■ ORIGINAL ARTICLES

$GABA_B$ receptor immunopositive cells found in the mouse pulmonary alveolar epithelium were Type II cells.

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ABSTRACT：The γ-amonobutyrate (GABA) system, a major inhibitory regulator in the central nervous system, may also play important roles in peripheral nonneuronal tissues and cells. Our previous study showed the presence of GABA system composed of GABA synthesize enzyme glutamate decarboxylase and GABA in the bronchial epithelium of GAD67-GFP knock-in mice. The cells were pulmonary neuroendocrine cells. Furthermore, cells expressing $GABA_B$ receptors were found in the alveolar epithelium. Present study was undertaken to identify the type of the alveolar cells by electron microscopic immunocytochemistry. The cells were identified as type II cells. Type II cells thought to be function as secretory cells of the lung surfactant. The other important function of type II cells is repair of the alveolar epithelium by proliferate and differentiate into type I cells. Since GABA system thought to be involved in cell proliferation and migration in the neural cells, the result of this study may suggest that GABA system has certain physiologic functions on maintenance of pulmonary epithelium.

Key words： Alveolar type II cells, GABA, $GABA_B$ receptors

INTRODUCTION

Gamma -aminobutyric acid (GABA), a major inhibitory neurotransmitter in the central nerve system, is also located in many peripheral non-neuronal tissues.[1, 2)]

GABA is synthesized by a decarboxylation reaction of glutamic acid catalyzed by glutamate decarboxylase (GAD). [1)] Mammalian species express two isoforms of GAD, GAD65 and GAD67, where the numbers refer to the molecular mass in kDa. In the brain, GAD67 is responsible for synthesis of >90% of GABA. The two GAD isoforms are produced by many of the same GABAergic neurons. Since, GABA is synthesized by GAD, the GAD 67-green fluorescent protein (GAD67-GFP) knock-in mouse is a useful model for studying the distribution of GABAergic cells in many tissues and organs.[3, 4)] By using of this model mouse, we found that the lungs of these mice contained cells with an intense GFP signal exclusively in the airway epithelium.[5)] These cells identified as pulmonary neuroendocrine cells (PNECs) by immunohistochemistry and by transmission electron microscopy.[5)] This study revealed that the GFP-positive PNECs are actually GABAergic cells since these cells expressed GABA in addition to the GAD65/67.

The effects of GABA are exerted through ionotropic $GABA_A$ and $GABA_C$ receptors, as well as metabotropic $GABA_B$ receptors.[1)] $GABA_B$ receptors are coupled to G proteins, and active functional $GABA_B$ receptors are heterodimers that are composed of two subunits, R1 and R2. In a previously reported paper, we described that reverse transcription-polymerase chain reaction analyses revealed mRNAs encoding the $GABA_B$ receptor subunits necessary for the assembly of functional receptors, R1 and R2 in the lung. [5)] $GABA_B$ receptor subunit R1 and R2 proteins were expressed in alveolar epithelial cells. However, GFP-positive PNECs were not immunoreactive for $GABA_B$ receptor R1 and R2 subunits. These results provide evidence that PNECs produce GABA, which may act upon $GABA_B$ receptors expressed in certain alveolar epithelial cells in mice. The pulmonary alveolar epithelium is comprised of two morphologically distinct types of cells, called type I and type II cells. [6)] This study, therefore, was undertaken to identify the alveolar epithelial cells expressed $GABA_B$ receptors.

MATERIALS AND METHOD

Animals

GAD67-GFP mice (Δneo) were generated as described by Tamamaki et al.[7)] Here, we refer to these animals as GAD67-GFP knock-in mice. These mice are maintained at the Department of Anatomy and Cell Biology, Osaka Medical College (Osaka, Japan). All animals were housed in a temperature-controlled room (23˚C) with free access to water and regular food (CE-2, Clea Japan) under a standard dark/light schedule of 12/12 hours. All animal experiments were reviewed and approved by the Ethics Review Committee for Animal Experimentation of Osaka Medical College.

