The characteristics of hematopoietic stem cells from autoimmune-prone mice and the role of neural cell adhesion molecules in abnormal proliferation of these cells in MRL/lpr mice

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ABSTRACT

Background and Objectives

Using various animal models for autoimmune diseases, we have previously shown that such diseases are *stem cell disorders*.[1] In order to understand how autoimmune diseases develop, we investigated the distinct qualitative differences between hematopoietic stem cells (HSC) from normal and autoimmune-prone mice.

Design and Methods

We studied the major histocompatibility complex (MHC) restriction between HSC and stromal cells *in vitro* and *in vivo*. We also examined the ability of HSC to adhere to a stromal cell line and, using flow cytometry, analyzed the expression of various adhesion molecules in HSC before and after the onset of autoimmune disease. In addition, the effect of antibodies to anti-adhesion molecules on the proliferation of HSC was investigated.

Results

The abnormal HSC of MRL/lpr mice showed no MHC restriction (or preference) with stromal cells either *in vitro* or *in vivo*, although there was MHC restriction between normal HSC and stromal cells, as we previously reported.[2,3] The abnormal HSC of MRL/lpr mice exhibited enhanced adhesion to stromal cells *in vitro* and expressed a higher amount of adhesion molecules such as neural cell adhesion molecule (NCAM). Interestingly, the proliferation of HSC in MRL/lpr mice was significantly suppressed by anti-NCAM monoclonaal antibodies.

Interpretation and Conclusions

Abnormal HSC of MRL/lpr mice are more resilient than normal HSC. Furthermore, among various adhesion molecules, only NCAM shows increased expression on HSC of MRL/lpr mice after the onset of autoimmune diseases, and these molecules contribute to the enhanced proliferation capacity of abnormal HSC in MRL/lpr mice. The present findings suggest that there are intrinsic qualitative differences between HSC from normal and autoimmune-prone MRL/lpr mice.

Key words: hematopoietic stem cells, MRL/lpr mice, bone marrow transplantation, NCAM.

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We have previously found that allogeneic bone marrow transplantation (BMT) from normal mice to autoimmune-prone mice can be used to prevent and treat autoimmune diseases,[4,5] and that BMT from autoimmune-prone mice to normal mice leads to the development of autoimmune diseases in normal mice.[1 6,7]

Based on these findings, we have proposed that autoimmune diseases are *stem cell disorders*.[1,7,8] Thus, conventional allogeneic BMT can be used to treat autoimmune diseases in various autoimmune-prone mice.[8] However, in MRL/lpr mice, which are radiosensitive (< 8.5 Gy), we found that conventional intravenous BMT (IV-BMT) had only a transient effect on autoimmune diseases, which recurred.[9] Therefore, we concentrated on how we could prevent and treat autoimmune diseases in radiosensitive and chimeric-resistant MRL/lpr mice. We found that stromal cells are essential for successful allogeneic BMT: stromal cells play a crucial role in preventing graft failure,[10,11] since there is a major histocompatibility complex (MHC) restriction between hematopoietic stem cells (HSC) and stromal cells.[2,3] In order to prevent the recurrence of autoimmune diseases in MRL/lpr mice, we therefore carried out BMT plus bone grafts to replace not only hematopoietic cells but also stromal cells with donor cells. MRL/lpr mice that had been irradiated (8.5Gy) and then reconstituted with C57BL/6 bone marrow cells (BMC) plus bone grafts survived more than 48 weeks.[12,13] However, this strategy (8.5Gy/bone/BMT) was not found to be beneficial for the treatment of florid autoimmune diseases in MRL/lpr mice. MRL/lpr mice with proteinuria (<+) can endure 8.5Gy irradiation, whereas MRL/lpr mice with proteinuria (≥ ++) are more radiosensitive and are unable to endure 8.5Gy irradiation due to the development of uremic enterocolitis. Therefore, we devised a new strategy that reduces the toxic effects of radiation (fractionated irradiation: 5.5Gy x 2) and prevents graft rejection. Finally, we found that *IBM-BMT* (injection of whole BMC [containing both hematopoietic and mesenchymal stem cells] into the bone marrow cavity) is the best strategy for allogeneic BMT.

In order to further understand how the autoimmune diseases develop, in the present study we investigated the distinct qualitative difference of HSC between normal and autoimmune-prone MRL/lpr mice. Furthermore, since we have recently shown that neural cell adhesion molecule (NCAM) contributes to the hematopoiesis-supporting capacity of stromal cells in normal mice,[14] we attempted to investigate whether NCAM plays an important role in the abnormal proliferation of HSC from MRL/lpr mice.

