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Gastrin Releasing Peptide-like Immunoreactive Substance in Rat Mammary Glands during Pregnancy

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The concentration of gastrin releasing peptide-like immunoreactive substance (GRP-IS) was measured by enzyme immunoassay (EIA) in rat mammary glands during pregnancy and after delivery. The GRP-IS concentration was high in the middle stage of pregnancy (10-14 days of gestation) and decreased in late pregnancy to reach a plateau range. By using HPLC, it was shown that GRP(20-29) and GRP(16-29) were mainly present in rat mammary glands. Immunohistochemical study revealed that epithelial cells of rat mammary glands were stained intensely with antiserum GP-6201. These results suggest that GRP-IS is produced and secreted in epithelial cells of mammary glands and takes part in the proliferation, differentiation and hypertrophy of mammary glands.

Gastrin releasing peptide (GRP) was first isolated from the porcine gastric tissue by McDonald et al. (1) as a bombesin (BN)-like peptide (2). Later, amino acid sequences of canine GRP, human GRP (hGRP) and rat GRP (rGRP) (3) were determined. BN-GRP has been shown to be a growth factor; stimulation of proliferation of Swiss 3T3 fibroblasts (4), human normal bronchial epithelial cells (5), small cell lung cancer cells (6,7) and induction of gastrin cell hyperplasia and increasing pancreatic DNA content in rats (8-10). We previously reported that the high concentration of GRP-IS was existed in human milk during pregnancy (11). It is not clear whether the GRP-IS is transferred from plasma to mammary glands by active concentrating mechanism or it is produced in mammary glands

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and secreted into milk. In this study, we purpose to determine the level of GRP-IS and the origin of GRP-IS in mammary glands and to speculate the role of GRP-IS during pregnancy.

MATERIALS AND METHODS

Rat : Wistar rats at various stages of pregnancy were purchased from Seiwa Experimental Animals, Ltd.(Fukuoka, Japan). Rat mammary glands (upper three out of six mammary glands on the right side) were collected at various stages of pregnancy and post-partum (gestation: 10, 14, 18 and 21 days, post-partum: 1 and 4 days). The connective tissues and fats were removed from mammary glands. Tissues were immersed in 3 volumes of boiling water for 5 min. Mammary glands were cooled, and homogenized in 2 volumes of 5 % trifluoroacetic acid (TFA) with a glass tissue grinder. The homogenizer was rinsed with another 20 volumes of the same acid solution. The pooled mixtures were stirred for 6 h at 4°C, and centrifuged at 2000 x g for 30 min. Supernatants were loaded on reversed-phase C18 cartrige (SEP-PAK, Waters Associate Inc., Milford, USA). After washing with 10 ml of 4 % acetic acid (AcOH), pH 4.0, GRP-ISs were eluted with 3 ml of 70 % acetonitrile in 0.5 % AcOH, pH 4.0. Eluates were concentrated by spin-vacuum evaporation, and lyophilized. The lyophilized extracts were reconstituted to 100 µl with assay buffer (0.05 M phosphate buffer, pH 7.2, containing 1 mM $MgCl_2$, 0.5 % bovine serum albumin and 250 KIU/ml aprotinin) and submitted to EIA. Further, the protein concentration of the supernatants of the pooled mixtures was determined with the method of Lowry et al. (12). After extraction of rat mammary glands, rat blood samples were collected from the femonal artery and vein. They were obtained into chilled tubes containing aprotinin (500 KIU/ml) and 1mg/ml EDTA. After centrifugation (3000 x g, 4°C, 20 min), plasma samples were stored at -40°C until use. Extraction of GRP-IS in plasma samples was performed with use of SEP-PAK similar to mammary glands.

EIA : EIA was performed previously described (13). The antiserum (GP 6201) raised against hGRP was specific to the carboxy-terminal (C-terminal) portion of hGRP as examined with synthetic hGRP and its fragments. A hGRP(12-27) was labeled with β-D-galactosidase by use of N-(ε-maleimidecaproyloxy)succinimide. EIA was performed by delayed addition. B/F separation was performed by the double antibody solid phase method. The detectable minimum amount of GRP-IS by this method was 10 pM.

