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A Novel Concentrating System of Chicken Stem Cells by Bone Marrow Side Population Cells

Fumitake Usui[1], Yoshiaki Nakamura[1,2], Yasuhiro Yamamoto[3], Kouichi Tatsumi[1], Kazue Tominari[1], Tamao Ono[1] and Hiroshi Kagami[1]

[1] Faculty of Agriculture, Shinshu University, Minamiminowa, Nagano, 399–4598, Japan
[2] Animal Breeding and Reproduction Research Team, National Institute of Livestock and Grassland Science (NILGS), Tsukuba, Ibaraki, 305–0901, Japan
[3] Division of Human Genetics, Department of Integrated Genetics, National Institute of Genetics, Research Organization of Information and Systems, Mishima, Shizuoka, 411–8540, Japan

Numerous studies in mammalian species have recently been reported that many stem cells have an ability to efficiently efflux the vital DNA-binding dye Hoechst 33342, and it is called side population (SP) cells. However, few study have been reported on the avian SP cells. It could be possible that concentration of hematopoietic stem cells (HSCs) in birds since the characteristic of SP cells should be shared in various tissues and species. In this study, we first attempted the isolation of SP cells from chicken bone marrow and the assessment by gene expression and morphologic analyses. Bone marrow cells (BMCs) were flushed from the femurs and tibias of chicks aged at 10 days with PBS. The BMCs were layered on lymphocyte separation medium and centrifuged for excluding the erythrocytes. The separated cells were adjusted to 10^6/m*l* in HBSS. Hoechst 33342 were added (1.25 μg/m*l*) and incubated 60 to 90 minutes at 37℃. Propidium iodide was added (2 μg/m*l*) to exclude dead cells. The SP cells were isolated with flow cytometer. The sorted cells were stained with May-Gruenwald Giemsa (MG) for morphological analysis and RNA was extracted for gene expression analysis. The avian SP cells which was vanished by addition verapamil counld be separated. The percentage of SP cells in chicken bone marrow was about 2.6%. The morphological analysis by MG staining indicated that the SP cells had a larger nuclear and little cytoplasm which were typical characterisation of mouse HSCs. The pattern of gene expressions (*CD34, c-Kit, CD4* and *CD8*) in SP cells also resembled that of the mouse HSCs. These results suggested that the HSCs could be enriched from avian bone marrow cells. Together with these results, it was concluded that SP is one of powerful tools for concentration of avian stem cells.

Key words: bone marrow cells, chicken, flow cytometric analysis, hematopoietic stem cells, side population

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Introduction

Stem cells is the cells from the embryos, fetus, or adult that has the ability to reproduce itself for long time in a certain conditions. It also can give rise to specialized cells that make up the tissues and organs of the body. If necessary, the stem cells can proliferate or differentiate to maintain the constancy of the organ and can cause restoration and reproduction in the case of the damage. The hematopoietic stem cells (HSCs) in bone marrow is one of the most important cells for stem cell researches (Blau *et al.,* 2001). It was proposed that the HSCs which had both self-reproduction ability and many differentiation ability to maintain a limited maturity blood corpuscle are kept for life (Till and McCulloch, 1961), and fundamental researches and the clinical study of the HSCs have been piled. As the results, the cell transplant medical care has already been established with the HSCs in the human. In addition, the concentration technology of the HSCs which used monoclonal antibody is developed. Restoration of hematopoietic function of mouse irradiated with fatal dose radioactive rays was conducted by transplanting of a single HSC (Osawa *et al.,* 1996).

However, few study of the origin of HSCs developed by the transplant experiment using avian embryos has been reported. It was proved that hematopoiesis had two phase; primitive phase and definitive phase, by transplantation of the part of the quail embryos to the chick embryos (Dieterlen-Lievre, 1975). It was shown in chicken embryos that the definitive hematopoiesis derived from hemangioblast which was common progenitor of bloods (Jaffredo *et al.,* 2000).

However, little was known of adult HSCs in the birds.

