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Immunohistochemical localization of basic fibroblast growth factor in the healing stage of mouse gastric ulcer

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Abstract. The aim of this study was to clarify the involvement of basic fibroblast growth factor (bFGF) in gastric ulcer healing. For this purpose, light and electron microscopic immunohistochemical studies for bFGF were performed using an experimental gastric ulcer model of mice. Ulceration was induced by the application of acetic anhydride to the serosal surface of the body of the stomach. Stomach tissues were investigated of mice at 5 days and 3 weeks respectively after treatment and also of untreated normal mice. Five days after treatment an ulcer was seen in the stomach of the experimental mice. Immunohistochemistry revealed that bFGF was localized in fibroblasts in the ulcer bed. The growth factor was distributed throughout the cytoplasm excluding organelles involved in the usual secretory system, such as rough endoplasmic reticulum, Golgi apparatus and secretory vacuoles. bFGF was also detected in the nucleus. Three weeks after treatment the surface of the ulcer lesion was completely covered by regenerated epithelium. The stomach tissues were immunohistochemically negative for bFGF both inside and outside the scar region; untreated normal stomach tissues were also negative for bFGF. These results suggest that the growth factor plays important roles in gastric ulcer healing.

Introduction

Basic fibroblast growth factor (bFGF) is a single-chain polypeptide with a molecular weight of about 17 kDa. bFGF is a pluripotent growth factor; for example, it is a mitogen for many types of cultured mesoderm- and neuroectoderm-derived cells such as fibroblasts and vascular endothelial cells (Baird and Böhlen 1990; Gospodarowicz 1990). Also, in vivo it has angiogenic activity as revealed by implantation of the factor into avascular tissues such as the rabbit cornea (Gospodarowicz et al. 1979) and the chick chorioallantoic membrane (Esch et al. 1985).

Folkman et al. (1991) have reported the presence of bFGF in the bed of rat duodenal ulcer induced by cysteamine-HCl. bFGF was detected using the assay for mitogenic activity on 3T3 fibroblasts and Western blot analysis, although the localization of the factor in the lesion was not mentioned. In the present communication, an immunohistochemical study for bFGF was performed at the light and electron microscopic levels using an experimental gastric ulcer model of mice induced by acetic anhydride. The aim of the investigation was to clarify whether or not bFGF is concerned in gastric ulcer healing and, if so, to determine the precise localization of the factor.

Materials and methods

Gastric ulcer induction

Gastric ulceration was induced by acetic anhydride using the method of Okabe et al. (1971) with some modifications. Six-week-old male ddy mice were used in this study. The abdomen of each mouse was incised under anesthesia by peritoneal pentobarbital injection. A round piece of membrane filter, about 3 mm in diameter, was soaked in acetic anhydride, then placed upon the anterior serosal surface of the body of the stomach for 20 s and the abdomen was closed.

Light and electron microscopy

In this study, stomach tissues of mice at 5 days and 3 weeks respectively after treatment and also of untreated normal mice were investigated. Light and electron microscopy was performed as previously described (Yabu et al. 1991); 2.5-μm-thick sections embedded in JB4 were used for light microscopy and ultrathin Epon sections for electron microscopy with the modification that JB4 sections were observed after staining by hematoxylin and eosin.

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Western blot analysis

Antiserum against bFGF used in this immunohistochemical study was a kind gift from Dr. S. Shiosaka (Department of Neuroanatomy, Biomedical Research Center, Osaka University Medical School, Osaka, Japan). The antiserum was raised in rabbits against synthetic peptide representing the amino-terminus of bovine bFGF, and has been shown not to crossreact with acidic fibroblast growth factor (Iwata et al. 1991). In order to verify the specificity of the antiserum in the mouse stomach tissues, Western blot analysis was performed. Fresh stomach tissues containing the ulcer region 5 days after treatment were homogenized on ice in 50 m*M* TRIS-HCl, pH 7.4, 200 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid, 1 m*M* phenylmethylsulfonyl fluoride, 0.05% benzethonium chloride. The tissue extracts were centrifuged at 20000 *g* for 60 min, and the supernatants were collected. Aliquots of the supernatants, containing 50 μg of total protein, were diluted in sample buffer and subjected to 14% sodium dodecyl sulphate-polyacrylamide gel electrophoresis according to the method of Laemmli (1970).

