Impairment of B lymphopoiesis in precocious aging \((klotho)\) mice

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Abstract

Inactivation of the \(klotho\) gene in mice results in multiple disorders that resemble human aging after 3 weeks of age. Because hematopoiesis, especially B lymphopoiesis, is affected in humans and mice by aging, we analyzed the hematopoietic state in homozygous \(klotho\) \((kl/kl)\) mice. The \(kl/kl\) mice showed thymic atrophy and a reduced number of splenocytes. These mice had almost the normal number of myeloid cells, erythroid cells, IL-3-responsive myeloid precursors and colony forming units in spleen (CFU-S) in bone marrow (BM), but had a substantially decreased number of B cells in BM and peripheral blood as compared with wild-type mice. IL-7-responsive B cell precursors and all of the maturation stages of B cells in BM were also reduced. However, the function of hematopoietic stem cells including their capacity of B lymphopoiesis \(in vivo\) and \(in vitro\) was normal. Early B cell development was also normal in neonates and young \(kl/kl\) mice until 2 weeks old without aging phenotypes. RT-PCR analysis revealed that the level of IL-7 gene expression was significantly reduced in freshly isolated \(kl/kl\) BM cells. However, injection of IL-7 in \(kl/kl\) mice could not rescue the B lymphopenia. These findings indicate that Klotho protein may regulate B lymphopoiesis via its influence on the hematopoietic microenvironment.

Introduction

Aging induces the deterioration of physiological functions necessary for the survival and fertility of an organism, and results in common age-related diseases such as arteriosclerosis, cancer, dementia and osteoporosis (1,2). Aging also affects the immune system (3,4). Both B cell function and development are modulated in humans and mice (3,5,6), and the generation of pre-B cells is impaired in bone marrow (BM) by aging (7–10). Since early B cell development is highly dependent on functions of stromal cells (11,12), it has been postulated that the impairment of pre-B cell development by aging is due to functional abnormalities of stromal cells including the production of IL-7 (5,9,13). However, Kirman et al. have recently shown that IL-7 production by BM cells from aged mice is not impaired and that apoptotic cell death of pre-B cells with a lower level of \(bcl-x_l\) mRNA increases in BM from aged mice (14). Because aging is a complex process influenced by many genes (2), mechanisms of the age-associated alteration of early B cell development are not clearly understood yet.

A novel mouse mutant, termed \(klotho\), that exhibits multiple disorders resembling human aging has recently been established (15). Defect of \(klotho\) gene expression \((kl/kl)\) in mice results in multiple age-related disorders such as arteriosclerosis, osteoporosis, pulmonary emphysema, short lifespan and infertility after 3 weeks of age. Furthermore, \(kl/kl\) mice showed atrophy of the anterior pituitary gland which regulates the other endocrine organs. These results suggest that the \(klotho\) gene product is involved in the suppression of multiple aging phenotypes at a young age. The \(klotho\) gene encodes a novel cell surface protein of 1014 amino acids that has a cleavage...
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site to generate a secreted form (15–17). The extracellular domain consists of two internal repeats which exhibit 20–40% sequence identity to β-glucosidases of bacteria and plants as well as to mammalian lactase glycosylceramidase. The klotho gene is expressed in various organs, but not in lymphatic organs including BM, thymus and spleen. Because some organs without endogenous expression of the klotho gene were severely affected in kl/kl mice, it is considered that the secreted form of Klotho protein mainly works as a humoral factor to protect against precocious aging (15,17). Since kl/kl mice show the multiple phenotypes resembling human B220− IgM− CD24+ cells, we discuss abnormalities of the hematopoietic microenvironment in BM from kl/kl mice.

Methods

Mice

Heterozygous klotho mutant (kl/+) mice (C3H) were maintained in our animal facility and intercrossed, and the resulting littersmates were used for this study. Genotypes were confirmed by Southern blot analysis of tail DNA (15). SCID mice were purchased from Japan SLIC (Shizuoka, Japan).

