKAHASHI†, T. MATSUMOTO‡§, Y. AKIBA¶, H. MATSUI‡, K. TSUCHIMOTO*, H. ISHIÌ¶ & H. YAMADA†‡§

*Center for Clinical Pharmacy and Clinical Sciences, School of Pharmaceutical Sciences, Kitasato University, Tokyo;
†Graduate School of Pharmaceutical Science, Kitasato University, Tokyo; ‡Kitasato Institute for Life Sciences & Graduate School of Infection Control Science, Tokyo; §Kitasato University Oriental Medicine Research Center, the Kitasato Institute, Tokyo; ¶Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan

SUMMARY

Background: Our recent histochemical studies have revealed an increase in myofibroblasts and in leptin and its receptor in endothelial cells, and myofibroblasts in Helicobacter pylori-infected human and Mongolian gerbil fundic mucosa.

Aim: The present study was undertaken to clarify the H. pylori–induced interaction between leptin in cultured gastric surface mucous cells and fibroblasts.

Methods: GSM06 cells were incubated with an air–liquid interface on a collagen gel layer containing mouse fibroblast cell line L929. Medium containing H. pylori bacilli (ATCC43504) at 10–100 times higher concentration than the GSM06 cells was added from the luminal side and the localization of leptin was observed by immunohistochemistry. The transformation of L929 cells to myofibroblasts was detected by electron microscopy and PR 2D3 immunoreactivity.

Results: L929 cells in the control group showed a spindle shape with scarce cytoplasm. In the H. pylori-treated group, L929 cells showed features characteristic of myofibroblasts, and most GSM06 and L929 cells showed leptin immunoreactivity. In contrast, L929 cells incubated with H. pylori alone did not undergo this differentiation.

Conclusions: Attachment of H. pylori to surface epithelial cells caused conversion of fibroblasts to myofibroblasts. We suggest that leptin plays a role in this transformation.

INTRODUCTION

Our recent histochemical studies have revealed a marked increase in the number and size of myofibroblasts in Helicobacter pylori-infected human and Mongolian gerbil fundic mucosa. However, mediators that stimulate the conversion of undifferentiated mesenchymal cells or fibroblasts to myofibroblasts remain to be identified. In our studies in Mongolian gerbil fundic mucosa, leptin and its receptor were detected in endothelial cells of the microcirculatory network and very weakly in glandular cells in the control group, whereas in the H. pylori-infected group, in contrast, leptin was markedly visible in both myofibroblasts and endothelial cells. This finding led us to focus on the role of leptin in the transformation of fibroblasts to myofibroblasts.

Aim

The present study was undertaken to clarify the binding of H. pylori to GSM06 cells and morphological changes in L929 cells using a two-layered culture of gastric surface mucous cells and fibroblasts.
addition, the alteration of leptin localization was also investigated.

MATERIALS AND METHODS

Cell culture

GSM06 cells, a gastric surface mucous cell line from a primary culture of gastric fundic mucosal cells of a transgenic mouse, were provided by Daiichi Pharmaceutical Co Ltd (Tokyo, Japan).4, 5 The cells were cultured in Dulbecco’s modified Eagle’s medium / Ham’s F-12 medium (Sigma Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 1% SITE (5 mg / mL selenium, 10 mg / mL insulin, 5.5 mg / mL transferrin, 2 mg / mL ethanolamine) and 10 ng / mL mouse epidermal growth factor (Sigma) at 37 °C in a humidified atmosphere of 5% CO2 in air. The mouse fibroblast cell line L929 was cultured in Dulbecco’s modified Eagle’s medium / Ham’s F-12 medium supplemented with 10% fetal bovine serum, 1% SITE and 10 ng / mL mouse epidermal growth factor at 37 °C in a humidified atmosphere of 5% CO2 in air for 2 weeks before experiments.6 Medium containing CagA- and VacA-positive H. pylori bacilli (ATCC43504) at a multiplicity of infection of 10–100 was added from the luminal side and incubated with 5% CO2 for 1–24 h. No alteration in pH of the medium was detected. To clarify whether the effect of H. pylori is mediated by direct attachment or by water-soluble components from H. pylori, the effect of H. pylori water extract was prepared according to the method of Yoshida et al.7 and added to the medium instead of H. pylori-containing culture medium. For pre-treatment with leptin antibody, monoclonal antibodies against leptin (YC-040; Yanaihara Institute Inc., Shizuoka, Japan) was used at a final concentration of 1 μg / mL.

