Mesenchymal expression of Foxl1, a winged helix transcriptional factor, regulates generation and maintenance of gut-associated lymphoid organs

Katsuyuki Fukuda, a,b Hisahiro Yoshida, c,f Toru Sato, a,b Taka-aki Furumoto, a
Yoko Mizutani-Koseki, a Yasuo Suzuki, b Yasushi Saito, b Toshitada Takemori, d
Motoko Kimura, e Hiroshi Sato, e Masaru Taniguchi, e,f Shin-ichi Nishikawa, c
Toshinori Nakayama, c and Haruhiko Koseki a,f,*

a Department of Molecular Embryology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
b Clinical Cell Biology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260–8670, Japan
c Department of Molecular Genetics, Graduate School of Medicine, Kyoto University, Shogoin-Kawaharacho 53, Sakyo, Kyoto 606–8507, Japan
d Department of Immunology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162–8640, Japan
e Department of Molecular Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
f RIKEN Research Center for Allergy and Immunology, 1-7-22 Suchiro, Tsurumi-ku, Yokohama, 230-0045, Japan

Received for publication 30 May 2002, revised 22 November 2002, accepted 25 November 2002

Abstract

The Foxl1 gene, which encodes a winged helix transcriptional regulator, is expressed in the mesenchymal layer of developing and mature gastrointestinal tract. Foxl1-deficient mice exhibit various defects not only in the epithelial layer of the gastrointestinal tract but also in gut-associated lymphoid tissues. In the small intestine of Foxl1-deficient mice, the formation of Peyer’s patches is affected, particularly in the caudal region. This alteration is shown to be due to the delayed formation of Peyer’s patches organizing centers as revealed by the expressions of VCAM1 and IL-7 receptor α-chain at 17.5 days postcoitus. Peyer’s patch defects are concordant with the significantly decreased expression of Lymphotoxin β-receptor in the caudal region of fetal intestine. Foxl1 is suggested to regulate the responsiveness of fetal intestinal mesenchymal cells to inductive signals mediated by Lymphotoxins during Peyer’s patch organogenesis. In addition, constitutive outgrowth of colonic patches due to defects in radioresistant stromal components of colonic patches are seen in Foxl1-deficient mice. Because of the functional similarities of hypertrophic colonic patches to those seen in hapten-induced experimental colitis, this hypertrophy is suggested to involve Lymphotoxin β-receptor signaling. Together, the data suggest that Foxl1 might be involved in cellular responses of gut-associated lymphoid tissues dependent upon the Lymphotoxins/Lymphotoxin β-receptor axis.

© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Foxl1; Peyer’s patch; Colonic patch; Gut mesenchyme; Lymphotoxins; Mouse

Introduction

The local immune system protects mucosal surfaces independent of circulating antibodies, and the lymphoid elements distributed in different mucosal tissues form part of a unique immune system that confers local immunity in a regulated fashion. In mammals, the gut-associated lymphoid tissues (GALT) comprise organized lymphoid aggregates represented by Peyer’s patches (PPs), the appendix, mesenteric lymph nodes, and solitary lymphoid nodules, including crypt patches and colonic patches (CPs) (Croitoru and Bienenstock, 1994).

The cytoarchitectural components are remarkably similar between PPs and CPs. Both contain germinal centers with B cells in the central areas of the follicle interspersed with T cells and dendritic cells and macrophages that function as
antigen-presenting cells (APCs). The presence of M cells and germinal centers suggests that transepithelial particle transport and antigen recognition can take place in these sites (Croitoru and Bienenstock, 1994; Owen et al., 1991). PPs and CPs may be sites for the local initiation of immune responses. CPs, indeed, are essentially involved in the onset of hapten-induced colitis in mice by regulating T helper 2 (Th2)-type responses (Dohi et al., 1999, 2001). In addition to their structural similarities, PPs and CPs resemble each other functionally in that both are maintained upon the constitutive activation of Lymphotokins (LTs)/Lymphotokin β-receptor (LTβR) axis (Dohi et al., 2001). Thus, PPs and CPs might be equivalent structures, despite their differences in surrounding mucosa and luminal microbial exposure.

