J Korean Med Sci 2001; 16: 20-4
ISSN 1011-8934

Copyright © The Korean Academy of Medical Sciences

Cell Cycling Status of Human Cord Blood CD34+ Cells during Ex Vivo Expansion Is Related to the Level of Very Late Antigen Expression

Very late antigen-4 (VLA-4), which binds to the extracellular matrix protein fibronectin, is an integrin molecule known to be modulated during mobilization of CD34+ cells, and to be involved in signaling the mobilization stimuli. On the hypothesis that cell cycling status might be different depending on the level of VLA-4 expression, we investigated the DNA contents of human cord blood CD34+ cells during ex vivo expansion by recombinant human thrombopoietin and flt3-ligand with simultaneous measurement of surface VLA-4 at the 1st and 4th week. During this ex vivo expansion, expression of VLA-4 increased and almost all cells became VLA-4+ until the 4th day of culture. Expression of VLA-4 was maintained in the major population of the cultured cells until the 4th week. The cells in S/G2/M phase were greater in number in VLA-4 high fraction than in VLA-4 low fraction (n=4, $p<.001$). Furthermore, the fraction of cells in S/G2/M phase increased as the expression of VLA-4 became higher. These results suggest that cord blood CD34+ cells expressing high levels of VLA-4 have more proliferative activities.

Key Words : *VLA-4; Receptors, Very Late Antigen; Cell Cycle; Fetal Blood; Antigens, CD34; Ex Vivo Expansion*

Ju-Young Seoh, Hae-Young Park*, Wha-Soon Chung†, Seung-Cheol Kim‡, Myong-Joon Hahn§, Kyung-Hyo Kim||, Hee-Young Shin¶, Hyo-Seop Ahn¶, Kwi-Won Park, Kyung-Ha Ryu||**

Departments of Microbiology, Biochemistry*, Clinical Pathology†, Obstetrics & Gynecology‡, Pediatrics||, Medical Research Center, College of Medicine, Ewha Womans University, Seoul; Department of Microbiology§, College of Medicine, Sungkyunkwan University, Suwon; Departments of Pedistrics¶, Pediatric Surgery**, Seoul National University College of Medicine, Seoul, Korea

Received : 9 October 2000
Accepted : 1 December 2000

Address for correspondence
Kyung-Ha Ryu, M.D.
Department of Pediatrics, Dongdaemun Hospital, College of Medicine, Ewha Womans University, 70 Chongro 6-ga, Chongro-gu, Seoul 110-120, Korea
Tel : +82.2-760-5074, Fax : +82.2-765-3855
E-mail : ykh@mm.ewha.ac.kr

*This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (HMP-00-CH-04-0001).

INTRODUCTION

Normal hematopoiesis as well as mobilization and homing of stem cells is regulated by microenvironments. Several lines of evidence support that very late antigen-4 (VLA-4) comprises one of the microenvironments. VLA-4, which binds to the extracellular matrix protein fibronectin, is widely distributed on hematopoietic and non-hematopoietic tissues (1). The main function of VLA-4 is related to interactions between hematopoietic cells and the ligands or counter receptors for this molecule present in hematopoietic microenvironments (2). VLA-4 is an integrin molecule in heterodimer structure. Common β1-chain subunit (CD29) combines with the α1 to α6 subunits (CD49a to CD49f) of the α-chain to form VLA-1 to VLA-6 (CD49d for VLA-4). Blockage of VLA-4 markedly inhibited human hematopoietic cellular proliferation including granulopoiesis and B-lymphopoiesis (3-5). VLA-4 expression on CD34+ cells is significantly lower in normal peripheral blood than in bone marrow (6). Mobilization with granulocyte-colony stimulating factor (G-CSF) and/or chemotherapy results in reduction of VLA-4 expression on CD34+ cells in bone marrow as well as in peripheral blood, and seems to be a selective process involving downregulation of VLA-4 and c-kit (7). Treatment with antibodies to VLA-4 led to mobilization of stem/progenitor cells in mice and primates (8). VLA-4 is involved in signaling integrin/cytokine crosstalk (9) while VLA-4 itself can be modulated by other adhesion molecule such as platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) (10). Taken together, cellular status may be different depending on the expression level of VLA-4. In the present study, we investigated both the surface expressions of VLA-4 and cell cycling status of

human cord blood CD34+ cells during ex vivo expansion using thrombopoietin and flt3-ligand.