Syntheses of rGRP, rGRP(20-29) and rGRP(16-29) : rGRP, its C-terminal peptides, rGRP(20-29) and rGRP(16-29), were synthesized by 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase method according to Atherton et al. (14). Fmoc-amino acid derivatives bearing protecting groups based on tert-butyl alcohol were employed, together with Arg(Mtr) (Mtr; 4-methoxy-2,3,6-trimethylphenylsulfonyl). As the starting resin, 5-(4-(9-fluorenylmethyloxycarbonyl)aminomethyl-3,5-dimethoxyphenoxy)valeric acid (PAL) handle anchoring resin was employed. The first C-terminal residue, Fmoc-Met-OH, was loaded on the PAL resin by 1,3-diisopropylcarbodiimide (DIPCDI) procedure with the aid of 1-hydroxybenzotriazole (HOBt). The combination of piperidine treatment and DIPCDI plus HOBt procedure served to elongate the peptide chain manually.

Cleavages and deprotections of the peptide amides (rGRP and C-terminal peptides) from PAL resin were performed by 1M trimethyl-

silyl bromide (TMSBr)-thioanisole/TFA treatment in the presence of m-cresol and ethandithiol. C-terminal deca- and tetradecapeptides, rGRP(20-29) and rGRP(16-29) were obtained by gel-filtration on Sephadex G-15, followed by HPLC purification. The purities of rGRP(20-29) and rGRP(16-29) were ascertained by amino acid analyzer after 6N HCl hydrolysis and leucine aminopeptidase digestion. rGRP was purified by gel-filtration on Sephadex G-15, followed by HPLC purification. Purity of rGRP was ascertained by amino acid analyzer and FAB-MS (fast atom bombardment mass spectrometry) m/z:2908.6 $(M+H)^+$ for rGRP (calcd.2907.5).

Immunohistochemical study : Fresh frozen rat mammary tissue was sectioned (4 μm) in a cryostat and thow-mounted on a egg albumin-coated slide. The sections were air-dried and fixed with acetone for 10 min at 4°C. They were incubated with antiserum GP-6201 (13) at dilution of 1/200 for 60 min and the slides were thoroughly washed with PBS. This was followed by a secondary antibody using DAKO PAP kit (DAKOPATTS, CA, USA). The color reaction was performed with diaminobenzidine.

HPLC : Chromatography was performed using a reversed phase C-18 column (Cosmosil 5C18-AR, 4.6 x 150 mm, Nacarai Tesque, Inc., Kyoto, Japan). The column was equilibrated with 0.1 % TFA. Extract of rat mammary glands (1 rat mammary glands at 14 days of gestation) was applied to the column and eluted with a linear gradient of acetonitrile (15 % in 6 min and 15-40 % in 50 min) in 0.1 % TFA at a flow rate of 1 ml/min. The fraction size was 1 ml. Each fraction was concentrated by spin vacuum evaporation, and lyophilized. The residue was submitted to EIA. Synthetic rGRP and its fragments were applied to the column under the same conditions.

Statistical analysis : Comparisons between two groups were performed using Student's unpaired t test.

RESULTS

The displacement curves for synthetic rGRP(20-29), rGRP(16-29), rGRP(1-29) and extracts of mammary glands with this EIA were shown in Fig. 1. As displacement curves obtained with extracts of rat mammary grands were parallel to those of standard rGRP and its fragment peptides, the GRP-IS in mammary glands had the structure similar to the C-terminal portion of rGRP(1-29).

Immunohistochemical staining of mammary glands with GP-6201 was shown in Fig. 2. Only epithelial cells of mammary glands were positive. We suggested that the origin of GRP-IS in mammary glands was the epithelial cells.