Recent histological investigations have revealed that PP organogenesis is segregated into at least three distinct steps (Adachi et al., 1997). First, PP organizing centers are generated upon the cosegregation of VCAM1+ICAM1+ mesenchymal cells and inteleukin-7 receptor α-chain positive (IL-7α+) lymphoid cells, and their subsequent mutual interactions (Adachi et al., 1998; Honda et al., 2001; Yoshida et al., 1999). Genetic and epigenetic approaches have demonstrated that this interaction involves LTs derived from IL-7α+ lymphoid cells and chemokines from VCAM1+ICAM1+ mesenchymal cells. LTs from IL-7α+ lymphoid cells promote the differentiation and activation of VCAM1+ICAM1+ mesenchymal cells as demonstrated by defects in PP formation in IL-7α−, LTα−, LTβ−, LTβR−, Nfkb inducing kinase (nik), and Inhibitor of κB kinase α (Iκκα) mutant mice (Adachi et al., 1998; Alimzhanov et al., 1997; Fütterer et al., 1998; Honda et al., 2001; Koni et al., 1997; Koni and Flavell, 1998; Matsushima et al., 2001; Shinkura et al., 1999; Togni et al., 1994; Yoshida et al., 1999). Chemokines from VCAM1+ICAM1+ cells promote the accumulation of lymphoid cells (Ansel et al., 2000; Honda et al., 2001). Second, 1 to 2 days later, a set of hemopoietic cells accumulates in PP precursors as identified by the expression of CD45, IL-7α, CD11Cc, and c-fms, which promotes architecture formation. This second process is still independent of the entry of mature lymphocytes. Finally, mature lymphocytes expressing CD3 or CD45R enter the PP precursors after 18.5 days postcoitus (dpc). Thus, PP development is shown to be initiated by sequential interactions between mesenchymal cells derived from the splanchnopleure and hemopoietic cells from the fetal liver (Yoshida et al., 2001). In contrast to PP development, the molecular and cellular basis of CP development is not well understood. Recently, activation of the LTs/LTβR cascade has been shown to be essential for the generation of CP as well as PP (Dohi et al., 2001). Therefore, activation of the LTs/LTβR axis in mesenchymal cells is presumed to be required for the initiation of PP and CP development as well as for their maintenance.

Fox11, a member of the winged helix/forkhead family of DNA binding proteins, is expressed in the mesenchymal layer of developing and adult gastrointestinal tract and is required for the maintenance of the gut (Kaestner et al., 1996). Initial investigations by Kaestner and his colleagues on Fox11-deficient mice indicated that Fox11 regulates the proliferation and differentiation of gastrointestinal epithelium (Kaestner et al., 1997). Thus, it is probable that Fox11 controls the expression of factors that mediate epithelial–mesenchymal interactions, and that epithelial defects are induced by the disruption of the precisely controlled expression of factors in the mesenchyme. Indeed, the expressions of BMP2 and BMP4 are significantly reduced in Fox11-deficient mice. These previous observations led us to evaluate the role of Fox11 in mesenchymal–hemopoietic cell interactions essential for the development and maintenance of PP and CP.

We have independently established Fox11-deficient mice and investigated PP and CP development (Fukamachi et al., 2001). In this study, we report defects in PP development and the hypertrophy of CPs in Fox11-deficient mice. PP defects are correlated with altered responsiveness of mesenchymal cells to LTs as revealed by the down-regulation of LTβR in the fetal intestine. Fox11 is also involved in repressing the outgrowth of CPs in adult mice by regulating the functions of radioresistant stromal components of CPs.

Materials and methods

Mice

Fox11-deficient mice were generated as described, backcrossed onto a C57BL/6 background 10 times, and maintained under specific pathogen-free conditions in the animal facility of Chiba University Graduate School of Medicine.
Fig. 3. Distribution of VCAM1+ and IL-7Rα+ spots in developing small intestines. (A, B) VCAM1 expression in whole-mount fetal intestines of 17.5 dpc wild-type (Aa) and Foxl1-deficient mice (Ba). Higher magnification views of individual spots are also shown in the craniocaudal direction (Ab to j, Bb to f). Scale bars indicate 100 μm. (C, D) IL-7Rα expression in whole-mount fetal intestines of 17.5 dpc wild-type (Ca) and Foxl1-deficient mice (Da). Higher magnification views of individual spots are also shown in the craniocaudal direction (Cb to i, Db and c). (E, F) VCAM1 expression in whole-mount intestines of newborn wild-type (Ea) and Foxl1-deficient mice (Fa). Higher magnification views of individual spots are also shown in the craniocaudal direction (Eb to k, Fb to j). (G) Sections of VCAM1+ spots from newborn wild-type (Ga) and Foxl1-deficient mice (Gb). (H) Distribution of VCAM1+ spots in 17.5 dpc, 18.5 dpc, and newborn (Day 1) mice. Fetal and newborn small intestines of wild-type (+/+) and Foxl1-deficient mice (−/−) are arbitrarily divided into three regions cranio-caudally as shown in Fig. 2E, namely, regions I (closed bars), II (shaded bars), and III (open bars). PP numbers in each region are summarized.