Electron Microscopic Immunocytochemistry

Sections were preincubated with 20% normal donkey serum in 10 mM phosphate-buffered saline (PBS, pH 7.4) for 10 min at room temperature, followed by overnight incubation at 4°C with rabbit polyclonal antibody against

$GABA_{B(2)}$ subunit (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) as described before.[8] Sections were then rinsed in PBS and incubated with biotinylated donkey anti-goat IgG (diluted 100×; Chemicon International, Temecula, CA, USA) overnight at 4°C. After rinses with PBS, sections were processed for 3, 3'-diaminobenzidine tetrahydrochloride (DAB) staining. The sections were incubated with avidin-biotin-horseradish peroxidase complex (diluted 50×; Vector Laboratories, Burlingame, CA, USA) for 3 h at room temperature rinsed with PBS, and then incubated with 0.02% DAB in 50 mM Tris-HCl (pH 7.6) containing 0.002% H_2O_2 for 30 min at room temperature. The sections were rinsed in distilled water and then washed in 0.1 M phosphate buffer (PB; pH 7.4). Sections were treated with 1% OsO_4 in 0.1 M PB for 40 min. Sections were rinsed in distilled water and counterstained with 1% (w/v) uranyl acetate for 30 min. Sections were then dehydrated through a graded ethanol series and flat-embedded in Epoxy-resin (Luveak; Nacalai Tesque, Kyoto, Japan). Ultrathin sections were prepared on an ultramicrotome (Reichert-Nissei Ultracut S; Leica, Vienna, Austria) and observed under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

RESULTS

Identification of cell type of GFP-mouse alveolar cells which possess $GABA_B$ receptors by transmission electron microscopic immunocytochemistry

Alveolar type I cells are flattened cells contain just a few organelles.[9] The presence of lamellar bodies, which is the storage granule for pulmonary surfactant, is a criterion for the identification of alveolar type II cells.[10] Immunoreactive products indicating $GABA_{B(2)}$ subunit were detected in the rough endoplasmic reticulum and vesicles of alveolar epithelial cells (Fig. 1 and 2). These cells exhibited ultrastructural features characteristic of type II cells, including cytoplasmic lamellar bodies.

Furthermore, the immunoreactive products were observed in the cytoplasm of type II cells in close apposition to the capillary (Fig. 3). In contrast to type II cells, $GABA_{B(2)}$ subunits were not expressed in type I cells (data not shown).

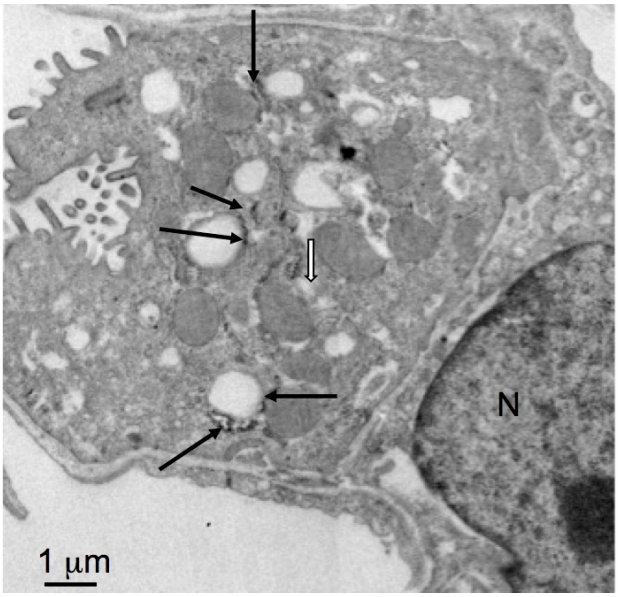

Fig. 1
Electron micrograph of immunocytochemistry of $GABA_{B(2)}$ subunit in GAD67-GFP mouse alveolar epithelial cells. Immunoreactive products are detected in rough endoplasmic reticulum and vesicles of alveolar epithelial cells (black arrows). These cells are type II cells. Hallmarks of the type II cell morphologic appearance are lamellar bodies indicated by white arrows. N: nucleus

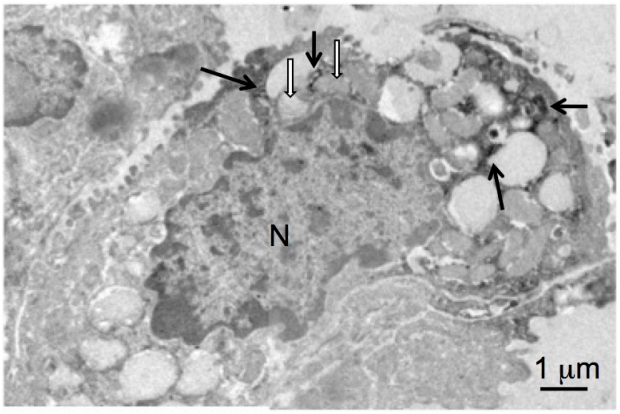

Fig. 2
Electron micrograph of immunocytochemistry of $GABA_{B(2)}$ subunit in GAD67-GFP mouse alveolar epithelial cells. Alveolar type II cells contain immunoreactive products for $GABA_{B(2)}$ subunits indicated by black arrows. White arrows indicate lamellar body that is a characteristic morphologic appearance of type II cells. N: nucleus

