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Cells expressing GABA synthetic enzyme, glutamate decarboxylase, in stomach and intestine: RT-PCR and immunohistochemistry studies

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Expression of glutamate decarboxylase in mouse stomach and intestine was studied by RT-PCR and immunohistochemical techniques. The RT-PCR study showed GAD mRNA expression in fetus and postnatal ages of 4 and 11 weeks old mice. A strong positive staining against GAD65 was observed at both stomach and intestine of the 4- and 11-week old mice. GFP positive cells were found in the fetus at both stomach and intestine, suggesting that GAD67 is expressed early in life. In this study, we present preliminary results of our findings on GAD expressing cells in stomach and intestine and discuss the significance of GAD expression and role(s) of these cells.

Key words: GABA, GAD, stomach, intestine

Introduction

Glutamate decarboxylase (GAD) is an enzyme [E.C. 4.1.1.15] to catalyze the synthesis of γ-aminobutyric acid (GABA) [1-3]. GABA functions as an inhibitory neurotransmitter in the nervous system [4]. In addition, GABA is also found in the non-neural tissues [5] where the role(s) of GABA in these tissues has been vigorously investigated [6]. There are accumulating evidences that GABA plays several significant physiological roles, such as lowering blood pressure, and giving relaxation and diuretic effects [7,8].

Mammalian species were found to have two independent genes for GAD and they express two isoforms, GAD65 and GAD67 [9]. Interestingly, both GAD isoforms were reported to be expressed within the same cells, a rare example that enzymes catalyzing the same chemical reaction are co-expressed in the same cells [3]. The GAD isoforms have been investigated in terms of structural differences, cellular localization, physiological functions, and regulation [1,10]. Studies using knock-out and/or knock-in mice suggested that GAD isoforms have different biological activities [11-13]. The knock-in mouse, GAD67/GAD mouse, developed by Obata and his colleagues, is a useful tool in studying the

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Abbreviations: Ab, antibody; CNS, central nervous system; GABA, γ-amino butyric acid; GAD, glutamate decarboxylase; GFP, green fluorescence protein; PBS, phosphate buffered saline

Table 1. Primers for RT-PCR

	direction	primer sequence	starting base at	bp
GAD65	forward	AGCCTTAGGGATTGGAACAG	942	258
	reverse	TTCCGGGACATCAGTAAC	1199	
GAD67	forward	GATACTTGGTGTGGCGTAGCCC	85	575
	reverse	ACGGGTGCAATTTCATATGTGAACATA	633	
β-actin	forward	AGCTGAGAGGGAAATCGTGC	569	492
	reverse	GATGGAGGGGCCGGACTCAT	1048	

exact localization of GAD67 isoform.

Spice extracts have been studies in order to discriminate the catalytic functions of GADs [14,15]. Such studies have revealed that various spice extracts could affect enzymatic activity of GADs. It is suggested that if GAD is located in the digestive system, GAD activity could be affected by food components. Recently, immunohistochemical and RT-PCR studies made it possible to detect GAD expression on skins, tongue, submandibular gland and others [16,17]. In the present study, we have used GAD67/GFP mice and applied techniques such as immunohistochemistry and RT-PCR to study the distribution of GAD isoforms in order to confirm if GADs are expressed in the digestive system.

Material and Methods

Animals - ICR mice (Nihon Clea, Osaka, Japan) was purchased from local breeder. GAD67/GFP knock-in mice were originally raised at the National Institute of Physiological Sciences, Okazaki, and has been kept at Osaka Medical College [18]. The animals were housed in cages under the environmental temperature of 24±2 °C, humidity of 80±5 %, and dark/light schedule of 12/12 hr. Water and normal food were allowed freely.

RNA isolation and RT-PCR - Male ICR mice of fetus at 17 days and postnatal at ages of 4 and 11 weeks old were anesthetized and their stomach and jejunum were dissected. The stomach and jejunum were removed and their contents were washed with RNase free water. The total RNA was prepared by using RNeasy mini kit (Qiagen) and conversion of RNA to cDNA was carried out by adding Omniscript reverse transcriptase (Qiagen). PCR was then performed by using ExTaq DNA polymerase (Takara, Japan). Primer pairs for the mice GAD65 and GAD67 were prepared as described [19] (Table 1). PCR cycle was programmed as 94 °C, 58 °C, and 72 °C for 45 sec, 45 sec, and for 1 min, respectively and carried out for 38 cycles with a GeneAmp PCR system 9700 (Applied Biosystems).