Proteins were electrophoretically transferred to Immobilon-P transfer membrane (Millipore Corporation, Bedford, Mass., USA) using the method of Towbin et al. (1979). The membrane was incubated in TRIS-buffered saline, pH 8.0 (TBS) containing 5% nonfat dry milk for 1 h at room temperature (RT), followed by incubation overnight at 4° C in a 1:1000 dilution of anti-bFGF antibodies in the same buffer. The membrane was then washed in a washing solution (TBS containing 0.1% polyoxyethylene sorbitan laurate) three times for 10 min each, and treated for 1 h at RT with goat biotinylated anti-rabbit IgG (Vector laboratories, Burlingame, Calif., USA) diluted 1:200 in TBS containing 1% nonfat dry milk. After washing the membrane as described above, the membrane was reacted with avidin-biotinylated alkaline phosphatase complex (Vector laboratories) in TBS for 30 min at RT, washed in TBS and finally developed in a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium in 50 m*M* TRIS-HCl, pH 9.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$.

Immunohistochemistry for bFGF

Under anesthesia by pentobarbital, mice were perfused intracardially with 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4. Stomach tissues were excised and fixed in the same fixative for 6 h at 4° C. The specimens were then immersed consecutively in 10%, 15% and 20% solutions of sucrose in phosphate-buffered saline, pH 7.4 (PBS), and 6-μm-thick frozen sections were prepared. Light microscopic immunohistochemistry was performed by the indirect immunofluorescent method. After incubation with normal goat serum (1:10) for 30 min at RT, sections were reacted with rabbit anti-bFGF antibodies (1:1000) overnight at 4° C and washed in PBS. Slides were then treated with affinity purified fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:100 dilution; Seikagaku kogyo, Tokyo, Japan) for 1 h at RT, washed in PBS and examined with a Nikon fluorescence microscope.

For immunoelectron microscopy, the pre-embedding technique was adopted because of its excellent preservation of tissue antigenicity. Embedding in Lowicryl K4M resulted in a deterioration of the immunoreactivity to the antiserum used in this study. The frozen sections were first stained by the indirect immunoperoxidase method using diaminobenzidine tetrahydrochloride as described previously (Yabu et al. 1992). After treatment with 1% osmium tetroxide in 0.1 *M* phosphate buffer, pH 7.4 for 1 h at RT, the tissues were dehydrated through an ethanol gradient and embedded in Epon 812. Ultrathin sections, cut and stained with lead citrate for 30 s, were observed with a JEOL 1200EX electron microscope. For controls, normal rabbit serum was applied instead of the first antibodies in both light and electron microscopic immunohistochemical studies.

Results

On the mucosal surface of the stomach 5 days after treatment, a sharply defined round ulcer was observed corresponding to the serosal surface where acetic anhydride had been applied. By light microscopy the ulcer was observed to reach to the subserosa (Fig. 1 a). After 3 weeks the ulcer of the stomach was completely healed, being covered by regenerated epithelium (Fig. 1 b).

Western blot analysis

As shown in Fig. 2, a predominant immunoreactive band was observed of 17 kDa apparent molecular weight. The molecular weight is almost the same as that