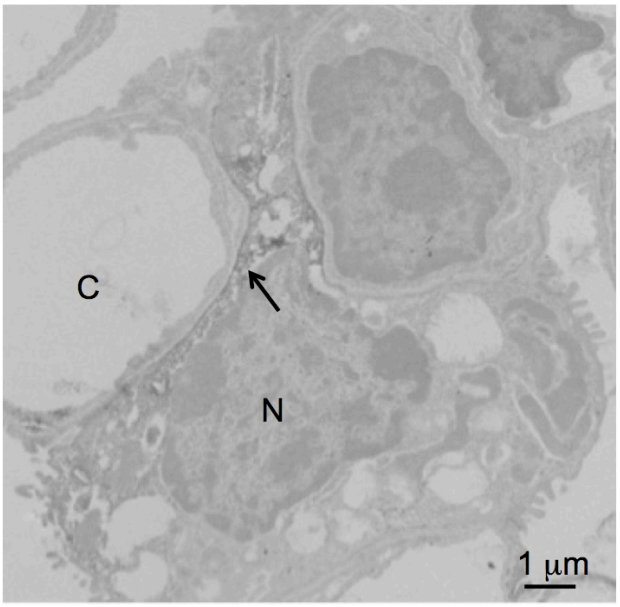

Fig. 3
Electron micrograph of immunocytochemistry of $GABA_{B(2)}$ subunit in GAD67-GFP mouse alveolar epithelial cells. Close apposition between immunoreactive products in the cytoplasm of type II cells and capillary (C). N: nucleus

DISCUSSION

The alveolar epithelium covers greater than 99% of the internal surface area of the lung and is composed of two major cell types, the alveolar type I cell (pneumocytes I) and the alveolar type II cell (pneumocytes II). Alveolar type I cells are large flat cells through which exchange of CO_2/O_2 takes place. They cover approximately 95% of the alveolar surface with their thin cytoplasmic extensions. In contrast, alveolar type II cells are small, cuboidal cells that cover approximately 5% of the alveolar surface.[10)]

The present electron microscopic immunocytochemistry clarified that $GABA_B$ receptor is present specifically in the alveolar type II cells. According to the review by Andreeva et al.[9)], a number of functions are known for type II cells. The most important function of type II cells is secretion of the lung surfactant. The other well known function of type II cells is repair function of the alveolar epithelium. By proliferating and migrating to damage areas, type II cells repair the alveolar epithelium. Type II cells are able to divide and to differentiate into type I cells.[11)]

Although GABA is the principal inhibitory neurotransmitter in the adult mammalian brain, it is thought to be involved in cell proliferation, migration, and in the promotion of cell survival in the neural cells.[12-16)] In the nonneuronal cells located outside the central nerve system, there has been little research on the participation of the GABA system on the proliferation both via $GABA_A$ receptors[17-24)] and $GABA_B$ receptors. Recent findings in non-neuronal peripheral cells suggest that, via $GABA_B$ receptors, GABA contributes to cell

proliferation in osteoblasts[25] and in murine embryonal carcinoma-derived ATDC5 cells.[26] Signal transduction pathway from the activation of $GABA_B$ receptors result in cell proliferation may through the modulation of adenylyl cyclase activity or Ca^{2+} or K^+ channels.[27-29] Additional downstream signal transduction pathways have been proposed elsewhere.[2]

We previously reported that GABAergic cells found in the GAD67-GFP knock-in mouse airway epithelium were PNECs, and it was ascertained that the cells synthesizes GABA.[5] These PNECs were exclusively located in the bronchial epithelium, and $GABA_{B(2)}$ receptor subunit positive type II cells were exclusively located in the alveolar epithelium. There are certain distance between the PNECs and alveolar type II cells. The PNECs are located mainly in the basal part of the epithelium, although their apical processes can reach the luminal surface.[30] Several researchers have stated that PNECs play an important role in lung biology by the paracrine/endocrine pathway. [31-33] The present study showed that $GABA_{B(2)}$ receptor subunits found in the cytoplasm of type II cells were situated in close apposition to the capillary.