Hematopoietic growth factors

Murine rIL-3 and rIL-7 were provided by Dr. Tetsuo Sudo (Toray Industries, Kamakura, Japan). The concentration of the cytokines used in vitro was as follows: rIL-3, 200 U/ml; rIL-7, 20 U/ml. For the in vivo injection study, murine rIL-7 was purchased from Genzyme (Cambridge, MA) and used at 500 ng/day.

mAb and FACS analysis

Biotinylated, FITC-conjugated or phycoerythrin (PE)-conjugated mAb against B220 (RA3-6B2), CD43 (S7), CD24 (HS-A; J11d), BP-1 (Ly-51; 6C3), IgM, Mac-1 (M1/70), Gr-1 (RB6-8C5), Thy-1.2 (S3-2.1), CD3 (145-2C11), CD4 (GK1.5), CD8 (S3-6.72), CD5 (Ly-1; S3-7.3) or TER119 (Ly-76) were purchased from PharMingen (San Diego, CA). PE-conjugated anti-Sca-1 (Ly 6A/E) mAb was purchased from PharMingen, and FITC-conjugated anti-c-kit (CD117) mAb was a gift from Dr. S.-I. Nishikawa (Kyoto University, Japan) and used to purify the primitive hematopoietic stem cell fraction (18). Biotinylated mAb were visualized using streptavidin–allophycocyanin or streptavidin–CyChrome (PharMingen).

Cell suspensions were treated with ACK lysing buffer (0.155 M ammonium chloride, 0.1 M disodium EDTA and 0.01 M potassium bicarbonate) to lyse erythrocytes before staining. Single-cell suspensions were prepared in staining medium (PBS with 3% FCS and 0.1% sodium azide) and were stained with the mAb described above. After 20 min incubation on ice, cells were washed twice with staining medium and resuspended in staining medium supplemented with propidium iodide (PI; 1 µg/ml). Stained cells were analyzed by FACScalibur (Becton Dickinson, San Jose, CA).

B-lineage cells were resolved into various fractions (A–F) based on the technique initially reported by Hardy et al. (19,20). Fraction A (B220+CD43+CD24+) cells were distinguished from Fraction B + C (B220+CD43−CD24+) cells on the basis of CD24 expression. Fraction D cells were defined as B220+CD43−IgM− cells and Fraction E + F were defined as B220+IgM+ cells.

Isolation of Lin−c-kit+Sca-1+ BM cells and co-culture with stromal cells

Total BM cells from kl/kl mice or littermates were stained with a cocktail of biotinylated mAb against lineage markers (Lin; B220, Mac-1, Gr-1, CD4, CD8 and TER119) for 20 min at 4°C. After washing the cells 3 times with staining medium, the cells were treated with streptavidin-conjugated immuno-magnetic beads (BioMag; Perceptive Diagnostics, Cambridge, MA) for 30 min to remove Lin highly positive cells. The remaining cells were collected and stained with PE–anti-Sca-1 mAb, FITC–anti-c-kit mAb and streptavidin–CyChrome for 20 min at 4°C. After washing, the cells were resuspended in staining medium supplemented with PI (1 µg/ml). Stained cells were analyzed by FACS Vantage (Becton Dickinson), and the Lin−c-kit+Sca-1− cells were sorted and used as a primitive hematopoietic stem cell fraction (18). Murine stromal cell line, OP-9 (21) was obtained from RIKEN cell bank (Tsukuba, Japan). OP-9 was seeded in six-well plates (Becton Dickinson Labware, Lincoln Park, NJ) 1 day before co-culture. Whole BM cells or sorted Lin−c-kit+Sca-1+ BM cells were cultured on a OP-9 stromal layer with 3 ml of RPMI 1640 containing rIL-7 (20 U/ml), 10% heat-inactivated FCS and 5×10−5 M 2-mercaptoethanol.

In vitro colony assay

Methylcellulose culture was carried out using a modified method of Iscove et al. (22–24). Briefly, 1 ml of culture medium contained an adequate number of total BM cells, 1.2% methylcellulose (Shin-etsu Chemical, Tokyo, Japan), α-medium (Gibco, Grand Island, NY), 30% FCS (Sigma, St Louis, MO), 1% deionized BSA (Sigma), 0.1 mM 2-mercaptoethanol (Eastman Organic Chemical, Rochester, NY) and the appropriate concentration of growth factors. The cultures were prepared in 35 mm non-tissue culture dishes (Becton Dickinson Labware) and incubated at 37°C in a humidified atmosphere of 5% CO2. The number of colonies was scored after 7 days of culture using an inverted microscope.