MATERIALS AND METHODS

CD34+ cell purification and stroma-free liquid culture

Umbilical cord blood was obtained at the end of full-term delivery. Mononuclear cells were isolated from cord blood using Ficoll-Hypaque (density, 1.077; Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. After two cycles of plastic adherence for 60 min, CD34+ cell fraction was isolated with superparamagnetic microbead selection using monoclonal antibody (QBEND10) and mini-MACS columns (Miltenyi Biotech, Bergisch Gladbach, Germany). The efficiency of purification was verified by the flow cytometry counterstaining with fluorescein isothiocyanate (FITC)-anti-human CD34 antibody (HPCA-2; Becton Dickinson (BD), Mountain View, CA, U.S.A.). In the obtained cell fractions, the purity of CD34+ cells ranged from 94% to 98%. The purified CD34+ cell fractions were cultured in the density of 0.5-1.0$\times 10^5$ cells/mL in IMDM (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD, U.S.A.), recombinant human (rh) thrombopoietin (10 U/mL; Chemicon, Temecula, CA, U.S.A.) and rh flt3-ligand (50 ng/mL; Chemicon). Twice a week, the cells were demipopulated by removal of one half the culture volume, which was replaced with fresh medium and growth factors. The cells were maintained for up to four weeks.

Simultaneous measurement of cell surface VLA-4 and DNA content

DNA contents and VLA-4 expression of the cultured cells were measured for relationship between cell cycle and VLA-4 expression at the 1st and the 4th week. After blocking with human serum, aliquots of purified or cultured cells were stained at 4℃ for 30 min with FITC-anti-human CD34 (anti-HPCA-2, BD) and phycoerythrin (PE)-anti-human CD49d (44H6, Serotec, Oxford, U.K.) or isotype-matched irrelevant antibodies. For simultaneous measurement of DNA content (9), the cells were fixed and permeabilized in 1 mL of phosphate-buffered saline (PBS) with 1% paraformaldehyde and 0.05% Nonidet P40 by overnight incubation at 4℃. After washing in PBS with 0.1% bovine serum albumin, the samples were stained for DNA by adding 500 μL of 10 μg/mL 7-amino-actinomycin D (7-AAD; Sigma, St Louis, MO, U.S.A.) in PBS for 90 min at 4℃ in the dark. The stained samples were analyzed on a FACSCalibur flow cytometer (BD). At least 20,000 events were acquired for each analysis and data were analyzed using CellQuest (BD) software.

Statistical analysis

Descriptive statistics were represented as mean±SD and statistical comparisons were done using paired Student t-test.

RESULTS

Immunophenotype of purified or cultured cells

The fraction of VLA-4-expressing cells ranged from 32.1% to 78.2% (n=4) of the purified cord blood CD34+ cells (data not shown). Until the 4th day of culture with thrombopoietin and flt3-ligand, expression of VLA-4 increased and almost all cells became VLA-4+ (data not shown). Until the 4th week of culture thereafter, expression of VLA-4 was maintained, while CD34-expressing cell fractions decreased (data not shown).

DNA content of the cultured cells and expression of VLA-4

The cell fractions in S/G2/M phase were 25.8±1.5% (n=4) and 21.2±1.9% (n=4) at the 1st and the 4th weeks of culture respectively (Fig. 1). When the cultured cells were arbitrarily divided into VLA-4^{hi} and VLA-4^{lo} cells for high and low levels respectively according to the level of VLA-4 expression, cells in S/G2/M phase were significantly greater in number in VLA-4^{hi} cells than in VLA-4^{lo} cells on both the 1st and the 4th weeks (n=4, $p<.001$) (Fig. 1). The fractions of cells in S/G2/M phase among in VLA-4^{hi} and VLA-4^{lo} cells were 30.0±1.11% and 24.3±1.56% on the 1st week, and were 27.4±1.64% and 9.9±1.10% on the 4th week respectively. When the cultured cells on the 4th week were divided into multiple fractions according to the level of VLA-4 expression, the fraction of cells in S/G2/M phase increased depending on the level of VLA-4 expression (Fig. 2).