Fluorescent immunohistochemistry Four- -and 11-week-old mice were anesthetized and their stomach and jejunum were dissected while 4% phosphate buffered formalin solution was perfused. The specimens were cut to 12 μm thickness with a Leica CM3050 cryomicrotome. The sections

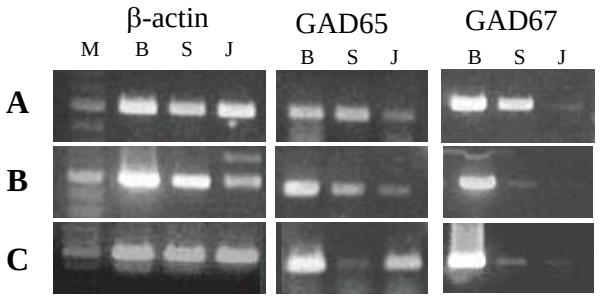

Fig. 1. RT-PCR analysis of GAD65 and GAD67 mRNAs from ICR mouse stomach, jejunum and brain. **A:**fetus at 17 days mRNA, **B:** 4 weeks, **C:**11 weeks. M : Maker, B : Brain, S : Stomach, J : Jejunum

were incubated at 4 °C for overnight with rabbit polyclonal GAD65 antibody (No. G5038, Sigma) at 1000-fold dilution or GAD67 antiserum (Alpha Diagnostic) at 1000-fold dilution with PBS. Then, sections were incubated with second antibody, Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) at 300-fold dilution with PBS. Propidium iodide (Molecular Probes) was used for staining nuclei. Fluorescent images were acquired with a Nikon ECLIPSE E600 fluorescent microscope.

Observation of GFP-positive cells - Stomach and jejunum of GAD67/GFP knock-in mouse of fetus at 17days old, and those of 1, 4, and 11weeks old were dissected as described above, except the thickness was 30 μm. The sections were washed with PBS 3 times, and then GFP-positive cells were observed on a fluorescent microscope.

Results

RT-PCR analysis of GAD65 and GAD67 - RT-PCR analyses were carried out to examine the expression of mRNA for GAD65 and GAD67. When mRNA was isolated from ICR mouse stomach, jejunum and brain, we have found all animals express GAD65 and GAD67 mRNAs (Fig.1). Although all samples gave positive results, the band intensity of GAD67 from jejunum was significantly weaker than those from the others. GAD65 appears to be a predominant form in both stomach and jejunum as the mRNA expression levels indicated.

Immunohistochemical analysis - Anti-GAD65 Ab staining was carried out on stomach and jejunum specimens from the 4- and 11- week-old ICR mice. As shown in Figs. 2 and 3, both stomach and jejunum showed GAD65 immunoreactivity. The immunohistochemical results agreed well with the results of RT-PCR. Thus, it is likely that GAD65 is expressed in both stomach and jejunum. Another immunohistochemical study was carried out with anti-GAD67 Ab. However, we were unable to detect any stains in both stomach and jejunum specimens. It is also noteworthy that immunohistochemical study on fetus was unsuccessful due to difficulties in handling the specimen. The location of GAD65 stains was carefully examined. It is probable to assume that the principal cells (Figs. 2a and 3a) and surface of mucosa (Figs. 2b and 3b) of stomach are likely to be stained. Also, the endocrine cells at epithelium (Figs. 2c and 3c) and crypts (Figs. 2d and 3d) of jejunum are assumed to be the GAD65 positive cells.

Expression of GFP-positive cells - GAD67/GFP knock-in mice were used in order to locate the cells expressing GAD67. Because these knock-in mouse express GFP in place of GAD67, it has been demonstrated that the direct visualization of GAD67 can be possible by following green fluorescent [12,18]. The knock-in mice of fetus at 17days and postnatal ages of 1, 4, and 11weeks were used. When the fetus stomach specimens were examined, we found GFP-positive cells at the surface of mucous that appeared as a line (Fig. 4a). Also found in stomach was a dense fluorescent spot at the serous membrane that appears to dote at submucosa (Fig. 4b). When the fetus jejunum was examined, there were no GFP-positive cells in the epithelium. We found a few GFP-positive cells in the muscular layer (Fig. 4c) where those fluorescent cell distributions were scattered. While GFP-positive cells were found in the fetus, we were unable to detect any GFP-positive cells for the mice after the birth.