Colony forming unit in spleen (CFU-S) assay

Female wild-type mice were X-irradiated (9.5 Gy). After 24 h, the mice were injected i.v. with 1×105 cells suspended in PBS. Mice were anesthetized and sacrificed by cervical dislocation at 12 days after injection. The spleens were removed, fixed in Bouin’s solution and colonies were counted (25).
**Long-term reconstitution assay**

Female wild-type mice and SCID mice were X-irradiated (9.5 and 2.0 Gy respectively). After 24 h, male BM cells (1×10^6) suspended in PBS were injected into the tail vein of female recipients. Peripheral blood (PB) of SCID mice was obtained from the retro-orbital venous plexus 20 weeks after transplantation and lysed with ACK lysis buffer. The samples were then used for flow cytometric analysis. The wild-type recipient mice were anesthetized and sacrificed by cervical dislocation at 20 weeks after injection. Thymus, spleen and BM were obtained, and genomic DNA was extracted from these organs. DNA samples (100 ng) were used for the PCR reaction. The sequence of the Y chromosome-specific primers (pY2) was 5'-GCATTTGCCTGTCAGAGAGAG-3' and 5'-AC-TGCTGCTGCTTTCCAACTA-3' (26,27). After an initial 7 min incubation at 95°C, the 30 cycles of PCR reactions were carried out using the following conditions: denaturation at 95°C for 1.0 min, annealing at 62°C for 2.0 min and polymerization at 72°C for 3.0 min. The amplified DNA was analyzed by agarose gel electrophoresis and stained with ethidium bromide.

**Histological analysis**

Femurs were fixed in 10% neutral-buffered formalin and then treated for decalcification using formic acid. Paraffin-embedded tissues were subjected to hematoxylin & eosin staining.

**RT-PCR analysis**

Total RNA was extracted from BM cells using an ISOGEN total RNA isolating kit (Waco, Tokyo, Japan). RNAs were reverse-transcribed using Superscript (Life Technologies, Grand Island, NY) and oligo(dT) (Pharmacia, Piscataway, NJ), in a final volume of 20 µl, and 1 µl of cDNAs was used for PCR. After an initial 7 min incubation at 95°C, the 30 cycles of PCR reactions were carried out using the following conditions: IL-7, macrophage colony stimulating factor (M-CSF) and stem cell factor (SCF) cDNA: denaturation at 95°C for 1.5 min, annealing at 55°C for 1.5 min, and polymerization at 72°C for 1.5 min. G3PDH cDNA: denaturation at 95°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 1 min. PCR primers for the cDNA amplification were as follows: IL-7 primers, 5'-ACATCATCTGAGTGCACA-3' and 5'-CTCTCACTGATGTTTAG-3' (28); M-CSF primers, 5'-CATGATAAGAAGACAACCC-3' and 5'-ATGGTACATCCACGTCCAT-3' (29); SCF primers, 5'-GACTGTGTGCTCTCTCAACA-3' and 5'-CTTGCAAMAATCTCCAGATC-3' (29); and the G3PDH primers, 5'-TGAAGGTAGGTGTAACCG-ATGGG-3' and 5'-CTGATAGAGGCCTAGTGCTACCAACAC-3' (30). The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

**IL-7 treatment in vivo**

Female 4-week-old kl/kl and +/- mice were given s.c. 500 ng/day of mouse rIL-7 dissolved in PBS containing 0.1% BSA for 6 days (31). Control mice were treated with vehicle solution (PBS/0.1% BSA) according to the same protocol. The mice were anesthetized and sacrificed by cervical dislocation on day 7. Flow cytometric analysis was performed with BM cells collected from the vehicle- and the IL-7-treated mice.

**Statistical analysis**

Data were analyzed using a single-tailed Student's t-test. Data are presented as mean ± SD.

**Results**

**Hematopoiesis in homozygous kl/kl mice with precocious aging**

Many aging phenotypes develop in homozygous kl/kl mice after 3 weeks of age (15). To examine hematopoiesis in kl/kl mice with aging phenotypes, percentages of T (Thy1+) cells, B (B220+) cells, myeloid (Mac-1+) cells and erythroid (TER119+) cells were analyzed in hematopoietic organs from kl/kl mice at 6 weeks by FACS (Fig. 1). It is noteworthy that the B cell:T cell ratio in the kl/kl spleen was normal (Fig. 1B), although the B cell:T cell ratio in the kl/kl PB was altered (Fig. 1A).