Design and Methods

Mice

Five- to eight-week-old C3H (H-2^k), C57BL/6 (B6: H-2^b), MRL/lpr (H-2^k), (NZB*NZW) F1 (B/WF1: H-2^d/H-2^z), (NZW*BXSB) F1 (W/BF1: H-2^z/H-2^b) mice and pregnant C3H mice with 14-day fetuses were purchased from Shizuoka Experimental Animal Laboratory (Hamamatsu, Japan). Five- to eight-week-old NOD/LTJ mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA, *http://jaxmice.jax.org*). All mice were maintained in a pathogen-free environment.

Purification of HSC

BMC were collected from normal young (5-7 weeks) or old (>4 months) C3H and B6 mice and from MRL/lpr, B/WF1, W/BF1 and NOD/LTJ mice before or after the onset of autoimmune diseases (proteinurea >++). All mice were treated with 5-fluorouracil (5-FU, 150 mg/kg) 3 days before being sacrificed by cervical dislocation. The animals' femora and tibiae were removed and cleaned of all connective tissue. BMC were collected by flushing the femora and tibiae with 1*phosphate-buffered saline containing 2% fetal bovine serum (FBS) using a 26-guage needle, then filtered, and washed twice. Low-density BMC were purified by discontinuous density gradient centrifugation using Percoll (Pharmacia; Uppsala, Sweden; http://www.pnu.com). The low density cells ($1.066 < \rho < 1.077$) were incubated with biotinylated monoclonal antibody (rat IgG class) cocktails against lineage markers (Mac-1, Gr-1, B220, CD4, CD8, NK1.1 and TER119) (BD Biosciences Pharmingen, San Diego, CA, USA), and mature hematopoietic cells were then removed using magnetic beads (Dynabeads M-280 Streptavidin; Dynal Biotech ASA, Oslo, Norway; *http://www.dynal.no*) with gentle agitation at a 3:1 bead/cell ratio. Thus, Lin$^-$ BMC were obtained; stem cell antigen 1 (Sca-1)-positive cells at a concentration of 26%. Sca-1$^+$ cells were separated from Lin$^-$ BMC using a magnetic cell separation system (MACS, Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany, *http://www.miltenyibiotec.com*). Lin$^-$ Sca-1$^+$ cells were considered to be HSC.

Preparation of fetal bone marrow adherent cells from C3H mice

The femora, tibiae, and humeri from C3H fetuses at day 16 of gestation were removed and diced. The resultant bone pieces were inoculated in a 25 cm^2 flask containing DMEM (low glucose) supplemented with 10% FBS and cultured at 37°C in 5% CO_2 in air. Half the medium in the culture flask was replaced with fresh medium every 3 days. When the adherent cells reached 70-80% confluence, they were collected by trypsin-EDTA treatment and subcultured once only.

Long-term culture of various HSC on stromal cells

FMS/PA6-P, a stromal cell line, established from BMC from 16-day fetal B6 mice in our laboratory,[14] and fetal bone marrow adherent cells from C3H mice express a large amount of MHC molecules. Therefore, we investigated the proliferative capacity of various HSC *in vitro* by culturing them on FMS/PA6-P cells or fetal bone marrow

adherent cells from C3H mice. Monolayers of FMS/PA6-P cells or fetal bone marrow adherent cells of C3H mice were prepared in 25 cm^2 flasks and irradiated (20 Gy), and then the HSC from >4-month-old C3H, B6 or MRL/lpr mice (with proteinurea >++) were inoculated (1×10^5 in 8 mL of 10% FBS IMDM for FMS/PA6-P cells and 1.5×10^5 in 8 mL of 10% FBS IMDM for fetal bone marrow adherent cells from C3H mice). After 1 week of culture, cobblestone colonies in the flask were counted. Each week, the medium in the flask containing non-adherent cells was completely removed and replaced with fresh medium. The non-adherent cells were then counted and 1×10^4 of these cells were assessed in methylcellulose culture assay (Methocult GF M3434, Stem Cell Technologies INC., Vancouver, BC) for the growth of colony-forming units in culture (CFU-C).

Colony-forming units-spleen assay

Recipient mice (8 to 9 weeks old) were lethally irradiated (9.5 Gy for B6 and C3H mice, 8.5 Gy for MRL/lpr mice) and then injected with 2×10^4 HSC the next day. The recipient mice were sacrificed 12 days after BMT. Their spleens were removed and weighed, and then fixed in Bouin's solution. The surface colonies were counted as colony-forming units-spleen (CFU-S).

Adhesion ability of various HSC to stromal cells

In order to evaluate the adhesion of HSC to stromal cells, 1×10^5 HSC from >4-month-old B6, C3H and MRL/lpr mice (with proteinurea >++) were cultured on monolayers of FMS/PA6-P cells for 2 hours and then non-adherent cells were removed. Adherent cells were collected by repeated pipetting and then assessed in methylcellulose culture assay (Methocult GF M3434, Stem Cell Technologies Inc., Vancouver, BC, Canada) for CFU-C, which includes BFU-E, CFU-GM, CFU-GEMM, CFU-G and CFU-M.

