Lymphopenia in Ednrb-deficient rat was strongly modified by genetic background

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ABSTRACT

The endothelin signaling pathway plays an important role in the migration, proliferation, and differentiation of neural crest cells. Mutations in the gene encoding the endothelin receptor type B (EDNRB) cause three symptoms: aganglionosis, pigmented disorder and hearing loss. In addition, the Ednrb null mice show abnormal splenic microarchitecture with lymphopenia. In this study, we examined whether similar phenotypes are reproduced in three Ednrb-null rat strains that we established previously. AGH-Ednrb<sup>sl/sl</sup> strain showed a low white blood cell count, significant size reduction and abnormal microarchitecture of spleen. Thymus displayed a marked reduction in the size, but maintained a normal CD4/CD8 ratio. In contrast, splenic cellularity was reduced to <15%, and splenic B and T cell numbers were reduced, showing a splenic lymphopenia. Interestingly, Ednrb-null rats in the LE and F344 genetic background did not show these abnormalities. These data show that proper T and B cell development is dependent on the endothelin signaling pathway, however, modifier gene(s) might be differentially expressed in these strain to modulate or compensate for the effect of the Ednrb deficiency.

The endothelin receptor type B (EDNRB) is a G protein-coupled receptor that activates a phosphatidylinositol-calcium second messenger system. The receptor interacts with a family of ligands known as endothelins to regulate several critical biological processes, including the development and function of blood vessels, the production of certain hormones, and the stimulation of cell growth and division (9). Endothelin 3 is one of the proteins that interacts with EDNRB. During embryonic development, endothelin 3/EDNRB interaction plays an important role in the migration, proliferation, and differentiation of neural crest cells (2, 8). These cells migrate from the neural tube to the specific regions in the embryo, where they give rise to many different types of cells including the neurons and glia of the enteric ganglia, melanocytes, facial cartilage and bone, and chromaffin cells of the adrenal medulla (7). Mutations in the EDNRB gene caused Waardenburg-Shah Syndrome 4 (3), which is characterized by changes in skin, hair, and eye coloring, hearing loss, and the Hirschsprung disease that causes severe constipation or intestinal blockage. We established the AGH-Ednrb<sup>sl/sl</sup> rat harboring null allele of the Ednrb gene (5). Homozygous mutant rats showed hearing loss with pigmentation disturbance in addition to Hirschsprung disease, which is an authentic model of Waardenburg-Shah Syndrome 4.

The Ednrb null mice show abnormal splenic microarchitecture with lymphopenia, and relative reduction of B cells compared to T cells (4). To test whether similar phenotypes are produced in three rat strains bearing null mutation of Ednrb, we examined the abnormality in the thymus and spleen. Ge-
Fig. 1  (A) AGH-Ednrb<sup>++</sup> and LEH-Ednrb<sup>sl/sl</sup> at the postnatal 8 day. (B) Representative picture showing the small spleen and thymus of the AGH-mutant rat comparing to the wild type littermate. (C) Hematoxylin and eosin staining shows significantly reduced white pulp (WP), red pulp (RP), trabecular in the AGH-mutant compared to wildtype rats at the postnatal 8 day. No distinct difference was found in thymus between both rats. Scale bar, 100 μm.
noting that the Ednrb alleles in AGH/Hkv-Ednrb, LEH/Hkv-Ednrb and F344-Ednrb rats was performed as described previously (5). All research and experimental protocols were conducted according to the Regulation for the Care and Use of Laboratory Animals of Hokkaido University (Approval ID: No. 110226). Single cell suspensions (1 × 10⁶) of the spleen and thymus at the postnatal 8 day were analyzed by FACS. The FITC-labeled anti-rat CD4, PE-labeled anti-rat CD8α and FITC-labeled anti-rat CD45RA (Biolegend, CA, USA) were used for detection. FITC-labeled Mouse IgG1 isotype and PE-labeled Mouse IgG1 isotype (Biolegend) were used for negative control. Flow cytometry was performed with an EPICS XL ADC flow cytometer (Beckman Coulter, CA, USA). The number of CD4⁺, CD8⁺, and CD45RA⁺ cells was calculated from the frequency of each positive staining against total cell number of spleen. Whole blood samples were collected from the postcava of all rat groups under intraperitoneal anesthesia by the use of EDTA-2K. Complete blood counts were obtained by the use of a MEK-6450 hematology analyzer (Nihon Kohden, Japan).