Immunohistochemistry and electron microscopy

The cultured cells were treated with Zamboni’s fixative. The indirect immunofluorescence method was performed using monoclonal antibodies against leptin (YC-040; Yanaihara Institute Inc., Shizuoka, Japan), Helicobacter pylori (Biogenesis, Poole, UK), and PR 2D3,8 which specifically detects the intermediate filaments of myofibroblasts and smooth muscle cells. Counterstaining was done with Alexa Fluor 594 phalloidin (Molecular Probes, Leiden, the Netherlands) and obser-

vation by confocal laser microscopy (Leica TCS NT). For electron microscopy, the cultured cells were treated by immersion in a fixative composed of 4% formaldehyde and 1% glutaraldehyde in 0.06 mol / L phosphate buffer (pH 7.4), followed by postfixing with 2% osmium tetroxide solution containing ruthenium red in the dark for 3 h. The samples were dehydrated in ethanol solutions, then embedded in Epon mixture. Semi- and ultra-thin sections were made using an LKB ultramicrotome. The specimens were stained with lead citrate and uranyl acetate and examined under a JEOL 1200EXII electron microscope at an accelerating voltage of 75 KV.

RESULTS

Confocal microscopic observation of two-layered culture of GSM06 and L929 cells

On Alexa®-phallodin staining, GSM06 cells appeared as several layers on the collagen gel layer containing L929 (Figure 1a–d). The base of the GSM06 cells showed strong Alexa–phallodin reactivity.

Localization of H. pylori near GSM06 cells

In the H. pylori-cocultured group, H. pylori immunoreactivity was mostly detected near the luminal surface of the GSM06 cells (Figure 1e,f). On electron microscopy, many bacilli were seen near the microvilli of the GSM06 cells (Figure 1G,H).

Alteration of L929 cell ultrastructure by H. pylori coculture

In the control group, few microfilaments were noted in the cytoplasm of the L929 cells (Figure 2h). In contrast, many bundles of microfilaments as well as well-developed rER were recognized in the L929 cells in the H. pylori-cocultured group (Figure 2i). L929 cells incubated with H. pylori alone did not show differentiation to myofibroblasts.

Alteration of Leptin and PR 2D3 immunoreactivity

In the control group, leptin immunoreactivity in the GSM06 cells was very weak (Figure 2a,b). H. pylori coincubation brought about a marked increase in leptin immunoreactivity in these cells (Figure 2c,d). PR 2D3 immunoreactivity was clearly recognized in these fibroblasts (Figure 2f), while L929 cells plus H. pylori
Figure 1. (a–d) Confocal laser micrographs of two-layered culture of GSM06 and L929 cells. On Alexa–phalloidin fluorescence, actin filaments are strongly recognized in the luminal and basal portion of the GSM06 cell layer (g) and the L929 cells (L) (a, c). On DAPI fluorescence, the localization of the nuclei is clearly seen (b, d). Slides A-D were all obtained from the same visual field. Original magnification: a, b × 100; c, d × 400. (e, f) Confocal laser micrographs of *H. pylori* immunoreactivity located at the luminal portion of GSM06 cells. *H. pylori* immunoreactivity is exclusively found near the luminal surface of GSM06 cells (arrows) (e). (f) Alexa–phalloidin staining showing the localization of actin filaments near the basolateral surface of GSM06 cells. Original magnification, × 2000. (g, h) Electron micrographs showing *H. pylori* bacilli near the luminal surface of GSM06 cells. Many bacilli are seen near the microvilli of the GSM06 cells. Ruthenium red positive substances are located between the microvilli and bacillus (arrow). Ruthenium red *en bloc* staining.
or pre-treatment with leptin antibody inhibited the increase in PR 2D3 immunoreactivity (Figures 2g and 3).

DISCUSSION

In the present study, a two-layered culture consisting of GSM06 and L929 cells with an air–liquid interface was used. This model has been reported to promote the differentiation of gastric surface mucous cells compared with the ordinary immersed model, in which no differentiation of the cultured cells is detected. Our biochemical studies have also shown the significance of this method in the maturation of the mucus component. Thus, this method is a useful tool in investigating the effect of H. pylori on surface epithelial cells and underlying fibroblasts. With the addition of H. pylori from the luminal side, the accumulation of H. pylori on the apical surface of GSM06 cells was clearly shown. This observation also shows that the GSM06 cells used in the present study possess functional polarity. In this study, the transformation of L929 cells to myofibroblasts was detected by electron microscopy and PR 2D3 immunoreactivity. Few previous studies have reported this transformation, even though the L929 cell line is one of the oldest in use. In addition, the lack of effect of H. pylori water extract suggests that this transformation is mediated by H. pylori attachment to the GSM06 cells and not by luminal factors. The role of ammonia and urease in this model is negligible because the medium does not contain urea and the pH of the incubation medium did not change. Other studies are necessary to confirm this observation including those using CagA-negative H. pylori and primary cultured fibroblasts.

