In animal studies involving observation of experimental animals for two or more generations, no effects of EDCs have been demonstrated. The question therefore arises as to why window effects are observed during the short period mentioned above. It is unknown whether or not these effects really do occur, and if they do, how they are produced.

Delayed growth of the thalamic nucleus specific to males (called sexual dimorphic nucleus) is seen in male rats treated with female hormones. We may say that under conditions of homeostasis of the physiological hormones in mature individuals, exposure to dose levels that usually cause only reversible changes can lead to irreversible changes, if the exposure occurs during genesis, morphogenesis, or functional development. However, there are no ample data endorsing this view in humans.

Considering the biological plausibility inferred from the experimental data accumulated to date*, we may say that there are no sufficient data that clearly rule out this view. Close attention has therefore been paid to these effects in children.

New theories of methodology, focusing on effects in fetuses and children, are now being developed, primarily in the United States, or the World Health Organization, within the framework of children's program, etc.

HEALTH HAZARDS AT LOW LEVELS OF EXPOSURE

Chemicals used for agriculture or industrial purposes are marketed, in general, only after their effects on living beings have been investigated. We may therefore understand that they are used on the premise that the possibility of these chemicals exerting hazardous effects on health at relatively high-dose levels has been almost ruled out. Nevertheless, problems with EDCs have begun to be highlighted. These problems may not be confined to those related to the accumulation of these substances through food chains in the ecosystem, but also to the additional possibility that these chemicals may exert effects at low-dose levels even if they have been declared safe at high-dose levels. The latter possibility may apply, however, only to some cases and not to others.

We may say that a major issue pertaining to EDCs that must be resolved urgently is whether or not they pose health hazards at low-dose levels. This issue can be summarized into the following three questions:

- presence/absence of threshold level;
- presence/absence of synergistic or additive effects; and
- possibility of extrapolation of high-dose effects to low-dose levels (i.e., presence/absence of a linear dose–response relationship).

No clear-cut answers have as yet emerged to these questions. Considering the above-mentioned characteristics of the effects of hormones, it is plausible to imagine how difficult it may be to resolve these questions.

To determine if these chemicals exerted hazardous effects on health at low-dose levels, the following basic questions may need to be considered; their biological plausibility is hardly denied.

- Regarding the presence or absence of threshold levels, it seems likely that many chemicals suspected of being EDCs can easily permeate across the cell membrane, which is composed of phospholipids. Therefore, assuming that one receptor molecule reacts with one chemical molecule, the lower limit of the dose level exerting the chemical's effects would be extremely low.

  Of course, since the probability of the binding of a ligand to the receptor will be low if the dose level is low, we cannot say that there is no threshold level for the effects seen in the low-

*Biological plausibility: Likelihood of a phenomenon as judged by considering the difference or similarity of elements of reactions in individual organisms, on the basis of the results of a series of related biological experiments. (Cf. probability)

dose-level range. In fact, for bisphenol A (which has been attracting close attention because of its hazardous effects on health at low-dose levels), the presence/absence of a threshold level has not yet been reported. It seems rational, therefore, to assume that these health hazards occur in a very low-dose-level range.

- If we consider not only the affinity of each substance for the receptor, but also the nonlinearity of responses (e.g., waveform responses as a result of reduced receptor expression following an increase in dose level), it is possible to assume that there are U-shaped or reverse U-shaped reactions, or oscillational dose–response curves. Interim data endorsing such a view are being accumulated.
- Regarding the possibility of synergistic or additive effects, the observation of additive effects among different nuclear receptors has been reported. Data yielded by analysis of interactions between receptor signals also suggest such a possibility. In fact, the dose–response curves for some composite materials were reported to be additive, but not synergistic.

Thus, the questions on health hazards at low-dose levels have several aspects:

- type of receptor-mediated actions of the hormone mimics;
- diverse reactive characteristics on the part of the receptors;
- diverse modification during expression of intracellular signals; and
- factors involved in irreversible changes related to morphogenesis and functional development.

Resolution of all these aspects of the question will lead to clarification of the mechanism of actions of the substances from each of the aforementioned standpoints. While these questions are among the hottest research themes at present, they are certainly unlikely to be resolved easily.

At a workshop held in North Carolina, USA, in October 2000, health hazards of chemicals at low-dose levels were discussed. Investigators for and against the possibility of these substances posing health hazards at low-dose levels gave detailed accounts of their studies, and no definitive conclusions could be reached, as the arguments of both sides appeared to be tenable.

This means that reports affirming the plausibility of these substances posing health hazards at low-dose levels in animal experiments cannot be immediately rejected. The workshop concluded by pointing out the necessity of paying attention to the possible hazards on fetuses and neonates.

HEALTH HAZARDS OF HORMONE-MIMICS TO HUMANS

The possibility of health hazards of hormone-mimics to human beings have not been supported by adequate epidemiological data, and the number of cases for which the data clearly endorse such effects is quite small. The U.S. National Research Council (NRC) emphasizes the necessity of conducting further epidemiological studies on this topic (NRC, 1999).