This mutation of Ednrb gene results in skin pigmentation disturbance in rats due to abnormal differentiation and migration of melanocytes. Wildtype rats have pigmented heads, backs, and tails. However, homozygous mutant rats almost have no pigmentation on their heads (5). As early as 3 days after birth, homozygous AGH-Ednrb<sup>sl/sl</sup> rats had no pigmentation on their heads, thus, we distinguished mutants from wildtype littermates (Fig. 1A). Mutant rats show serious aganglionic phenotype and most of them died by 10 days after birth as reported previously (5). In the AGH-mutant rats, the thymus and spleen at postnatal 8 days were reduced in size and weight (Fig. 1B, 2). The thymuses in AGH-mutants revealed no abnormalities in positioning, lobulation and architecture. A normal separation of cortical and medullary areas was present, implying that thymocyte precursors had entered the tissue and assisted in the organization. In contrast, AGH-mutants revealed a strong size reduction of the spleen and a dramatic reduction of the white pulp and lacked the clear follicular structure compared with those of wildtype rats (Fig. 1C). Ednrb is not important for proper splenic lobe migration and positioning; however, it seems to be dispensable for the lymphopoietic function of the spleen. On the other hand, LEH-Ednrb<sup>sl/sl</sup> and F344-Ednrb<sup>sl/sl</sup> rats at postnatal 8 days did not have any abnormality of size and histology in spleen and thymus (data not shown).

Next, to assess the abnormality of the blood in AGH strains, the mutant and wildtype rats were examined for peripheral red blood cells, white blood cells, platelets, hematocrit and hemoglobin levels. Table 1 shows a significant decrease in white blood cell typing of the Ednrb alleles in AGH/Hkv-Ednrb, LEH/Hkv-Ednrb and F344-Ednrb rats was performed as described previously (5). All research and experimental protocols were conducted according to the Regulation for the Care and Use of Laboratory Animals of Hokkaido University (Approval ID: No. 110226). Single cell suspensions (1 × 10⁶) of the spleen and thymus at the postnatal 8 day were analyzed by FACS. The FITC-labeled anti-rat CD4, PE-labeled anti-rat CD8α and FITC-labeled anti-rat CD45RA (Biolegend, CA, USA) were used for detection. FITC-labeled Mouse IgG1 isotype and PE-labeled Mouse IgG1 isotype (Biolegend) were used for negative control. Flow cytometry was performed with an EPICS XL ADC flow cytometer (Beckman Coulter, CA, USA). The number of CD4⁺, CD8⁺, and CD45RA⁺ cells was calculated from the frequency of each positive staining against total cell number of spleen. Whole blood samples were collected from the postcava of all rat groups under intraperitoneal anesthesia by the use of EDTA-2K. Complete blood counts were obtained by the use of a MEK-6450 hematology analyzer (Nihon Kohden, Japan).

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<tr>
<th>Table 1</th>
<th>Peripheral white blood cells (WBC), red blood cells (RBC), platelets (PLT), hemoglobin (HGB) and hematocrit (HCT) levels from AGH-mutant and wildtype rats</th>
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<td>WBC 1₀⁶/μL</td>
<td>196.9 ± 74.7</td>
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<td>RBC 1₀⁴/μL</td>
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<td>PLT 1₀⁴/μL</td>
<td>38.6 ± 13.4</td>
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<td>HGB g/dL</td>
<td>9.8 ± 1.5</td>
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<td>HCT %</td>
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Values represent mean ± SD. *: P < 0.001, versus wildtypes. n = 10 each.

Fig. 2 The organ weight as a percent of body weight of AGH-mutant (n = 10 each) was significantly lower than that of wildtype, but not of the LEH (n = 8 each) and F344 (n = 13 each) mutants. The kidney was used as the control and no significant difference in the kidney weight was found between the homozygous and heterozygous in all three strains. Data presented as Mean ± SD. t-test was performed to compare the mean values for data sets. *: P < 0.001.
cell counts in the mutant animals. All other blood parameters were within the normal ranges. Spleen cellularities were reduced to < 15% of controls. Collectively, these data show a strong dependence on Ednrb for proper expansion of lymphoid lineages. Additionally, this phenotype has shown little animal-to-animal variation.