DISCUSSION

Simple combination of thrombopoietin and flt3-ligand has been reported to be capable of sustaining proliferation of cord blood hematopoietic progenitor cells for more than six months (12). For analysis of cell cycling status simultaneously with VLA-4 expression of cord blood cell during ex vivo expansion, we applied the liquid

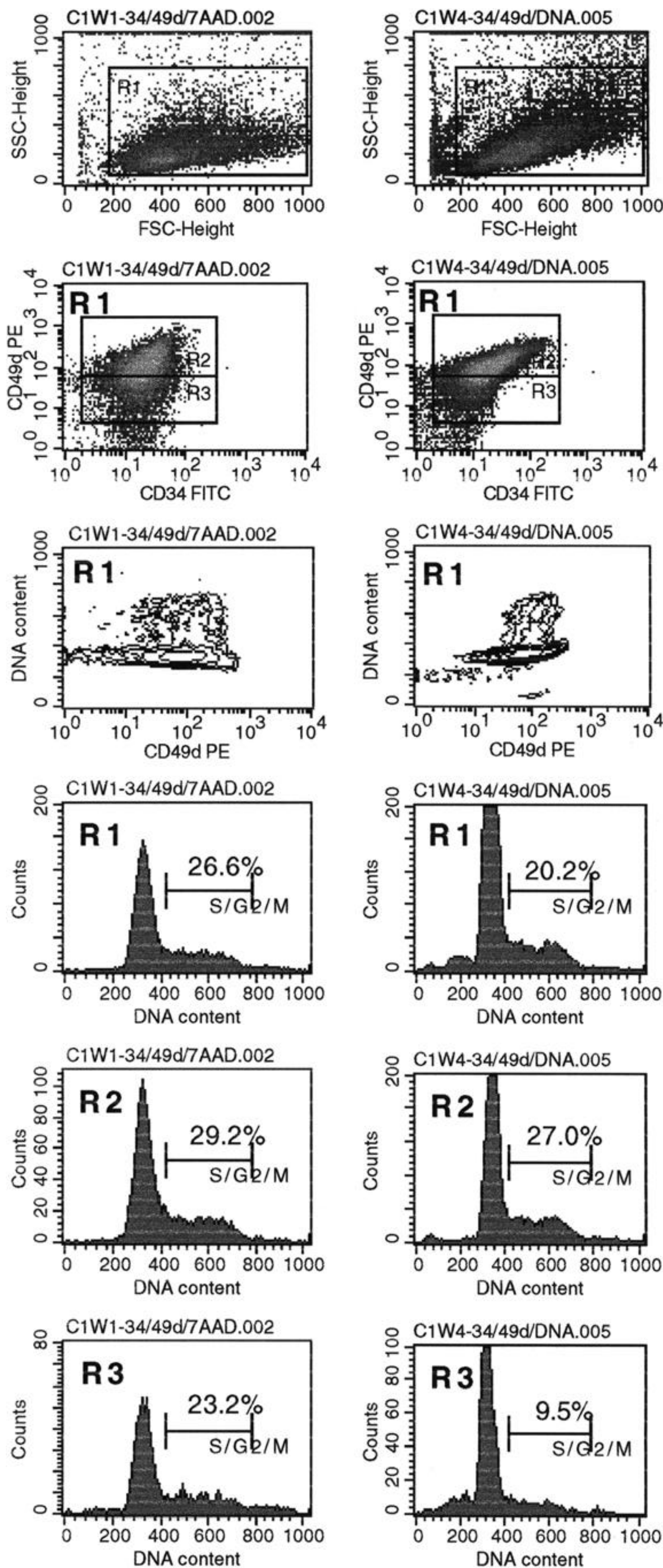

Fig. 1. Representative flow cytometric analysis for simultaneous measurement of cell surface VLA-4 and DNA contents. Cord blood CD34+ cells cultured for 1 week **(left)** or 4 weeks **(right)** with thrombopoietin and flt3-ligands were stained with fluorescein isothiocyanate (FITC)-anti-CD34 and phycoerythrin (PE)-anti-CD49d. Total cellular fractions (R1), gated on the scattergram of FSC vs. SSC, were analyzed for expression of CD34, VLA-4, and DNA contents. VLA-4^{hi} (R2) and VLA-4^{lo} (R3) cell fractions, gated on the scattergram of CD34 vs. VLA-4 fluorescence, were separately analyzed for DNA contents. The total cell fractions in S/ G2/M phase were 26.6% (R1-left) and 20.2% (R1-right) at the 1st and the 4th weeks of culture respectively. When cultured cells were arbitrarily divided into VLA-4^{hi} and VLA-4^{lo} cell fractions, the proportion of cells in S/G2/M phase were higher in VLA-4^{hi} cells (R2) than in VLA-4^{lo} cells (R3).