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Correspondence: Prof. H. Kagami, Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Nagano 399–4598, Japan.
(E-mail: kagami@shinshu-u.ac.jp)

Monoclonal antibody is generally used for the concentration of the stem cell. But there are few antibodies which are effective for concentration of HSCs in the chicken. The concentration of HSCs by the conventional monoclonal antibody is extremely difficult in birds. Therefore this study was conducted to concentrate stem cells by side population (SP) method. The SP method was established by Goodell *et al.* (1996). Many stem cells have an ability to efficiently efflux the vital DNA-binding dye Hoechst 33342, and it is called SP cells. SP cell purification has been extensively conducted to enrich mammalian HSCs by the Hoechst 33342 staining and the subsequent analysis with FACS. These SP cells were isolated from not only bone marrow but also skin (Terunuma *et al.,* 2003), a skeletal muscle (Meeson *et al.,* 2004), a breast (Behbod *et al.,* 2006). Furthermore, it is confirmed that the exhaust ability of Hoechst 33342 in the SP cell is saved in not only the mouse but also human, monkey, dog, pig, and zebra fish (Goodell *et al.,* 1997). However, few study have been reported on the avian SP cells. In this study, we attempted the isolation of SP cells from chicken bone marrow cells and their assessment by gene expression and morphological analyses.

Materials and Methods

Experimental Animals

White Leghorn chickens (10-day age) were used in the present studies. All procedures described here were reviewed and approved by the Animal Care and Use Committee of Shinshu university, and were performed in accordance with the Guiding Principle for the Care and Use of Laboratory Animals.

Preparation for Bone Marrow Cells (BMCs)

BMCs were flushed from the femurs and tibias of chicks aged at 10 days with phosphate-buffered saline (PBS) with a 21-gauge needle (Terumo, Tokyo). The BMCs were gently filtered through nylon mesh (Falcon; Bedford). The BMCs were washed (200 x*g* for 10 minutes) and resuspended in 0.9% NaCl. The resuspended cells were layered on NycoPrep 1.077 (COSMOBIO USA, Inc.) and centrifuged at 800 x*g* for 20 minutes. The mononuclear cells were separated from the serum-lymphocyte separation medium interface. The mononuclear cells were then washed and adjusted to 10^6/m*l* in HBSS.

Hoechst Staining

BMCs were stained with 1.25 μg/m*l* Hoechst 33342 for 60 to 90 minutes at 37℃. For inhibition experiments, BMCs were stained with Hoechst 33342 in the presence of 50 μM verapamil (Sigma), an inhibitor of ATP-binding cassette transporters. After staining, cells were washed and propidium iodide (PI) was added (2 μg/m*l*) to exclude dead cells. The samples were kept on ice until flow cytometric (FCM) analysis.

FCM Analysis

FCM analysis and sorting were performed on a dual laser (488nm and UV argon laser) flow cytometer (FACS Vantage SE, Becton Dickinson). The Hoechst 33342 dye was excited by the UV laser, and its fluorescence was measured at two wavelengths using a 410/20 (Hoechst blue) band-pass (BP) filter and a 575/20 (Hoechst red) BP filter. PI fluorescence was excited by the 488 nm laser, and detected after passing through a 675/20 BP filter. For an analysis, a gate was drawn to exclude PI positive dead cells and unstained debris. A Hoechst blue vs. Hoechst red dot-plot was obtained for the gated region. The SP cells, identified by their typical location, and main population (MP) cells, identified by their high frequency location, were sorted into polypropylene tubes containing HBSS.

May-Gruenwald Giemsa (MG) Staining

SP cells and MP cells were smeared onto glass slides and air dried. May-Grunwald staining solution (Wako) was then applied for 3 minutes at 37℃. Phosphate buffer (pH 6.4) of 1/15 M for 3 minutes and Giemsa solution (Wako) was applied for 15 minutes. Slides were then briefly washed in PBS and photographed.