Fig. 4. Distribution of CD11c+ spots in newborn small intestines. (A, B) CD11c expression in whole-mount intestines of newborn wild-type (Aa) and Foxl1-deficient mice (Ba). Higher magnification views of individual spots are also shown in the craniocaudal direction (Ab to m, Bb to f). Scale bars indicate 500 μm. Several sections of CD11c+ spots from newborn wild-type (An to p) and Foxl1-deficient mice (Bg and h) are shown. (C) Localization of CD11c+ cells in VCAM1+ spots in the caudal region of intestines of newborn wild-type (a) and Foxl1-deficient mice (b). Dendritic cells are colored red.
(Fukamachi et al., 2001). Backcrossed heterozygous mice were bred to generate homozygous mutants. Genotypes were determined by PCR as described (Fukamachi et al., 2001).

Mice harboring an aly mutation in the nik gene were purchased from SLC (Shizuoka, Japan) and interbred with Foxl1+/− mice to generate double heterozygotes. Whole-mount in situ hybridization was performed on 15.5 dpc fetal gut using a digoxigenin-labeled riboprobe as described by Wilkinson (1992) with slight modifications. Briefly, maleic acid buffer was used for washing instead of Tris-buffered saline. After the chromogenic reaction, specimens were refixed, dehydrated, embedded in paraffin, and sectioned into 10-μm-thick slices.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was performed on 15.5 dpc fetal gut using a digoxigenin-labeled riboprobe as described by Wilkinson (1992) with slight modifications. Briefly, maleic acid buffer was used for washing instead of Tris-buffered saline. After the chromogenic reaction, specimens were refixed, dehydrated, embedded in paraffin, and sectioned into 10-μm-thick slices.

**Antibodies**

The following monoclonal antibodies were used in this study: anti-CD11Cc (HL3; BD PharMingen, San Diego, CA), anti-VCAM1 (429 MLCAM.A; PharMingen), anti-ICAM1 (3E2; PharMingen), anti-CD45 (30F11.1; PharMingen), anti-CD45R/B220 (RA3-6B2; PharMingen), anti-ICAM1 (3E2; PharMingen), anti-CD45 (30F11.1; PharMingen), and anti-IL-7Rα (A7R34) (Sudo et al., 1993).

**Preparation of VCAM1+ ICAM1+ mesenchymal cells from fetal small intestines**

Small intestines of 17.5 dpc fetuses were dissected out under a stereomicroscope, chopped into small pieces with scissors, and dissociated by incubating with 200 μl dispase (Godo Shusei) in PBS containing 0.5% mouse serum for about 30 min at 37°C. After brief pipetting on ice, the dissociated tissues were washed with Heps-buffered saline containing 10% fetal calf serum (FCS) and 5 U/ml of DNase I. After another cycle of pipetting on ice, the suspended cells were filtered through nylon mesh to remove cell clusters, centrifuged, and washed twice. The cells were stained with monoclonal antibodies against VCAM1 and ICAM1 conjugated with biotin and FITC, respectively. Anti-VCAM1 antibody was developed by PE. Six million stained cells were separated by a FACScaliber (Becton–Dickinson) as shown in Fig. 1D.

**RNA isolation and RT-PCR analyses**

Total cellular RNA was isolated from 4000 VCAM1+ ICAM1+ cells and 3 × 10^5 VCAM1+ ICAM1− cells using Trizol (Gibco-BRL) according to manufacturer’s recommendations and further treated with DNase I to avoid contamination of the genomic DNA. cDNA was generated by reverse transcriptase (RT) using oligo(dT) primers. For analysis of Foxl1 expression, cDNA representing the amount in 100 cells was used for PCR. The following primers were used: Foxl1F, 5’-GGAGCCCGGGGATCCTAAG-3’ and Foxl1R, 5’-TAGGAAACGACACTGTTG-3’. PCR products were resolved in 1.5% agarose gels and subjected to DNA blot hybridization analysis using a radiolabeled Foxl1 cDNA probe. β-Actin was used as a positive control. For the detection of IL-7Rα, LTα, LTβ, LTβR, SLC, BLC, ELC, and Id2 expression, total cellular RNA was isolated from the oral side (prospective region I in Fig. 2E) and anal side (prospective region III in Fig. 2C) of 17.5 dpc fetal small intestine. cDNA equivalent to 0.1, 0.033, 0.011, and 0.0037 μg RNA was used for RT-PCR. In the PCR reactions for IL-7Rα, LTα, LTβ, LTβR, SLC, BLC, ELC, and Id2 gene expression, the following sets of primers were used: 5’-CTCAAGAATGATGGCTTGGTATAGA-3’ and 5’-AGATACATGGCTCCAGTTCTTTTC-3’ for IL-7Rα; 5’-TCACTGTGTTGGGTACCACAAGA-3’ and 5’-ATACACAGATCTCTGGACAC-3’ for LTα; 5’-TTGTTGGGAGTGCTCTACACTGTTCC-3’ and 5’-CTCGTGTACAACTAAACGACCGTAC-3’ for LTβ; 5’-TGGGACTCAGCGGGCTTCT-3’ and 5’-AAATGTGGGTCGGCTCTTGG-3’ for LTβR; 5’-ATGGCTCAGATGACTCTGCTTG-3’ and 5’-GTGTTGTCTTGACGTCGTC-3’ for SLC; 5’-TGGAGCTCAGACAGCAACG-3’ and 5’-CTTGAGATTGCCCTTCTCAG-3’ for ELC; and 5’-TCTGAGCATTACATCTGTC-3’ and 5’-CTGTTTCTCCCTTGATGC-3’ for Id2. Thirty cycles of PCR reactions were carried out except for β-actin, which was analyzed by 20 cycles. Three different pools of RNA were subjected to this analysis and provided almost identical results (K. Fukuda, T.S. Sato, and H. Koseki, unpublished observations).

