Senescent B Lymphopoiesis Is Balanced in Suppressive Homeostasis: Decrease in Interleukin-7 and Transforming Growth Factor-β Levels in Stromal Cells of Senescence-Accelerated Mice

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The suppression of the B cell population during senescence has been considered to be due to the suppression of interleukin-7 (IL-7) production and responsiveness to IL-7; however, the upregulation of transforming growth factor-β (TGF-β) was found to contribute to B cell suppression.To investigate the mechanism of this suppression based on the interrelationship between IL-7 and TGF-β during senescence, senescence-accelerated mice (SAMs), the mouse model of aging, were used in this study to elucidate the mechanisms of B lymphopoietic suppression during aging. Similar to regular senescent mice, SAMs showed a decrease in the number of IL-7–responding B cell progenitors (i.e., colony-forming unit pre-B [CFU-pre-B] cells in the femoral bone marrow [BM]). A co-culture system of B lymphocytes and stromal cells that the authors established showed a significantly lower number of CFU-pre-B cells harvested when BM cells were co-cultured with senescent stromal cells than when they were co-cultured with young stromal cells. Interestingly, cells harvested from a senescent stroma and those from the control culture without stromal cells were higher in number than those harvested from a young stroma, thereby implying that an altered senescent stromal cell is unable to maintain self-renewal of the stem cell compartment. Because TGF-β is supposed to suppress the proliferative capacity of pro-B/pre-B cells, we added a neutralizing anti-TGF-β antibody to the co-culture system with a pro-B/pre-B cell-rich population to determine whether such suppression may be rescued. However, unexpectedly, any rescue was not observed and the number of CFU-pre-B cells remained unchanged when BM cells were co-cultured with senescent stromal cells compared with the co-culture with young stromal cells, which essentially showed an increase in the number of CFU-pre-B cells ($P < 0.001$ in 5 μg/ml). Furthermore, TGF-β protein level in the supernatant of cultured senescent stroma cells was evaluated by enzyme-linked immunoabsorbent assay, but surprisingly, it was found that TGF-β concentration was significantly lower than that of cultured young stromal cells. Thus, TGF-β activity was assumed to decline particularly in a senescent stroma, which means a distinct difference between the senescent suppression of B lymphopoiesis and secondary B lymphocytopenia. Concerning proliferative signaling, on the other hand, the level of *IL-7* gene expression in cells from freshly isolated BM decreased significantly with age. Therefore, the acceleration of proliferative signaling and the deceleration of suppressive signaling may both be altered and weakened in a senescent stroma (i.e., homeosupression). Exp Biol Med 229:494–502, 2004

Key words: aging; B-lymphopoiesis; interleukin-7; transforming growth factor-β; senescence-accelerated mice; homeosuppression

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Introduction

Aging is accompanied by changes in the immune system, leading to a decrease in the overall cellular and humoral responsiveness (1). Because the most marked age-associated change in the immune system is the rapid involution of the thymus after puberty, most of the decline in humoral immunity has been attributed to changes in the T-cell compartment rather than to an intrinsic primary B cell

deficit. Consequently, attention has been focused on age-associated changes in T lymphocytes and their functions (2). However, it has recently been clarified that there are also deficiencies in B cell development in the bone marrow (BM) of aged animals (3–6). Alterations in B cell development may include both the skewing of *V*-gene utilization, particularly in cells responsive to phosphoryl choline, and the decrease in the generation of various developmental B cell subsets. The altered representation of these subsets appears to be a consequence of a developmental arrest of the maturation of pro-B cells and the earliest stage of surface Ig-positive cells (7). Age-related changes in the B cell development may account for the deterioration of the immune system in senescent mice.

B lymphopoiesis is suppressed during senescence not only in mice but also in humans. A decrease in interleukin-7 (IL-7) production by stromal cells and a simultaneous reduction in B lymphocyte reactivity to IL-7 are considered as a possible background for this negative senescent regulation. Furthermore, in addition to senescence, B cells were noted to be regulated by two pathways not only for IL-7 but also for transforming growth factor-β (TGF-β) in regular mice; that is, not only the downregulation of the former but also the upregulation of the latter simultaneously play a role in suppression regulation (8). This is in good agreement with the observation that the supplementation of IL-7 could not compensate for the B cell suppression. In this study, possible senescence-associated alterations in the productions of IL-7 and TGF-β are examined in senescence-accelerated mice (SAMs).

Senescence-accelerated mice provide a unique model system for studying senescence or aging in higher organisms, because they exhibit a marked acceleration of aging, which has been confirmed to be the same manner as that observed in the regular mice. Senescence-accelerated mice are characterized by the early onset of aging (mean life span of 40 weeks under conventional conditions), loss of general behavioral activity, increased skin coarseness, and spinal lordokyphosis (9). Because the number of splenic cells starts to decrease at approximately 30 weeks old, the SAMs used were, in general, 30 weeks old or slightly older. Although one must carefully interpret the results of studies using SAMs because the mechanism of "accelerated aging" may not be associated with that of "normal aging," results of previous studies conducted by other researchers and ourselves indicate that SAMs are a suitable model for predicting the possible mechanism of aging in hematopoietic systems (10–14).