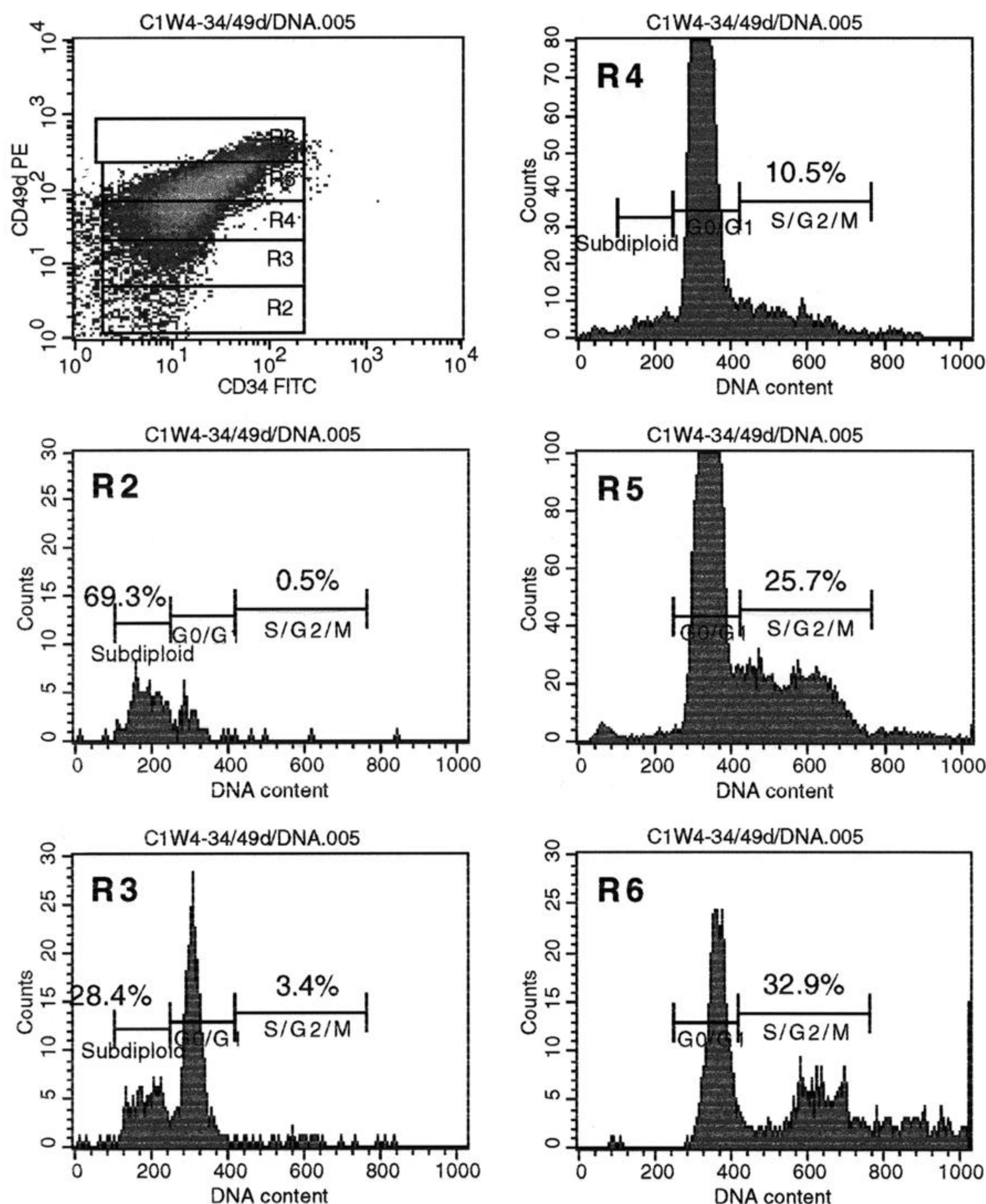

Fig. 2. Representative flow cytometric analysis for simultaneous measurement of cell surface VLA-4 and DNA contents. Cord blood CD34+ cells cultured for 4 weeks with thrombopoietin and flt3-ligands were stained as in Fig. 1. Cellular fractions, gated on the scattergram of FSC vs. SSC, were analyzed for expression of CD34, VLA-4, and DNA contents. The total cell fractions were divided from R2 to R6 in order low expression of VLA-4, and were separately analyzed for DNA contents. The results showed that proportions of cells in S/G2/M phase were dependent on the level of VLA-4 expression.

culture system using thrombopoietin and flt3-ligand. This system produced to approximately 1,000-fold expansion of total cells for 4 weeks (data not shown). During this ex vivo expansion, expression of VLA-4 increased and almost all cells became VLA-4+ until the 4th day of culture. Expression of VLA-4 was maintained in the major population of the cultured cells until the 4th week, while the minor fraction of VLA-4− cells appeared at the 1st week. These VLA-4− cells seemed to be apoptotic in nature as their DNA contents were revealed to be sub-

diploid (Fig. 2).