In conclusion, this paper summarizes the current knowledge concerning the health hazards of hormone-mimics to humans. Reports dealing with the effects of these substances on humans are confined to those pertaining to the effects of dioxins and polychlorinated biphenyls (PCBs); the validity and usefulness of these results have not yet been established.

The following information is based on case studies conducted to date.

Health hazards of dioxins

Regarding health hazards of dioxins, two-year dosing studies revealed weight loss and liver damage, and three-generation reproductive studies in rats disclosed intrauterine death and a decrease in litter size. Onset of endometriosis in rhesus monkeys has also been reported.

A causal relationship of EDCs to the following episodes in humans has been suggested: biased male-to-female ratio in children born in the dioxin-exposed Seveso area of Italy, and increased inci-

dence of cleft palate in the Diemerzeedijk district of the Netherlands, probably due to steroids. In both of these cases, the U.S. Environmental Protection Agency (USEPA) did not affirm a causal relationship, and classified them as cases requiring special attention.

No consensus has been reached concerning the relationship of hypothyroidism observed in the inhabitants along Lake Michigan to the ingestion of PBB- (polybrominated biphenyls-) contaminated fish.

Effects on mature females (e.g., increased incidence of breast cancer)

No reports affirm the effects of dioxins on mature human females (e.g., effects on breast cancer or endometriosis as discussed below). There are many unresolved questions on this topic. However, none of the studies conducted in mature experimental animals revealed data endorsing the plausibility of occurrence of such effects. On the other hand, it is known that the age at menarche is lower and the incidence of breast cancer higher in females exposed to dioxins. Some investigators cite these data when discussing the health hazards of dioxins. It is also known that females exposed to dioxins are often taller.

In European countries, a height increase of about 3.5 mm per year and an approximately one-year decrease in the age at menarche have been reported during the past 30 years. It is difficult to identify the influence of extrinsic endocrine factors on these changes, and no studies addressing this issue have been reported to date. Although several studies have been published concerning the effects of female hormone preparations, including pills used for contraception and hormone replacement therapy in postmenopausal women, no studies have provided data that establish the effects of EDCs.

Endometriosis

Endometriosis is a disease of unexplained origin that is seen in primates with sexual cycles. It has been pointed out that this disease tends to be more severe in individuals exposed to dioxins (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD] and to PCBs). Data yielded from experiments in rhesus monkeys are used as evidence to corroborate the causal relationship between dioxins and endometriosis. Thus, we cannot rule out the biological plausibility of these effects. However, no reports affirming the causal relationship in humans have been published.

Possibility of other effects on humans

Biological plausibility has also been considered for the following effects of hormone-mimics on humans: qualitative dysfunction of human sperm, effects on neurobehavior of neonates, and immune functions. The effects on immune functions have been suggested by reports of cases with Yu-sho (PCB intoxication).

CONCLUSION

The International Program of Chemical Safety (IPCS), a section of the World Health Organization, has released a Web site publication "Global Assessment of the State-of-the Science of Endocrine Disruptors" (GAED), June 2002 (URL: <http://ehp.niehs.hih.gov/who/>). WHO/IPCS started the GAED program in March 1998 after the publication of *Our Stolen Future* (Theo Colbone et al., 1996). The publication took three years to edit; covering a policy to document all the published pertinent literatures, to summarize them as descriptive manner solely based on those published literatures. Twenty-seven expert scientists and 20 independent peer-reviewers participated in editing the GAED.

Other reports on nonylphenol and octylphenol, released by the Japanese Ministry of Environment (MoE), revealed an "ovotestes" formation that was observed in the assay of the laboratory experimen-

tal fish (*Medaka*) exposed to doses close to those recorded in the monitoring fields in the MoE surveillance. Further, phthalates, such as di-(2-ethylhexyl)phthalate, di-cyclohexylphthalate, and butylbenzylphthalate, as selected and prioritized chemicals by the MoE, showed some unique data in different endpoints, including mRNA expression, in dose ranges lower than those no observed effect levels (NOELs) and/or no observed adverse effect levels (NOAELs) reported previously.

The effects of EDCs on human health are unknown at this moment. However, due to the biologically plausible data currently accumulated, the existence of endocrine disruptions under certain circumstances seems to be a reality. Thus, by the time of the SCOPE/IUPAC symposium, the EDC research for the next stage may shift from plausibility to possibility, and put forward further mechanistic research.