The purposes of this study are to confirm the status of B lymphopoiesis in SAMs compared with that in other regular strains and to elucidate the mechanism of age-related changes in B lymphopoiesis in SAMs. Here, we examined age-related changes in the number and function of B cell progenitors in the BM and their supportive microenvironment.

Materials and Methods

Mice. A senescence-prone substrain of the AKR/J mouse, SAMs/-P-1 (9), from The Jackson Laboratory in Bar Harbor, ME, was kindly provided by Dr. Toshio Takeda, Emeritus, the Chest Disease Research Institute, Kyoto University. The mice were bred and maintained at the experimental animal facility of the National Institute of Health Sciences under pathogen-free conditions. Male SAMs designated as "young (8–12 weeks old)" or "senescent (30–36 weeks old)" were used in the present study; these ages were selected because the number of splenic cells and/or hemopoietic progenitor cells start to decrease at approximately 30 weeks of age (11).

Preparation of BM Cells. The BM cell suspensions were prepared by repeatedly flushing the cells from femurs and dispersing them by trituration through a 23-gauge hypodermic needle with the Iscove-modified Dulbecco medium (IMDM; Invitrogen Corp., Carlsbad, CA) or RPMI 1640 medium (Invitrogen).

***In Vitro* Colony Assays.** Colony-forming unit pre-B (CFU-pre-B) cells were assayed by suspending mononuclear cells in 1-ml aliquots of the recombinant IL-7 (rIL-7)–supplemented MethoCult M 3630 medium (Stem Cell Technologies Inc., Vancouver, Canada) in 35-mm, plastic petri dishes. Femoral BM cells from three mice per group were pooled and assayed. A MethoCult M 3630 medium consisting of 1 ml of the semisolid IMDM medium containing 1% methylcellulose, 30% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT), 0.1 m*M* 2-mercaptoethanol (2-ME), 2 m*M* L-glutamine, and 10 ng/ml of rIL-7 (R&D Systems, Inc., Minneapolis, MN) was used. Granulocyte-macrophage colony-forming units (GM-CFUs) were assayed by suspending mononuclear cells in the alpha medium containing 1% methyl cellulose, 30% FBS, 1% bovine serum albumin, 1 m*M* 2-ME, and 10 ng/ml of granulocyte-macrophage colony-stimulating factor (Genzyme, Cambridge, MA) and plating 1-ml aliquots in 35-mm, plastic dishes. Both CFU-pre-B cells and GM-CFUs in culture plates in triplicate were incubated at 37°C in a fully humidified atmosphere of 5% carbon dioxide in air. Aggregates of 50 or more cells in 7-day cultures were counted as colonies. Aggregates ranging from 10 to 49 cells were counted as clusters.

Co-culture of Stromal Monolayers and Pro-B/Pre-B Cell-Rich Populations. Stromal monolayers were prepared by culturing BM cells derived from young or senescent SAMs at 1×10^6/ml in 96-well Coster 3596 or 24-well Falcon 3047 flat-bottomed plates in 0.2 or 1 ml of the RPMI 1640 medium supplemented with 20% FBS. Confluent adherent layers were formed after 7 days. To obtain pro-B/pre-B cell-rich populations, the bulk culture of pooled BM cells from young SAMs stimulated with rIL-7 was performed as described previously (4). Briefly, BM cells from young SAMs were cultured at 1×10^6 cells/ml in RPMI 1640 supplemented with 20% FBS, 2×10^{-5} *M*

2-ME, 1% L-glutamine, and 2 ng/ml of murine rIL-7 (Genzyme) and plated in six-well Coster 3516 culture trays. Nonadherent cells were harvested after 4 days of culture. This bulk culture provided a highly rich (>10-fold) source of IL-7–responsive $B220^+$, $CD43^+$, IgM^-, pro-B/pre-B cells (data not shown). Pro-B/pre-B cell-rich populations were suspended at 5×10^4/ml in RPMI 1640 supplemented with 20% FBS, 2×10^{-5} *M* 2-ME, 1% L-glutamine, and 1 ng/ml of murine rIL-7. Aliquots (0.1 or 1.0 ml) of this cell suspension were added to established stromal cell monolayers in 96- and 24-well flat-bottomed trays, respectively, and co-cultured at 37°C in a fully humidified atmosphere of 5% carbon dioxide in air. Nonadherent cells were harvested after 3 days, counted, and cloned using the CFU-pre-B colony assay system.