**Whole-mount immunohistochemical analysis of fetal and adult gut**

Specimens were dissected and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS), first under microwave irradiation (three times for 20 s each) and then for another 90 min on ice. After washing in PBS and dehydrating in methanol, the specimens were treated with 0.3% H2O2 to bleach intrinsic peroxidase and then with PBSMT (2% skim milk/0.3% Triton X-100 in PBS) twice for 1 h to avoid nonspecific antibody binding, and incubated overnight with the first antibodies. After washing in PBSMT four times for 30 min, the specimens were incubated with horseradish peroxidase (HRP) conjugated second antibodies for 6 h. After washing twice in PBSMT for 30 min each, the specimens were washed overnight. Then after washing in PBST (0.3% Triton X-100 in PBS) once for 20 min, the antigens were detected by color reaction with dianiben-
zine. When a biotinated antibody was used as the first antibody, the specimens were incubated with HRP-streptavidin for 4 h in PBST after washing with PBST.

After the chromogenic reactions, the prospective PPs represented by VCAM1/H11001 or CD11c/H11001 spots were embedded in paraffin and sectioned for histological examination. For the detection of CD11c/H11001 cells in the prospective PPs, VCAM1/H11001 spots were embedded in OTC compound, cryosectioned, and subjected to immunostaining with an anti-CD11c monoclonal antibody.

RNase protection assay

Cytokine RNA levels were determined by RNase protection assay using a RiboQuant multiprobe kit (PharMingen) according to the instructions of the manufacturer.

Serum Igs

The concentrations of serum IgM, IgG1, IgG2a, and IgA were measured by ELISA and statistically analyzed by Student’s t-test. Results were considered significant at P < 0.05. In brief, 96-well plates were coated with a purified anti-mouse IgM, IgG1, IgG2a, or IgA at 37°C for 1 h and then incubated with samples at 37°C for 1.5 h. The plates were washed and further developed by adding horseradish peroxidase conjugated second antibody and its substrate, o-phenylenediamine. The absorption was measured on a 490 nm spectrophotometer. Results were analyzed by using the Microplate Manager III system (Bio-Rad, Hercules, CA).

Bone marrow transplantation

BDF1 wild-type and mutant mice with C57BL/6 background, 8–11 weeks old were used for transfer experiments. Bone marrow cells flushed out from femurs were suspended in RPMI containing 10% FCS. Then a single cell suspension was prepared by filtration through a nylon mesh membrane, and the erythrocytes were removed. Approximately 2 × 10^6 mononuclear cells were transferred into lethally irradiated (9.0 Gy) host mice through the tail vein. To detect cells of donor origin, anti-H-2Kb and anti-H-2Kd monoclonal antibodies were used. Enlargement of CPs and the repopulation of donor cells in CPs and PPs were analyzed 11 weeks after bone marrow transfer.