Since the thymus was atrophic and was almost not detected...
indicate that B cell development is impaired in BM from kl/kl/H11001 mice. The number of B cells (B-I cells) in the 6-week-old kl/kl/BM was drastically reduced (16.2 versus 7.4%), whereas it was of comparable frequency in kl/kl/H11001 mice (Fig. 1A). The number of total nucleated cells in spleen from kl/kl/H11001 mice was significantly lower than that from +/+ mice (P < 0.001). The number of total nucleated cells in BM from kl/kl/H11001 mice was slightly lower than that from +/+ mice (P = 0.03). The number of B lymphocytes in the kl/kl/H11001 BM was significantly lower than that in the +/+ BM (P < 0.001), whereas the number of myeloid cells, erythroid cells and T cells in the kl/kl/H11001 BM was slightly higher without statistical significance. Interestingly, the percentage of TER119+ erythroid cells in the kl/kl/H11001 spleen was drastically reduced (16.2 versus 7.4%), whereas it was normal or slightly elevated in the kl/kl/H11001 BM. These results indicate that B cell development is impaired in BM from kl/kl/H11001 mice with precocious aging. On the other hand, no significant difference in the proportion of CDS+ B cells (B-I cells) in the peritoneal cavity of kl/kl/H11001 mice was observed (Fig. 1C).

Analysis of B progenitors in BM from kl/kl/H11001 mice

Development of early B-lineage cells in BM from kl/kl/H11001 mice at 6 weeks was further analyzed by FACS with cell surface staining for stage-specific cell surface markers. Pro-B, pre-B and mature B cells were thus defined as B220+CD43+IgM- (Fraction A–C), B220+CD43+IgM- (Fraction D) and B220+IgM- (Fraction E + F) B cells respectively (19). The number of each stage of B-lineage cells in kl/kl/H11001 mice was lower than that in +/+ mice (Fig. 2A) and reduction of the pre-B cells was more than that of the mature (IgM+) B cells. Subpopulations among B220+CD43+IgM- (Fraction A–C) pro-B cells were further analyzed by their expression of BP-1 or CD24 to distinguish between Fraction A and Fraction B + C (Fig. 2B). The percentage of BP-1+ or CD24high subpopulations among pro-B cells was apparently lower in kl/kl/H11001 mice, although all stages of B subpopulations were reduced in kl/kl/H11001 mice. These results were summarized in Table 2.

The number of clonogenic progenitors in BM from kl/kl/H11001 mice at 6 weeks was examined by the in vitro colony assay and the in vivo CFU-S assay (Table 3). The number of IL-7-responsive clonogenic progenitors in kl/kl/H11001 mice was reduced up to 5% of that in +/+ mice. These results are consistent with the reduction in the number of pro-B cells (Fraction B + C) in kl/kl/H11001 mice, since this population contains the vast majority of IL-7-responsive B-lineage cells (32). In contrast, the number of IL-3-responsive clonogenic progenitors (committed myeloid progenitors) and day 12 CFU-S (more primitive hematopoietic stem cell fraction) in kl/kl/H11001 mice was almost the same as that in +/+ mice, suggesting the presence of primitive hematopoietic stem cells in BM from kl/kl/H11001 mice with aging phenotypes.

In order to examine the capacity for B lymphopoiesis by primitive hematopoietic stem cells, total BM cells or Lin–c–kit+Sca-1+ BM cells (primitive hematopoietic stem cell fraction) of kl/kl/H11001 mice at 6 weeks were cultured in the presence of confluent layers of OP-9 plus rIL-7 (20 U/ml) (Fig. 3). When total BM cells were used, the emergence of B cells (both B220+IgM- immature B cells and B220+IgM+ mature B cells) was delayed in kl/kl/H11001 mice compared with that in +/+ mice. When Lin–c–kit+Sca-1+ BM cells were used, the emergence of B cells in kl/kl/H11001 and +/+ mice was almost the same, indicating a normal capacity of B lymphopoiesis by hematopoietic stem cells in BM from kl/kl/H11001 mice.