Extraction of Total RNA and Polymerase Chain Reaction (PCR). Total RNA was extracted from BM cells using the TRIsol reagent (Invitrogen) according to the manufacturer's instructions. First, messenger RNA (mRNA) was reverse transcribed using superscript (Life Technologies, Grand Island, NY) and random hexamers. Next, PCR amplification of complementary DNA (cDNA) was performed with the graded dilution of cDNA for semi-quantitative evaluation of IL-7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression under the following conditions: IL-7 cDNA, 95 °C for 1 min, 55°C for 2 mins, and 72°C for 3 mins for 35 cycles; and GAPDH cDNA, 94°C for 30 secs, 60°C for 30 secs, and 72°C for 1 min for 20 cycles. Murine IL-7 and GAPDH primers were synthesized based on a published cDNA sequence (15):

IL-7 (sense) 5'-GCCTGTCACATCTGAGTGGC-3'

IL-7 (antisense) 5'-CAGGAGGCATCCAG-GAACTTCTG-3'

GAPDH (sense) 5'-TGAAGGTCGGTGTGAACG-GATTTGGC-3'

GAPDH (antisense) 5'-CATGTAGGCCATGAGGTC-CACCAC-3'

The expected amplified PCR products were 496 and 982 base pairs long for IL-7 and GAPDH, respectively. The PCR products were photographed using the Bio-Rad 2000 gel documentation system (Bio-Rad Laboratories, Hercules, CA), and intensities of expressions were evaluated by ImageGauge version 3.11 (Science Lab 98 for Windows; Fuji Film, Tokyo, Japan). In this experiment, GAPDH expressions were not altered among the experimental groups.

Effect of Anti–TGF-β Antibody on Growth of Pro-B/Pre-B Cell-Rich Population Co-Cultured with Stromal Cells. To examine the effect of TGF-β produced by stromal cells on the growth of pro-B/pre-B cells, a neutralizing monoclonal antibody (mAb) to TGF-β (mouse IgG_1 isotype, R&D Systems) at dilutions ranging from 1–10 μg/ml was added to the co-culture system. The mouse IgG_1 isotype (R&D Systems) was used as mock control. The number of nonadherent cells in the co-cultures was determined 3 days later.

Determination of Level of TGF-β Protein Produced by Cultured Stromal Cells. Stromal monolayers were prepared by culturing BM cells from young and senescent SAMs at 1×10^6/ml in 24-well Falcon 3047 flat-bottomed plates in 1 ml of the RPMI 1640 medium supplemented with 20% FBS. Confluent adherent layers were obtained after 7 days. The supernatant in the culture plates was removed; and then 1 ml of RPMI 1640, supplemented with 20% FBS, 2×10^{-5} *M* 2-ME, and 1% L-glutamine were added to the culture plates. The culture medium was collected after 7 days of culture and was used for the determination of the level of the TGF-β protein produced by stromal cells. The TGF-β concentration in the culture medium was determined using a TGF-β–specific enzyme-linked immunoabsorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions. All the samples were assayed in triplicate. The samples were acid activated (16) by adding 1/5 vol of 1 *N* hydrochloride at room temperature and neutralized after 10 mins by adding of 1/5 vol of 1.2 *N* NaOH in 0.5 *M* HEPES, and the mixture was diluted with the same volume of calibrator diluents in the ELISA kit.

Statistical Analysis. Data were analyzed using the analysis of variance (ANOVA). Values were considered significantly different at $P < 0.05$.

Results

Decrease in Number of B Cell Progenitors (Pre-B Cells). Age-related changes in the numbers of B lymphocytes and hematopoietic progenitors differ from each other. Table 1 summarizes the results of the triplicate experiments. The number of femoral GM-CFU cells from 30-week-old and 36-week-old senescent mice assayed on the basis of their colony-forming ability increased to 112% and 109%, respectively, that of GM-CFU from 12-week-old mice. In contrast, the CFU-pre-B colony assay, using Day 7 B cell colonies as the end point, was used to determine the number of IL-7–responsive B cell progenitors in young and senescent BM cells. The numbers of femoral CFU-pre-B cells from 30-week-old and 36-week-old mice decreased to 75.7% and 65.0%, respectively, that from 12-week-old mice. Furthermore, the decrease in the number of CFU-pre-B cells from femoral BM in senescent mice could not be counteracted by increasing IL-7 concentration in the culture medium 4-fold or by extending the culture period (data not shown).

Significant Decrease in Number of Large Pre-B Colonies. Among B cell colonies of various sizes, we noted that the number of relatively larger B cell colonies decreased significantly with aging (Fig. 1). The number of cells per colony ranged from 50–5000. Therefore, CFU-pre-B cell colonies in Table 1 were categorized according to their size, namely, small (50–200 cells), intermediate (201–

Table 1. Age-Related Changes in Number of Hematopoietic Progenitor Cells in Senescence-Accelerated Mice[a]

	Mean ± SEM of triplicate experiments (%)		
	12-week-old mice	30-week-old mice	36-week-old mice
Femoral GM-CFU cells	72,762 ± 672	81,250 ± 2811 (112%)	79,058 ± 4763 (109%)
Femoral CFU-pre-B cells	13,868 ± 516	10,505 ± 1083* (75.7%)	9017 ± 220** (65.0%)

[a] GM-CFU, granulocyte-macrophage colony-forming unit; CFU-pre-B, colony-forming unit pre-B.
* $P < 0.05$; ** $P < 0.001$.