Adhesion molecule expression on various Lin^- $Sca\text{-}1^+$ HSC

As described above, Lin^- BMC were purified using magnetic beads binding to biotinylated monoclonal antibody (rat IgG class) cocktails against lineage markers. The purified Lin^- BMC were further stained with anti-mouse NCAM monoclonal antibody (Clone: N-CAM13, BD Biosciences Pharmingen), labeled with fluoroscein isothiocyanate (FITC) using a commercially available kit (American Qualex, San Clemente, CA, USA) and other monoclonal antibodies including anti-CD62L, anti-VCAM-1, anti-VLA-4, anti-CD44 and anti-ICAM. The cells were also stained with anti-Sca-1 monoclonal antibodies (all the monoclonal antibodies were labeled with FITC or phycoerythrin, BD Biosciences Pharmingen, San Diego, CA, USA). The stained cells were analyzed by a FACScan (BD, Mountain View, CA, USA).

Analyses of the effect of anti-NCAM antibody on the proliferation of HSC

HSC were first incubated with anti-NCAM monoclonal antibody (1 μg/mL) or corresponding isotype antibodies for 2 hours, and after washing were added to an irradiated (20 Gy) confluent FMS/PA6-P cell layer (3×10^3 HSC/well). The culture was then incubated for 6 days and pulsed with 0.5 μCi of ^{3}H-thymidine (^{3}H-TdR) for the last 20 hours of the culture period. Results are presented as the mean cpm ± standard deviation (SD) of seven wells.

All experiments were carried out three or more times, and reproducible results obtained. Representative data are shown in the tables and figures.

Statistics

Statistical differences in all experiments were analyzed by the two-tailed Student's t test.

Results

No MHC restriction exists between abnormal HSC from MRL/lpr mice and stromal cells in vitro

When the HSC of old (i.e. > 4 months old) B6 mice were cultured with an MHC-matched stromal cell line (FMS/PA6-P: H-2^b) (Figure 1A) or the HSC of old C3H mice were cultured with MHC-matched stromal cells (fetal bone marrow adherent cells from C3H mice: H-2^k) (Figure 1B), they showed a good proliferative response. In contrast, when the HSC of old B6 mice were cultured with MHC-mismatched stromal cells (fetal bone marrow adherent cells from C3H mice: H-2^k) (Figure 1B) or the HSC from old C3H mice were cultured with an MHC-mismatched stromal cell line (FMS/PA6-P: H-2^b) (Figure 1A), they showed a poor proliferative response. However, the HSC of autoimmune-prone MRL/lpr mice (proteinurea >++) proliferated on a MHC-mismatched stromal cell line (FMS/PA6-P: H-2^b) (Figure 1A) to a similar extent as those on MHC-matched stromal cells (fetal bone marrow adherent cells from C3H mice: H-2^k) (Figure 1B); there was no significant difference in total non-adherent cell counts after 4 weeks of culture (*data not shown*). Moreover, they proliferated to a much greater extent on MHC-mismatched stromal cells (FMS/PA6-P: H-2^b) than did the HSC of B6 mice on MHC-matched stromal cells (FMS/PA6-P: H-2^b) (Figure 1A). This phenomenon was also observed when adult bone marrow adherent cells were used instead of fetal stromal cells. There was no difference in the proliferation of HSC from MRL/lpr mice (H-2^k) on adult C3H (H-2^k) and B6 (H-2^b) bone marrow adherent cells, whereas the proliferation of HSC from C3H mice (H-2^k) decreased greatly on adult B6 bone marrow adherent cells (H-2^b) [0.36 of control (C3H HSC on adult C3H bone marrow adherent cells)] (*data not shown*). These findings suggest that no MHC restriction exists between abnormal HSC from MRL/lpr mice and stromal cells *in vitro*.