IUPAC
Official Journal of the International Union of Pure and Applied Chemistry

Pure and Applied Chemistry

Implications of Endocrine Active Substances for Humans and Wildlife—a SCOPE/IUPAC Project

SCOPE

IUPAC

Volume 75, Nos. 11–12, November–December 2003

Special Topic Issue

Health Hazards of Endocrine-Disrupting Chemicals on Humans as Examined from the Standpoints of Their Mechanisms of Action

JMAJ 46(3): 97–102, 2003

Tohru INOUE*, Katsuhide IGARASHI and Jun SEKIZAWA

Chairperson, Biological Safety Research Center, National Institute of Health Sciences*

Reprinted from JMAJ (Japan Medical Association Journal) Vol. 46, No. 3, March, 2003

Health Hazards of Endocrine-Disrupting Chemicals on Humans as Examined from the Standpoints of Their Mechanisms of Action

JMAJ 46(3): 97–102, 2003

Tohru INOUE*, Katsuhide IGARASHI and Jun SEKIZAWA

Chairperson, Biological Safety Research Center, National Institute of Health Sciences*

Abstract: Hormonally active compounds were first recognized in "*Silent Spring*" by Rachel Carson in 1962, which implicated pesticides, such as DDT and derivatives. Nearly four decades later, the book "*Our Stolen Figure*," by Theo Colborn *et al.*, and other pertinent publications have revisited and broadened the issue to a variety of chemicals and areas exposed. Translations of these books have just become available in Japan in the past three or four years, and since then Japan has started to join the debate and/or discussion of the global issue of endocrine disruptors—"Environmental Hormones." Although significant numbers of chemicals possessing a hormone-mimicking action have been recognized for many years and based on biological plausibility their receptor-mediating effects strongly suggest effects in humans similar to those seen in wildlife, little is known about the experimental evidence related to human adverse effects. The key issue in resolving the dilemmas posed by the biological plausibility and poor experimental evidence may be to clarify their mechanism of actions at low levels. In other words, the mechanisms of the possible low-dose effects may be resolved simultaneously by defining three major properties threshold, oscillation, and additive-synergism.

Key words: Receptor; Hormone mimics; Homeostasis; Effects at low dosage; Human hazards

Introduction

The objective of this paper is to summarize all the currently available information on the possible hazards of endocrine-disrupting chemicals (EDs) on human health from the stand-

This article is a revised English version of a paper originally published in the Journal of the Japan Medical Association (Vol. 127, No. 2, 2002, pages 197–201). The Japanese text is a transcript of a lecture originally aired on October 12, 2001, by the Nihon Shortwave Broadcasting Co., Ltd., in its regular program "Special Course in Medicine".

points of the mechanisms of actions of these chemicals.

It is not uncommon to come across agrochemicals and industrial chemicals that have hormone-mimicking effects. These chemicals, the so-called "environmental hormones," often accumulate at detectable levels in the environment, and it has been feared that they may have adverse effects on living beings. Following reports of feminization and decreased colony size of wild creatures, and reports suggesting a possible association of these chemicals with abnormalities of reproductive organs and oncogenesis in human, attention has been focused on the possibility that these occurrences may be associated with exposure to EDs. In this connection, a Japanese translation of the book entitled "*Our Stolen Future*," written by Theo Colborn *et al.*, was published some time ago.

This paper will review the problems related to EDs, the courses of arguments regarding the harmful effects of these chemicals, and current medical topics pertaining to them.

Chemicals with Hormone-Mimicking Actions

Substances with hormone-mimicking effects can be divided into four groups: (1) hormones found *in vivo*, (2) medicines with hormone-mimic actions manufactured for use in hormonal therapy, etc., (3) plant hormones known to exert phytoestrogen-like actions, and (4) chemicals found in environments that can interact with hormone receptors.

In addition, substances which do not interact with hormone receptors but exert effects on gonads by their modifying effects on steroid metabolism may be deemed as hormone-mimics in the broader sense of the term. In this paper, however, emphasis shall be placed on the hormone-mimicking actions mediated by receptors which play essential roles in the mechanism of actions of hormone-mimics.

Characteristics of the Receptor-Mediated Actions of Hormone-Mimics

The receptor-mediated actions of hormone-mimics are fundamentally characterized by the similarity in the structures of the receptors involved, crossing the barrier of species. This characteristic allows us to estimate the possibility of the actions of these chemicals exerted in nature also occurring in humans.

Secondly, since similarities in the structure to various sex steroids and hormones are also known, it is possible that each individual hormone-mimic exerts diverse effects by acting on male hormone receptors, female hormone receptors, receptors in the nuclei (including some unknown receptors), etc.

Thirdly, many of these chemicals are eliminated from the living body in the form of conjugated inactive substances instead of as degraded metabolites. They may also be eliminated in the unchanged form. Therefore, if feces and urine containing these substances are eliminated into river water, it is plausible to imagine that even inactivated hormones can sometimes become active and exert hormone-mimic actions in the environment. This is one of the characteristics unique to this class of chemicals.

Receptor-mediated responses involve many unresolved questions. Various undefined elements may be involved, including the relationship between receptor binding and signals, the relationship between receptor-ligand binding (ligand: substances that can bind to receptors) and the dissociation of ligands from receptors, signal cross-talks, involvement of unknown nuclear receptors, etc.

The actions of these chemicals add to the effects of intrinsic hormones. For this reason, these chemicals may exert their actions in a way different from that known for other chemicals which do not have structural or functional counterparts *in vivo*. For example, stimulation of hormone receptors by these extrinsic chemicals may modify homeostasis *in vivo*, leading

to weakening of the physiological stimulation of these receptors by the intrinsic substances. Therefore, the influence of the continued effects of environmental hormones needs special study.