3000), and large (>3000 cells). As shown in Figure 1, the numbers of large, intermediate, and small B cell colonies for all groups decreased with age (58.3% for large colonies, 75.8% for intermediate colonies, 78.4% for small colonies in 30-week-old mice relative to those in 12-week-old mice; 22.1% for large colonies, 52.0% for intermediate colonies, and 76.5% for small colonies in 36-week-old mice relative to those in 12-week-old mice). The decrease in the numbers was statistically most significant for large colonies in 30- and 36-week-old mice relative to those to 12-week-old control ($P < 0.001$ and $P < 0.005$, respectively) and also for intermediate colonies in 36-week-old mice relative to those in 12-week-old control ($P < 0.05$).

Decrease in IL-7 Expression Level in BM. As observed in the regular senescent mice, the number of pre-B cell progenitors also decreased in SAMs. Therefore, the expression level of IL-7, which is known to be a pre-B cell stimulator, was evaluated in BM cells. IL-7 expression level decrease during senescence (17–19). In this study, IL-7 mRNA expression level was evaluated by reverse transcriptase (RT)–PCR. The BM stromal cell–derived IL-7 is a positive regulator of *in vivo* B lymphopoiesis. As shown in Figure 2, the IL-7 mRNA expression level in BM cells from senescent mice was 6.2% that from young mice. These findings are comparable to those in the literature (17–19). Further experiments using SAMs were designed.

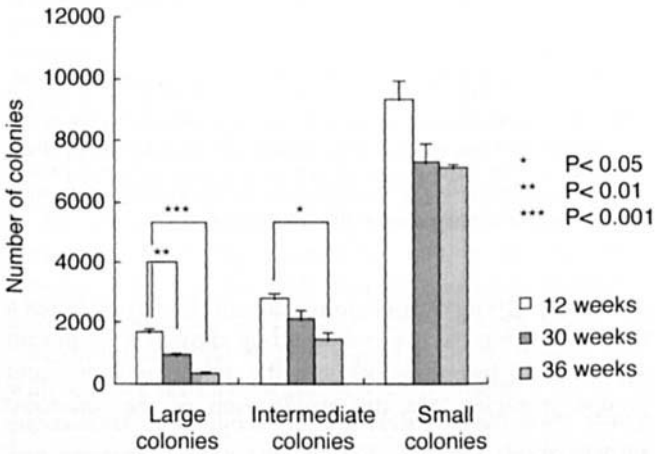

Figure 1. Age-related changes in number of B cell colonies of large, intermediate, and small sizes (mean ± SEM of three replicate experiments). Femoral bone marrow (BM) cells from three mice per group were harvested and pooled. The preparation of BM cell suspensions is described in the "Materials and Methods" section. Colony-forming unit pre-B (CFU-pre-B) cells were assayed by suspending mononuclear cells in 1-ml aliquots of the recombinant interleukin-7 (rIL-7)–supplemented MethoCult M 3630 medium (Stem Cell Technologies Inc., Vancouver, Canada) in 35-mm, plastic petri dishes. Culture plates in triplicate for CFU-pre-B cells were incubated at 37 °C in a fully humidified atmosphere of 5% carbon dioxide in air. According to the size of CFU-pre-B cell colonies shown in Table 1, colonies were categorized as follows: small (50–200 cells), intermediate (201–3000), and large (>3000 cells).

Decrease in Proliferative Capacity of B Cell Progenitors, Pre-B Cell Response to IL-7. The decrease in IL-7 expression level is also associated with the decrease in the responsiveness of pre-B cells to IL-7. To evaluate such responsiveness, we used a recloning assay to determine the proliferative capacity of the progeny of CFU-pre-B cells. Cells derived from 36 large colonies were pooled and recloned for 7 days in a semisolid medium supplemented with rIL-7. Table 2 shows the results of the recloning study. The numbers of secondary colonies, which included small (50–200 cells) and intermediate (201–3000) colonies and clusters (10–49 cells) generated from individual large primary colonies and derived from 30-week-old and 36-week-old femoral BMs, decreased significantly to 69.0% and 2.7%, respectively, that of secondary colonies grown from large primary colonies derived from 12-week-old femoral BM. Furthermore, cells from small primary colonies derived from either young or senescent mouse BM formed no secondary colonies. In B lymphopoiesis, unlike in the case of myelogenous progenitors, the results indicate that the responsiveness of CFU-pre-B cells to IL-7 decreases with age.

Decrease in Maintenance Capacity of Stromal Cells for B Lymphopoiesis. Although the pre-B progenitor cells were altered during senescence, a decrease in the maintenance capacity of stromal cells for B cell lineages may also be of importance in association with hematopoietic senescence (17, 18, 20). Using a co-culture system in 24-well flat-bottomed trays, we determined whether the capacity of stromal cells to support B lymphopoiesis is altered with age. Interestingly and unexpectedly, the number of lymphocytes recovered from the coculture of pro-B/pre-B cells with young stromal cells was significantly lower than that recovered from senescent stromal cells (Fig. 3A). In contrast, the total number of CFU-pre-B cells recovered from the co-culture with young stromal cells was significantly higher than that recovered from the co-culture with senescent stromal cells (Fig. 3B).