Although the morphology, histochemistry, distribution, differentiation, and possible function of PNECs in the mammalian lungs have been reviewed, [34, 35] their functions are not fully understood. Proposed functions include airway epithelial remodeling,[32, 36] and control of growth and morphogenesis of the fetal lung.[37-39] The results of the former and present experiments prompt one to speculate the GABA system in the mouse pulmonary epithelium has a certain functional role in pulmonary epithelium especially in alveolar epithelium. Further research is necessary to elucidate the functional roles of GABA in epithelial cells of the lung.

REFERENCES

1) Watanabe M, Maemura K, Kanbara K et al: GABA and GABA receptors in the central nervous system and other organs. Int. Rev. Cytol. 213: 1-47, 2002
2) Watanabe M, Maemura K, Oki K et al: Gamma-aminobutyric acid (GABA) and cell proliferation: focus on cancer cells. Histol. Histopatho. 21: 1135-1141, 2006
3) Abe H, Yanagawa Y, Kanbara K et al: Epithelial localization of green fluorescent protein-positive cells in epididymis of the GAD67-GFP knock-in mouse. J. Androl. 26: 568-577, 2005
4) Maemura K, Yanagawa Y, Obata K et al: Antigen presenting cells expressing glutamate decarboxylase 67 were identified as epithelial cells in glutamate decarboxylase 67- GFP knock-in mouse thymus. Tissue Antigens 67: 198-206, 2006
5) Yabumoto Y, Watanabe M, Nakamura Y et al: Expression of GABAergic system in pulmonary neuroendocrine cells and airway epithelial cells in GAD67-GFP knock-in mice. Med. Mol. Morphol. 41: 20-27, 2008
6) Dobbs L.G and Gonzalez R.F: Isolation and culture of pulmonary alveolar epithelial type II cells. In: *Culture of Epithelial Cells, Second Edition.* Edited by R. Ian Freshney and Mary G. Freshney, pp 277-301, Wiley-Liss, Inc. 2002

7) Tamamaki N, Yanagawa Y, Tomioka R et al: Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. J. Comp. Neurol. 467: 60-79, 2003
8) Kanbara K, Okamoto K, Nomura S et al: Cellular localization of GABA and $GABA_B$ receptor subunit proteins During spermiogenesis in rat testis. J. Androl. 26: 485-493, 2005
9) Andreeva AV, Kutuzov MA and Voyno-Yasenetskaya TA: Regulation of surfactant secretion in alveolar type II cells. Am. J. Physiol. 293: L259–L271, 2007
10) Wang D, Haviland D.L, Burns A.R et al: A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. Proc. Natl. Acad. Sci. USA 104: 4449-4454, 2007
11) Evans M.J, Cabral L.J, Stephens R.J et al: Transformation of alveolar type 2 cells to type 1 cells following exposure to NO_2. Exp. Mol. Pathol. 22: 142–150, 1975
12) Barker J.L, Behar T, Li Y.X et al: GABAergic cells and signals in CNS development. Perspect. Dev. Neurobiol. 5: 305-322, 1998
13) Waagepetersen H.S, Sonnewald U and Schousboe A: The GABA paradox: Multiple roles as metabolite, neurotransmitter, and neurodifferentiative agent. J. Neurochem. 73: 1335-1342, 1999
14) Owens D.F and Kriegstein A.R: Is there more to GABA than synaptic inhibition? Nat. Rev. Neurosci. 3: 715-727, 2002
15) Ben-Ari Y: Excitatory actions of GABA during development: the nature of the nurture. Nat. Rev. Neurosci. 3: 728-739, 2002
16) Ben-Yaakov G and Golan H: Cell proliferation in response to GABA in postnatal hippocampal slice culture. Int. J. Dev. Neurosci. 21: 153-157, 2003
17) Culiat C.T, Stubbs L.J, Nicholls R.D et al: Concordance between isolated cleft palate in mice and alterations within a region including the gene encoding the b3 subunit of the type A γ-aminobutyric acid receptor. Proc. Natl. Acad. Sci. USA 90: 5105-5109, 1993
18) Culiat C.T, Stubbs L.J, Woychik R.P et al: Deficiency of the b3 subunit of the type A γ-aminobutyric acid receptor causes cleft palate in mice. Nat. Genet. 11: 344-346, 1995
19) Homanics G.E, DeLorey T.M, Firestone L.L et al: Mice devoid of gamma-aminobutyrate type A receptor beta3 subunit have epilepsy, cleft palate, and hypersensitive behavior. Proc. Natl. Acad. Sci. USA 94: 4143-4148, 1997
20) Ding R, Tsunekawa N and Obata K: Cleft palate by picrotoxin or 3-MP and palatal shelf elevation in GABA-deficient mice. Neurotoxicol. Teratol. 26: 587-592, 2004
21) Geigerseder C, Doepner R.F, Thalhammer A et al: Stimulation of TM3 Leydig cell proliferation via GABA(A) receptors: a new role for testicular GABA. Reprod. Biol. Endocrinol. 2 : 13, 2004
22) Minuk G.Y and Gauthier T: The effect of g-aminobutyric acid on hepatic regenerative activity following partial hepatectomy in rats. Gastroenterology 104: 217-221, 1993
23) Zhang M, Song G and Minuc G.Y: Effects of hepatic stimulator substance, herbal

