Effects of Neopterin on the Hematopoietic Microenvironment of Senescence-Accelerated Mice (SAM)

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The pteridine neopterin (NP) is produced by monocytes and is known to be a useful marker of immunological activation, although, it remains elusive whether neopterin itself exhibits biological functions. Recently, we found that NP stimulates hematopoietic cell proliferation and differentiation by activating bone marrow stromal cell function. In order to elucidate the biological effect of NP on stromal cells, its effects on hematopoiesis was determined in the mouse model of age-related stromal impairment, senescence-accelerated mice (SAMs). An intraperitoneal administration of NP increased the number of peripheral leukocytes and CFU-GM in the bone marrow and spleen of young SAMs, however, no increase of CFU-GM in old SAMs (stromal impairment) was observed when compared with young SAMs. NP also increased the CFU-GM colony formation of bone marrow and spleen cells from young SAMs in a soft agar culture system, but it did not enhance CFU-GM colony formation of cells from old SAMs cultured in this system. Treatment with NP induced the production of hematopoietic stimulating factors, including IL-6 and GM-CSF, by bone marrow stromal cells from young SAMs but stromal cells from old SAMs did not respond to NP stimulation. Further studies will be required to clarify the mechanism by which NP stimulates the production of hematopoietic growth factors from stromal cells, the results of this study indicate that NP is a potent hematopoietic regulatory factor by activating stromal cell function(s).

Key words neopterin; hematopoiesis; senescence-accelerated mice (SAM); stromal cell; hematopoietic microenvironment

Neopterin (NP) is a metabolite of guanosine triphosphate in the synthetic pathway of biopterin[1] and large amounts are produced by monocytes/macrophages in response to stimulation with interferon-γ (IFN-γ).[2] Increased NP levels accompany immune responses *in vivo* and *in vitro* and considerable interest has focused on measuring the levels of NP as a marker of immunological activation.[2—4] It has been suggested that NP and its derivatives modulate the redox balance in biological systems, but little is known about the biological activity of NP. Schobersberger *et al.* reported that NP induced apoptosis in the rat alveolar epithelial cell line L2.[5] Kojima *et al.* reported that NP inhibited NADPH-oxidase in peritoneal macrophages.[6] NP was also found to inhibit erythropoietin gene expression[7] and to induce proto-oncogene c-fos[8] and inducible nitric oxide synthetase (iNOS)[9] gene expression. Recently, we demonstrated that exogenously administered NP affected hematopoiesis *in vivo*.[10] The intraperitoneal administration of NP increased the number of blood leukocytes about 2-fold over the control level and increased the number of granulocyte-macrophage progenitor cells (CFU-GM) in both the bone marrow and spleen of mice.[10] Furthermore, a stimulatory effect of NP on hematopoiesis was found in the murine long-term bone marrow culture (LTMC) system, which reproduces hematopoiesis well *in vitro*.[11] NP also stimulated CFU-GM colony formation in a soft agar culture system, but no enhancing activity of NP on CFU-GM colony formation was observed when purified hematopoietic stem cells were cultured in this system. These findings suggest that NP dose not affect hematopoietic progenitors directly, but stimulate hematopoiesis by activating stromal cell function.[11] It has been demonstrated that stromal cells, a heterogeneous collection of mesenchymal cells that grow as an adherent layer in LTMC, are an essential component of hematopoietic microenvironment.[12—15] Stromal cells have been shown *in vitro* to regulate the proliferation and differentiation of hematopoietic stem cells by producing diffusible factors and through direct cellular interactions with adherent molecules. However, no suitable animal model has been developed for studying the hematopoietic regulatory function(s) of stromal cells *in vivo* during the process of hematopoiesis.