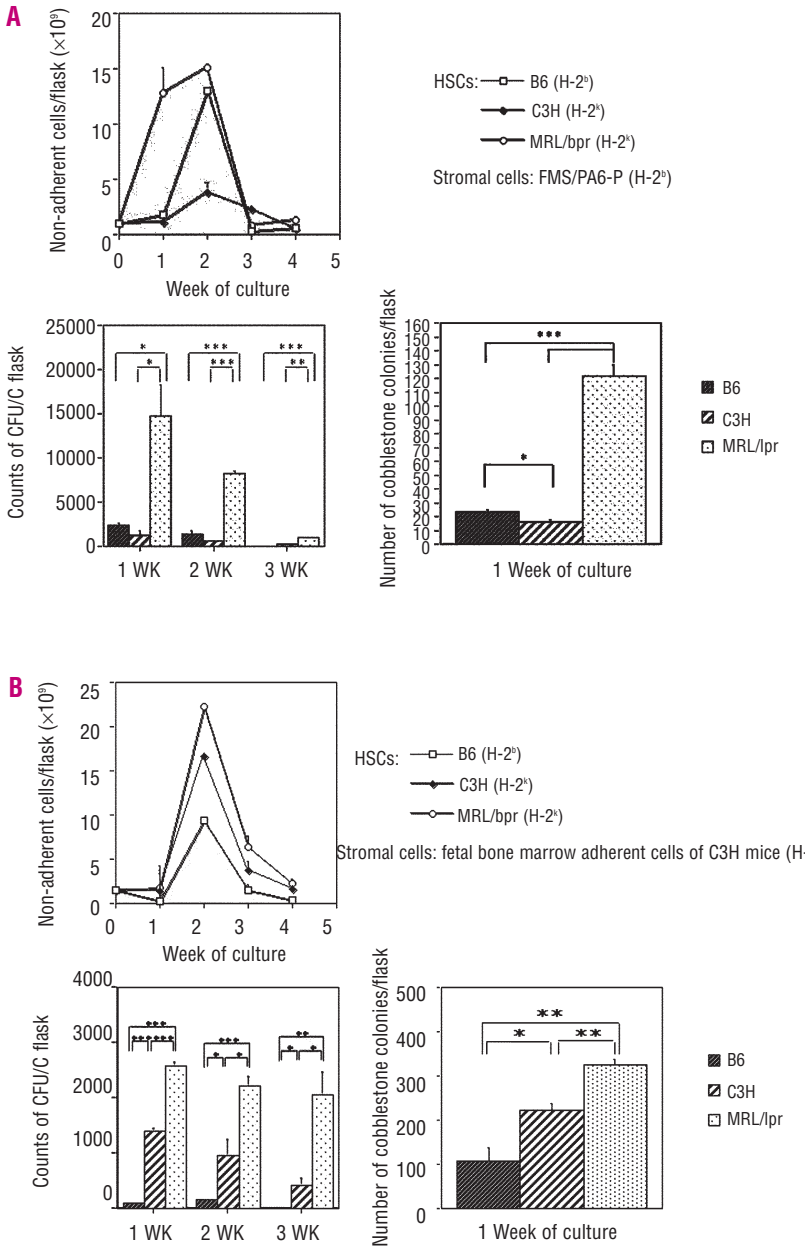

Figure 1. No MHC restriction exists between abnormal HSC from MRL/lpr mice and stromal cells *in vitro*. HSC from >4-month-old MRL/lpr (proteinurea >++, H-2ᵏ), normal B6 (H-2ᵇ) or normal C3H (H-2ᵏ) mice were obtained as described in the *Design and Methods*. HSC from various mice were then cultured on MHC-matched or –mismatched stromal cells. Briefly, 1×10^5 of these HSC were cultured on a monolayer of FMS/PA6-P stromal cell line (H-2ᵇ) (A) and 1.5×10^5 were cultured on a monolayer of fetal bone marrow adherent cells from C3H mice (H-2ᵏ) (B). Each week, the medium in the flask containing non-adherent cells was completely removed and replaced with fresh medium. The non-adherent cells were then counted. ***: $p<0.001$; **: $p<0.01$; *: $p<0.05$. CFU-C: colony-forming unit in culture.

No MHC restriction exists between abnormal HSC from MRL/lpr mice and stromal cells in vivo

The CFU-S assay has long been used for characterizing multi-lineage progenitor cells *in vivo*.[15] The colonies observed on day 12 are indicative of HSC, and their colony formation is dependent on MHC matching.[16] Therefore, we next investigated the ability of HSC from MRL/lpr mice to produce CFU-S in allogeneic (B6) or syngeneic (MRL/lpr) recipients. As shown in Figure 2, normal HSC of B6 mice formed significantly higher CFU-S in syngeneic combinations (HSC from B6 mice→B6 mice) than those in allogeneic combinations (HSC from B6 mice→C3H mice). The same phenomenon was also observed when normal HSC from C3H mice were investigated (Figure 2). However, abnormal HSC from MRL/lpr mice showed similar CFU-S counts in an allogeneic combination (HSC from MRL/lpr mice→B6 mice) to those in a syngeneic combination (HSC from MRL/lpr mice→MRL/lpr mice). These findings suggest that there is no MHC restriction between abnormal HSC from MRL/lpr mice and stromal cells *in vivo*. Furthermore, abnormal HSC from MRL/lpr mice produced significantly higher CFU-S counts in allogeneic combination (HSC of MRL/lpr mice→B6 mice) than did HSC from normal mice (HSC from B6 mice→C3H mice or HSC of C3H mice→B6 mice). Moreover, the spleens of allogeneic recipients injected with abnormal HSC from MRL/lpr mice weighed significantly more than those of animals in which a combination of normal HSC and syngeneic stromal cells was used (HSC from B6 mice→B6 mice). These findings indi-

