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Prenatal administration of neuropeptide bombesin promotes lung development in a rat model of nitrofen-induced congenital diaphragmatic hernia

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ABSTRACT

Background/purpose: Fetal medical treatment to improve lung hypoplasia in congenital diaphragmatic hernia (CDH) has yet to be established. The neuropeptide bombesin (BBS) might play an important role in lung development. The present study aims to determine whether prenatally administered BBS could be useful to promote fetal lung development in a rat model of nitrofen-induced CDH.

Methods: Pregnant rats were administered with nitrofen (100 mg) on gestation day 9.5 (E9.5). BBS (50 mg/kg/day) was then daily infused intraperitoneally from E14, and fetal lungs were harvested on E21. The expression of PCNA was assessed by both immunohistochemical staining and RT-PCR to determine the amount of cell proliferation. Lung maturity was assessed as the expression of TTF-1, a marker of alveolar epithelial cell type II.

Results: The lung-body-weight ratio was significantly increased in CDH/BBS(+) compared with CDH/BBS(−) ($p < 0.05$). The number of cells stained positive for PCNA and TTF-1 was significantly decreased in CDH/BBS(+) compared with CDH/BBS(−) ($p < 0.01$). The TTF-1 mRNA expression levels were significantly decreased in CDH/BBS(+) compared with CDH/BBS(−) ($p < 0.05$).

Conclusions: Prenatally administered BBS promotes lung development in a rat model of nitrofen-induced CDH. Neuropeptide BBS could help to rescue lung hypoplasia in fetal CDH.

The treatment of newborn babies with congenital diaphragmatic hernia (CDH) remains challenging for pediatric surgeons [1]. Recently, we have introduced new therapeutic approaches in addition to a gentle ventilation strategy and achieved a survival rate of more than 90% in cases of isolated CDH [2]. However, it continues to be impossible to rescue babies with extreme pulmonary hypoplasia. In order to solve this problem, several surgical fetal therapies, including tracheal occlusion, have been developed to promote fetal lung growth [3]. However, the risk of premature birth remains a serious problem with no improvements.

Neuropeptide bombesin (BBS) is a 14-amino acid peptide originally identified in skin of the frog *Bombina bombina*[4]. Its mammalian homologue, which has been identified as gastrin-releasing peptide (GRP), and BBS are referred to collectively as "bombesin-like peptides" (BLPs). The receptors of BBS are known to be widely distributed in the central nervous system and gut [5]. The authors have previously noticed the concept of the brain–gut axis and reported that BBS maintained intestinal mucosal structures and exhibited an immunomodulatory effect in transplanted intestinal allografts while preserving the graft microcirculation and preventing ischemic reperfusion injury [6–10]. In addition, this multipotent neuropeptide has been reported to promote the growth and maturation of the developing fetal lung in both humans and nonhuman primates [11–13]. It was also reported that the highest level of bombesin-like peptide occurred in mid-gestation human fetal lung [14]. However, there have been no reports that exogenously administered BBS could promote growth and maturity of immature lung in pathological condition like immature lungs in CDH.

To evaluate lung maturity, immunohistochemical staining against proliferating cell nuclear antigen(PCNA) has been widely used and reported that PCNA-positive cells in the fetal lung decrease during the late stage of pregnancy in rats [15]. TTF-1 is known as a marker of alveolar epithelial cells type II (AECs-II) and is considered to play an important role in stem cell production in the alveolar epithelium [16]. The differentiation from AECs-II into alveolar epithelial cells type I (AECs-I) should be one of the key processes in lung development in late gestation and the number of TTF-1-positive cells was reported to increase in immature lungs. TTF-1 should be appropriate to evaluate lung maturity in addition to PCNA.

The aim of this study was to investigate whether BBS promotes lung growth and maturity in a rat model of nitrofen-induced CDH.