Decrease in TGF-β Production by Senescent Stromal Cells. On the basis of the above-mentioned co-

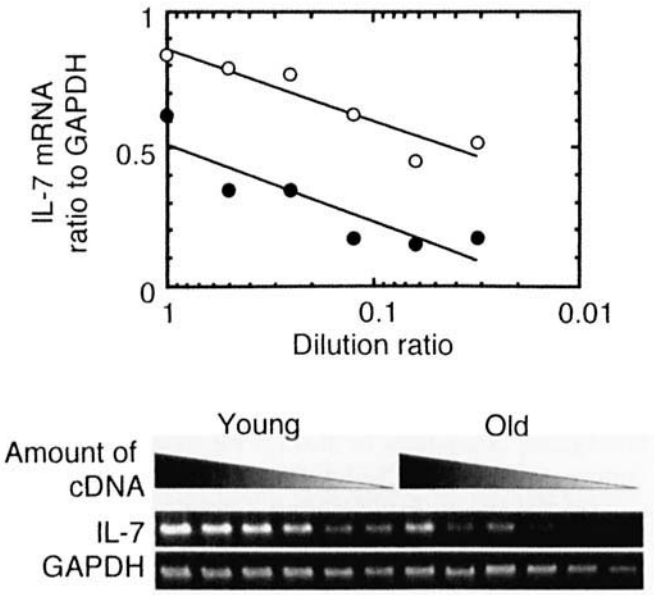

Figure 2. Expression level of interleukin-7 (IL-7) messenger RNA (mRNA) in bone marrow (BM) cells freshly isolated from young and old senescence-accelerated mice (mean ± SEM of three replicate experiments). Vertical bars for SEM are within the symbols. Total RNA was extracted from BM cells using the TRIsol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The extracted mRNA was reverse transcribed using Superscript (Life Technologies, Grand Island, NY) and random hexamers. The reverse-transcribed complementary DNAs (cDNAs) are then amplified by polymerase chain reaction (PCR) using specific primers for murine IL-7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The conditions and primer sequences used for the PCR amplification of cDNAs are shown in the "Materials and Methods" section of reference 15. The expected amplified PCR products were 496 and 982 base pairs long for IL-7 and GAPDH, respectively. In this experiment, GAPDH expressions were not altered among the experimental groups.

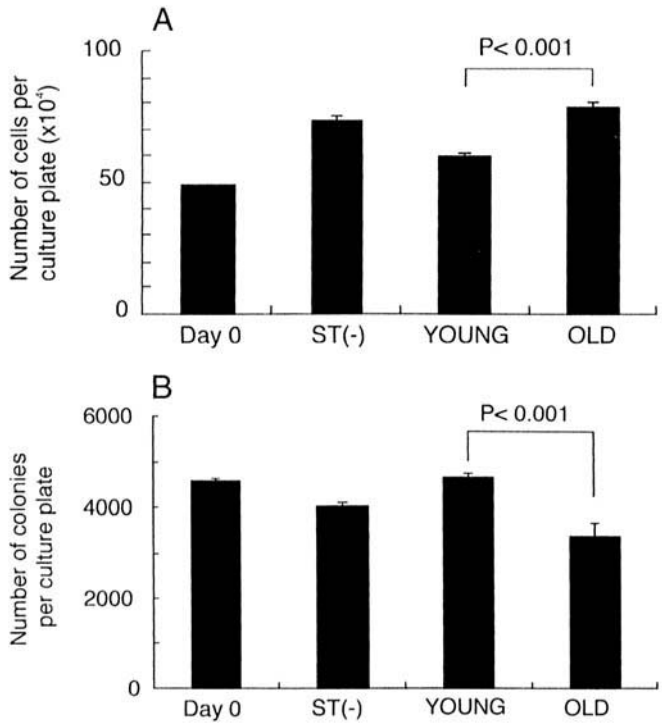

Figure 3. (A) Co-culture of pro-B/pre-B cell-enriched populations with stromal cells: change in the number of nonadherent cells (mean ± SEM of three replicate experiments). (B) Co-culture of pro-B/pre-B cell-rich populations with stromal cells: change in total number of CFU-pre-B cells (mean ± SEM of three replicate experiments). Stromal monolayers were prepared by culturing bone marrow (BM) cells from young or senescent senescence-accelerated mice (SAMs) at 1×10^6/ml in 96-well Coster 3596 or 24-well Falcon 3047 flat-bottom plates in 0.2 or 1 ml of the RPMI 1640 medium supplemented with 20% fetal bovine serum. Confluent adherent layers were formed after 7 days. To obtain Pro-B/pre-B cell-rich populations (>10-fold) (i.e., interleukin-7 [IL-7]–responsive $B220^+$, $CD43^+$, IgM^-, pro-B/pre-B cells), pooled BM cells from young SAMs stimulated with recombinant IL-7 were cultured, as described in the "Materials and Methods" section (4). Nonadherent cells were harvested and counted. Day 0 indicates nonadherent cell number at the beginning of co-culture; ST (-), nonadherent cell number after culture with IL-7 alone; YOUNG, nonadherent cell number after co-culture with young stromal cells in the presence of IL-7; OLD, nonadherent cell number after co-culture with senescent stromal cells in the presence of IL-7.