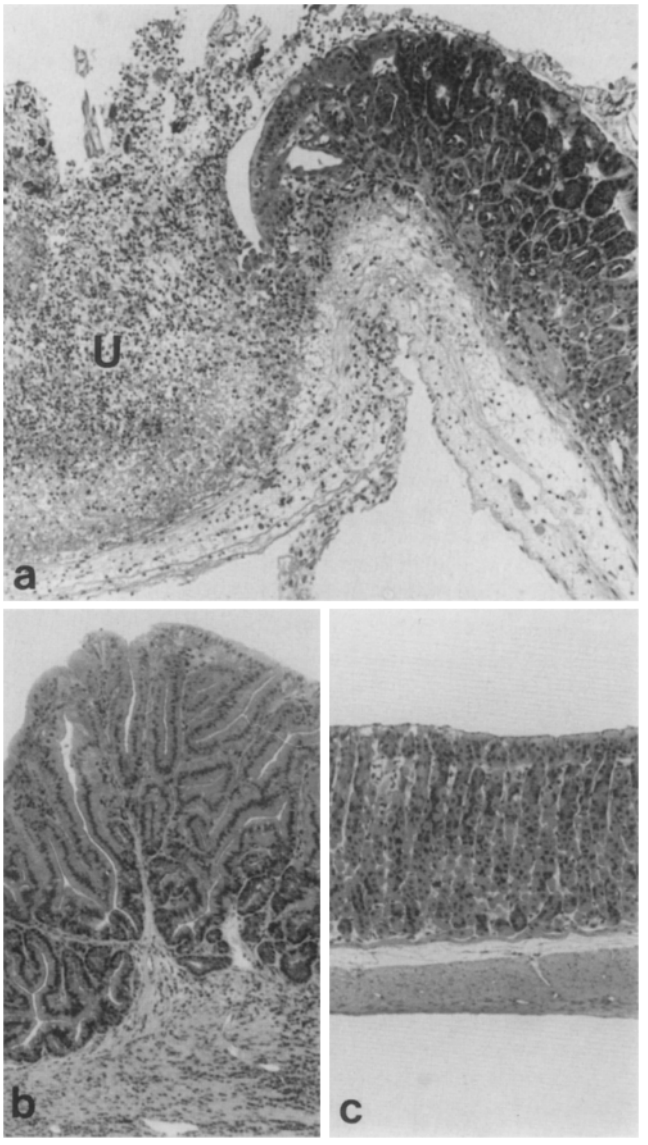

Fig. 1 a–c. Light micrographs of mouse stomach tissues. **a** An ulcer (*U*) is seen in the stomach 5 days after treatment with acetic anhydride. **b** After 3 weeks the ulcer lesion is covered by regenerated epithelium. **c** Untreated normal stomach tissue. × 100

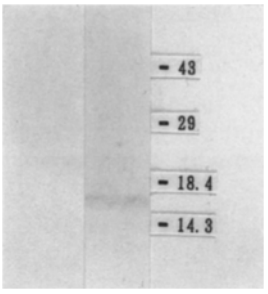

Fig. 2. Western blot analysis of extracts of the stomach tissues 5 days after treatment utilizing anti-basic fibroblast growth factor (bFGF) antibodies. Molecular weights in kDa are indicated at the *right*

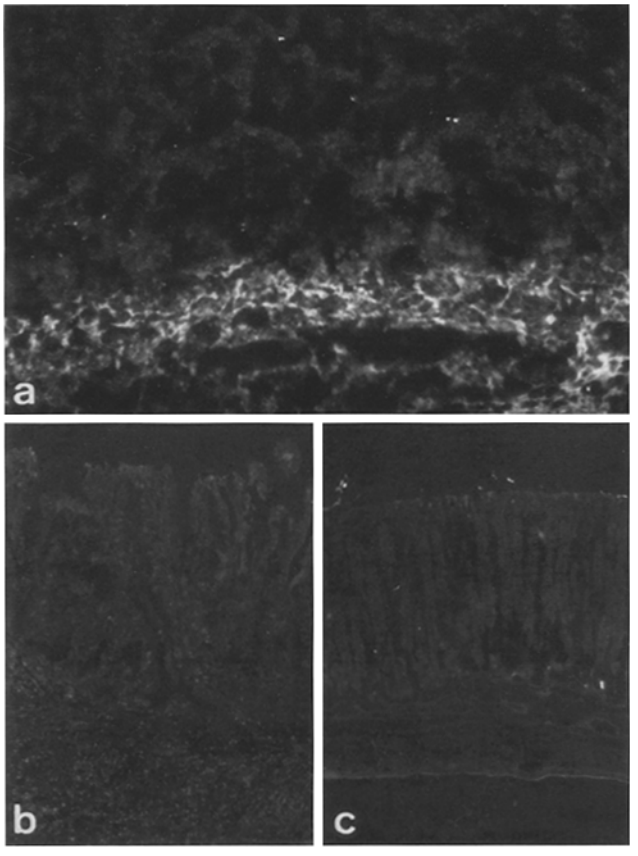

Fig. 3a–c. Light microscopic localization of bFGF in mouse stomach tissues. **a** In the gastric ulcer region 5 days after treatment, bFGF is localized in spindle-shaped cells in the ulcer bed. **b** The ulcer scar region convered by regenerated epithelium 3 weeks after treatment is immunohistochemically negative for bFGF. **c** Normal stomach tissue is devoid of immunoreactivity for bFGF. **a** ×240; **b, c** ×100