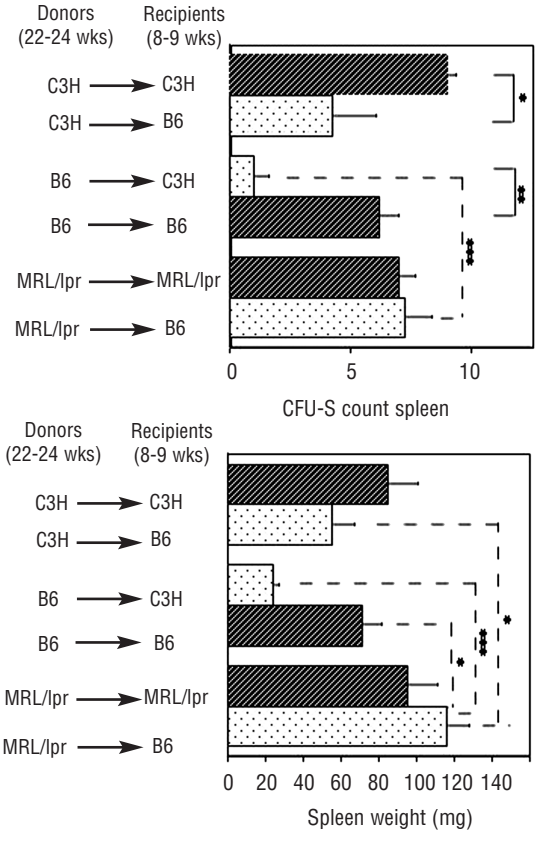

Figure 2. CFU-S (Day 12) assay of HSC obtained from autoimmune-prone MRL/lpr and normal mice. Normal HSC produced significantly higher CFU-S counts in syngeneic combinations than those in allogeneic combinations. However, abnormal HSC from MRL/lpr mice produced similar CFU-S counts in allogeneic combination to those in syngeneic combination. Furthermore, abnormal HSC from MRL/lpr mice produced significantly higher CFU-S counts in allogeneic combination than did HSC from normal mice. ***:$p<0.001$; **: $p<0.01$; *: $p<0.05$. CFU-S, colony-forming unit-spleen.

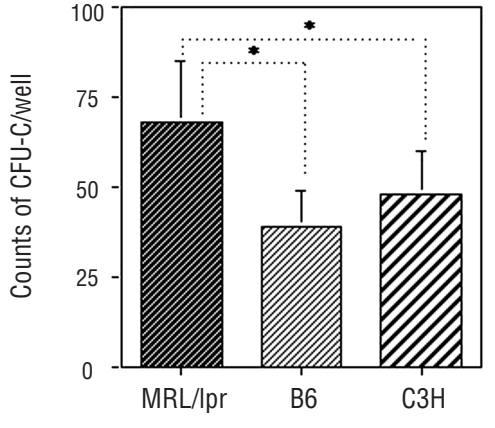

Figure 3. Abnormal HSC from MRL/lpr mice exhibit enhanced adhesion to FMS/PA6-P cells *in vitro*. HSC (1×10^5) were cultured on a monolayer of FMS/PA6-P cells for 2 hours, and the adherent cells were then assessed in a methylcellulose assay for CFU-C. The total CFU-C counts developed from the HSC of MRL/lpr mice (proteinurea >++) that adhered to the FMS/PA6-P cells were significantly higher than those from the HSC from B6 and C3H mice (>4 months old). *:$p<0.05$. CFU-C: colony-forming unit in culture.

cate that abnormal HSC from MRL/lpr mice are more resilient than normal HSC.

Abnormal HSC from MRL/lpr mice exhibit enhanced adhesion to FMS/PA6-P cells in vitro

The ability of HSC to adhere to stromal cells correlates strongly with homing ability *in vivo*.[17-20] We cultured 1×10^5 HSC on a monolayer of FMS/PA6-P cells for 2 hours, and then assessed the adherent cells in a methylcellulose assay for CFU-C, which includes BFU-E, CFU-GM, CFU-GEMM, CFU-G and CFU-M. The total CFU-C counts developed from the HSC from abnormal MRL/lpr mice which adhered to the FMS/PA6-P cells were significantly higher than those from the HSC of normal B6 and C3H mice (Figure 3), indicating an enhanced adhesion (or homing) ability of HSC in MRL/lpr mice.