Pitfall in the Effects of Hormone-Mimics

We must distinguish the interactions of endocrine hormone-mimics with hormone receptors from the hazards caused to endocrine tissue. Bearing this in mind, let us now summarize the problems related to the effects of hormone-mimics.

1. Antagonistic effects on the maintenance of homeostasis

The endocrine system is regulated by homeostatic mechanisms. It is not uncommon for the effects of small amounts of hormone-mimics to interfere slightly with these mechanisms, often with no adverse influence; this is well-known. However, this is not always the case. There seems to be a group of genes that act antagonistically to each other in the maintenance of homeostasis.

With the uterus growth test, which is used to check for estrogenic activity, the ovary is removed in advance and the blood level of the intrinsic female hormone is reduced to the minimum. Under the thus-created extremely undeveloped state of the uterus, the test substance (a chemical or hormone) is administered to check for its effects on the growth of the uterus. This test (checking for growth of the uterus in ovariectomized animals) is designed to evaluate the hormone activity and effects of hormone-mimics under conditions of blockade of homeostasis.

This test method itself is valid. However, there is no sufficient rational evidence that indicates that the responses observed under such indirect control conditions of the living body can serve as an indicator of the health hazards of hormone mimics. Although the ovotestes seen in lower vertebrates may be used if the effects observed were to be valid as such an indicator, there is no consensus on what is valid as an indicator of the health hazards of ED's when mammals are used as experimental animals.

2. Down-regulation of the expression of receptors

It is known that the expression of genes encoding receptors is down-regulated by stimuli, leading to reduced receptor sensitivity. This can lead to a paradoxical outcome wherein the effects observed in the presence of low levels of a substance are not seen at high levels of the same substance. If this phenomenon occurred in individual organisms, the dose-response relationship will be non-linear.

This means that extrapolation of results obtained at high levels of the chemicals to conditions where low levels of the same substance are present would be difficult. It is needed to test the validity of this hypothesis, and analysis of the mechanisms underlying this phenomenon if the hypothesis were indeed valid, are thus important. Studies to resolve these questions are now under way.

3. Data gap concerning the effects of female hormones

In mature women, there are high levels of physiological hormones *in vivo*, and these are subject to cyclic control. It has been proposed that girls with inadequate physical growth begin menstruation at lower ages and undergo sexual maturation earlier than usual, and that hormone-mimics in these subjects can precipitate breast cancer.

The weak links in this hypothesis have been pointed out, and it has been shown experimentally that estrogen by itself may be teratogenic, although this tendency has been shown to be weak. It is known that organisms are programmed such that excessive exposure to estrogens during the intrauterine period or other developmental stages is avoided.

There are many open questions as to the

mechanism by which mature females remain physiologically stable, even when exposed daily to high levels of estrogen (400 pM/l). Some dramatic effects are probably needed to disturb this physiology.

4. Multi-generation tests and effects on fetuses

It has been shown that exposure to hormones or hormone-mimics during intrauterine or early neonatal periods can lead to irreversible changes in the pattern of development. This susceptibility period is short, extending from the 13th gestational day to about one week after birth. These effects are the so-called "intrauterine window effects."

In animal studies involving observation of experimental animals for two or more generations, no effects of EDs have been demonstrated. The question therefore arises as to why window effects are observed during the short period mentioned above. It is unknown whether or not these effects really do occur, and if they do, how are they produced.

Delayed growth of the thalamic nucleus specific to males (called sexual type II nucleus) is seen in male rats treated with female hormones. We may say that under conditions of homeostasis of the physiological hormones in mature individuals, exposure to dose levels that usually cause only reversible changes can lead to irreversible changes, if the exposure occurs during genesis, morphogenesis or functional development. However, there are no ample data endorsing this view in humans.

Considering the biological plausibility inferred from the experimental data accumulated to date,[*1] we may say that there are no sufficient data that clearly rule out this view. Close attention has therefore been paid to these effects in children.

New theories of methodology, focusing on the effects in fetuses and children, are now being developed, primarily in the United States, within the framework of children's program, etc.

Health Hazards at Low Levels of Exposure

Chemicals used for agriculture or industrial purposes are marketed, in general, only after their effects on living beings have been investigated. We may therefore understand that they are used on the premise that the possibility of these chemicals exerting hazardous effects on health at relatively high dose levels has been almost ruled out. Nevertheless, problems with EDs have begun to be highlighted. These problems may be not confined to those related to the accumulation of these substances through food chains in the ecosystem, but also to the possibility additionally that these chemicals may exert effects at low dose levels even if they have been declared safe at high dose levels. The latter possibility may apply, however, only to some cases and not to others.

We may say that a major issue pertaining to EDs that must be resolved urgently is whether or not they pose health hazards at low dose levels. This issue can be summarized into the following three questions: (1) presence/absence of threshold level, (2) presence/absence of synergistic or additive effects, and (3) possibility of extrapolation of high-dose effects to low-dose levels (i.e., presence/absence of a linear dose-response relationship). No clear-cut answers have as yet emerged to these questions. Considering the above-mentioned characteristics of the effects of hormones, it is plausible to imagine how difficult it may be to resolve these questions.

