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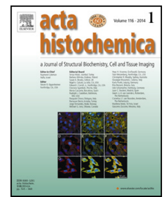

Spatial distribution of osteoblast activating peptide in the rat stomach

Ahmed E. Noreldin[a,b], Maina Sogabe[a], Yoshiaki Yamano[c,d], Masato Uehara[a], Mohamed A.A. Mahdy[d,e], Mohamed A. Elnasharty[b], Ahmed Sayed-Ahmed[f], Katsuhiko Warita[a], Yoshinao Z. Hosaka[a,d,*]

[a] *Department of Veterinary Anatomy, Faculty of Agriculture, Tottori University, Tottori, Japan*
[b] *Department of Histology and Cytology, Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt*
[c] *Department of Veterinary Biochemistry, Faculty of Agriculture, Tottori University, Tottori, Japan*
[d] *Department of Basic Veterinary Science, United Graduate School of Veterinary Science, Yamaguchi University, Yamaguchi, Japan*
[e] *Department of Anatomy and Embryology, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt*
[f] *Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt*

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ABSTRACT

Osteoblast activating peptide (OBAP) was previously reported to be expressed in the rat stomach and to have a vital role in osteogenesis, but its distribution in rat stomach has not been determined. Thus, the aim of the present study was to identify the cell types expressing OBAP in the rat stomach. The stomachs of twelve 10-to-11-week-old male Jc1:SD rats were used. Samples were collected for immunohistochemistry, immunoelectron microscopy and dot blot assay. Immunohistochemical investigation revealed that OBAP was distributed mainly in parietal cells without any expression in chief cells, X/A-like cells or enterochromaffin-like cells. Moreover, OBAP-immunopositive cells were observed mainly in the upper and lower parts of the gastric gland. Significantly high optical density of immunopositive cells was observed in the upper and lower gastric gland regions. The dot blot assay confirmed that OBAP is secreted by parietal cells and that it is present in the gastric gland lumen. Immunoelectron microscopy demonstrated that OBAP was confined to the mitochondrial inner membrane within parietal cells and that the number of mitochondria in the upper and lower parts of the gastric epithelium was significantly larger than the number in the middle part of the gastric epithelium. Based on the results, it was concluded that OBAP is mainly produced by mitochondria of parietal cells in the upper and lower parts of the gastric epithelium. Moreover, the presence of OBAP in the gastric gland lumen suggests an exocrine mechanism of release.

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Introduction

Experimental induction of osteoporosis was previously performed by surgery to remove either the whole stomach (gastrectomy) or the acid-secreting portion of the stomach (fundectomy) (Bussabarger et al., 1938). The mechanism by which bone loss is induced by gastrectomy or fundectomy has remained unclear (Andersson et al., 2002). Many theories for bone loss including dietary deficiency (calcium, vitamin D) and lack of gastric acid have been tested, but none of them were considered to be the main cause of osteoporosis (Persson et al., 1993; Klinge et al., 1995; Davies et al., 1997; Adachi et al., 1998; Schmiedl et al., 1999). Studies were therefore carried out to find novel secretions that are produced by the stomach and affect bone metabolism directly or indirectly.

The distribution of stomach secreting-hormones and peptides that play an important role in osteogenesis was investigated in many studies in order to determine the main mechanism of bone loss induced by gastrectomy (Larsson et al., 2001; Fukushima et al., 2005, 2010; Liu et al., 2011). One of these hormones that were investigated is a hypothetical hormone called "gastrocalcin", which is produced by enterochromaffin-like (ECL) cells. ECL cells were found to be the predominant endocrine cells in the oxyntic mucosa of the stomach and to be involved in bone metabolism (Persson et al., 1989; Larsson et al., 2001). It was also found that the granule extract of ECL cells increased a typical Ca^{2+}-mediated second messenger

Abbreviations: ECL, enterochromaffin like cells; HE, hematoxylin and eosin; H^+/K^+ ATPase, Hydrogen potassium ATPase; IHC, immunohistochemistry; IF, immunofluorescent; OBAP, osteoblast activating peptide; PTHLH, parathyroid hormone-like hormone; TEM, transmission electron microscope.