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1. Materials and methods

1.1. Animal model

Pregnant Sprague–Dawley rats were purchased from Shimizu laboratory (Kyoto, Japan). The day in which the vaginal plug was confirmed was considered to be day 0 of gestation (E0). In order to produce the fetal CDH rat model, 100 mg of nitrofen (2,4-dichorophenyl-p-nitrophenylether: WAKO Chemical, Osaka, Japan) dissolved in 1 ml of olive oil was administered via an orogastric tube under short anesthesia on E9.5 (term, 22 days). As a control, some rats were given the same dose of olive oil without nitrofen. The animals were divided into three groups on E14: 1) CDH/BBS(+) group, in which the nitrofen-treated rats were administered BBS (50 μg/kg/day: Peptide Institute, Inc., Osaka, Japan) using an osmotic minipump (Alzet 2002: Palo Alto, CA, USA) implanted in the peritoneal cavity under general anesthesia; 2) CDH/BBS(−) group, in which the nitrofen-treated rats were administered normal saline instead of BBS using an osmotic minipump; and 3) control group, in which rats treated without nitrofen were administered normal saline instead of BBS using an osmotic minipump. The fetuses were harvested via cesarean section and weighed on E21. The peritoneal cavity of each fetus was opened and a defect in the diaphragm was confirmed with a visual inspection of the diaphragm. The bilateral lungs were removed and weighed, and the lung-body-weight ratio (both lungs (mg)/body (g) weight: LBWR) was measured. The expression of proliferating cell nuclear antigen (PCNA) was assessed using both immunohistochemical staining and real-time polymerase chain reaction (PCR) in order to determine the amount of cell proliferation. The degree of lung maturity was assessed as the expression of thyroid transcript factor-1 (TTF-1), a marker of alveolar epithelial cell type II.

1.2. Immunohistochemical staining

The left lungs were immersed and fixed in 4% paraformaldehyde for eight hours and embedded in paraffin. The samples were cut into 5-μm-thick sections and deparaffinized. Subsequently, antigen retrieval was performed by boiling the sections in a 10 mmol/L of sodium citrate solution at a pH of 6.0 for two periods of five minutes in a microwave at medium heat. After rinsing the slides in PBS, the endogenous peroxidase activity was blocked by exposing the slides to a 3% hydrogen peroxide in methanol solution for a period of 10 minutes.

To detect positive cells of PCNA or TTF-1 in the lungs, immunohistochemical staining was performed using a primary antibody to rat PCNA (PC10: Nichirei, Tokyo, Japan) or TTF-1 (SPT24: Nichirei, Tokyo, Japan). The sections were incubated with the primary antibodies for one hour at room temperature. The primary antibodies were visualized using the Histofine Simple Stain MAX-PO (M) kit (Nichirei, Tokyo, Japan) according to the instruction manual. The slide was counterstained with hematoxylin. Using a Dynamic Cell Count (BZ-H1C: Keyence, Tokyo, Japan) with high power field (×400), the number of positive cells was counted and averaged for five sites in each group.

1.3. RT-PCR and real-time RT-PCR

The left lungs obtained from the three groups, control (n = 8), CDH/BBS(−) (n = 7) and CDH/BBS(+) (n = 7), were analyzed. Total RNA was extracted according to the guanidinium acid phenol method using ISOGEN II (Nippon gene, Toyama, Japan). In addition, total RNA was reversed transcribed using ReverTra Ace® qPCR RT Master Mix (Toyobo, Tokyo, Japan) according to the manufacturer's instructions.

Real-time reverse transcription-PCR (RT-PCR) was performed using the Real-time PCR Master Mix (Toyobo, Tokyo, Japan) and the 7500 Real-Time PCR Systems (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions. The matching primers for PCNA (Rn01514538_g1), TTF-1 (Rn01436110_m1) and β-actin (Rn014244440_s1) were purchased from Applied Biosystems.

1.4. Statistical analysis

The statistical analysis was performed using Student's *t*-test for unequal variances. A *p* value of less than 0.05 was considered to be statistically significant.