Results

Foxl1 expression in gastrointestinal tract

We and Kaestner and his colleagues have previously reported that Foxl1 is expressed exclusively in the mesenchymal layer of the gastrointestinal tract (Fukamachi et al., 2001; Kaestner et al., 1996). We reexamined Foxl1 expression in 15.5 dpc fetal gut because PP organogenesis begins between 13.5 and 15.5 dpc (Adachi et al., 1997). Foxl1 expression was seen in the mesenchymal layer, and particularly, at the zone immediately beneath the epithelial layer (Fig. 1A, B, C). Foxl1 expression in VCAM1+ICAM1+ cells in 17.5 dpc fetal small intestine was also examined by RT-PCR. Foxl1 is apparently expressed in VCAM1+ICAM1+
Fig. 6. Constitutive outgrowth of CPs in FoxI1-deficient mice. (A) External appearance of the luminal sides of the colon of 10-week-old FoxI1-deficient (a) and wild-type mice (b). Note bleeding in the colon of FoxI1-deficient mice. Sections reveal the generation of lymphoid follicles in the colon of FoxI1-deficient mice (c). A section of wild-type colon is shown (d). (B) Germinal center formation in colonic lymphoid follicles of 10-week-old FoxI1-deficient mice. The
cells, while the relative amount of expression is lower than that in VCAM1+ICAM1− cells (Fig. 1D).

**Defects in PP formation in Foxl1 mutants**

We observed a significant reduction in the number of PPs in Foxl1 homozygotes. There were 3.4 PPs on average in adult Foxl1 mutants, while the average was 9.2 in the wild-type (Fig. 2A and C). The size of an individual PP was also reduced in Foxl1 mutants as revealed by visual inspection and the expression of CD45 (Fig. 2A, B, and D). We further found that PP defects were prominent in the caudal region of the small intestine in Foxl1 homozygotes. We divided the small intestine arbitrarily into three regions based on the length of the entire small intestine (Fig. 2E, left). PP numbers were more obviously reduced in the caudal regions than in the cranial regions (Fig. 2E, right). We did not observe significant alterations in either the thymus, the spleen, or the lymph nodes other than mesenteric lymph nodes. The number of lymphocytes resided in each mesenteric lymph node in Foxl1 homozygotes was almost double that of the wild-type (K. Fukuda, unpublished observations).

We next investigated which process in PP organogenesis is affected in Foxl1-deficient mice. First, the formation of PP organizing centers, which is completed 15.5–16.5 dpc in the wild-type, was investigated by the expression of VCAM1 and IL7R-α at 17.5 dpc. The formation of VCAM1+ spots was impaired in Foxl1-deficient mice, especially, in the caudal region of the fetal intestine (Fig. 3A and B). Moreover, the VCAM1+ spots in Foxl1 mutants were irregular and smaller compared to the wild-type (Fig. 3Bb, c, d, e, and f). Sections of the spots revealed differences in localization of VCAM1+ cells between wild-type and Foxl1-deficient mice (Fig. 3G). VCAM1+ cells were juxtaposed with the muscle layer in the wild-type, while they extended toward the luminal side in Foxl1-deficient mice. Similarly, the formation of IL7R-α+ spots was affected in the caudal region of the fetal intestine of Foxl1-deficient mice (Fig. 3C and D). Thus, the formation of PP organizing centers was significantly affected in Foxl1-deficient mice. Interestingly, the number of VCAM1+ spots in Foxl1 mutants was almost the same as that in wild-type 18.5 dpc fetus and newborn mice, although the VCAM1+ spots in newborn Foxl1-deficient mice were smaller and irregular compared with the wild-type as observed at 17.5 dpc (Fig. 3E, F, H, and K. Fukuda, unpublished observations). Since VCAM1+ spots are generated in the craniocaudal sequence, our observations indicate that the formation of PP organizing centers due to the cosegregation of VCAM1+ cells and IL7R-α+ is significantly delayed in Foxl1 homozygotes (Adachi et al., 1997).

The second step of PP organogenesis is represented by the accumulation of hemopoietic cells in PP organizing centers. We examined the expression of CD11c, which demarcates dendritic cells in newborn small intestine. The number of CD11c+ spots was significantly reduced in Foxl1-deficient mice, particularly in the caudal region of the small intestine (Fig. 4A and B). In addition, the CD11c+ spots were much smaller in Foxl1-deficient mice than in the wild-type. Sections of visible spots revealed a significantly reduced number of CD11c+ cells in Foxl1-deficient mice (Fig. 4An, o, p, Bg, h). Accumulation of CD11c+ cells into VCAM1+ spots in the caudal region was examined. VCAM1+ spots were cryosectioned and subjected to CD11c staining with different colored chromogenic reagents. Few CD11c+ cells were seen in the VCAM1+ spots of Foxl1-deficient mice (Fig. 4C). Therefore, the accumulation of hemopoietic cells within VCAM1+ spots is also affected in Foxl1 mutants and is more strongly affected in the caudal than the cranial region. Together, the data suggest that a delay in the formation of PP organization centers might lead to defects in the accumulation of hemopoietic cells required for the architecture formation of PPs.