RNA Isolation and RT-PCR

Total RNA samples were isolated from SP cells, MP cells and BMCs. The samples were placed in to a 1.5-mL RNase free microcentrifuge tube with TRIzol reagent (Invitrogen, Carlsbad). A DNase reaction was performed to digest any contaminating DNA. Oligo (dt)-primer first-strand cDNA was prepared with the use of a Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad) from 1.0 μg total RNA of each cells. Primer pairs were (1) *CD34* forward primer: GGGACTTGAACTCCAACCAA; reverse primer: ACCTCACTGTGCAGAACACG (International Chicken Genome Sequencing Consortium, 2004); (2) *c-Kit* forward primer: AAGTGGATGGCACCTGAAAG; reverse primer: CTCCCTCTCTCACCCCCTAC (Zhou *et al.,* 1993); (3) CD4 forward primer: CAGCATGAAGCAAAAGTGGA; reverse primer: TTATACATCCGGCGTTGACA (Koskinen *et al.,* 2002); (4) CD8 forward primer: AATGGTGTCTCCTGGATTCG; reverse primer: ATGTTCTCACCGCTTGTTCC (Luhtala *et al.,* 1997). PCR was performed 2 times, first PCR was performed using the following program: 30 cycles at 94℃ for 1 min, 66℃ for 1 min and 72℃ for 1 min. Second PCR was performed using first PCR product as a template and above program. Amplification of DNA fragments was verified by 1.5% agarose gel electrophoresis.

Results and Discussion

This is the first attempts to isolate avian bone marrow SP cells. The chicken bone marrow SP cells which were vanished on FACS analysis by the verapamil addition could be separated in the condition of 1.25 μg/m*l* Hoechst 33342 in density with incubation time for 60 minutes at 37℃ (Fig. 1A and B). On the other hand, the most suitable condition of the mouse BMCs is 5.00 μg/m*l* Hoechst 33342 density for 60 minutes incubation at 37℃. From here onwards, chicken BMCs may be easy to be dyed to Hoechst 33342. The percentage of SP cells in

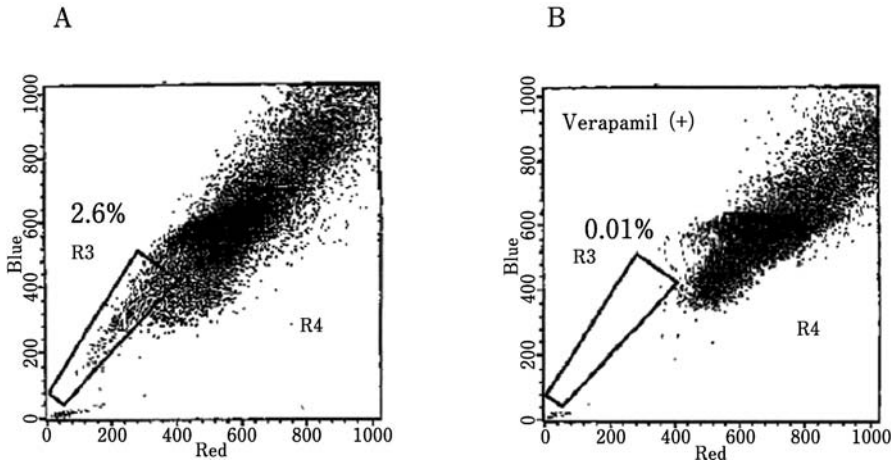

Fig. 1. **Flow cytometric analysis of chicken bone marrow SP cells. Chicken BMCs were stained with Hoechst 33342 and analysed by flow cytometry.** A: Hoechst fluorescence (Hoechst blue vs Hoechst red) of chicken BMCs are shown. Gated region indicates SP population. B: Hoechst fluorescence of chicken BMCs that were stained with Hoechst in the presence of 50μM verapamil.