To investigate what cell types were affected and quantify these differences of spleens between AGH wildtype and mutant rats, we performed the FACS analysis on spleens (n = 5, each group) for CD45RA (B cells), CD4 and CD8 (T cells) (Fig. 3). The mean total cell number of the wildtype spleens was 19.20 ± 2.59 × 10^6, significantly higher than 2.88 ± 1.75 × 10^6 for the mutant spleens (P < 0.001). Relative cell numbers of the T and B cell in the mutant rats were significantly reduced (Fig. 3). On the other hand, the relative CD4/CD8 ratios of thymic subpopulations were normal (data not shown), and only overall numbers of cells were reduced.

Previously we established mutant showing three symptoms, intestinal aganglionosis, pigmented disorder and hearing loss, reproduced features of Waardenburg-Shah Syndrome 4. Furthermore, we introgressed this mutation into LEH and F344 strains to produce two congenic strains, LEH-Ednrb<sup>sl/sl</sup> and F344-Ednrb<sup>sl/sl</sup>. When bred onto two different genetic backgrounds, these features were differently modified. Among three strains of homologous rats, the AGH-mutant rats showed the most serious aganglionosis, followed by the LEH and F344 mutants, which corresponded to the order of their average survival time (5). Thus, we concluded that variations in penetrance and survival period among the three strains of Ednrb<sup>sl/sl</sup> rats were attributable to distinct differences in the severity of aganglionosis, and modifier genes in the genetic backgrounds of these strains significantly modulated the severity of the aganglionosis phenotype. In the previous study, to identify loci responsible for strain differences in the severity of aganglionosis, we performed quantitative trait locus (QTL) analysis using intercross progenies from susceptible AGH and resistant F344 strains. QTL analysis mapped a major QTL showing a highly significant linkage to the distal portion of chromosome 2, is designated Lrag1 (Locus of resistance to aganglionosis 1) (6).

Development of the primary and secondary lymphoid organs is a tightly controlled process. Many mutations in laboratory rodents have led to better understanding of the molecular interactions and signaling pathways essential to the development and organization of lymphoid tissues, and the functional consequences of loss or disruption of the normal structures. It has been determined that the Foxn1, Phx1, Tbx1, Hoxa3, Pax1 and Pax9 are required for normal thymic development and function (1). The Tlx1, Bapx1, Tcf21, Wt1 and Dh genes are essential for development of the spleen, while mutations of Nkx2–3, Lta, Ltib, Ltbr, Map3k14, Relb, Tnf, Tnfrs, Cxcl13, Cxcr5, or Sharpin result in disruption of normal splenic microarchitecture (10). Since Ednrb mRNA is widely distributed in various cell types of many tissues (11), endothelin might activate several types of cells in lymphoid organs, especially spleen. Thus, the transcription of these genes above and related signaling pathways might be modulated by the activation of endothelin signaling pathway directly or indirectly. We did not examine more mature splenic function including the ability of cell maturation and immune responses due to the early lethality of mutant rats. However, these data show that proper T and B cell development is dependent on endothelin signaling pathway.

Previously we established three congenic rat strains with same Ednrb null mutation. We found that genetic background strongly modified the symptoms of aganglionosis but not hearing loss (5). In this study, we also investigated the impact of genet-

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**Fig. 3** FACS analysis of splenocytes from AGH-mutant and wildtype rats at 8 days of age. The mean total cell number of the wildtype spleens was 19.2 ± 2.59 × 10^6, significantly higher than 2.88 ± 1.75 × 10^6 for the mutant spleens (n = 10 each. *: P < 0.001). CD45RA<sup>+</sup>, CD4<sup>-</sup> and CD8<sup>-</sup> cell counts in the AGH-wildtypes with 1.15 ± 0.16 × 10^6, 0.44 ± 0.11 × 10^6, and 0.19 ± 0.05 × 10^6, respectively, whereas in AGH-mutants were 0.17 ± 0.1 × 10^6, 0.10 ± 0.06 × 10^6, and 0.06 ± 0.04 × 10^6, respectively. Data presented as Mean ± SD. *t-test was performed to compare the mean values for data sets. (n = 10 each. *: P < 0.01).
ic background on the development of the spleen. The reason for the normal splenic and thymic phenotypes in LEH and F344 mutant rats or whether Lrag1 is involved with lymphopenia remains unclear; however, modifier genes might be differentially expressed in these strains to modulate or compensate for the effect of the Ednrb deficiency. Therefore, identification of the resistant loci to lymphopenia in both rat strains will provide new important information regarding gene interactions controlling the development of spleen.

REFERENCES