Senescence-accelerated mice (SAMs) were found in an inbred colony of the AKR/J strain.[16] SAMs provide a unique model system for studying senescence or aging in higher organisms because they exhibit marked acceleration of aging, which has been confirmed to occur in the same manner as that observed in regular mice. SAMs 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, spinal lordo-kyphosis and anemia.[16,17] As the number of white blood cells (WBC) and reticulocytes in peripheral blood start to decrease at approximately 30 weeks old, the SAMs used were 30 weeks old or slightly older (old SAMs). Although the study results must be carefully interpreted using SAMs ("accelerated aging" may not be associated with "normal aging"), the results of previous studies conducted by us and other researchers indicate that SAMs are a suitable model for predicting the possible mechanism of aging in hematopoietic systems.[17—20] A number of studies have shown no effect on the aging process on hematopoiesis with respect to the absolute number of hematopoietic stem cells in bone marrow.[21] Recently, Sakuma *et al.* found a latent defect in the hematopoietic microenvironment in old SAMs.[20] Furthermore, Kumagai *et al.* reported the low ability of colony-stimulating factor production from the bone marrow stromal cells induced by bacterial

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lipopolysaccharide (LPS) in old SAMs.[21] These observations suggest that the age-related impairment of hematopoietic microenvironment in SAMs appears to be responsible for the age-related decrease of hematopoiesis. Therefore, this suppressive hematopoiesis in old SAMs based on the impairment of stromal cells may contribute to developing a good model for evaluating the role of NP in the stromal cell function.

The aim of this study was to test whether exogenously administrated NP affects the regulatory function(s) of hematopoietic microenvironment in the mouse model of age-related stromal impairment, SAMs. Groups of young (8—12 weeks old) and old (30—36 weeks old) SAMs were injected intraperitoneally with NP, and the changes in the number of peripheral blood cells as well as hematopoietic progenitors in bone marrow and spleen were investigated. Furthermore, the effects of NP on the proliferation and differentiation of hematopoietic stem cells and hematopoietic supportive functions of stromal cells were examined using *in vitro* culture system.

MATERIALS AND METHODS

Mice and Cells SAMs/P-1,[16] a senescence-prone substrain of the AKR/J mice from the Jackson Laboratory in Bar Harbor, Maine, were kindly provided by Dr. Toshio Takada, Emeritus, the Chest Disease Research Institute, Kyoto University. The mice were bred and maintained under pathogen-free conditions. Male SAMs designated as "young (8—12 weeks old)" or "old (30—36 weeks old)" were used in this study because the number of WBC and reticulocytes in peripheral blood start to decrease at 30 weeks old.[18] Old SAMs were used as the mouse model of stromal impairment.

The spleen specimens were minced in ice-cold Iscove's modified Dulbecco's Medium (IMDM), gently homogenized in a Potter's glass homogenizer and a single cell suspension was obtained. Bone marrow cells were obtained aseptically from femurs and tibiae by flushing with IMDM into 15-ml plastic tubes using a 21-gauge needle and a 1-ml syringe, and clumps of cells were dispersed by repeated passage through a 21-gauge needle. Bone marrow or spleen cells were washed once with IMDM, and aliquots of spleen and bone marrow cells were diluted in Turk's solution and the nucleated cells were counted.

Agents and Antibodies D-(+)-neopterin (NP) was obtained from SIGMA (St. Louis, MO, U.S.A.). It was dissolved in 1 N HCl to produce a concentration of 1 mg/ml, then diluted 10-fold for use with IMDM. Hamerlinck reported the concentration of neopterin was 5.89 ± 1.78 nM/l (14.9 ± 4.5 ng/ml) in normal human serum.[22] Castenskiold *et al.* showed the elevation of serum neopterin level more than 70 nM/l (177.1 ng/ml) in the immunological activated patients, such as veno-occlusive disease after allogenic bone marrow transplantation.[23] Thus, we used neopterin at the concentration of 1 ng/ml to 10 μg/ml for *in vitro*, and 500 μg/kg to 5 mg/kg *in vivo* experiments.

Monoclonal antibodies against mouse Sca-1, Mac-1, TER119, Gr-1, Thy1.2 and B220 were purchased from BD PharMingen (San Diego, CA, U.S.A.). Sheep anti-rat IgG-coated immunomagnetic beads (Dynabeads M450 sheep anti-rat IgG) were purchased from Dynal (Great Neck, NY, U.S.A.).

Administration of NP Various concentrations of NP were injected intraperitoneally for 3 d, then the number of peripheral leukocytes, CFU-GM in bone marrow and spleen were counted on days 1, 7 and 14. At least 5 animals per group were examined.