culture data, we propose a hypothesis that the CFU-pre-B inhibitory activity of BM cells may reside predominantly in young stromal cells rather than in senescent stromal cells. Because stromal cells produce BM-derived TGF-β (21) and are also a negative regulator of B lymphopoiesis (22–24), we investigated whether TGF-β production by stromal cells is reduced with age. Figure 4 shows percent changes in the number of the same seeded BM cells cocultured with young stromal cells (open circles) or senescent stromal cells (closed circles) in 96-well flat-bottomed trays after adding a graded dose of a neutralizing mAb to TGF-β. The high-dose group (10 μg/ml and more; data not shown) exhibited a toxic effect, but the lower-dose group showed a significant difference of responses between the young and senescent groups, implying that the proliferation of the senescent

Table 2. Secondary B Cell Colonies Derived From One Large Colony-Forming Unit B Cell Colony[a]

Donor mouse age (week)	Mean ± SEM of triplicate experiments					
	Large	Intermediate	Small	Cluster	No. of total colonies[b] (with cluster)[c]	% to a/% to b
12	ND	1.4 ± 0.6	5.8 ± 0.7	7.2 ± 0.6	7.2 ± 0.6 (14.4 ± 1.0)	100%/100%
30	ND	ND	2.3 ± 0.5	7.4 ± 1.7	2.3 ± 0.5* (9.7 ± 1.4)**	24%/69%
36	ND	ND	ND	0.4 ± 0.2	ND (0.4 ± 0.2)**	—/2.7%

[a] Percentages for 30-week-old or 36-week-old mice compared with 12-week-old mice are shown in parentheses. ND, not detected.
[b] Number of total colonies by large, intermediate, and small B cell colonies without clusters.
[c] Number of total colonies with clusters.
* $P < 0.005$; ** $P < 0.01$.

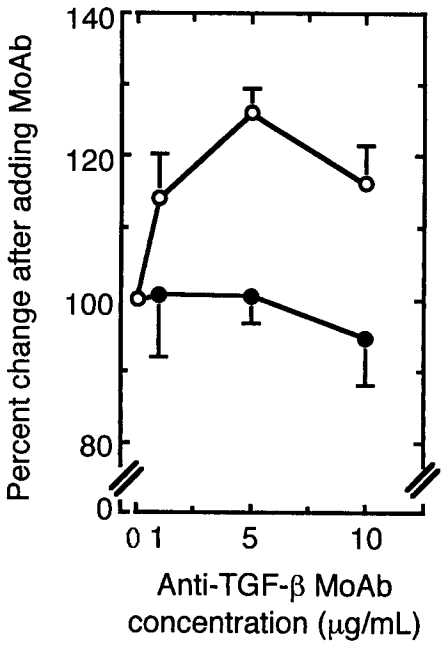

Figure 4. Effect of neutralizing anti–transforming growth factor-β (TGF-β) monoclonal antibody (mAb) on proliferation of pro-B/pre-B cells from young senescence-accelerated mice co-cultured with femoral stromal cells from young mice (open circles) and those from senescent mice (closed circles). The effect of TGF-β on the growth of pro-B/pre-B cells was evaluated on the basis of the number of nonadherent cells after the supplementation of the neutralizing antibody to TGF-β (mouse IgG_1 isotype: R&D Systems, Inc., Minneapolis, MN) at dilutions ranging from 1–10 μg/ml to the coculture system. The mouse IgG_1 isotype (R&D Systems) was used as the mock control. The senescent group shows no substantial rescue effect of mAb, whereas the young group shows significant rescue effect of mAb by repeated-measure analysis of variance testing (P = 0.0111). (Vertical bars indicate SEM of triplicate experiments).