Next, we measured the content of GRP-IS in rat mammary glands from middle stage of pregnancy to post-partum. The content of GRP-IS (GRP-IS level was expressed as pmol GRP-IS/mg protein) in mam-

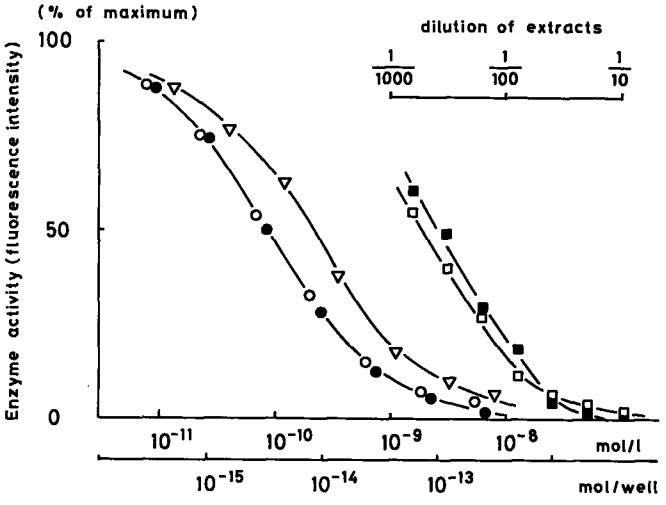

Fig. 1. Displacement curves with rGRP, its fragment peptides and rat mammary glands. Extracts from one rat mammary gland (14 days of gestation) were diluted. rGRP(▽), rGRP(20-29)(○), rGRP(16-29)(●), and extracts of rat mammary glands(□, ■).

mary glands was high in middle stage of pregnancy (700 nmol/g wet weight, 80 pmol/mg protein), compared with those in late pregnancy (15 pmol/mg protein, $P < 0.01$) and after delivery (16 pmol/mg

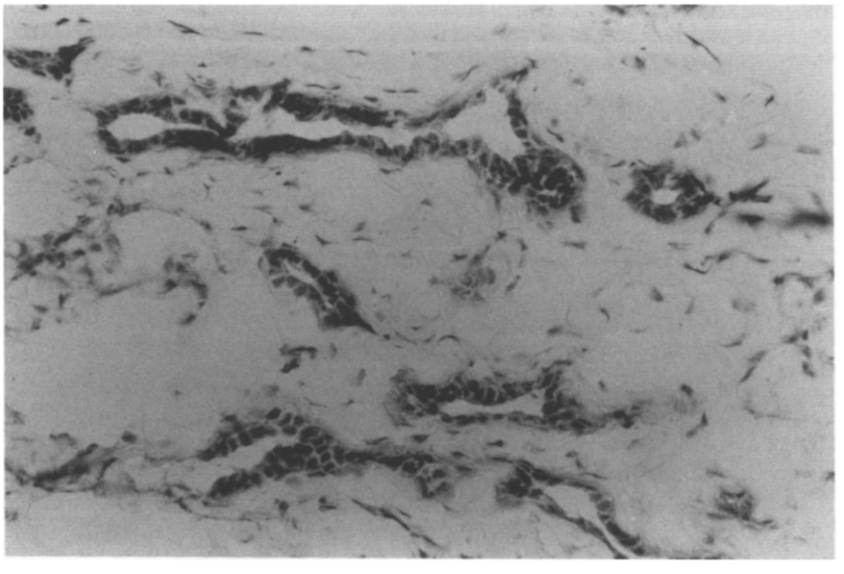

Fig. 2. Immunohistochemical study of GRP-IS-positive cells in rat mammary glands. Section was counterstained with hematoxylin. 14 days of gestation. x180.

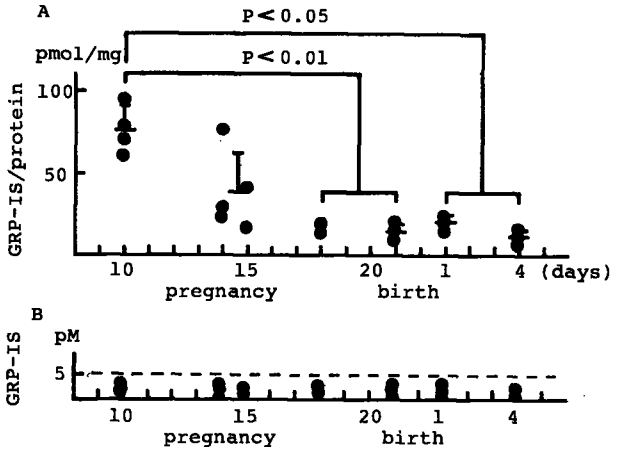

Fig. 3. Level of GRP-IS in rat mammary glands and plasma during pregnancy and post-partum. Mean and standard deviation values were calculated in 10, 14-15, 21 days of gestation and 0, 4 days of post-partum. A: mammary glands, B: plasma.