Discussion

Glutamate decarboxylase (GAD) catalyzes decarboxylation reaction of L-glutamate to produce GABA. In the CNS of higher mammals, GABA plays as an inhibitory neurotransmitter. Those neuronal cells have been shown to express two GABA-producing enzymes, GAD65 and GAD67. With the advancement in detecting mRNA and protein by RT-PCR and fluorescent labeled Ab, respectively, it has become possible to detect protein expression in various

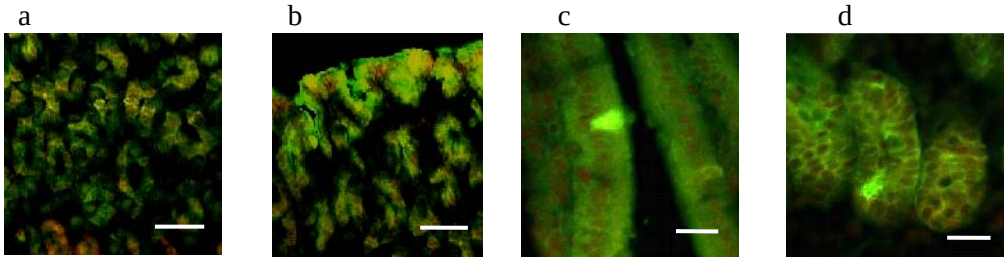

Fig. 2. Immunohistochemistry for GAD65 on 4 weeks old mice. GAD65 immunostaining is shown in green, whereas propidium iodide (PI) staining is shown red. Photos cover general region to contain principal cells in stomach (a), surface of mucosa in stomach (b), endocrine cells in jejunum (c), and crypts in jejunum (d). Bar is indicated 50μm.

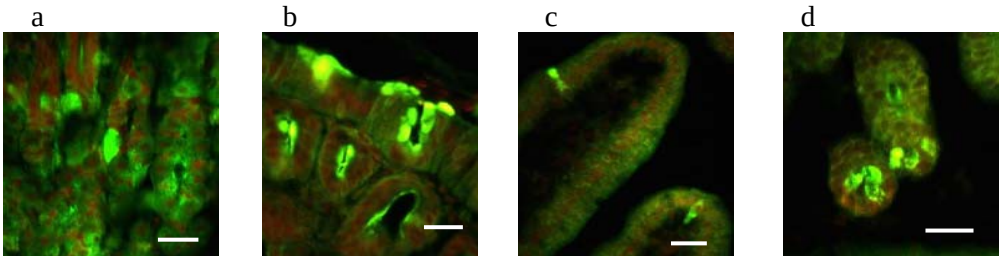

Fig. 3. Immunohistochemistry for GAD65 on 11 weeks old mice. GAD65 immunostaining is shown in green, whereas PI staining is shown red. Photos cover general region to contain chief cells in stomach (a), surface of mucosa in stomach (b), endocrine cells in jejunum (c), and crypts in jejunum (d). Bar is indicated 50μm.

tissues. In this study, we have focused on an expression of GAD isoforms in stomach and jejunum.

We have found that GAD65 and GAD67 mRNAs are expressed in both stomach and jejunum at all age groups we have examined. However, GAD65 appears to be expressed stronger than GAD67. We have assumed that the difficulty in detecting GAD67 with antibody was attributed to the quality of the antibody we have used; therefore we have used the GAD/GFP knock-in mice. The knock-in mice showed positive fluorescence only for the fetus specimens. The absence of fluorescence

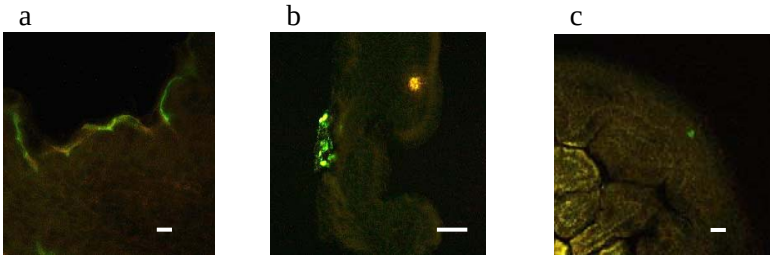

Fig. 4. GFP positive cells in 17days fetus mice. GFP positive cells are shown in green. Photos cover the region containing the surface of mucosa in stomach (a), serous membrane and submucosa in stomach (b), and muscular layer in jejunum (c). Bar is indicated 50μm.