2. Results

The incidence of CDH in this study was 50% (13/26 fetuses) among the nitrofen-treated rats not administered BBS and 49% (24/49) in those administered BBS. The defect in the diaphragm was observed on the left side in all CDH fetuses, and no other anomalies were found.

There were no significant differences in body weight between the CDH/BBS(−) group and the CDH/BBS(+) group. The LBWR values were compared between the three groups: control (nitrofen(−), BBS(−)) (n = 23), CDH/BBS(−) (nitrofen(+), BBS(−)) (n = 13), and CDH/BBS(+) (nitrofen(+), BBS(+)) (n = 24). Consequently, the LBWR in the CDH/BBS(−) group was significantly less than that observed in the control group (16.05 ± 2.32 v.s. 23.86 ± 2.78; $p < 0.01$). On the other hand, the LBWR in the CDH/BBS(+) group was significantly greater than that observed in the CDH/BBS(−) group (18.29 ± 3.03 v.s. 16.05 ± 2.32; $p < 0.05$) (Fig. 1).

Regarding the immunohistochemical stainings, both PCNA- and TTF-1-positive cells were localized to the alveolar endothelium in the fetal rats. The number of PCNA-positive cells in the CDH/BBS(−) group was significantly greater than that observed in the control group (877.0 ± 87.8 v.s. 290.4 ± 50.65; $p < 0.01$). Meanwhile, the number of PCNA-positive cells in the CDH/BBS(+) group was less than that observed in the CDH/BBS(−) group (501.8 ± 72.7 v.s. 877.0 ± 87.8; $p < 0.01$) (Fig. 2). In addition, the number of TTF-1-positive cells in the CDH/BBS(−) group was significantly greater than that observed in the control group (664.0 ± 90.5 v.s. 238.4 ± 52.8; $p < 0.01$). Conversely, the number of TTF-1-positive cells in the CDH/BBS(+) group was significantly less than that observed in the CDH/BBS(−) group (267.6 ± 30.0 v.s. 664.0 ± 90.5; $p < 0.01$) (Fig. 3).

On RT-PCR, the mRNA expression levels of PCNA (PCNA/β-actin) were low in the CDH/BBS(+) group. However, there were no significant differences between the CDH/BBS(−) and CDH/BBS(+) groups (2.49 ± 1.11 v.s. 1.69 ± 0.58) (Fig. 4). On the other hand, the mRNA expression levels of TTF-1 (TTF-1/β-actin) in the CDH/BBS(+) group were significantly decreased compared with those observed in the CDH/BBS(−) group (2.55 ± 1.21 v.s. 1.45 ± 0.23; $p < 0.05$) (Fig. 5).

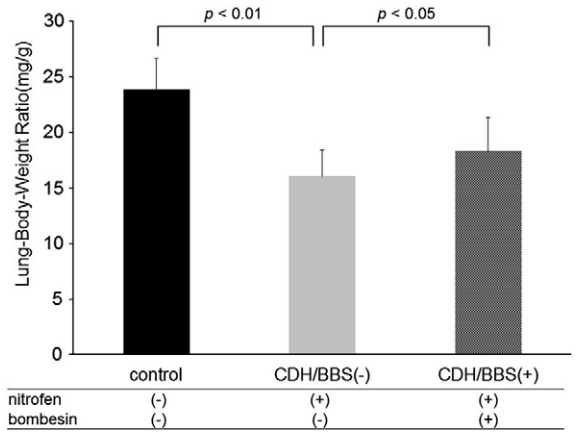

Fig. 1. Lung-body-weight ratio (LBWR) values in the control, CDH/BBS(−) and CDH/BBS(+) groups. The LBWR values in the control group were significantly greater than those observed in the CDH/BBS(−) group ($p < 0.01$). The LBWR values in the CDH/BBS(+) group were also significantly greater than those observed in the CDH/BBS(−) group ($p < 0.05$).