Enrichment of Hematopoietic Stem Cells The nucleated cells from bone marrow or spleen were washed once with IMDM, and the cell pellet was suspended in 10 ml of 0.86% NH_4Cl for 5 min at 4 °C to rupture the erythrocytes. The cells were then washed twice with IMDM and nuclear cells of bone marrow or spleen were obtained.

For the enrichment of bone marrow hematopoietic stem cells, two steps of cell separation were performed. Firstly, whole bone marrow nucleated cells were suspended in IMDM with 10% FCS at a concentration of 2×10^6/ml, and then cultured at 37 °C for 2 h. The non-adherent cells were harvested and washed with IMDM. Secondly, negative cells against anti-murine TER119, Gr-1, Mac-1, Thy1.2 and B220 antibodies were collected by immunomagnetic cell-separation method according to the protocol of the manufacturer. Non-adherent bone marrow cells were incubated with a mixture containing 20 μl (0.1 mg/ml) of each anti-murine TER119, Gr-1, Mac-1, Thy1.2 and B220 antibodies for 30 min at 4 °C. After rinsing once with IMDM, cells were treated with 400 μl of Dynabeads M-450 sheep anti-rat IgG for 30 min at 4 °C with slow rotation. Positive cells for each monoclonal antibody were rosetted with Dynabeads and removed with a magnet (Lineage positive fraction; Lin(+)), and negative cells (Lin(−)) were collected and washed twice with IMDM. In the Lin(−) fraction, mature lineage committed cells were depleted and the number of CFU-GM was about 15 to 20-fold higher than cells in the whole bone marrow fraction. Using this procedure, about 5×10^6 Lin(−) cells that originated from 10^8 nucleated bone marrow cells were collected and used as a source of hematopoietic stem cells. Lin(−) cells were resuspended in IMDM and their viability was 96—98% as measured by trypan blue dye exclusion.

Progenitor Cell Colony Assay CFU-GM was assayed in the presence of various concentrations of NP using a Methocult GF M3534 kit (Stem Cell Technologies, Vancouver, Canada). The assays were performed according to the protocols supplied by the manufacturer. Whole bone marrow, spleen nucleated cells or Lin(−) bone marrow cells with NP were cultured in a humidified incubator at 37 °C and 5% CO_2, and the colonies were counted using a dissecting microscope. Seven days after plating, colonies containing more than 50 cells were considered to be CFU-GM and were counted.

Determination of the Level of IL-6 and GM-CSF Produced by Cultured Bone Marrow Stromal Cells Stromal monolayers were prepared by culturing bone marrow cells from young and old SAMs at 1×10^6/ml in 24-well Falcon 3047 flat-bottomed plates in 1-ml of IMDM supplemented with 10% FCS. Confluent adherent layers were obtained after 14 d. The supernatant in the culture plates was removed and 1-ml of IMDM supplemented with 10% FCS in the presence of various concentrations of NP was added to the culture plates. The culture medium was collected after 48 and 72 h of culture and was used to determine the level of IL-6 and GM-CSF produced by stromal cells. The concentration of

each IL-6 and GM-CSF in the culture medium was determined using IL-6 and GM-CSF-specific ELISA kit (R&D Systems, Inc., Minneapolis, MN, U.S.A.) according to the manufacturer's instructions. All samples were assayed in triplicate.

Statistical Analysis The results are expressed as the mean±S.D. of triplicate experiments. Differences between the means were determined using analysis of variance, ANOVA, and $p<0.05$ was considered significant.

RESULTS

Effects of Intraperitoneally Administrated NP on Hematopoiesis *in Vivo* Young and old mice were injected intraperitoneally with 5 mg/kg and 500 μg/kg of NP for 3 d and the number of peripheral leukocytes and CFU-GM in the bone marrow and spleen were counted after 1, 7 and 14 d. A group of mice was injected with PBS as a control. The results were expressed as a percentage of the control. Figure 1 shows that the number of peripheral leukocytes in both young and old SAMs was significantly increased at days 1 and 7 after NP treatment (absolute number of leukocytes in control animals of young and old SAMs were 5580±1200 and 3850±1010, respectively). After 14 d of NP treatment, an increased number of peripheral leukocytes in young SAMs was observed, but leukocytes in old SAMs declined and reached the control level.