* Corresponding author at: Department of Veterinary Anatomy, Faculty of Agriculture, Tottori University, 4-101 Koyama Minami, Tottori 680-8553, Tottori, Japan. Tel.: +81 857 31 5419; fax: +81 857 31 5419.

E-mail address: y-hosa@muses.tottori-u.ac.jp (Y.Z. Hosaka).

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response in different osteoblast-like cell lines (Larsson et al., 2002). Parathyroid hormone-like hormone (PTHLH) was also detected in rat ECL cells (Andersson et al., 2005), and it was suggested that gastrocalcin and PTHLH are different names for the same hormone that has a role in treatment of osteoporosis (Andersson et al., 2005; Liu et al., 2011).

Another hormone that was investigated is ghrelin, which is secreted by X/A-like cells in the stomach (Sakata et al., 2002). Ghrelin is thought to be a good candidate for the treatment of osteoporosis (Kojima et al., 1999). Also, there is a direct relationship between ghrelin and bone formation, which is an increase in calcium accumulation in the bone matrix and increase in bone mineral density by ghrelin administration, was found in SD rats (Fukushima et al., 2005).

Hydrogen potassium (H^+/K^+) ATPase is a vital enzyme for hydrochloric acid production by parietal cells in the gastric gland. H^+/K^+ ATPase is present in the cell membrane of parietal cells and mediates the exchange of intracellular H^+ and extracellular K^+ to secrete gastric acid under the condition of stimulation of secretagogues on parietal cells (Yao and Forte, 2003). Therefore, the activity of gastric H^+/K^+ ATPase can be used as an indicator of the ability of gastric acid secretion by parietal cells (Soumarmon and Lewin, 1986).

Recently, osteoblast activating peptide (OBAP), novel 24-amino-acid peptide that induces increases in several osteoblast differentiation markers, including alkaline phosphatase and osteocalcin, in osteoblast cell lines has been reported (Fukushima et al., 2010). Moreover, the recovery rate from osteoporosis in gastroectomized animals was significantly increased by OBAP administration in same study. The sequence of amino acids of OBAP encodes the C-terminal side domain of NADH dehydrogenase (ubiquinone) flavoprotein 3 (Ndufv3) transcript variant 2, which is located in the mitochondrial membrane, and has been suggested to be a preprotein of OBAP (preOBAP) (Pilkington and Walker, 1989; Runswick et al., 1989; Kitahara et al., 1996; De Coo et al., 1997). Although, the function of Ndufv3 is still unknown, OBAP was reported to be effective for treatment of induced osteoporosis in gastroectomized rats. Therefore, OBAP is considered to be a good candidate for treatment of osteoporosis (Fukushima et al., 2010).

The effect of OBAP has been partially clarified, but the cells that synthesize and secrete OBAP in the stomach have not been identified. Thus, to obtain an insight into the localization of OBAP in the stomach, we tried to identify OBAP-immunopositive cells and determine the secretion routes of OBAP by using immunohistochemical and immunocytochemical techniques.

Materials and methods

Animals

Twelve 10-to-11-week-old male Jc1: SD rats (250–300 g; Shimizu Kagaku, Kyoto, Japan) were maintained in a 12:12 h, light-dark cycle with free access to food and water throughout the experiment. All of the experiments followed the protocols approved by the Ethics Animal Care Committee of Tottori University, Japan (Approval No. 13-T-19) and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals [DHEW (DHHS) Publication No. (NIH) 85-23, revised in 1985, Office of Science and Health Report, DRR/NIH, Bethesda, MD 20205].

After anaesthetizing by ether inhalation, the animals were euthanized by cervical dislocation. Then stomachs were surgically removed and flushed with phosphate buffer saline (PBS, pH 7.4). Stomach tissues were divided into three groups: (1) tissues fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) overnight at 4 °C for immunohistochemistry (IHC) and immunofluorescence (IF), (2) tissues fixed in 4% PFA and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at 4 °C for immunoelectron microscopy and transmission electron microscopy (TEM), and (3) tissues preserved at −20 °C for a dot blot assay.