medicine selenium/vitamin E, and ciprofloxacin on cirrhosis in the rat. Gastroenterology 110: 1150-1155, 1996

24) Kaita K.D.E, Assy N, Gauthier T et al: The beneficial effects of ciprofloxacin on survival and hepatic regenerative activity in a rat model of fulminant hepatic failure. Hepatology. 27: 533-536, 1998

25) Fujimori S, Hinoi E and Yoneda Y: Functional $GABA_B$ receptors expressed in cultured calvarial osteoblasts. Biochem. Biophys. Res. Commun. 293:1445-1452, 2002

26) Tamayama T, Maemura K, Kanbara K et al: Expression of GABAA and GABAB receptors in rat growth plate chondrocytes: Activation of the GABA receptors promotes proliferation of mouse chondrogenic ATDC5 cells. Mol. Cell. Biochem. 273: 117-126, 2005

27) Cunningham M.D and Enna S.J: Evidence for pharmacologically distinct $GABA_B$ receptor associated with cAMP production in rat brain. Brain Res. 720: 220-224, 1996

28) Couve A, Moss S.J and Pangalos M.N: $GABA_B$ receptors: a new paradigm in G protein signalling. Mol. Cell. Neurosci. 16: 296-312, 2000

29) Onali P, Mascia F.M and Olianas M.C: Positive regulation of $GABA_B$ receptors dually coupled to cyclic AMP by the allosteric agent CGP7930. Eur. J. Pharmacol. 471: 77-84, 2003

30) Williams P.T: Gray's anatomy. 38th ed. Churchill Livingstone, New York pp 1664-1673, 1995

31) Goniakowska-Witalinska L: Neuroepithelial Bodies and Solitary Neuroendocrine Cells in the Lungs of Amphibia. Microsc. Res. Tech. 37: 13-30, 1997

32) Reynolds S.D, Giangreco A, Power J.H.T et al: Neuroepithelial bodies of pulmonary airways include a reservoir of progenitor cells capable of epithelial regeneration. Am. J. Path. 156: 269-278, 2000

33) Cutz E and Jackson A: Neuroepithelial bodies as airway oxygen sensors Resp. Physiol. 115 (2): 201-214, 1999.

34) Ito T: Differentiation and proliferation of pulmonary neuroendocrine cells. Prog. Histochem. Cytochem. 34: 247-322, 1999

35) Van Lommel A, Bolle T, Fannes W et al: The pulmonary neuroendocrine system: The past decade. Arch. Histol. Cytol. 62: 1-16, 1999

36) Gosney J.R: Pulmonary neuroendocrine cell system in pediatric and adult lung disease. Microsc. Res. Tech. 37: 107-113, 1997

37) Van Lommel A: Pulmonary neuroendocrine cells (PNEC) and neuroepithelial bodies (NEB): chemoreceptors and regulators of lung development. Paediatr. Resp. Rev. 2: 171-176, 2001

38) Hoyt R.F. Jr, McNelly N.A, McDowell E.M et al: Neuroepithelial bodies stimulate proliferation of airway epithelium in fetal hamster lung. Am. J. Physiol. 260: L234-L240, 1991

39) Sunday M.E, Hua J, Reyes B et al: Antibombesin monoclonal antibodies modulate fetal mouse lung growth and maturation in utero and in organ cultures. Anat. Rec. 236: 25-32, 1993