An increased number of CFU-GM in the bone marrow and spleen of young SAMs were observed with NP treatment (Fig. 1) (the absolute number of CFU-GM per tibia and spleen in control young SAMs were 37725±3150 and 25928±2060, respectively). On the other hand, a rapid increase of CFU-GM in spleen of old SAMs was found 1 d after NP treatment (absolute number of CFU-GM per spleen in control old SAMs was 2510±427), however, CFU-GM was decreased and reached the control level after 7 d of treatment (Fig. 1). In the bone marrow of old SAMs, an increased number of CFU-GM was found only 7 d after treatment (absolute number of CFU-GM per tibia in control old SAMs was 26274±2436), and declined to the control level at 14 d after treatment (Fig. 1). In addition, treatment with 5 mg/kg of NP was necessary to enhance hematopoiesis in old SAMs, though 500 μg/kg of NP was sufficient to enhance hematopoiesis in young SAMs (Fig. 2), suggesting the low responsiveness of old SAMs to NP treatment as compared with young SAMs.

Effects of NP on CFU-GM Colony Formation of Bone Marrow and Spleen Cells from Young and Old SAMs In order to determine whether NP affected hematopoietic stem cell proliferation and/or differentiation, various concentrations of NP were added to soft agar cultures of bone marrow or spleen cells from young or old SAMs. Figure 3 shows the CFU-GM colony formation in the culture with bone marrow cells from young and old SAMs and the results were expressed as a percentage of the control. When bone marrow cells from young SAMs were cultured in this system, the addition of NP (from 1 μg/ml to 10 ng/ml) significantly increased the number of CFU-GM ($p<0.05$) (absolute number of CFU-GM in control was $234\pm10/10^5$ bone marrow cells). 100 ng/ml of NP was necessary to enhance the CFU-GM colony formation in the culture with bone marrow cells from old SAMs (Fig. 3) (absolute number of CFU-GM in the control was $213\pm15/10^5$ bone marrow cells). Introduction of

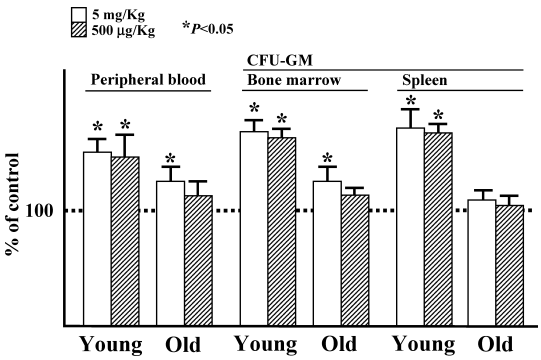

Fig. 2. The Number of Peripheral Leukocytes, and CFU-GM in Bone Marrow or Spleen of Young and Old SAMs 7 d after NP Treatment

The number of peripheral leukocytes, and CFU-GM in bone marrow or spleen was assayed at 7 d after intraperitoneal administration of 5 mg/kg or 500 μg/kg of NP. The results were expressed as a percentage of control. Each bar represents the mean±S.D. obtained from five mice individually analyzed. * Significant difference ($p<0.05$) with respect to NP non-treated mice.

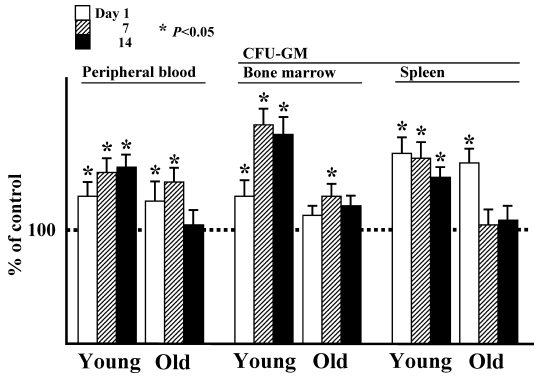

Fig. 1. The Number of Peripheral Leukocytes, and CFU-GM in Bone Marrow or Spleen of Young and Old SAMs after Treatment with 5 mg/kg of NP

The number of peripheral leukocytes, and CFU-GM in bone marrow or spleen was assayed at 1, 7 and 14 d after intraperitoneal administration of 5 mg/kg of NP. The results were expressed as a percentage of control. Each bar represents the mean±S.D. obtained from five mice individually analyzed. * Significant difference ($p<0.05$) with respect to NP non-treated mice.