In vivo B lymphopoiesis by hematopoietic stem cells from kl/kl/H11001 mice

In order to confirm the capacity of B lymphopoiesis in vivo by hematopoietic stem cells from kl/kl/H11001 mice, we examined the long-term reconstitution of BM cells. We injected BM cells (2×106) obtained from 6-week-old male kl/kl/H11001 mice into lethally irradiated female wild-type mice. All of the BM transplanted mice survived for 20 weeks, whereas all of the irradiated mice without BM transplantation were dead within 2 weeks. After 20 weeks, mice were sacrificed and DNA was extracted from BM, spleen and thymus. PCR study using a mouse Y chromosome-specific primer (pY2) (26,27) clearly showed that BM, spleen and thymus were reconstituted with cells derived from kl/kl/H11001 mice (Fig. 4A). Next, we injected kl/kl/H11001 BM cells (2×106) into 2 Gy irradiated SCID mice which have no mature lymphocytes in BM. After 20 weeks, PB was obtained and stained with anti-CD3–FITC and anti-B220–PE respectively. As shown in Fig. 4(B), the frequency of B220+ B lymphocytes [16.1 ± 7.2% (+/+)] versus 15.6 ± 4.0% (kl/kl/H11001),
mice cells from BM were obtained from 6-week-old +/+ and kl/kl mice. Colony formations were determined as described in Methods. Values significantly different from their respective control groups at ”P < 0.01.

### Table 2. Frequency of B cell compartments in BM from kl/kl mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fraction A (%)</th>
<th>Fraction B + C (%)</th>
<th>Fraction D (%)</th>
<th>Fraction E + F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>1.49 ± 0.49</td>
<td>5.19 ± 1.46</td>
<td>23.0 ± 5.6</td>
<td>10.1 ± 2.2</td>
</tr>
<tr>
<td>kl/kl</td>
<td>0.36 ± 0.22a</td>
<td>0.43 ± 0.11b</td>
<td>2.4 ± 0.54b</td>
<td>2.8 ± 1.1b</td>
</tr>
</tbody>
</table>

BM cells were obtained from 6-week-old +/+ and kl/kl mice. Values significantly different from their respective control groups at ”P < 0.01 and "P < 0.001.

### Table 3. Colony formation by BM cells from kl/kl mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CFU-IL-7 (10^5)</th>
<th>CFU-IL-3 (10^5)</th>
<th>Day 12 CFU-S (10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>11.5 ± 1.8</td>
<td>33.7 ± 4.3</td>
<td>12.5 ± 1.3</td>
</tr>
<tr>
<td>kl/kl</td>
<td>1.7 ± 1.5a</td>
<td>31.0 ± 8.4</td>
<td>13.6 ± 1.5</td>
</tr>
</tbody>
</table>

BM cells were obtained from 6-week-old +/+ and kl/kl mice. Colony formations were determined as described in Methods. Values significantly different from their respective control groups at ”P < 0.01.

### Fig. 2. Flow cytometric analysis of B-lineage cells in BM from 6-week-old kl/kl mice. (A) BM cells from +/+ and kl/kl mice were stained with mAb for anti-IgM-FITC, anti-B220-allophycocyanin and anti-CD43-PE. (B) B220^+CD43^+ BM cells were further analyzed with expression of BP-1 and CD24 (HSA). Both BP-1^+ and CD24^+ fraction within B220^+CD43^+ BM cells were severely reduced. The number in each square represents the percentage within each subset. One representative result from six mice.

Normal B lymphopoiesis was further confirmed in BM from young kl/kl mice. Since many aging phenotypes are displayed in kl/kl mice after 3 weeks (15), we examined B lymphopoiesis in liver from newborn kl/kl mice and in BM from kl/kl mice at 2 weeks. As shown in Fig. 5, normal B cell development was observed in those organs from kl/kl mice, suggesting that the klotho gene product is not involved in B lymphopoiesis at least until 2 weeks after birth and that B lymphopenia in BM from kl/kl mice is due to the secondary effect of aging phenotypes.