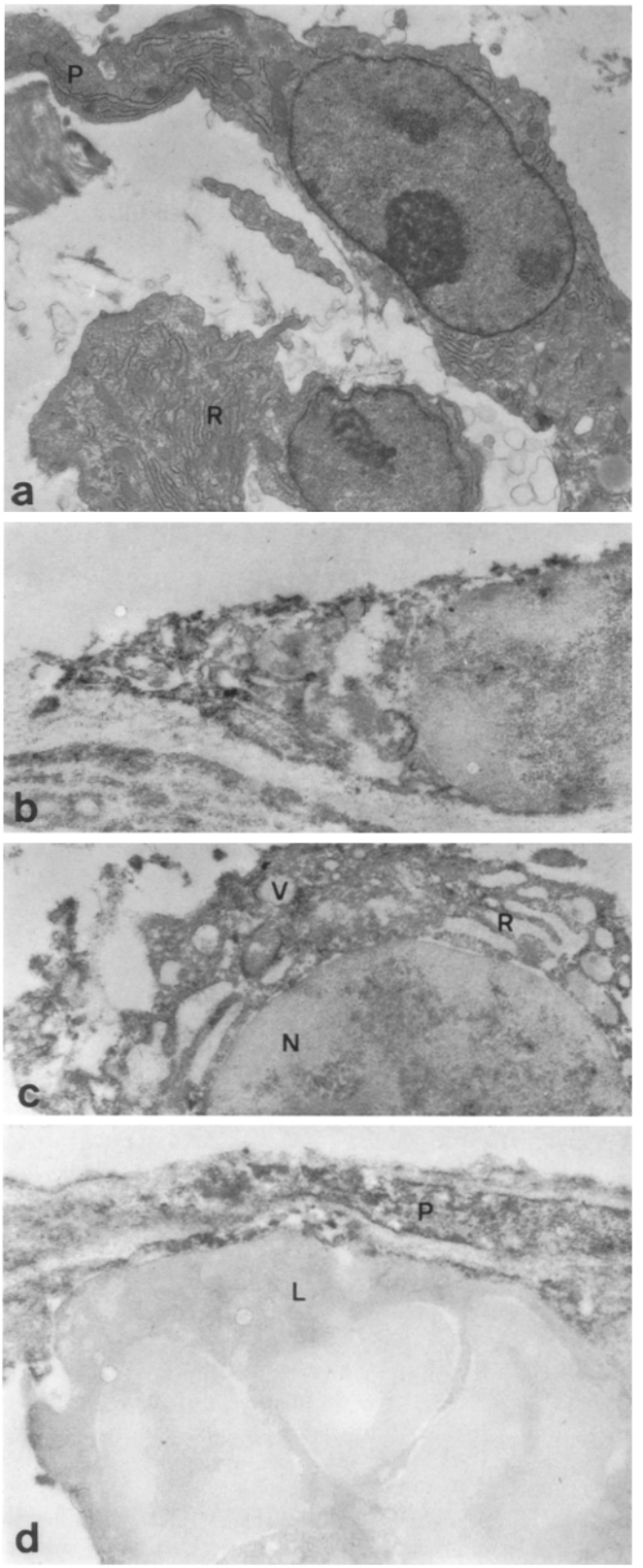

Fig. 4a–d. Electron microscopic observations of the gastric ulcer bed 5 days after treatment. **a** Electron micrograph of fibroblasts in the ulcer bed showing long cytoplasmic processes (*P*) and fairly well-developed intracellular organelles such as rough endoplasmic reticulum (*R*). **b–d** Immunoelectron micrographs for bFGF. **b** Black precipitates showing the localization of bFGF are observed in fibroblasts in the ulcer bed. **c** The cytoplasm of a fibroblast in the ulcer bed shows positive immunoreactivity to bFGF, but cytoorganelles such as rough endoplasmic reticulum (*R*) and vacuoles (*V*) are free of staining. The growth factor was visualized in the nucleus (*N*) as a spotted pattern of immunoprecipitation. **d** In the long cell process (*P*) of a fibroblast, cytoplasmic staining for bFGF is seen. A polymorphonuclear leukocyte (*L*) is devoid of staining for bFGF both in the nucleus and in the cytoplasm. **a** ×5800; **b** ×7500; **c** ×11800; **d** ×11300

of bFGF and the specificity of the antiserum in the mouse stomach tissues was thus confirmed.

Light microscopic localization of bFGF

In the mouse stomach tissues after 5 days, the immunoreactivity for bFGF was detected in the ulcer region. However other stomach regions, including the area surrounding the ulcer crater covered by regenerated mucosal epithelium, were negative for bFGF. Observations at higher magnifications revealed that the growth factor was localized in spindle-shaped cells in the ulcer bed (Fig. 3a). Sections obtained after 3 weeks were negative for bFGF both inside and outside the ulcer scar region (Fig. 3b). Normal stomach tissues (Fig. 1c) were also devoid of immunoreactivity for bFGF (Fig. 3c). All of the control studies using normal rabbit serum instead of anti-bFGF antibodies gave negative results.