To determine if these chemicals exerted hazardous effects on health at low dose levels, the following basic questions may need to be considered; their biological plausibility is hardly denied.

[*1] Biological plausibility: Likelihood of a phenomenon as judged by considering the difference or similarity of elements of reactions in individual organisms, on the basis of the results of a series of a related biological experiments. (cf. probability)

(1) Regarding the presence or absence of threshold levels, it seems likely that many chemicals suspected of being EDs can easily permeate across the cell membrane, which is composed of phospholipids. Therefore, assuming that one receptor molecule reacts with one chemical molecule, the lower limit of the dose level exerting the chemical's effects would be very low.

Of course, since the probability of the binding of a ligand to the receptor will be low if the dose level is low, we cannot say that there is no threshold level for the effects seen in the low dose level range. In fact, for bisfenol A, which has been attracting close attention because of its hazardous effects on health at low dose levels, the presence/absence of a threshold level has not yet been reported. It seems rational, therefore, to assume that these health hazards occur in a very low dose level range.

(2) If we consider not only the affinity of each substance for the receptor, but also the non-linearity of responses (e.g., waveform responses as a result of reduced receptor expression following an increase in dose level), it is possible to assume that there are U-shaped or reverse U-shaped reactions or oscillational dose-response curves. *Interim* data endorsing such a view are being accumulated.

(3) Regarding the possibility of synergistic or additive effects, the observation of additive effects among different nuclear receptors has been reported. Data yielded by analysis of interactions between receptor signals also suggest such a possibility. In fact, the dose-response curves for some composite materials were reported to be additive, but not synergistic.

Thus, the questions on health hazards at low dose levels have several aspects: (1) the type of receptor-mediated actions of the hormone mimics, (2) diverse reactive characteristics on the part of the receptors, (3) diverse modification during expression of intracellular signals, and (4) factors involved in irreversible changes related to morphogenesis and functional development. Resolution of all these aspects of the question will lead to clarification of the mechanism of actions of the substances from each of the aforementioned standpoints. While these questions are among the hottest research themes at present, they are certainly unlikely to be resolved easily.

At a workshop held in North Carolina, USA, in October 2000, health hazards of chemicals at low dose levels were discussed. Investigators for and against the possibility of these substances posing health hazards at low dose levels gave detailed accounts of their studies, and no definitive conclusions could be reached, as the arguments of both sides appeared to be tenable.

This means that reports affirming the plausibility of these substances posing health hazards at low dose levels in animal experiments cannot be immediately rejected. The workshop concluded by pointing out the necessity of paying attention to the possible hazards on fetuses and neonates.

Health Hazards of Hormone-Mimics on Humans

The possibility of health hazards of hormone-mimics on humans have not been supported by adequate epidemiological data, and the number of cases for which the data clearly endorse such effects is quite small. The US National Research Council emphasizes the necessity of conducting further epidemiological studies on this topic (National Research Council, 1999).

In conclusion, this paper summarizes the current knowledge concerning the health hazards of hormone-mimics on humans. Reports dealing with the effects of these substances on humans are confined to those pertaining to the effects of dioxins and PCB, and the validity and usefulness of these results have not yet been established.

The following are based on case studies conducted to date.

1. Health hazards of dioxins

Regarding health hazards of dioxins, two-year dosing studies revealed weight loss and liver damage, and three-generation reproductive studies in rats disclosed intrauterine death and a decrease in litter size. Onset of endometriosis in rhesus monkeys has also been reported.

A causal relationship of EDs to the following episodes in humans has been suggested: biased male-to-female ratio in children born in the dioxin-exposed Seveso area of Italy, and increased incidence of cleft palate in the Diemerzeedijk district of the Netherlands, probably due to steroids. In both of these cases, the Environmental Protection Agency (EPA) of the United States did not affirm a causal relationship, and treated classified them as cases requiring special attention.

No consensus has been reached concerning the relationship of hypothyroidism observed in the inhabitants along Lake Michigan to the ingestion of PBB (polybrominated biphenyls)-contaminated fish.

2. Effects on mature females, e.g., increased incidence of breast cancer

No reports affirming the effects of dioxins on mature human females (e.g., effects on breast cancer or endometriosis as discussed below). There are many unresolved questions on this topic. However, none of the studies conducted in mature experimental animals revealed data endorsing the plausibility of occurrence of such effects. On the other hand, it is known that the age at menarche is lower and the incidence of breast cancer higher in females exposed to dioxins. Some investigators cite these data when discussing the health hazards of dioxins. It is also known that females exposed to dioxins are often taller.

In European countries, a height increase of about 3.5 mm per year and an approximately one-year decrease in the age at menarche have been reported during the past 30 years. It is difficult to identify the influence of extrinsic endocrine factors on these changes, and no studies addressing this issue have been reported to date. Although a number of studies have been published concerning the effects of female hormone preparations, including pills used for contraception and hormone replacement therapy in postmenopausal women, no studies have provided data that establish the effects of EDs.