Increase in adhesion molecule expression on Lin⁻ Sca-1⁺ HSC from MRL/lpr mice after the onset of autoimmune disease

The adhesion of HSC to stromal cells is mediated by adhesion molecules expressed on HSC. We, therefore, next investigated adhesion molecule expression on various HSC. As shown in Table 1 and Figure 4, there was no difference between NCAM expression in Lin⁻ Sca-1⁺ HSC of normal young (5 to 7 weeks old) and old (22 to 24 weeks old) mice. However, Lin⁻ Sca-1⁺ HSC from old MRL/lpr mice (with proteinuria >++) expressed higher amounts of NCAM (4.92 ± 0.45%) than those from young MRL/lpr mice without proteinuria (1.52±0.24%) and normal old mice (old B6: 2.96±0.21%; old C3H: 1.57±0.14%). For other adhesion molecules, such as CD44, CD62L, very late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1), no differences were found between young and old MRL/lpr mice (VCAM-1 expression in various mice is shown in Table 2 as a control; other data are not shown). These findings suggest that the increased ability of abnormal HSC from MRL/lpr mice to adhere to stromal cells may be attributable to the increased expression of adhesion molecules such as NCAM. Increased expression of NCAM was also found in HSC from other autoimmune-prone mice, such as W/B F1, B/W F1 and NOD/LTJ mice, after the onset of autoimmune diseases (*data not shown*).

Anti-NCAM monoclonal antibodies inhibit the proliferation of HSC from MRL/lpr mice

Very recently, we have found that NCAM contributes to the hematopoiesis-supporting capacity of stromal cells through homophilic or heterophilic binding.[14] We, therefore, investigated whether the higher expression of NCAM has some effects on the abnormal proliferation of HSC from MRL/lpr mice. After preincubating HSC with anti-NCAM monoclonal antibody, $3*10^3$ HSC were co-cultured with irradiated (20 Gy) FMS/PA6-P cells for 6

Table 1. Increases in NCAM expression on Lin⁻ Sca-1⁺ HSC from MRL/lpr mice after the onset of autoimmune diseases.

Mice	*Young (5-7 weeks) Proteinurea (-)*	*Old (22-24 weeks) Proteinurea (-)*	*Old (22-24 Weeks) Proteinurea (>++)*
C3H	1.43±0.14*[a]	1.57±0.14[c]	–
B6	2.53±0.62[a]	2.96±0.21[d]	–
MRL/lpr	1.52±0.24[b]	–	4.92±0.45

**:percentage of NCAM expression on Lin⁻ Sca-1⁺ HSCs; [a]:p>0.05 (old C3H vs. young C3H or old B6 vs. young B6); [b]:p<0.01 (old MRL/lpr vs. young RL/lpr); [c]: p<0.01 (old MRL/lpr vs. old C3H); d:p<0.05 (old MRL/lpr vs. old B6).*

days. The cultures were then pulsed with ^{3}H-TdR for the last 20 hours of the culture period. We found that the proliferation of HSC from MRL/lpr mice was significantly suppressed by the addition of anti-NCAM monoclonal antibody, whereas there was no suppression in the proliferation of HSC from B6 and C3H mice (Figure 5). The same phenomenon was also observed in autoimmune-prone W/B F1, B/W F1 and NOD/LTJ mice (*data not shown*). These results suggest that the interaction between HSC and stromal cells through NCAM plays an important role in the proliferation of HSC from MRL/lpr mice.

We also found that the HSC from MRL/lpr mice showed significant proliferation ability in comparison with those from B6 and C3H mice using the HSC-proliferation assay mentioned above.

Discussion

MRL/lpr mice, which spontaneously develop massive lymphadenopathy, are well known as an animal model for systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). We have previously proposed that autoimmune diseases are stem cell disorders.[2,4-6,9,10,21-24] In the present study, we confirmed this concept using MRL/lpr mice, and found that a distinct qualitative difference exists between normal and abnormal HSC from MRL/lpr mice.

We have reported previously that donor-derived stromal cells play a crucial role in successful BMT across MHC barriers[13,25] and that an MHC restriction exists between P-HSC and stromal cells not only *in vivo* but also *in vitro*. Recently, we have found that an MHC class I (D and S loci) determines the restriction between HSC and stromal cells.[3] In contrast to HSC from normal mice, the HSC from autoimmune-prone MRL/lpr mice proliferated on MHC-mismatched stromal cells to a similar extent to those on MHC-matched stromal cells (Figure 1). Moreover, they proliferated to a much greater extent on MHC-mismatched stromal cells than did the HSC from B6 mice cultured on MHC-matched stromal cells (Figure 1), suggesting that there is no MHC restriction between abnormal HSC from MRL/lpr mice and stromal cells *in vitro*. This phenomenon was also seen *in vivo*. Namely, abnormal HSC from MRL/lpr mice gave rise to similar CFU-S counts in allogeneic combination to those in syngeneic combination and produced significantly higher CFU-S counts in allogeneic combination than did HSC from normal mice (Figure 2). These findings indicate that abnormal HSC are more *resilient* than normal HSC.