**The expression of genes essential for PP organogenesis in Foxl1 mutants**

We next investigated the expression of IL-7Rα, LTα, LTβ, LTβR, SLC, BLC, ELC, and Id2, which are functionally involved in PP development (Adachi et al., 1998; Honda et al., 2001; Yokota et al., 1999; Yoshida et al., 1999). Since defects in PP formation were seen mainly in the caudal region of the small intestine of Foxl1 homozygotes, we compared the expressions of these genes in the cranial (prospective region I) and caudal (prospective region III) regions between wild-type and Foxl1-deficient mice at 17.5 dpc (Fig. 5). The expression of IL-7Rα was not significantly affected in the caudal region of Foxl1 mutant intestine compared with the wild-type. IL-7Rα expression in the cranial region was slightly less than in the caudal region in both the wild-type and the Foxl1 mutants, although
IL7Rα+ spots were seen in the cranial region. Significant numbers of IL-7Rα+ cells might reside in the caudal region of the fetal intestine in Foxl1 homozygotes but fail to form aggregates. The expression of LTα and LTβ exhibited three times reduction in the caudal region of the small intestine of Foxl1 homozygotes compared to the wild-type. The expression of LTβR decreased almost 10 times in the caudal region of the fetal intestine of Foxl1 mutants, while its expression in the cranial region was unaffected. The expressions of ELC and BLC also decreased around three times in the caudal region of the fetal intestine of Foxl1 mutants compared to the wild-type, while the expression of SLC was not significantly altered. Id2 expression was not affected either.

**Hypertrophy of CPs in Foxl1-deficient mice**

Multiple polyp-like structures were reproducibly observed in the colon of Foxl1 mutant mice older than 10 weeks (Fig. 6A). Histological examinations revealed these polyp-like structures to be submucosal lymphoid follicles (Fig. 6Ac and d). Flow cytometric analyses indicated that 85% of the mononuclear cells included in these lymph follicles were CD45R+ mature B lymphocytes and the rest were CD3+ mature T lymphocytes (K. Fukuda, unpublished observations). PNA-positive activated B lymphocytes were found to have accumulated in the central region of the B cell zone, suggesting the presence of germinatal centers in the follicles (Fig. 6B). We next investigated the latent outgrowth of lymphoid follicles by immunostaining with anti-CD45R monoclonal antibody (Fig. 6C). In 2-week-old mice, the submucosal accumulation of CD45R+ lymphocytes was seen not only in Foxl1-deficient mice, but also in wild-type. These are identical to the structure previously described as CPs (Dohi et al., 1999). At this stage, the nodules were slightly larger in Foxl1−/− than the wild-type (Fig. 6Ca). In 5-week-old mice, a significant enlargement of the CPs due to lymphocyte accumulation was obvious only in Foxl1−/− mice (Fig. 6Cb). In 10-week-old mice, CPs in Foxl1−/− were much larger than those in the wild-type. The CPs in the wild-type were also slightly enlarged (Fig. 6Cc). In 9-month-old mutants, the luminal protrusion of enlarged CPs was prominent (Fig. 6Cd). We investigated the distribution of another component of CPs, CD11c+ dendritic cells, in the enlarged lymphoid follicles. CD11c+ dendritic cells localized densely in the marginal region of CPs in both wild-type and Foxl1-deficient mice (Fig. 6D). Thus, the submucosal lymphoid follicles in Foxl1-deficient mice appear to be due to the hypertrophy of CPs. This possibility was further addressed using a genetic approach. CPs, as well as PPs, are absent from the small intestine of nik<sup>aby</sup> homozygous mutants (Fig. 6Ea and b). In Foxl1/nik<sup>aby</sup> double-mutant mice, neither CPs nor enlarged lymphoid follicles were ever observed (Fig. 6Ec and d). Thus, the nik<sup>aby</sup> mutation suppresses the formation of lymphoid follicles by affecting the initial formation of CPs. Together, the results imply that latent mechanisms repressing the constitutive outgrowth of CPs might be impaired in Foxl1-deficient mice.

The lack of CPs in nik<sup>aby</sup> mutants suggests that this development might utilize similar genetic cascades as those required for PP development. Indeed, the enlarged CPs include CD11c+ dendritic cells in their marginal regions and are present in PPs, and a histological resemblance has been reported (H. Yoshida and K. Fukuda, unpublished observations and Fig. 6D; Owen et al., 1991). We further examined the distribution of IL-7Rα+ cells and c-kit+ cells. These cells were also allocated to the marginal regions of enlarged CPs as seen in PPs (Fig. 6F). Thus, the enlarged CPs seen in Foxl1 homozygotes are structurally similar to PPs, although they protrude toward the lumen (Fig. 6D and E).