for 1, 4, 11 weeks old mice suggests that these mice express little or no GAD67. It is of interest to note that our current observation indicates the age-dependent expression of GAD isoforms; GAD67 expressed predominantly during the prenatal stage while GAD65 in postnatal stage.

Distinctly different biological roles for GAD isoforms have been demonstrated previously. GAD65-deficient mice are shown to be highly susceptible to seizure and exhibit emotional behaviors such as anxiety and aggression [11,20,21]. GAD67-deficient mice are born with cleft palate and die shortly after the birth [22] which suggests GAD67 may produce GABA acting at the basal level in brain. Our results are consistent with an idea that GAD67 protein may be involved in the fetal development, probably in growth of the digestive system, while GAD65 protein may be involved in probably functional roles of digestive system, *i.e.*, excreting acid and/or hormones.

GAD65 protein was detected on the surface of stomach mucous of postnatal mice and GAD67 protein was at the similar region in the fetus stomach. Recently, the presence of G-protein coupled receptor (mGluR1), a candidate for umami receptor in taste bud, was reported at the stomach glandular [23]. It was also reported that GABA-immunoreactive cells were found at the stomach pylorus and upper part of the small intestine [24]. These observations and our current results have suggested that GABA at the gastrointestinal tract may act as a gut hormone, as well as another role as an enteric neurotransmitter. A subpopulation of mucosal endocrine cells may play this role.

Since GAD proteins are found at the surface of stomach and at the epithelium and crypts of jejunum, GAD may participate in the synthesis of GABA at these sites by utilizing dietary L-glutamate as a substrate. In addition to previously identified location of GAD-immunoreactive cells in stomach by Gilon *et al.*[23], we found that GAD is located at the pyloric and oxyntic mucosa, endocrine cells in mouse and rat stomach in this study. It has been suggested that GAD should be located at a part of endocrine and exocrine glands that participate in the secretion of gastric acid. We have also observed GAD on pancreas where various hormones and proteins are excreted. Pancreas is an organ where GAD is thought to be active and a depletion of GAD from β-cells was reported to cause type I diabetes and GAD65 is identified as an autoantigenic protein [25]. Further studies are need to explore the possibility of GAD being involved in the excretion mechanism. In the above described Gilon's study, isoforms of GAD were not discriminated. Since the presence of isoforms had been identified in the beginning of 1990, it was not clearly distinct which GAD isoforms were dealt with at the time of the Gilon's study and even the RT-PCR study was not carried out then. Our present study may suggest that GAD65 is the functional isoform since it is the major isoform expressed after the birth.

The endocrine cells found in digestive system are the likely cell types that are immunoreactive to GAD65 in epithelium and they are the ones typically found in the epithelium of absorbed cells and the goblet cells. Those endocrine cells form neuroendocrine complex in the intestine may have a close connection with nerve system. If GAD in the neuroendocrine complex synthesizes GABA, it is probable that GABA may participate in digestion of foods by acting as a hormone in the intestine in secreting digestive enzymes [26,27]. A part of endocrine cells at the crypt of intestine was known to move as these cells become a part of villi during the growth. Our current finding of the location of GAD in intestine is consistent with GAD being expressed in the endocrine cells. A significant amount of GABA was found in the duodenum where GABA may participate in the development of duodenum [28]. Along major curvature of the antrum pyloricum, a high density of GABA immunoreactive cells was exhibited [29]. Our present finding was consistent with the

previous description of GABA distributions. It is likely that those cells exhibiting GABA immunoreactivity contain GABA synthetic enzyme, GAD. Since it has been suggested that GABA plays a role in intestinal endocrine regulation, GAD should have some extensive roles in the digestive system. Moreover, the difference in expression of GAD isoforms in different developmental stages suggests that GAD may also be involved in the growth and development of stomach and intestine.

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