We also found that the MHC-restriction between the HSC from B6 mice and MS-5, a stromal cell line established by irradiating bone marrow adherent cells from C3H mice, was lower than that between the HSC from B6 mice and fetal bone marrow adherent cells from C3H mice (*data not shown*), suggesting that the MHC-restriction between the HSC and stromal cells exists in nature in the HSC and mesenchymal stem cells, because the FMS/PA6-P and fetal bone marrow adherent cells both show some

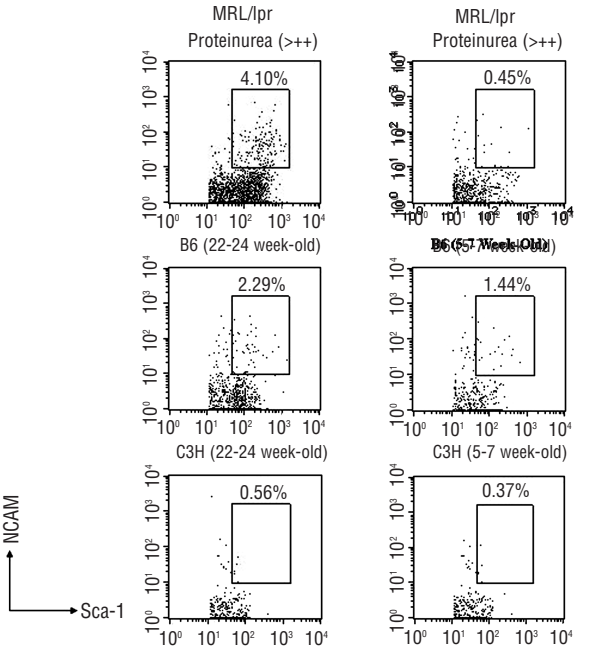

Figure 4. NCAM expression on Lin⁻ Sca-1⁺ HSC from B6, C3H and MRL/lpr mice. The frames indicate the percentages of NCAM expression in Lin⁻ Sca-1⁺ HSC. NCAM: neural cell adhesion molecule; Sca-1, stem cell antigen 1.

Table 2. No differences in VCAM-1 expression on Lin⁻ Sca-1⁺ HSC from various mice.

Mice	*Young (5-7 weeks) Proteinurea (-)*	*Old (22-24 weeks) Proteinurea (-)*	*Old (22-24 weeks) Proteinurea (>++)*
C3H	13.07±1.32*[a]	14.27±0.57[a]	–
B6	21.33±0.71[a]	17.5±1.39[a]	–
MRL/lpr	13.61±1.61[a]	–	14.23±0.29

**: Percentage of VCAM-1 expression on Lin⁻ Sca-1⁺ HSC; [a]: p>0.05 (old C3H vs. young C3H or old B6 vs. young B6 or old MRL/lpr vs. young MRL/lpr or old MRL/lpr vs. old C3H and B6).*

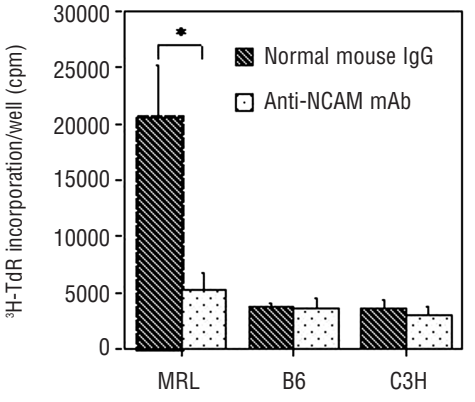

Figure 5. The inhibitory effect of anti-NCAM monoclonal antibody (mAb) on the proliferation of HSCs of MRL/lpr mice. The proliferation of HSC from MRL/lpr mice (proteinurea >++) was significantly suppressed by the addition of anti-NCAM monoclonal antibody, whereas there was no suppression in the proliferation of HSC from B6 and C3H mice. *:*p*<0.05. ^{3}H-TdR: ^{3}H-thymidine; cpm: count per minute; NCAM: neural cell adhesion molecule.

characteristics of mesenchymal stem cells, while the MS-5 cell line does not.