Previously, experimental hapten-induced colitis was proved to be associated with CP hypertrophy and Th2-type responses (Dohi et al., 1999, 2001). We frequently found blushing of the mucosa and prolapse of the rectum by visual inspection of Foxl1-deficient mice (K. Fukuda, unpublished observations). Histological examination revealed the transmucosal infiltration of mononuclear cells, but obvious erosion or ulceration was never seen. Goblet cells appeared normally. Hypertrophy of the crypts was seen. These colorectal changes in Foxl1-deficient mice suggest the presence of weak inflammatory responses, although they do not fulfill the clinical criteria indicating the onset of colitis (Dohi et al., 1999). We, thus, examined the expression of cytokines in enlarged CPs of 10-week-old mice. The expression of IL-4 was elevated in hypertrophic CPs while those of interferon-γ (IFN-γ), IL-2, IL-5, and IL-13 were not above those of wild-type (Fig. 7A and B). These observations suggest the involvement of IL-4 for CP outgrowth in Foxl1-deficient mice. This idea is supported by our other observation that the hypertrophy of CPs in Foxl1-deficient mice was more obvious in BALB/c than in C57BL/6 background (K. Fukuda and H. Fukamachi, unpublished observation). However, the serum levels of IgG1 was not significantly affected ($P = 0.208$), while those of total IgM, IgG2a, and IgA were significantly elevated ($P < 0.05$) (Fig. 7C). This implies some other profound mechanisms regulating Ig production could be affected in the GALT of Foxl1-deficient mice.

The enlargement of CPs in Foxl1 mutants could be attributed to defects in either stromal or hematopoietic lineage cells. To address this, we examined bone marrow chimeras of wild-type and Foxl1 mutants. To avoid lethal graft-versus-host reactions, we used a combination of C57BL/6 and BDF1. In Foxl1+/+→ Foxl1−/− chimeras, the CPs were apparently enlarged as observed in Foxl1 mutants and consisted mainly of Foxl1+/+ cells (Fig. 8A and E). Small PPs seen in these chimeras were also repopulated by H2K<sup>d</sup>+ donor cells (Fig. 8C and E). In Foxl1−/−→ Foxl1+/+ chimeras, no CP enlargement was seen, while the PPs were normally reconstituted. FACS analyses revealed that more than the 80% of circulating
lymphocytes in the chimeras were negative for H2Kd expression. Thus, it is presumed that most lymphocytes in the chimeras consisted of donor cells (Fukuda and Koseki, unpublished observations). This implies that Foxl1/− cells do not induce the enlargement of CPs, while they can repopulate into GALT as seen in PPs. These results indicate that the enlargement of CPs in Foxl1 mutants is due mainly to alterations in radiosensitive stromal fractions, but not mature lymphocytes.

Discussion

The role of the Foxl1 gene product in PP organogenesis

In this study, we indicate that defects in PP formation in Foxl1-deficient mice may be due to a significant delay in the formation of PP organizing centers (Honda et al., 2001; Yoshida et al., 1999). This defect could be attributed to functional abnormalities in either the lymphoid or the mesenchymal component, or both. It is notable that the expression of IL-7Ra mRNA is not significantly reduced, while IL-7Ra+ cells are not segregated into PP organizing centers in the caudal region of 17.5 dpc Foxl1 mutant intestine. This discordance suggests that IL-7Ra+ cells present in the caudal region of 17.5 dpc Foxl1 mutant intestine reside dispersely without cosegregating with VCAM1+ICAM1+ mesenchymal cells to form PP organizing centers. Although PP organizing centers are not formed, reduced amounts of LTα and LTβ are still expressed, consistent with the decrease in IL-7Ra expression. This suggests that IL-7Ra+ cells might be activated functionally because LTα and LTβ are produced mainly by IL-7Ra+ cells upon engagement of the IL-7 receptor by its ligands (Yoshida et al., 1999). Thus, the repopulation and activation of IL-7Ra+ lymphoid cells in the caudal region of fetal intestine might not be primarily affected in Foxl1 mutants, but the segregation of IL-7Ra+ cells to generate PP organizing centers is significantly delayed. Since the condensation of IL-7Ra+ cells is suggested to depend on inductive signals emanating from VCAM1+ICAM1+ mesenchymal cells mediated by chemokines including BLC, the intestinal mesenchymal component of PP organizing centers could primarily be affected in Foxl1 mutants. Indeed, Foxl1 is expressed in intestinal mesenchymal cells including VCAM1+ICAM1+ cells. The significant reduction of LTβR expression in the caudal region of 17.5 dpc Foxl1 mutant intestine together with the lack of VCAM1+ICAM1+ mesenchymal cells implies that the responsiveness of presumptive VCAM1+ICAM1+ mesenchymal cells to LTs derived from IL-7Ra+ cells could be significantly affected in Foxl1-deficient mice. The LT-dependent differentiation, proliferation, and/or condensation of presumptive VCAM1+ICAM1+ mesenchymal cells might be delayed in Foxl1-deficient mice. These observations suggest that Fox1 gene product might be involved in regulating the responsiveness of fetal intestinal mesenchymal cells to inductive signals that initiate PP organogenesis. It is, however, also possible that Foxl1 is primarily needed for efficient engagement of VCAM1+ICAM1+ mesenchymal cells and IL-7Ra+ cells that could be required for up-regulation of LTβR expression.