Immunoelectron microscopic localization of bFGF

In the ulcer bed after 5 days, most of the spindle-shaped cells were typical fibroblasts. They had long cytoplasmic processes, and the cytoplasmic organelles such as rough endoplasmic reticulum and Golgi apparatus were fairly well developed (Fig. 4a). By immunoelectron microscopy, black precipitates showing the localization of bFGF were observed in these cells in the ulcer bed (Fig. 4b). The growth factor was distributed throughout the cytoplasm of the fibroblasts (Fig. 4b–d), but intracellular organelles such as rough endoplasmic reticulum, Golgi apparatus and secretory vacuoles were devoid of staining (Fig. 4c). In the nucleus a spotted immunoprecipitation pattern was obtained (Fig. 4b and c). A clear deposition of immunoreactive bFGF was difficult to find in the extracellular matrix. Other cell types such as neutrophilic leukocytes and lymphocytes were not stained for bFGF (Fig. 4d). Control studies using normal serum instead of the first antibodies also showed negative staining.

Discussion

The present immunohistochemical study revealed that bFGF was localized in fibroblasts in the gastric ulcer bed. The growth factor was distributed throughout the cytoplasm, but the cytoorganelles involved in the usual secretory system, such as rough endoplasmic reticulum (ER), Golgi apparatus and secretory vacuoles were devoid of immunoreactivity to bFGF. This cytoplasmic localization of basic fibroblast growth factor is consistent with the previous immunohistochemical report on the localization of bFGF in cultured A431 human epidermoid carcinoma cells (Yamamoto et al. 1991). The present findings are in accordance with the fact that bFGF has no signal peptide sequence leading to conventional secretion by the ER-Golgi system. The production of bFGF by fibroblasts has also been reported in vitro (Moscatelli et al. 1986; Story 1989; Root and Shipley 1991) and fibroblasts are thus considered to have the potency to produce bFGF.

Fibroblasts are common connective tissue cells. In the present work, fibroblasts were positive for bFGF only in the ulcer region. Root and Shipley (1991) reported that the addition of fetal bovine serum to cultured fibroblasts stimulated the production of bFGF. Therefore factors secreted from infiltrated mononuclear and polynuclear cells in the ulcer region might cause fibroblasts to produce bFGF.

Ohtani et al. (1993) have reported that in normal human stomach tissues immunoreactivity to bFGF is present at the luminal surface of vascular endothelial cells and in nuclei of fibroblasts. In this study we could not detect any staining for bFGF in normal mouse stomach tissues. This could be due to differences in the sensitivity of the immunohistochemical technique used. From our results the quantity of bFGF in normal stomach tissues appeared to be very scanty in contrast to the abundance of the growth factor in the ulcer region. Mignatti et al. (1992) performed experiments using NIH 3T3 cells transfected with bFGF complementary DNA and also drugs or treatments affecting various pathways of protein secretion. They showed that bFGF can be released by cells via a mechanism of exocytosis which is independent of the ER-Golgi system.

In relation to the roles of bFGF in gastric ulcer healing, the following speculation is possible on the basis of its biological activities. Firstly, because bFGF has mitogenic activity to fibroblasts, the peptide might stimulate the growth of fibroblasts in the ulcer region by paracrine and autocrine mechanisms. Fibroblasts are known to produce extracellular matrix components such as collagen, and an increase in the number of fibroblasts is considered to compensate for the tissue defect due to ulceration, leading to ulcer healing.

The second possible role of bFGF in the ulcer bed is to induce neovascularization on the basis of its angiogenic effect. By electron microscopy capillaries were observed only sparsely in the ulcer bed; for healing of the ulceration, neovascularization is necessary for the provision of nutrients and oxygen as well as for the removal of useless metabolites. Concerning neovascularization, Rhodin and Fujita (1989) have reported detailed electron microscopic observations of capillary growth in the mesentery of normal young rats. Fibroblasts were observed to approach and settle down on the capillary sprouts, and were transformed into pericytes being surrounded by a basal lamina. Fibroblasts, thus, are considered to participate in neovascularization by producing bFGF as angiogenic factor and also by conversion into pericytes. In conclusion, this study has demonstrated immunohistochemically the existence of bFGF in fibroblasts in the gastric ulcer region. Possible roles of the growth factor in gastric ulcer healing have been discussed.

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