3. Endometriosis

Endometriosis is a disease of unexplained origin that is seen in primates with sexual cycles. It has been pointed out that this disease tends to be more severe in individuals exposed to dioxins (TCDD/PCBs). Data yielded from experiments in rhesus monkeys are used as evidence to corroborate the causal relationship between dioxins and endometriosis. We cannot thus rule out the biological plausibility of these effects. However, no reports affirming the causal relationship in humans have been published.

4. Possibility of other effects on humans

Biological plausibility has been pointed out also on the following effects of hormone-mimics on humans: qualitative dysfunction of human sperm, effects on neurobehavior of neonates, and immune functions. The effects on immune functions have been suggested by reports of cases with Yu-sho (PCB intoxication).

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

ISAO TSUBOI,*,† KOHJI MORIMOTO,* YOKO HIRABAYASHI,* GUANG-XUN LI,* SHIN AIZAWA,† KAZUHIRO J. MORI, JUN KANNO,* AND TOHRU INOUE§,[1]

Division of Cellular and Molecular Toxicology, National Institute of Health Sciences, Tokyo 158–8501, Japan; †Department of Anatomy and Developmental Biology, Nihon University School of Medicine, Tokyo 173–8610, Japan; Department of Molecular and Cellular Biology, Faculty of Science, Niigata University, Niigata 950–2181, Japan; and §Center for Biological Safety and Research, National Institute of Health Sciences, Tokyo 158–8501, Japan

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

This work was supported by a Grant-in-Aid (No. 13670236) for Scientific Research C from the Japan Society for the Promotion of Sciences.

[1] To whom requests for reprints should be addressed to Center for Biological Safety and Research, National Institute of Health Sciences, 1-18-1 Kamiyohga, Setagayaku, Tokyo 158–8501, Japan. E-mail: tohru@nihs.go.jp

Received August 1, 2003.
Accepted March 1, 2004.

1535-3702/04/2296-0001$15.00

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.

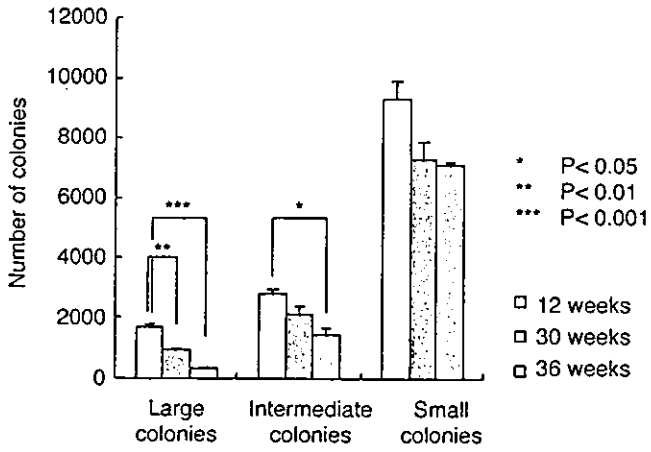

Figure 1. Age-related changes in number of B cell colonies of large, intermediate, and small sizes (mean ± SEM of three replicate experiments). Femoral bone marrow (BM) cells from three mice per group were harvested and pooled. The preparation of BM cell suspensions is described in the "Materials and 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-

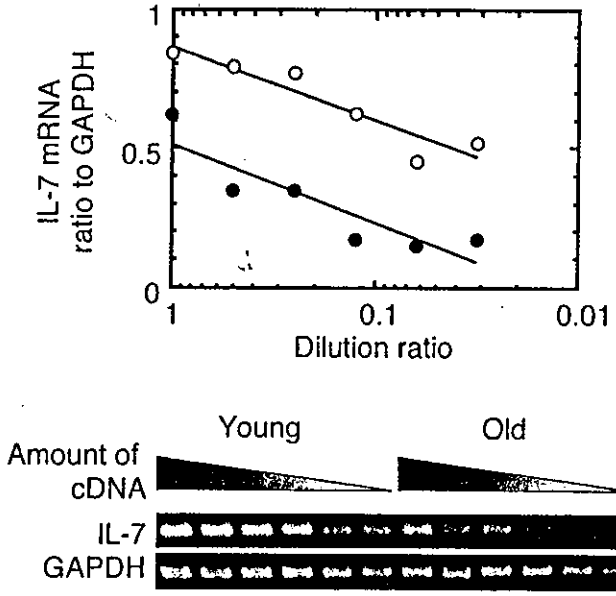

Figure 2. Expression level of interleukin-7 (IL-7) messenger RNA (mRNA) in bone marrow (BM) cells freshly isolated from young and old senescence-accelerated mice (mean ± SEM of three replicate experiments). Vertical bars for SEM are within the symbols. Total RNA was extracted from BM cells using the 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.