Here we also suggest that PP organizing centers generated after 17.5 dpc in Foxl1-deficient mice fail to undergo the subsequent accumulation of various hemopoietic cells that would lead eventually to the formation of mature PPs. The collapse of PP organogenesis might be due to defects in the formation of PP architecture rather than in the entry of mature lymphocytes, because the accumulation of CD11c+ cells in PP organizing centers is already impaired. In other words, PP organizing centers containing considerable numbers of CD11c+ cells are qualified to develop into mature PPs in Foxl1-deficient mice. Thus, the accumulation of various hemopoietic cells into PP organizing centers is also an essential process for PP organogenesis. It is intriguing that the accumulation of various blood cells must be completed by 17.5 dpc. Recently, IL-7Ra+CD3− cells residing in the fetal intestine have been shown to be potential progenitors of CD11c+ dendritic cells and to be generated in fetal liver and migrate to fetal intestine via the mesentery (Yoshida et al., 2001). The seeding of IL-7Ra+CD3− cells into the fetal intestine might not be affected since a significant amount of IL-7Ra is expressed even in the caudal region of Foxl1 mutant intestine. The delayed and inefficient cosegregation of IL-7Ra+CD3− cells and VCAM1+ICAM1+ mesenchymal cells might disturb the subsequent differentiation of IL-7Ra+CD3− cells into cells required to form the architecture of PPs, including dendritic cells.

Maintenance of CPs by the Foxl1 gene product

The large lymphoid follicles observed in the large intestine of Foxl1-deficient mice were revealed to be hypertrophic CPs. The Foxl1 gene product might be involved in maintaining the volume of CPs by regulating the functions of radiosensitive stromal components, but not radiosensitive bone marrow-derived components. Since enlarged CPs structurally resemble PPs and the generation of CPs, as well as PPs, is dependent on the LTβR/Nik axis, it is presumed that mesenchymal cells equivalent to VCAM1+ICAM1+ cells in PPs could primarily be affected. Indeed, Foxl1 is expressed in mesenchymal cells of the large intestine as well as stomach and small intestine (Fukamachi et al., 2001). Recently, the constitutive activation of LTβR/Nik axis was demonstrated to be required for the maintenance of PPs and CPs (Dohi et al., 2001). Epigenetic inactivation using soluble LTβR-Ig fusion protein leads to a significant reduction in the number of PPs and CPs associated with a decrease in the number of dendritic cells. Thus, it is likely that Fox1 is involved in the regulation of the proliferative response of various cells present in mature CPs upon activation of the LTβR/Nik axis. Alternatively, stromal cells in...
the CPs of Foxl1 mutants might overactivate the resident lymphocytes. A similar hypertrophy of CPs was observed in experimental hapten-induced colitis, which is associated with Th2-type responses of CPs and is prevented by the elimination of CPs by a soluble LTβR-Ig fusion protein (Dohi et al., 2001). This implies that the constitutive activation of resident lymphocytes by APCs in CPs also leads to CP hypertrophy, which also involves the LTβR/Nik axis. Indeed, the expression of IL4 is notably elevated in hypertrophic CPs. Thus, Foxl1 might also be involved in regulating functions of APCs in CPs, although it remains unclear whether APCs are affected in either an autonomous or a nonautonomous manner. Since CPs are overlaid by M cells as well as PPs, antigens derived from luminal microorganisms could play a role during the outgrowth of CPs in Foxl1 mutants (Owen et al., 1991).

Together, the data suggest that the cellular responses of GALT dependent upon the LTβR/Nik axis may be affected in Foxl1-deficient mice. It is, however, unclear whether the Foxl1 gene product functions in a signal-transducing cascade associated with the LTβR/Nik axis or in a parallel pathway, which is also needed for the functional manifestation of LT-dependent responses.

Acknowledgments

We are grateful to Mr. S. Sugimori, Ms. M. Uchida, and Ms. S. Takeda for help in many respects. This project was supported by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology, the Japanese Government, and a grant from Suzuken Memorial Foundation.

References