In the present study, we demonstrated that the proportions of cord blood CD34+ cells in S/G2/M phase were related to their level of VLA-4 expression. These results suggest that proliferative activities of cord blood hematopoietic progenitor cells may be related to the level of VLA-4 expression. The results are comparable with those of Eksioglu-Demiralp et al. (13) who showed that variable expression of VLA-4 in B cell chronic lymphocytic leukemia was related to disease stages. Further studies are needed to investigate whether the decrease in the level of VLA-4 expression in CD34+ cells during mobilization accompanies any changes in cell cycling status.

ACKNOWLEDGMENTS

The authors appreciate Kirin Brewery Co. for their heartful donation of the cytokines, thrombopoietin, and stem cell factor.

REFERENCES

1. Reuss-Borst MA, Ning Y, Klein G, Muller CA. *The vascular cell adhesion molecule (VCAM-1) is expressed on a subset of lymphoid and myeloid leukemias. Br J Hematol 1995; 89: 299-305.*
2. Papayannopoulou T, Nakamoto B. *Peripheralization of hematopoietic progenitors in primates treated with anti-VLA-4 integrin. Proc Natl Acad Sci USA 1993; 90: 9374-8.*
3. Warrens AN, Simon AR, Theodore PR, Sachs DH, Sykes M. *Function of porcine adhesion molecules in a human marrow microenvironment. Transplantation 1998; 66: 252-9.*
4. Iguchi A, Okuyama R, Koguma M, Obinata M, Yanai N. *Selective stimulation of granulopoiesis in vitro by established bone marrow stromal cells. Cell Struct Funct 1997; 22: 357-64.*
5. Ryan DH, Tang J. *Regulation of human B cell lymphopoiesis by adhesion molecules and cytokines. Leuk Lymphoma 1995; 17: 375-89.*
6. Dercksen MW, Gerritsen WR, Rodenhuis S, Dirkson MK, Slaper-Cortenbach IC, Schaasberg WP, Pinedo HM, von dem Borne AE, van der Schoot CE. *Expression of adhesion molecules on CD34+ cells: CD34+ L-selectin+ cells predict a rapid platelet recovery after peripheral blood stem cell transplantation. Blood 1995; 85: 3313-9.*
7. Steen R, Tjonnfjord GE, Gunnes Groseth LA, Heldal D, Egeland T. *Efflux of CD34+ cells from bone marrow to peripheral blood is selective in steady-state hematopoiesis and during G-CSF administration. J Hematother 1997; 6: 563-73.*
8. Craddock CF, Nakamoto B, Andrews RG, Priestley GV, Papayannopoulou T. *Antibodies to VLA-4 integrin mobilize long-term repopulating cells and augment cytokine-induced mobilization in primates and mice. Blood 1997; 90: 4779-88.*
9. Papayannopoulou T, Priestley GV, Nakamoto B. *Anti-VLA4/VCAM1-induced mobilization requires cooperative signaling through the kit/mkit ligand pathway. Blood 1998; 91: 2231-9.*
10. Leavesley DI, Oliver JM, Swart BW, Berndt MC, Haylock DN, Simmons PJ. *Signals from platelet/endothelial cell adhesion molecule enhance the adhesive activity of the very late antigen-4 integrin of human CD34+ hemopoietic progenitor cells. J Immunol 1994; 153: 4673-83.*
11. Holm M, Thomsen M, Hoyer M, Hokland P. *Optimization of a flow cytometric method for the simultaneous measurement of cell surface antigens, DNA content, and in vitro BrdUrd incorporation into normal and malignant hematopoietic cells. Cytometry 1998; 32: 28-36.*
12. Piacibello W, Sanavio F, Garetto L, Severino A, Bergandi D, Ferrario J, Fagioli F, Berger M, Aglietta M. *Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. Blood 1997; 89: 2644-53.*
13. Eksioglu-Demiralp E, Alpdogan O, Aktan M, Firatli T, Ozturk A, Budak T, Bayik M, Akoglu T. *Variable expression of CD49d antigens in B cell chronic lymphocytic leukemia is related to disease stages. Leukemia 1996; 10: 1331-9.*