**IL-7 production of stromal cells in BM from kl/kl mice**

To examine abnormalities in BM from kl/kl mice, the structure of BM in femurs from kl/kl mice at 6 weeks was histologically analyzed (Fig. 6). The thickness of cortical bone was thinner than that from +/+ mice as described (15). The BM space of femurs from kl/kl mice was retained or slightly extended. BM cellularity was not reduced (normocellular BM) and fatty changes which are often seen with aging were not observed in kl/kl mice. Furthermore, myeloid cells but not mononuclear cells were prominent in kl/kl mice, which is compatible with B lymphopenia in BM from kl/kl mice shown in Tables 1 and 2.

As IL-7 and SCF derived from stromal cells are known to be essential for early B cell development in BM (11,12), we measured expression of IL-7, SCF and M-CSF mRNA in BM cells from kl/kl mice at 6 weeks by a semiquantitative RT-PCR method. As shown in Fig. 7, expression of IL-7 mRNA in BM cells from kl/kl mice was significantly lower than that from +/+ mice. On the other hand, expression of SCF and M-CSF mRNA from the kl/kl BM was identical to that from the +/+ BM, which is consistent with the fact that myeloid lineage differentiation is not affected in kl/kl mice (Table 1 and 3).

We then tried to rescue B lymphopenia in BM from kl/kl mice at 4 weeks by administering IL-7 (Table 4). When +/+ mice were treated with rIL-7 for 6 days, the proportion of Fraction A + B + C and Fraction D in BM was significantly increased compared with the vehicle-treated mice (”P < 0.05
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Fig. 3. In vitro analysis of B lymphopoiesis by BM cells from 6-week-old +/+ and kl/kl mice. Whole BM or Lin−c-kit+Sca-1− BM cells were cultured over a confluent layer of OP-9 stromal cells supplemented with rIL-7 for 19 days. Expression of B220 and IgM on cultured BM cells was examined at different time intervals. Non-adherent cells harvested from four dishes by gentle pipetting at every medium change were rinsed once with medium, pooled and counted. The data presented are representative of two independent experiments.

Discussion

In this study, we examined the hematopoietic state of kl/kl mice with precocious aging in vivo and in vitro, focusing on the possible role of Klotho protein in proliferation and differentiation of hematopoietic stem cells. Early B cell development was preferentially impaired in BM from kl/kl mice, whereas myeloid cell development was not affected. However, hematopoietic stem cells in adult kl/kl mice were able to differentiate into mature B cells both in SCID mice in vivo, and in the presence of OP-9 stromal cells and IL-7 in vitro, and B cell development was not perturbed in neonates and young kl/kl mice without aging phenotypes. These results suggest that Klotho protein is not essential for B lymphopoiesis. This is supported by the evidence that B cell development is maintained by a nearly protein-free defined medium, i.e. Klotho protein-negative condition in vitro (34). Therefore, Klotho protein is considered to play a role in B cell differentiation indirectly and possibly to influence the hematopoietic microenvironment in BM.

The hematopoietic microenvironment in BM plays a critical role in B lymphopoiesis (11,12,35). It consists of a variety of cells including macrophages, fibroblasts, endothelial cells, pre-adipocytes, osteoblasts, osteoclasts and hematopoietic stem cells themselves. It is a complex, three-dimensional structure composed of these cells and abundant extracellular matrix (36), and these cells interact with each other to maintain constitutive hematopoiesis (36,37). In particular, the functional balance between osteoclasts and osteoblasts plays a critical...
**Fig. 4.** In vivo analysis of B lymphopoiesis by BM cells from 6-week-old *kl/kl* mice. BM cells (2×10⁶) from male +/+ or *kl/kl* mice were transplanted into the lethally irradiated (9.5 Gy) female +/+ mice (A) or irradiated (2 Gy) SCID mice (B). (A) Twenty weeks later, BM, spleen (Sp) and thymus (T) were harvested from each mouse and analyzed for the presence of donor-derived male cells by PCR. M, BM cells from +/+ male mice; F, BM cells from +/+ female mice. (B) Twenty weeks later, PB was obtained from each mouse, stained with mAb to CD3-FITC and B220-PE respectively, and analyzed for the presence of donor-derived lymphocytes. The number in each square represents the percentage within each subset. One representative result from three mice.