Antibodies and preparation for anti-OBAP antibody

The following antibodies were used: monoclonal mouse anti-rat H^+/K^+ ATPase β subunit antibody (1:2000; Novus Biologicals, Cat: NB300-583, Littleton, CO, USA) as a marker for parietal cells (Jones et al., 1991), polyclonal goat anti-rat ghrelin antibody (1:200; Abcam, Cat: ab104307, lot: GR45020-4, Cambridge, UK) as a marker for X/A-like cells (Stengel and Taché, 2009) and monoclonal mouse anti-rat calbindin antibody (detecting 28 kD subtype, 1:4000; Sigma-Aldrich, Cat: C9848, lot: 088k4799, St. Louis, MO, USA) as a marker for ECL cells (Andersson et al., 1998). An anti-OBAP antibody was obtained from a custom antibody producing service (1:800; Operon Biotechnologies, Tokyo, Japan). An synthetic OBAP sequence (LDLNLDLSKFRLPQPSSGRESPRH; Fukushima et al., 2010) was used to immunize rabbits, and sera were collected as a polyclonal anti-OBAP antibody. The immunospecific reaction of anti-OBAP antibody to OBAP peptide was confirmed (Supplementary data).

Immunohistochemistry

Four-μm-thick paraffin sections were prepared and were deparaffinized by xylene and rehydrated in graded alcohols. Some sections were stained with hematoxylin and eosin (HE). The other sections were used for immunohistochemical staining by the following procedure. Briefly, sections were treated with 0.5% TritonX-100 (Nacalai, Kyoto, Japan) for 20 min. Endogenous peroxidase activity was eliminated with 3% hydrogen peroxide in methanol for 5 min at room temperature. The stomach sections were blocked with 5% BSA (Sigma-Aldrich, Cat: A9647, lot: 41H0520, St. Louis, MO, USA) diluted in PBS for 1 h, and incubated with the OBAP antibody overnight at 4 °C. For negative control sections, PBS was used instead of the primary antibody. After washing with PBS, sections were incubated with biotin-conjugated goat anti-rabbit IgG antiserum (Histofine kit, Cat: 424032, lot: H1401, Nichirei, Tokyo, Japan) for 30 min at room temperature. Then the sections were washed in PBS, followed by incubation with streptavidin-peroxidase conjugate for 30 min at room temperature. The streptavidin-biotin complex with antibody binding was visualized using peroxidase/diaminobenzidine (DAB) (peroxidase/DAB ChemMate Detection Kit; Dako, Cat: K5007, lot: 00054660A, CA, USA). Sections were finally counterstained slightly with Mayer's hematoxylin. Micrographs of the sections were taken with a digital camera (DP-71, Olympus, Tokyo, Japan) attached to a microscope (Ix71, Olympus).

Immunofluorescence

Double IF for OBAP with H^+/K^+ ATPase, ghrelin and calbindin was performed to detect OBAP-immunopositive cells in parietal cells, X/A-like cells and ECL cells, respectively. For antigen retrieval of ghrelin with OBAP, the sections were heated in 10 mM citrate buffer (pH 6.0) for 20 min at 95 °C, and for calbindin with OBAP, proteinase K was applied for 3 min. No antigen retrieval procedure was used for OBAP and H^+/K^+ ATPase. The sections were treated with 0.5% TritonX-100, incubated with donkey serum (GeneTex, Cat: GTX30972, lot: 821403731, Irvine, CA, USA) for 30 min and incubated at 4 °C overnight with the combination of specific primary antibodies and dilutions as stated above. Antibody binding was localized by incubation for 30 min with Alexa-Fluor-488-labeled donkey anti-goat IgG (1:200, Invitrogen, Cat: NL001, lot: LZP0812031, Eugene, OR, USA), Alexa-Fluor-546-labeled donkey

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