Leptin, a protein product of an obese gene expressed primarily by adipocytes, provides feedback information on the size of energy stores to central Ob receptors controlling food intake, energy expenditure and body weight. Recently, this hormone was reported to be produced in the gastric mucosa of rats and humans. With regard to the role of leptin in the gastric mucosa, a nutritional effect has been detected and leptin has been suggested to be involved in early CCK-mediated effects activated by food intake. Leptin has also been reported to accelerate ulcer healing, and during this process to cause the upregulation of tumour necrosis factor-α and an increase in production of nitric oxide due to upregulation of constitutive and inducible nitric oxide synthase in the ulcer area. In most of these reports, leptin was shown to be localized in the epithelial cells, particularly in the chief cells, but not in the mesenchymal cells. Most studies have used standard immunohistochemical procedures with moderate sensitivity, making it difficult to identify cell types showing leptin immunoreactivity. The present study has demonstrated the localization of leptin immunoreactivity in both GSM06 and L929 cells under conditions of H. pylori coculture from the luminal side of this culture system. We employed confocal laser microscopic observation combined with fluorescent immunohistochemistry, which, because of its high sensitivity and possibility of higher magnification, allows localization of immunoreactivity in the small mesenchymal cells to be observed. Our previous in vivo experiments using H. pylori-induced Mongolian gerbil fundic mucosa also coincides with the present culture study, especially the increased immunoreactivity of leptin in the mesenchymal cells. Western blot analysis should be performed in the near future to confirm this data.

With regard to the relation of leptin to fibroblasts, leptin has been shown to induce the expression of matrix metalloproteinases. Further, fibroblast dysfunction in migration, vascular endothelial growth factor production and response to hypoxia has been reported in db/db leptin receptor-deficient mice. These data indicate that leptin may stimulate fibroblasts to differentiate into myofibroblasts. Many cytokines play a role in the regulation of fibroblast activities, including growth factors, interleukins and retinoic acid. The interrelation between leptin and these factors requires clarification. Further, the function of leptin may be mediated by other factors such as angiopoietin.

The role of the myofibroblast in the gastric mucosal damage has generally been thought to be the increased collagen deposition and shrinkage of the ulcer bed, but recent studies have shown that they have a broader and more refined function. Myofibroblasts can receive, regulate and transmit immune cell-derived signals to adjacent epithelial cells. The mesenchymal cells including myofibroblast can also prolong T-cell survival, which may have profound implications for the duration of an intestinal inflammatory process through the production of proinflammatory cytokines. Taken together, these factors indicate that the role of the myofibroblast in the persistent inflammation induced by H. pylori should be re-evaluated in future.

© 2004 Blackwell Publishing Ltd, Aliment Pharmacol Ther 20 (Suppl. 1), 125–130
Figure 2. (a–g) Confocal laser micrographs showing the localization of leptin and PR 2D3 immunoreactivity of the control, *H. pylori*-treated two-layered culture and L929-alone groups. (a, b) In the control group, leptin immunoreactivity was not detected either in the GSM06 or L929 cells. (a) Leptin immunoreactivity; (b) Alexa–phalloidin fluorescence. Original magnification, × 200. (c–e) In the *H. pylori*-treated group, leptin immunoreactivity is recognized in the GSM06 and in the L929 cells. (c, d) Leptin immunoreactivity; (e) Alexa–phalloidin fluorescence. (c, e) × 200, (d) × 400. (f) PR 2D3 immunoreactivity is clearly seen in L929 cells. Original magnification, × 400. (g) Only very weak PR 2D3 immunoreactivity is seen in L929 cells treated with *H. pylori*. Original magnification, × 400. (h, i) Electron micrographs showing L929 cells of the control and *H. pylori*-treated groups. Ruthenium red en bloc stain. (h) In the control group, the cytoplasm of fibroblasts is poor and few microfilaments are seen; (i) in the *H. pylori*-treated group, many microfilaments are seen in the cytoplasm.
Co-culture with *H. pylori* induced conversion of L929 cells to myofibroblasts. L929 cells were converted to myofibroblast-like cells under coexistence with *H. pylori* attached to GSM06 cells. It is suggested that leptin plays an important role in this transformation.

**REFERENCES**