**Fig. 5.** Flow cytometric analysis of newborn liver and BM cells from *kl/kl* mice. The number in each square represents the percentage within the immature (B220⁺IgM⁻) and mature (B220⁺IgM⁺) B cells. One representative result from five to eight mice.
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Fig. 6. Histological analysis of BM from 6-week-old +/+ and kl/kl mice. Hematoxylin & eosin-stained sections of femur are shown. (A and B) Original magnification ×2. (C and D) Original magnification ×100.

Table 4. Effect of IL-7 treatment on B lymphopoiesis in BM from kl/kl mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>Fraction A + B + C</th>
<th>Fraction D</th>
<th>Fraction E + F</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>saline</td>
<td>5.9 ± 1.4</td>
<td>32.2 ± 6.2</td>
<td>10.1 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>IL-7</td>
<td>10.5 ± 1.5</td>
<td>59.3 ± 5.0b</td>
<td>13.4 ± 1.6</td>
</tr>
<tr>
<td>kl/kl</td>
<td>saline</td>
<td>0.7 ± 0.2</td>
<td>2.8 ± 0.8</td>
<td>1.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>IL-7</td>
<td>0.8 ± 0.2</td>
<td>3.4 ± 1.2</td>
<td>1.5 ± 0.8</td>
</tr>
</tbody>
</table>

Mice were s.c. injected with 500 ng/day of rIL-7 for 6 days (n = 4). The total nucleated BM cell counts obtained were multiplied by the frequency of relevant cell populations, as determined by FACS, in order to obtain cell counts for individual cell populations. The data represented are expressed as mean cell number (×10⁵/femur) ± SD of four mice. Values significantly different from their respective control groups at *P < 0.05 and **P < 0.01.

Fig. 7. Expression of IL-7 mRNA in BM of 6-week-old kl/kl mice. Expression of IL-7, SCF and M-CSF mRNAs was measured by semiquantitative RT-PCR in freshly isolated BM cells from +/+ and kl/kl. cDNAs serially diluted 8 times were used for PCR and starting amounts of cDNAs for the dilution were normalized by the amount of G3PDH. M, size marker. One representative result from three mice.

The role in bone remodeling and maintenance of hematopoietic microenvironment in BM including early B lymphopoiesis (38). Previously we showed that B lymphopoiesis is impaired in osteopetrotic c-fos-deficient mice and c-src-deficient mice (39), and concluded that the defect in B cell development in these mice is due to abnormalities of the microenvironment in BM and spleen as a consequence of osteopetrosis. These mice show the extramedullary hematopoiesis in spleen and selective impairment of B cell development, suggesting that the splenic hematopoietic microenvironment without osteoclasts and osteoblasts may not be suitable for B cell development. Since the hallmark of osteoporosis is a reduction in skeletal mass caused by an imbalance between bone resorption and bone formation (40), osteoporosis may also perturb the hematopoietic microenvironment in BM. As B
lymphopoiesis is affected by aging (7–10), changes of the hematopoietic microenvironment caused by osteoporosis are considered to be critical for B lymphopoiesis.

As the development of early B cell precursors depends on IL-7 produced by stromal cells (11,12,35,41–43), it was reasonable to ascribe the age-associated decline in the number of pre-B cells to a decreased IL-7 production (9), release (13) or responsiveness in BM (10). We found that expression of IL-7 mRNA by fresh BM cells was reduced in kl/kl mice, but that stromal cells generated in Whitlock-Witte-type long-term BM cultures from kl/kl mice could maintain IL-7 mRNA expression and B lymphopoiesis (data not shown). Since expression of SCF and M-CSF mRNA and myeloid differentiation were not impaired in kl/kl mice, only the function of IL-7-producing subsets of stromal cells might be suppressed in vivo and IL-7 production of the subsets was recovered by in vitro culture. The reduced expression of BP-1 in the CD43+ compartment in kl/kl BM (Fig. 2B) was consistent with a reduction in IL-7 availability in kl/kl BM, since IL-7 can up-regulate BP-1 expression on pro-B cells and induce proliferation of early B-lineage cells (44). Furthermore, IL-7 administration could not rescue the B lymphopenia in vivo (Table 4), suggesting that IL-7 responsiveness is also impaired in BM from kl/kl mice.