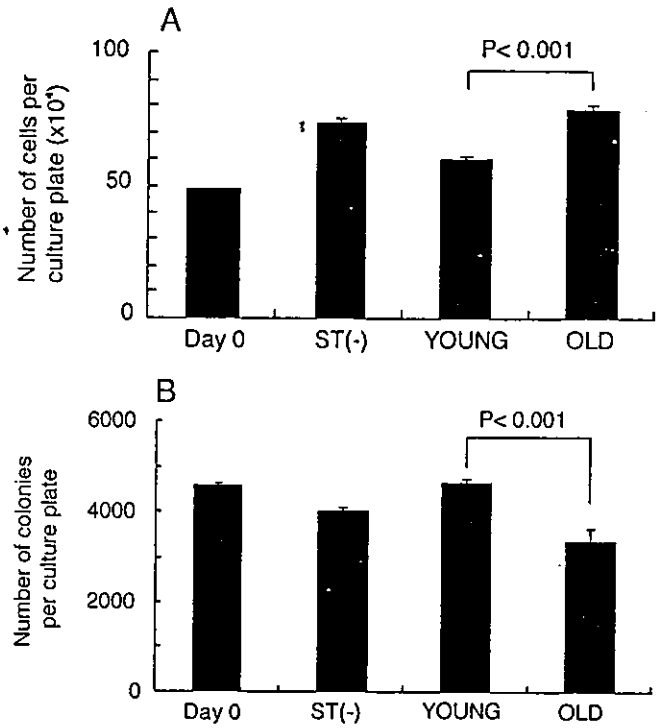

Figure 3. (A) Co-culture of pro-B/pre-B cell-enriched populations with stromal cells: change in the number of nonadherent cells (mean ± SEM of three replicate experiments). (B) Co-culture of pro-B/pre-B cell-rich populations with stromal cells: change in total number of CFU-pre-B cells (mean ± SEM of 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.
* $P < 0.005$; ** $P < 0.01$.

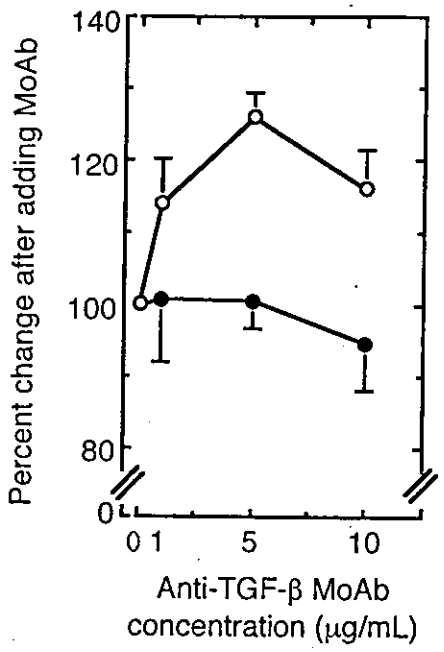

Figure 4. Effect of neutralizing anti–transforming growth factor-β (TGF-β) monoclonal antibody (mAb) on proliferation of pro-B/pre-B cells from young senescence-accelerated mice co-cultured with femoral stromal cells from young mice (open circles) and those from senescent mice (closed circles). The effect 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).

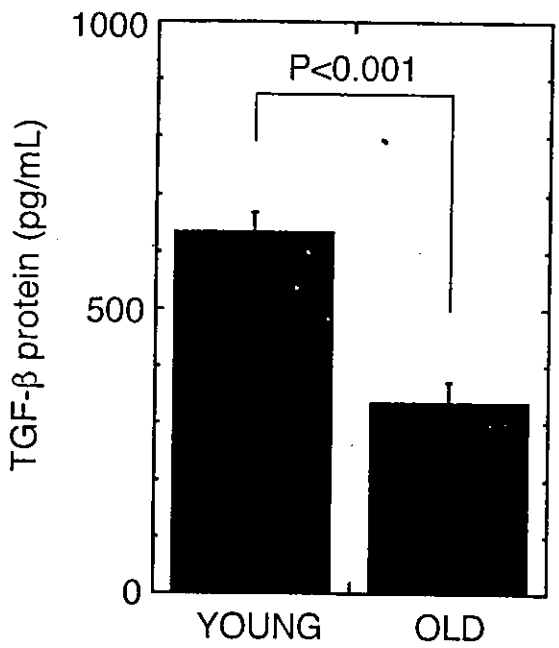

Figure 5. Transforming growth factor-β (TGF-β) protein level in the supernatant of cultured stromal cells from young and senescent senescence-accelerated mice (SAMs) (mean ± SEM of three replicate experiments). Stromal monolayers were prepared by culturing bone marrow (BM) cells from young or senescent 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.

We thank Dr. Yukio Kodama, Ms. Erika Tachihara, Ms. Yoshiko Usami, and Ms. Yasuko Shinzawa for assistance in the maintenance of SAMs at the experimental animal facilities and Ms. Noriko Katsu and Ms. Chiaki Kimura for secretarial help in the preparation of the manuscript.