The homing of HSC after transplantation is critical to successful engraftment, and is a complex process involving migration, diapedesis through the endothelium, and adhesion to stroma. Since the ability of HSC to adhere to stromal cells correlates strongly to homing ability *in vivo*,[17-20] we investigated the ability of abnormal HSC to adhere to stromal cells. We found that abnormal HSC from MRL/lpr mice were significantly more adhesive than normal HSC (Figure 3). Moreover, increased expression of an adhesion molecule, NCAM, was also found in Lin$^-$ Sca-1$^+$ HSC from old MRL/lpr mice with proteinuria (Table 1 and Figure 4), which may contribute to the enhanced adhesiveness of abnormal HSC from MRL/lpr mice.

NCAM, a neural cell adhesion molecule of the immunoglobulin superfamily, is a transmembrane glycoprotein in mice.[26] Most research concerning NCAM has focused on the neural system because of the expression of this molecule on the surface of almost all neural cell types throughout the central and peripheral nervous system, subserving neuron-neuron and neuron-glia adhesion.[27,28] NCAM plays a pivotal role in early brain development, synaptic plasticity, and memory consolidation[29,30] and promotes neurite outgrowth and fasciculation.[31,32] In the hematopoietic system, however, its expression and function are not well elucidated, except that NCAM is regarded as a NK cell marker. Very recently, we hypothesized that NCAM expressed by stromal cells in the normal murine hematopoietic system may contribute to the maintenance of normal HSC through heterophilic binding, since we found that NCAM is not expressed in any HSC/progenitor cells of the bone marrow.[14] However, NCAM is highly expressed by the abnormal HSC from MRL/lpr mice. We, therefore, investigated whether the higher expression of NCAM had some effect on the abnormal proliferation of HSC from these mice, and found that the proliferation of HSC from MRL/lpr mice was significantly suppressed by the addition of anti-NCAM monoclonal antibody, whereas there was no suppression of the proliferation of HSC from B6 and C3H mice (Figure 5). These results suggest that the homophilic interaction between HSC and stromal cells through NCAM plays a central role in the abnormal proliferation of HSC from MRL/lpr mice. We are in the process of investigating the mechanism underlying this phenomenon.

NCAM was not only highly expressed in HSC from MRL/lpr mice with autoimmune disease, but also in HSC from other autoimmune-prone mice after the onset of autoimmune disease (*data not shown*). These findings suggest that abnormal expression of NCAM may be one of the causes of accelerated proliferation of HSC from autoimmune-prone mice, although many mechanisms, including abnormalities of cytokine receptors and their production, may be involved in this abnormal proliferation. We will further investigate the relationship between the onset of autoimmune disease and the increase in NCAM expression on HSC from various autoimmune-prone mice.

There are three possibilities concerning the relationship between MHC-independent proliferation and NCAM overexpression of HSC in MRL/lpr mice: first, NCAM overexpression on HSC may shield MHC receptors and prevent the MHC receptors from interacting with putative molecules expressed on stromal cells. Second, the greater proliferation of HSC induced by NCAM overexpression may overcome the growth inhibition by the MHC restriction. Third, NCAM may only contribute to the abnormal proliferation of HSC from MRL/lpr mice, whereas other unknown molecules on the HSC may interact with MHC molecules on stromal cells and induce MHC-independent proliferation of the HSC from MRL/lpr mice. We will focus future research in this area. Since NCAM expressed by stromal cells plays an important role in supporting the hematopoietic stem cells through homophilic or heterophilic binding,[14] we were interested to determine whether NCAM expression was also enhanced in stromal cells of autoimmune-prone mice after the onset of autoimmune disease. However, no significant difference of NCAM expression in stromal cells of autoimmune-prone mice before and after the onset of autoimmune disease was observed (*data not shown*), suggesting that the enhanced expression of NCAM is limited to abnormal HSCs of autoimmune-prone mice and promotes the autonomous proliferation of the HSC.

In conclusion, we have shown that there is no MHC restriction between abnormal HSC from MRL/lpr mice and stromal cells either *in vitro* or *in vivo*. Moreover, abnormal HSC from MRL/lpr mice are significantly more adhesive. Furthermore, among various adhesion molecules, NCAM expression is only increased on HSC from

MRL/lpr mice after the onset of autoimmune diseases, and these molecules contribute to the enhanced proliferation capacity of abnormal HSC from these mice. The present finding – that there are intrinsic qualitative differences in HSC between normal and autoimmune-prone MRL/lpr mice – is a significant step toward further understanding how autoimmune diseases develop. Furthermore, our findings have also indicated the possibility that anti-NCAM monoclonal antibodies may be used in a clinical setting as a treatment for autoimmune disease.

Authors' Contributions

XW, HH and SI contributed to the conception and design of the study, and to the analysis and interpretation of data; XW performed the majority of the experiments and drafted the article; HH revised the article; SI drafted and profoundly revised the article, and obtained the necessary funding. The other authors contributed to some of the experiments, the analysis of the data and to drafting the article. All authors approved the final version of the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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