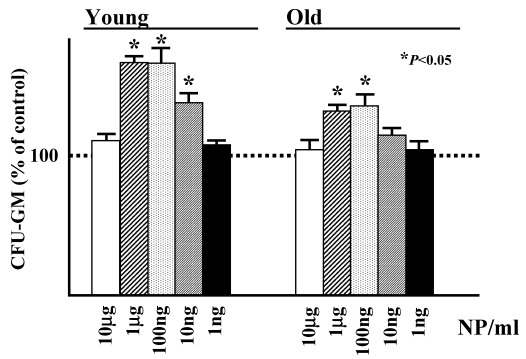

Fig. 3. CFU-GM Colony Formation of Bone Marrow Cells from Young and Old SAMs in the Presence of NP

Whole bone marrow nucleated cells from young or old SAMs were cultured in the presence of various concentration of NP, and the number of CFU-GM was counted 7 d after cultivation. The results were expressed as a percentage of the control. Each bar represents the mean±S.D. obtained from triplicate experiments. * Significant difference ($p<0.05$) with respect to culture without NP treatment.

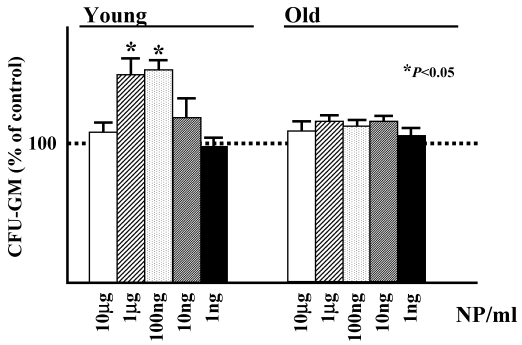

Fig. 4. CFU-GM Colony Formation of Spleen Cells from Young and Old SAMs in the Presence of NP

Spleen nucleated cells from young or old SAMs were cultured in the presence of various concentration of NP, and the number of CFU-GM was counted 7 d after cultivation. The results were expressed as a percentage of the control. Each bar represents the mean±S.D. obtained from triplicate experiments. *Significant difference ($p<0.05$) with respect to culture without NP treatment.

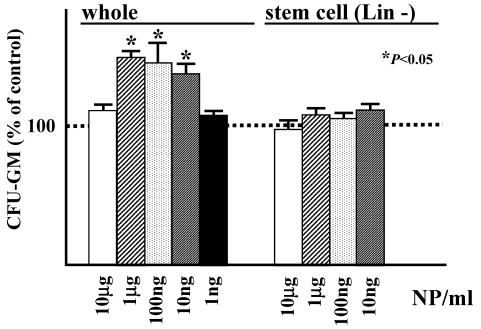

Fig. 5. CFU-GM Colony Formation of Lin(−) Cells from Young SAMs in the Presence of NP

Whole or Lin(−) bone marrow cells from young SAMs were cultured in the presence of various concentration of NP, and the number of CFU-GM was counted 7 d after cultivation. The results were expressed as a percentage of the control. Each bar represents the mean±S.D. obtained from triplicate experiments. *Significant difference ($p<0.05$) with respect to culture without NP treatment.

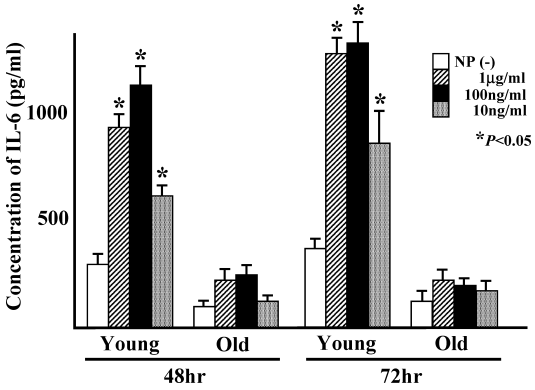

Fig. 6. Concentration of IL-6 in the Conditioned Medium of Stromal Cells Originated from Young or Old SAMs

Conditioned medium of bone marrow stromal cells was collected 48 and 72 h after treatment with various concentration of NP, and the concentration of IL-6 was assayed by ELISA methods as described in Materials and Methods. Each bar represents the mean±S.D. obtained from triplicate experiments.