protein, $P < 0.05$). The level of GRP-IS in mammary glands remained constant throughout late pregnancy and post-partum. But the level of GRP-IS in plasma was below 5 pM and not changed during pregnancy and post-partum (2 fmol/ml) (Fig. 3).

HPLC revealed that GRP-IS in rat mammary glands was composed of two main peaks. One was coeluted with rGRP(20-29) in the region of 21 % acetonitrile and the other was coeluted with rGRP(16-29) in the region of 23 % acetonitrile, with a minor peak in the region of 31 % acetonitrile which had a different retention time with those of rGRP and its fragments, rGRP(20-29) and rGRP(16-29) (Fig.4).

DISCUSSION

We previously reported that there was a high level of GRP-IS in human milk during pregnancy, and the GRP-IS in human milk during pregnancy was thought to be a processing intermediate generated by proteolytic cleavage of the proGRP. Since the epithelial cell of mammary glands was positive for GRP-IS (Fig. 2) and the concentration of GRP-IS was more than hundred million higher in mammary glands (700 nmol/g wet weight at 10 days of gestation) than in plasma (2 fmol/ml) (Fig. 3), GRP-IS is likely to be produced and

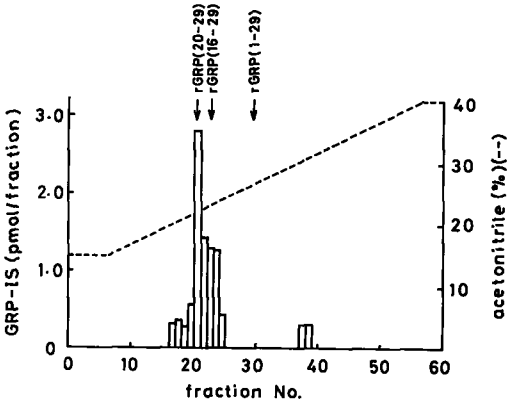

Fig. 4. HPLC of the extract of the rat mammary glands. Extract of rat mammary glands was applied to reversed-phase C18 HPLC column and eluted by a linearly increasing concentration of acetonitrile. Fractions (1ml) were assayed for the presence of GRP-IS. The arrows indicated the elution position of rGRP, rGRP(20-29) and rGRP(16-29).

secreted in mammary gland cells, rather than to be transferred from plasma to milk. By using HPLC there were two main GRP-ISs in rat mammary glands extract. One of them was rGRP(20-29) and the other was rGRP(16-29). These results concluded that the epithelial cells of mammary glands might be a source of GRP.

The physiological function of GRP-IS in mammary glands is not clear. The mammary glands develope during pregnancy, and the epithelial cells proliferate and differentiate into cells producing milk proteins. It was reported that many factors including estrogen, cortisol, prolactin and insulin were taken part in the proliferation, differentiation and hypertrophy of mammary glands in consecutive process (15, 16). Recently the factors which were produced and secreted in mammary glands were reported ; a new growth factor which was derived from mesenchyme had a growth activity to the epithelial cells (17) and epidermal growth factor (EGF)(18). As the preproEGF mRNA was found in mammary glands of confinement mouse (18), EGF might be produced and secreted in mammary glands.

GRP had a growth-stimulating activity on the various tissues. GRP, which is produced and secreted in mammary glands similarly to EGF, may have a mitogenic activity to mammary glands. As the GRP-IS concentration in rat mammary glands was high in middle stage of pregnancy (Fig. 3), the GRP-IS might take part in the change of mammary glands in middle stage of pregnancy, especially, when mammary glands were proliferated and differentiated rather than hypertrophied.

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