Although IL-7 is required for development of pre-B cells (35,41), it is not sufficient for survival and proliferation of B-lineage cells (35,42,43,45). Stromal cell contact is essential for B lymphopoiesis (11,12,42,43). Stromal cells are thought to elaborate many important factors for B lymphopoiesis (35,45,46) and molecules that promote adhesion of B cell precursors to stromal cells (47). As B cell precursors from kl/kl mice can proliferate in response to IL-7 in vitro (Fig. 3), it is possible that negative regulators are produced by stromal cells in vivo (48,49). In addition, the three-dimensional structure of BM plays a very important role in the in vivo organization of B lymphopoiesis. Progenitor cells are initially located in or near the endosteum of the BM and begin to differentiate in the peripheral region of BM (50). Thus, changes of these structures due to klotho deficiency (possible osteoporosis) may cause the altered B lymphopoiesis. Therefore, possible mechanisms to inhibit B lymphopoiesis in BM by klotho deficiency are as follows: (i) loss of Klotho protein modulates positive signals for B lymphopoiesis, (ii) Klotho protein may control synthesis of negative regulators and (iii) loss of Klotho protein changes the three-dimensional structure of hematopoietic microenvironment or suppresses some specific cell types important for B lymphopoiesis.

kl/kl mice show the abnormalities in the pituitary gland (15). The pituitary gland controls many other endocrine glands and is thus indirectly involved in many physiological functions (51) and its importance for the immune system has recently been recognized (52,53). Furthermore, it is known that only thyroid hormone is critical for normal B cell development among pituitary gland-related hormones (54,55). Although the free thyroxine level (0.68 ± 0.51 ng/dl) in serum of kl/kl mice was significantly lower than that (1.20 ± 0.19 ng/dl) of wild-type mice as expected (mean ± SD of four pools of four mice each, P < 0.01), thyroxine treatment failed to restore the frequency of B cell populations to normal (data not shown). These results indicate that hypothyroidism is not essential for B lymphopenia in kl/kl mice. One explanation for the observation is that Klotho protein may play an important role in the responsiveness to thyroxine and that the decreased responsiveness may be a part of the reason for B lymphopenia in kl/kl mice.

In contrast to the reduction of B lymphocyte progenitors, the frequency of myeloid progenitors and day 12 CFU-S did not decrease in BM from kl/kl mice. Furthermore, BM cells from kl/kl mice could reconstitute hematopoiesis in the lethally irradiated mice (Fig. 4A), indicating that primitive hematopoietic stem cells with long-term repopulating ability and myeloid cell differentiation appear to be normal in kl/kl mice. The function of hematopoietic stem cells is known to change with age (56,57). The proliferative potential of BM cells from aged mice is greater than or equal to that of BM cells from young mice (58–61). Recently, Morrison et al. showed that the frequency of hematopoietic stem cells and myeloid committed progenitors and the ratio of hematopoietic stem cells in cell cycling were increasing with age (62). However, it is unknown whether these changes are determined intrinsically or caused by changes of the hematopoietic microenvironment in aged mice. Since expression of the klotho gene cannot be detected in lymphocytes, BM cells and bone, the secreted isoform may play a major role in hematopoiesis (15–17). Further investigation is necessary to determine a receptor for the secreted form of Klotho protein to understand not only the mechanisms of aging in kl/kl mice but also the function of hematopoietic microenvironment in B lymphopoiesis.

In conclusion, we showed that adult kl/kl mice suffer from severe B lymphopenia according to the impaired B cell development in BM. Since the functions of hematopoietic stem cells are normal, changes of the hematopoietic microenvironment in BM may be responsible for this phenomenon. Further studies including target cells for the secreted form of Klotho protein in BM clarify the mechanisms of B lymphopenia in kl/kl mice.

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Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>CFU-S</td>
<td>colony forming unit in spleen</td>
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<td>k/lk mice</td>
<td>homozygous klotho mice</td>
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<td>Lin</td>
<td>lineage markers</td>
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<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
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<td>PB</td>
<td>peripheral blood</td>
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<td>PE</td>
<td>phycoerythrin</td>
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<td>PI</td>
<td>propidium iodide</td>
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<td>SCF</td>
<td>stem cell factor</td>
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<tr>
<td>+/- mice</td>
<td>control littermate mice</td>
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</tbody>
</table>

References
Impairment of B lymphopoiesis in klotho mice


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