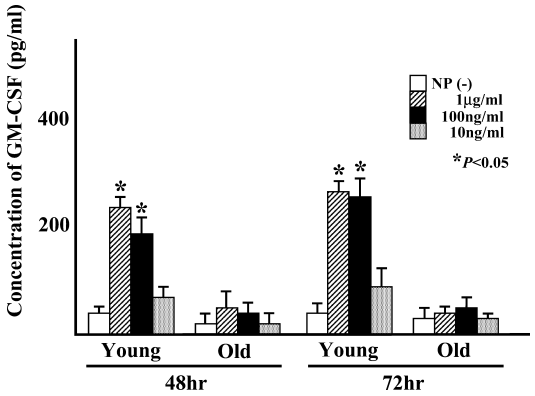

Fig. 7. Concentration of GM-CSF in the Conditioned Medium of Stromal Cells Originated from Young or Old SAMs

Conditioned medium of bone marrow stromal cells was collected 48 and 72 h after treatment with various concentration of NP, and the concentration of GM-CSF was assayed by ELISA methods as described in Materials and Methods. Each bar represents the mean±S.D. obtained from triplicate experiments.

1 μg and 100 ng/ml of NP increased the number of CFU-GM in the culture with spleen cells from young SAMs (Fig. 4) (absolute number of CFU-GM in the control was 124±10/10^6 spleen cells). On the other hand, NP did not affect the CFU-GM colony formation in the culture with spleen cells from old SAMs (Fig. 4) (absolute number of CFU-GM in the control was 18±2/10^6 spleen cells). No enhancing activity on CFU-GM colony formation of NP was observed when bone marrow Lin(−) cells (hematopoietic stem cell enriched fraction) of young SAMs were cultured in this system (Fig. 5), indicating that NP did not directly affect CFU-GM colony formation by hematopoietic stem cells.

Effects of NP on the Cytokine Production by Bone Marrow Stromal Cells In order to study the effects of NP on bone marrow stromal cell functions, the concentration of IL-6 and GM-CSF in conditioned medium of bone marrow stromal cells obtained from young or old SAMs after NP treatment was examined. The supernatants of bone marrow stromal cells were collected 48 and 72 h after the addition of various concentrations of NP. Figure 6 shows the IL-6 production by stromal cells after NP treatment. IL-6 production by stromal cells from young SAMs was remarkably stimulated by NP treatment, while the production of IL-6 by stromal cells originating from old SAMs was not. GM-CSF production by stromal cells originating from young SAMs was stimulated by NP treatment (Fig. 7). Again, GM-CSF production of stromal cells from old SAMs was not stimulated by NP treatment (Fig. 7). Neither the stimulatory nor inhibitory effects of NP on the proliferation of both stromal cells from young and old SAMs was found (data not shown).

DISCUSSION

NP is a pyrazino-pyrimidine compound derived from guanophosphate within the biosynthetic pathway of tetrahydrobiopterin (BH4), which is an essential cofactor for tyrosine and tryptophan hydroxylation and plays an important role in the biosynthesis of catecholamines and serotonin.[24] There is evidence that activation of the T lymphocyte/macrophage axis raises the NP level, and this leads to the assumption of its value as a biochemical marker for clinical assessment of the cell-mediated immune response.[2—4] However, the mode of action and the biological significance of NP are not yet known. Recently, we demonstrated that exogenously administered NP stimulated hematopoiesis in the murine bone marrow and spleen.[10] NP treatment increased the number of CFU-GM and CFU-S in murine bone marrow

culture system, indicating that NP stimulated the proliferation of hematopoietic stem cells.[11] NP also stimulated the CFU-GM colony formation in a soft agar culture system, but no enhancing activity of NP on CFU-GM colony formation was observed when purified hematopoietic stem cells were cultured in this system, suggesting that NP did not affect the proliferation of hematopoietic progenitors directly.[11]