1. Hodes RJ. Aging and the immune system. Immunol Rev 160:5–8, 1997.
2. Ghia P, Melchers F, Rolink AG. Age-dependent changes in B lymphocyte development in man and mouse. Exp Gerontol 35:159–165, 2000.
3. Zharhary D. Age-related changes in the capability of the bone marrow to generate B cells. J Immunol 141:1863–1869, 1988.
4. Riley RL, Kruger MG, Elia J. B cell precursors are decreased in senescent BALB/c mice, but retain normal mitotic activity in vivo and in vitro. Clin Immunol Immunopathol 59:301–313, 1991.
5. Jonsson JI, Phillips RA. Interleukin-7 responsiveness of B220+ B cell precursors from bone marrow decreases in aging mice. Cell Immunol 147:267–278, 1993.
6. Stephan RP, Sanders VM, Witte PL. Stage-specific alterations in murine B lymphopoiesis with age. Int Immunol 8:509–518, 1996.
7. Klinman NR, Kline GH. The B-cell biology of aging. Immunol Rev 160:103–114, 1997.
8. Tang J, Nuccie BL, Ritterman I, Liesveld JL, Abboud CN, Ryan DH. TGF-beta down-regulates stromal IL-7 secretion and inhibits proliferation of human B cell precursors. J Immunol 159:117–125, 1997.
9. Takeda T, Hosokawa M, Takeshita S, Irino M, Higuchi K, Matsushita T, Tomita Y, Yasuhira K, Hamamoto H, Shimizu K, Ishii M, Yamamuro T. A new murine model of accelerated senescence. Mech Ageing Dev 17:183–194, 1981.
10. Izumi-Hisha H, Ito Y, Sugimoto K, Oshima H, Mori KJ. Age-related decrease in the number of hemopoietic stem cells and progenitors in senescence accelerated mice. Mech Ageing Dev 56:89–97, 1990.
11. Tsuboi I, Morimoto K, Horie T, Mori KJ. Age-related changes in various hemopoietic progenitor cells in senescence-accelerated (SAM-P) mice. Exp Hematol 19:874–877, 1991.
12. Sakuma A, Tsuboi I, Morimoto K, Horie T, Sugimoto K, Mori KJ. Age-related decreases in the reconstituting ability of hemopoietic cells and the ability of hemopoietic microenvironment to support hemopoietic reconstitution in senescence accelerated (SAM-P) mice. Mech Ageing Dev 73:127–135, 1994.
13. Saitoh T, Morimoto K, Kumagai T, Tsuboi I, Aikawa S, Horie T. Comparison of erythropoietic response to androgen in young and old senescence accelerated mice. Mech Ageing Dev 109:125–139, 1999.
14. Kumagai T, Morimoto K, Saitoh T, Tsuboi I, Aikawa S, Horie T. Age-related changes in myelopoietic response to lipopolysaccharide in senescence-accelerated (SAM) mice. Mech Ageing Dev 112:153–167, 2000.
15. Yamamoto H, Hatano M, Iitsuka Y, Mahyar NS, Yamamoto M, Tokuhisa T. Two forms of Hox11 a T cell leukemia oncogene, are expressed in fetal spleen but not in primary lymphocytes. Mol Immunol 32:1177–1182, 1995.
16. Lawrence DA, Pircher R, Jullien P. Conversion of a high molecular weight latent beta-TGF from chicken embryo fibroblasts into a low molecular weight active beta-TGF under acidic conditions. Biochem Biophys Res Commun 133:1026-10-34, 1985.
17. Updyke LW, Cocke KS, Wierda D. Age-related changes in production of interleukin-7 (IL-7) by murine long-term bone marrow cultures (LTBMC). Mech Ageing Dev 69:109–117, 1993.
18. Stephan RP, Reilly CR, Witte PL. Impaired ability of bone marrow stromal cells to support B-lymphopoiesis with age. Blood 91:75–88, 1998.
19. Okada S, Yoshida T, Hong Z, Ishii G, Hatano M, Kuro OM, Nabeshima Y, Tokuhisa T. Impairment of B lymphopoiesis in precocious aging (klotho) mice. Int Immunol 12:861-871, 2000.
20. Li F, Jin F, Freitas A, Szabo P, Weksler ME. Impaired regeneration of the peripheral B cell repertoire from bone marrow following lymphopenia in old mice. Eur J Immunol 31:500-505, 2001.
21. Nemunaitis J, Tompkins CK, Andrews DF, Singer JW. Transforming growth factor beta expression in human marrow stromal cells. Eur J Haematol 46:140–145, 1991.
22. Lee G, Namen AE, Gillis S, Ellingsworth LR, Kincade PW. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-beta. J Immunol 142:3875–3883, 1989.
23. Kincade PW. B lymphopoiesis: global factors, local control. Proc Natl Acad Sci U S A 91:2888–2889, 1994.
24. Lebman DA, Edmiston JS. The role of TGF-beta in growth, differentiation, and maturation of B lymphocytes. Microbes Infect 1:1297–1304, 1999.
25. Stephan RP, Lill-Elghanian DA, Witte PL. Development of B cells in aged mice: decline in the ability of pro-B cells to respond to IL-7 but not to other growth factors. J Immunol 158:1598–1609, 1997.
26. Sherwood EM, Xu W, King AM, Blomberg BB, Riley RL. The