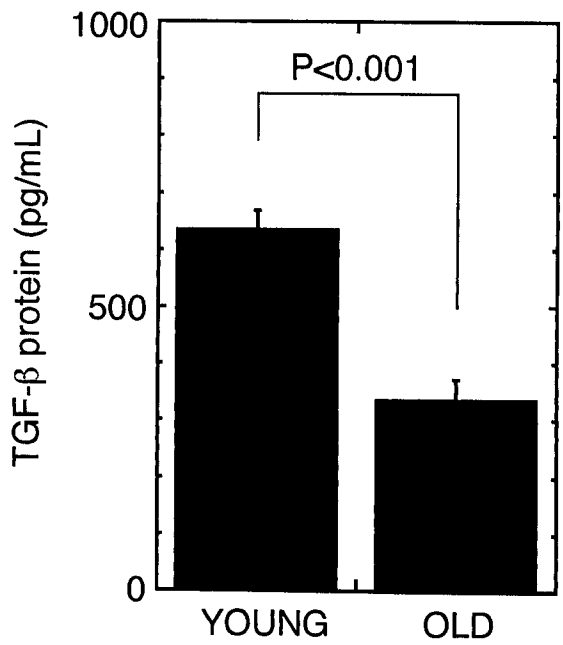

Figure 5. Transforming growth factor-β (TGF-β) protein level in the supernatant of cultured stromal cells from young and senescent senescence-accelerated mice (SAMs) (mean ± SEM of three replicate experiments). Stromal monolayers were prepared by culturing bone marrow (BM) cells from young or senescent SAMs at 1×10^6/ml in 24-well Falcon 3047 flat-bottom plates in 1 ml of the RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS). Confluent adherent layers were formed after 7 days. The supernatant of culture plates was removed, 1 ml of RPMI 1640 was supplemented with 20% FBS, and 2×10^{-5} *M* 2-ME and 1% L-glutamine were added to the culture plates. The culture medium was collected after culture for 7 days and was used for determination of the level of the TGF-β protein produced by stromal cells. The TGF-β concentration in culture medium was determined using a TGF-β–specific enzyme-linked immunoabsorbent assay kit.

group was unexpectedly not recovered by the neutralizing antibody, whereas (although it was not expected) the proliferation of the young group was prominently recovered at 1- and 5-μg/ml doses (statistical significance, $P < 0.001$ in the group of 5 μg/ml). Thus, despite the prominent decrease in IL-7 expression level and in the biological activity of IL-7 in the BM, TGF-β production seemed to have unexpectedly decreased in the senescent group. To confirm the decrease in TGF-β production by stromal cells with age, we directly measured TGF-β protein level in the supernatant of cultured stromal cells derived from young and senescent SAMs using ELISA. Figure 5 shows that the TGF-β protein level in the supernatant of cultured senescent stromal cells is markedly lower than that of cultured young stroma, thus implying that TGF-β production by stromal cells decreases with age (634.0 ± 36.0 vs. 337.0 ± 37.9, young and old, respectively, $P < 0.001$).

Discussion

It has long been questioned whether age-related alterations in B lymphopoiesis are mainly due to a functional impairment of B cell precursor cells or due to that of senescent stromal cells. Several studies showed steady-state B lymphopoiesis and focused on the decrease in B cell production due in part to the decreased IL-7 responsiveness (25, 26). Indeed, our initial experiments demonstrated that the numbers of femoral CFU-pre-B cells (3-6), particularly those forming large colonies, decrease with age, suggesting that age-related alterations of B lymphopoiesis seem to be based on the quality of B cell precursors (Table 1 and Fig. 1).

The expression level of IL-7, a pre-B cell stimulator, was evaluated in BM cells, because IL-7 production decreases during senescence (17–19). Mice in which the *IL-7* gene has been knocked out manifest a prominent decrease in the number of pre-B lymphocytes and a severe impairment in capacity for self-renewal in the pre-B cell compartment, even though the number of pro-B cells appears to be normal. Phenotypically, age-related changes seem closer to the changes in the B lymphopoiesis observed in aged mice (27). Our data showed that the IL-7 mRNA expression level in freshly isolated BM cells decreases with age (Fig. 2). We attempted to measure IL-7 expression level only in freshly isolated BM cells and BM stromal cells, because the expression of IL-7 is easily upregulated

immediately after the start of culture. Because of the limited materials for ELISA, protein expression level was not determined. These findings are consistent with those of Updyke *et al.* (17), who reported that the relative quantity of the IL-7 protein released into the medium for long-term B cell culture decreases with age. A study by Stephan *et al.* (18) suggested that the age-related decrease in the function of BM cells is associated with the impaired release of IL-7. Interestingly, a novel mutant mouse model of aging, *klotho*, was reported to exhibit a similar significant decrease in the level of *IL-7* gene expression in freshly isolated BM cells as determined by RT-PCR analysis (19); the mouse initially exhibits multiple disorders that resemble various aging phenotypes. Based on the findings by other researchers and ourselves, it seems likely that IL-7 production by BM stromal cells decreases with age. As seen in Figures 1–4, comparable results were obtained in reports available in the literature; hence, the present findings obtained through the experiment using SAMs may be applicable to the analysis of natural aging in regular mice.

Next, we demonstrated that the production of TGF-β by marrow stromal cells decreased with age, although the mechanism underlying this phenomenon is not yet known. Young stromal cells inhibited B cell proliferation in the co-culture system, and this inhibition was reversed by treatment with antibodies to TGF-β. The results of the co-culture system demonstrated that significantly fewer lymphocytes could be recovered from the co-culture system with young stromal cells than with senescent stromal cells; conversely, a significantly higher number of CFU-pre-B cells is maintained in the co-culture system with young stromal cells than with senescent stromal cells (Fig. 3A and B). Moreover, the neutralizing antibody to TGF-β restored the proliferative capacity of pro-B/pre-B cells co-cultured with young stromal cells but not that of those co-cultured with senescent stromal cells (Fig. 4). Furthermore, the TGF-β protein level in supernatant of cultured senescent stromal cells is markedly lower than that of young stromal cells (Fig. 5). These results imply that senescent stromal cells are not capable of producing TGF-β. These data agree with those reported by Dubinett *et al.* (28) that IL-7 downregulates both mRNA expression and protein production of TGF-β by murine macrophages. Thus, it seems unlikely that exogenous IL-7 added to our co-culture system would induce TGF-β production by stromal cells derived from a young stroma. Furthermore, Gazit *et al.* (29) have recently reported that fibroblast CFU (CFU-F) isolated from senescent mice produces less TGF-β *in vitro* than CFU-F from young mice and that the matrix of long bones of senescent mice contains less TGF-β than that of young mice. These data suggest that the production of the CFU-pre-B cell regulator TGF-β by stromal cells may decrease with age. Consequently, CFU-pre-B cells co-cultured with senescent stromal cells may proliferate and/or differentiate more rapidly than CFU-pre-B cells co-cultured with young stromal cells in the presence of IL-7.