The results of this study show that exogenous administrated NP increased the leukocytes in peripheral blood and CFU-GM in the bone marrow and spleen of young SAMs (Fig. 1). However, increases of CFU-GM in the bone marrow and spleen were not observed in old SAMs when compared to young SAMs (Figs. 1, 2). NP stimulates the CFU-GM colony formation in the soft agar culture of bone marrow and spleen cells from young SAMs (Fig. 3), but stimulatory activity of NP on CFU-GM colony formation was not found in a culture with spleen cells from old SAMs (Fig. 4). Although, both spleen and bone marrow are the organs of hematopoiesis in mice, these observations suggest a lack of responsiveness to NP, especially in spleen cells from old SAMs. Unfortunately, the mechanism why the splenic cells from old SAMs have low responsiveness to NP as compared with cells from bone marrow is not clarified in this study. It has been demonstrated that stromal cells, a heterogeneous collection of mesenchymal cells including fibroblasts, adipocytes, macrophages and endothelial cells are thought to be essential components of hematopoietic microenvironment. It is necessary to study which type of stromal cells are distributed in the spleen and bone marrow, quantitatively and qualitatively. And the response to NP stimulation of each cell should be studied, individually.

Similarly, low responsiveness of the splenic cells of old SAMs to hematopoietic stimulatory agent, bacterial lipopolysaccharide (LPS), was reported by Kumagai *et al.*[21] LPS is a major contributing factor in the initiation of generalized inflammation due to infection, and is often used as a hematopoietic stimulatory agent *in vivo*. These observations suggest a lack of responsiveness of stroma impairment old SAMs to hematopoietic stimulants, such as LPS and NP.

Whether changes in the functional properties of the hematopoietic system occur with age has not been clarified. There is general agreement with the concept that steady-state hematopoiesis in aged mammals is not demonstrably impaired.[21,25,26] However, inducible hematopoiesis occurs whenever there is an increased demand for mature blood cells, *e.g.* in the case of infection, after loss of blood cells and after myeloablative events caused by exposure to cytotoxic drugs,[27,28] a latent defect of hematopoietic regulatory system in the spleen (and bone marrow) is revealed in senescent mammals. Recently, an increase of neopterin levels in plasma during aging was reported[29] and the percentage of S-phase of CFU-GM in the bone marrow of senescent mice was found.[20] These findings suggest that hematopoiesis may already be accelerated in senescent mice under steady conditions.

Furthermore, no hematopoietic stimulating activity of NP was observed when Lin(−) cells isolated from whole bone marrow cells from young SAMs were cultured *in vitro* (Fig. 5). Lin(−) cells isolated from bone marrow have been considered as an enriched fraction of hematopoietic stem cells. Thus, the results may indicate that NP did not affect the proliferation and differentiation of hematopoietic stem cells directly, even though cells were obtained from young SAMs. Previously, it was observed that NP or LPS stimulated the proliferation and differentiation of hematopoietic stem cells *via* the modulation of stromal cell functions.[11,20] Stromal cells have been shown to be essential for the regulation of hematopoietic stem cell proliferation and differentiation through the production of humoral growth factors, cell-to-cell interactions or both.[12—15] In this study, we found that NP induced the production of GM-CSF and IL-6, which act as positive growth factors on the proliferation and differentiation of hematopoietic progenitors, by stromal cells from young SAMs. A slight but not significant increase of GM-CSF and IL-6 production from stromal cells of old SAMs induced by NP was observed (Figs. 6, 7). Previously, we have reported that the treatment with 100 ng/ml of LPS induced the production of IL-6 and GM-CSF by stromal cells from young SAMs, though, a slight increase of these cytokine production from stromal cells of old SAMs.[30] On the other hand, Tha *et al.* reported that LPS induced the IL-6 production by the brain cerebral cortex cells from old SAMs as seen in the cells from control young SAMs.[31] These observations suggest that stromal cells in old SAMs are specifically impaired in comparison with that of young SAMs. NP has been shown to induce the expression of iNOS gene expression through the activation of cellular transcription factor nuclear factor κB (NF-κB) in vascular smooth muscle cells.[32] It is well known that NF-κB plays a central role in controlling a variety of gene expressions, including IL-6 and GM-CSF.[33] Extensive studies are in progress to resolve the issue of the effects of NP on NF-κB gene expression and the activity of NF-κB in stromal cells from young and old SAMs. Further study is required to elucidate the mechanism(s) of action of NP, however, these findings taken together provide evidence that NP is a potent hematopoietic regulatory factor *via* activating the hematopoietic microenvironment.

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