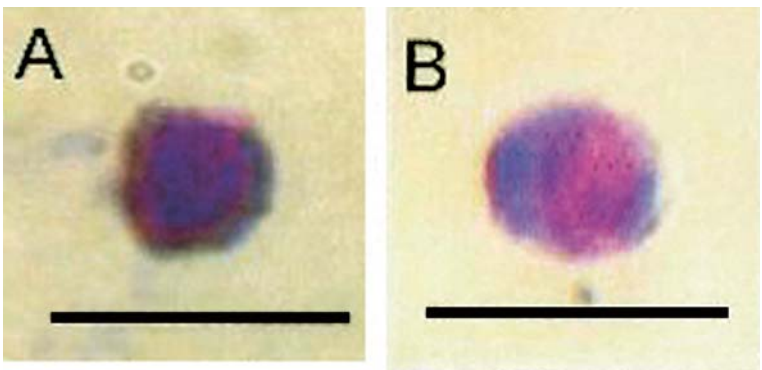

Fig. 2. **Morphologic analyses of bone marrow SP cells and MP cells.** A: The staining of bone marrow SP cells with May-Grunwald Giemsa is shown. B: The staining of bone marrow MP cells with May-Grunwald Giemsa is shown. The scale bar means 10μm.

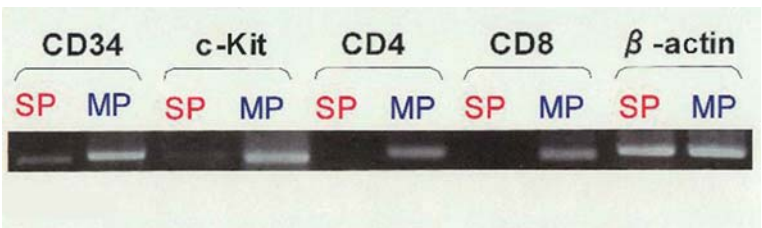

Fig. 3. **Gene expression analyses of bone marrow SP cells and MP cells.** The results of RT-PCR analysis of *CD34, c-Kit, CD4* and *CD8* in SP cells and MPcells are shown. The β-actin was used as an internal control.

chicken bone marrow was about 2.6%. It was very high frequency in comparison with other animal SP cells (Goodell *et al.,* 1996; Kobayashi *et al.,* 2008; Alt *et al.,* 2009). The chicken BMCs may have many stem cells, since stem cells are generally enriched in SP cells.

The morphological analysis by MG staining indicated that the SP cells had a larger nuclear and little cytoplasm in comparison with MP cells (Fig. 2A and B). The morphological characterisation of chicken bone marrow SP cells were typical to that of the mouse HSCs.

It has been known that mouse HSCs are enriched in CD34 (−) KSL [CD34 (−/low) c-Kit (+) Sca-1 (+) lineage marker (Mac-1, Gr-1, B220, CD4 and CD8) (−)] bone marrow cells (Osawa et al., 1996). We analyzed the gene expression of chicken bone marrow SP cell and the MP cell using markers (*CD34, c-Kit, CD4, CD8*) in chicken. Gene expression of SP cells represented *CD34* (low), *c-Kit* (low), *CD4* (−), *CD8* (−) (Fig. 3). On the other hand, the gene expression of MP cells represented *CD34* (+), *c-Kit* (+), *CD4* (+), *CD8* (+) (Fig. 3). It was different from SP cells in MP cells about the gene expression pattern. Differentiation antigen markers; CD4 and CD8, were only expressed in a MP cells. From here onwards, it was suggested that the MP cells were differentiated cell group and the SP cells were undifferentiated cell group. Furthermore, the pattern of gene expressions in SP cells also resembled that of the mouse HSCs except *c-Kit.* It was known that a lot of hematopoietic progenitor cells with a phenotype of CD34 (+) KSL were also included as well as HSCs with a phenotype of CD34 (−) KSL in a mouse marrow SP cell. And CD34 (−) KSL cells were not only in the SP fraction but also MP fraction (Morita *et al.,* 2006). It was supposed that chicken bone marrow MP fraction may have many c-Kit (+) cells which did not eject Hoechst 33342.

In conclusion, we developed a novel system for isolation of chicken bone marrow SP cells. These results suggested that the HSCs could be enriched in avian bone marrow SP cells. Together with these results, it was considered that the SP is one of powerful tools for concentration of avian stem cells.

Acknowledgments

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