In the present study, we observed that the CFU-pre-B cell number in the BM decreased with age, whereas, as we have observed previously (13), the total number of splenic B cells remained relatively unchanged. These findings are consistent with those observed in other murine strains by other researchers (3–6) and have been considered to be mediated by a decrease in B lymphocyte production in the BM and increased longevity of mature B cells (30). Furthermore, our data revealed an intrinsic defect in the B-progenitor-cell response to IL-7, as well as an age-related impaired production of not only IL-7 but also TGF-β by stromal cells. In SAMs, the arrest of pro-B cell maturation with advanced aging was evidently associated with the decrease in the number of pre-B cells. This may be explained by the coexistence of an intrinsic defect in the B-progenitor-cell response to IL-7 (i.e., pre-B cells and more immature pro-B cells); this interpretation is in good agreement with previous reports (6). Moreover, a decrease in IL-7 production by stromal cells during aging was confirmed (Fig. 2), which is evident in regular mice (17, 18).

The present study revealed that senescent B lymphopoiesis is suppressed, the background mechanism of which is unlikely different from mechanical B cell damage and its acute responses. Although B cell damage may be based on a positive circuit (i.e., an increase in IL-7 production associated with a decrease in TGF-β production) (28, 31, 32), our present data clearly show that TGF-β production is rather suppressed despite the prominent decrease in IL-7 production. Such homeostatic B lymphopoiesis balanced at the lower level may be a prominent characteristic of the basic mechanism of B lymphopoietic senescence, although the details of this mechanism are as yet unknown.

Another objective of our current study is to address the issue of using SAMs as an experimental mouse model for predicting the possible basic mechanism of senescence and B lymphopoiesis during aging. Aging is a physiological process and is likely controlled by a combination of many different factors. Whether the determinant of accelerated aging in SAMs is the same as that of normal aging in mice remains to be elucidated. However, the determinant factors for aging of an organism are, at present, poorly understood. Thus, different experimental approaches using animal models such as SAMs may provide an insight into such factors, because the study of abnormal systems has often led to the clarification of how a normal system functions.

Our present study, performed using SAMs and focusing on the quantity and quality of B lymphopoietic progenitor cells, suggests age-related alterations in lymphopoietic progenitor cells. Among them, the changes shown in Tables 1 and 2 and Figure 1 are essentially identical to those observed in regular mouse strains as previously reported. Namely, the decreased IL-7 responsiveness of BM cells from aged mice appears to be associated with both the decrease in the number of IL-7–responsive cells and the decrease in colony size and to correlate with findings in other strains (3–6). Furthermore, the number of secondary

colonies generated by the progeny of CFU-pre-B cells derived from large primary colonies was significantly smaller for the BM of senescent mice than for that of young mice (Table 2). Note that there seems to be an almost complete arrest in the production of secondary CFU-pre-B colonies from 36-week-old mice (Table 2), whereas comparable primary BM cells produced the same amount of GM-CFU (109%) from 12-week-old mice, and 65.0% of CFU-pre-B colonies was maintained (Table 1) in 12-week-old mice. The decrease in the number of secondary colonies was prominently observed in the most senescent age group (i.e., 36 weeks old) at the level of "cluster," because there seems to be a split between the time to senescence for spleen atrophy and that for substantial hemopoietic arrest in *in vitro* colonization. Stephan *et al.* (25) reported that a small percentage of BM pro-B cells from aged mice undergo cell cycle and that a large percentage of these cells enter G0/G1 after stimulation with IL-7, suggesting an impairment or delay in their ability to undergo cell division after IL-7 stimulation. The surrogate light chain is a component of the pre-B cell receptor, which is critical for Ig-variable heavy chain selection, cellular proliferation, and survival in the pre-B stage. Sherwood *et al.* (26) and Frasca *et al.* (33) reported that surrogate L chain mRNA and protein levels in IL-7–expanded B cell precursors decrease with age, which is associated with decreased protein levels of the E2A-encoded transcription factors, E47 and E12 (33). Based on these results, impairment in the IL-7 receptor function and its signal transduction in pro-B/pre-B cells may underlie the decrease in B cell production with age. It seems likely that the reduced generation of secondary colonies may be due in part to the deterioration of the proliferative capacity of B-cell progenitors from senescent mice in response to IL-7 rather than to the exclusive differentiation of B-cell progenitors to mature B cells in response to IL-7. In this regard, senescent changes observed in B lymphopoiesis in SAMs may be assumed to be identical to those reported in regular mice.

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