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# CKβ8-1 alters expression of cyclin E in colony forming units-granulocyte macrophage (CFU-GM) lineage from human cord blood CD34$^+$ cells

**Eui-Kyu Noh[1], Jae-Sun Ra[1], Seong-Ae Lee[1], Byoung S. Kwon[1] and In-Seob Han[1,2]**

[1]Immunomodulation Research Center
Department of Biological Science
University of Ulsan
Ulsan 680-749, Korea
[2]Corresponding author: Tel, 82-52-259-2352;
Fax, 82-52-259-1694; E-mail, hanis@ulsan.ac.kr

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Abbreviations: BFU-E, burst forming unit-erythroid; CB, cord blood; CFU-GEMM, colony forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte; CFU-GM, colony forming units-granulocyte/macorphage; EPO, erythropoietin; IMDM, Isscove's modified Dulbecco's medium

## Abstract

**A C6 β-chemokine, CKβ8-1, suppressed the colony formation of CD34$^+$ cells of human cord blood (CB). Molecular mechanisms involved in CKβ8-1-medicated suppression of colony formation of CD34$^+$ cells are not known. To address this issue, the level of various G1/S cell cycle regulating proteins in CKβ8-1-treated CD34$^+$ cells were compared with those in untreated CD34$^+$ cells. CKβ8-1 did not significantly alter the expression of the G1/S cycle regulation proteins (cyclin D1, D3, and E), CDK inhibitor (p27and Rb), and other cell proliferation regulation protein (p53) in CB CD34$^+$ cells. Here we describe an *in vitro* system in which CB CD34$^+$ cells were committed to a multipotent progenitor lineage of colony forming units-granulocyte/macrophage (CFU-GM) by a simple combination of recombinant human (rh) GM-CSF and rhIL-3. In this culture system, we found that cyclin E protein appeared later and disappeared faster in the CKβ8-1-treated cells than in the control cells during CFU-GM lineage development. These findings suggested that cyclin E may play a role in suppressing the colony formation of CFU-GM by CKβ8-1.**

**Keywords:** cell cycle; chemokine; cyclin E; granulocytes; macrophages; multi potent; stem cells

## Introduction

Hematopoietic stem cells (HSC) can differentiate into different lineages, including myeloid, megakaryocyte, lymphoid, and erythroid cells. Key issue in HSC biology has focused on how their commitment to a particular lineage and maturation is regulated at the cellular and molecular levels. Because HSC have been widely used in the treatment of leukemia (Miyamoto *et al.*, 2000) and are present in high frequency in umbilical cord blood (CB), human CB has been established as a source of hematopoietic repopulating cells (Broxmeyer and Smith, 1999; Gluckman, 2001; Kim *et al.*, 2003; Chung *et al.*, 2005). This issue is of great clinical relevance. During hematopoiesis, cytokines act as developmental signals that direct the commitment of progenitor cells into and through particular lineages.

It is of interest that all C6 β-chemokines so far analyzed, including CKβ8-1, have shown myelosuppressive activity on colony forming unit-granulocyte/macrophage (CFU-GM), burst forming unit-erythroid (BFU-E), and colony forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM). Most non-C6 β-chemokines do not have such suppressive activity. Although it is unknown what mechanism of CKβ8-1 inhibits proliferation of myeloid progenitor cells, the properties of myelosuppression can be used in a human chemotherapy setting. Lkn-1, a member of human C6 β-chemokine, protected bone marrow myeloid progenitor cells when cytotoxic chemotherapeutics were used in a preclinical experiment (Kim *et al.*, 2003). This may be due to the role of Lkn-1 in decreasing the number of myeloid progenitor cells that enter S phase of the cell cycle. Inhibition of human endothelial cell proliferation by a CXC chemokine, PF4, is associated with a decrease in cyclin E-cyclin dependent kinase 2 (CDK2) activity (Gentilini *et al.*, 1999). PF4 is known as an inhibitor of hematopoeitic progenitor cell proliferation (Han *et al.*, 1990; Broxmeyer *et al.*, 1993). As cells progress through the G1-phase, cyclin E is synthesized with a peak in late G1. Cyclin E associates with CDK2 and is essential for entry into the S phase (Ohtsubo *et al.*, 1995). Once cells enter

the S phase, cyclin E is degraded and CDK2 then associates the cyclin A (Fotedar and Fotedar, 1995).

In this study we sought to further understand the inhibitory effect of CKβ8-1 on colony formation of $CD34^+$ cells from human CB. As such, we examined whether CKβ8-1 played a role in regulating expression of cell-cycle related proteins in order to arrest the commitment to cell lineage, especially to CFU-GM.

## Materials and Methods

### Cells and cell culture

CB cells were obtained from normal human CB scheduled for discard after delivery. A $CD34^+$ cell-enriched fraction was prepared by density cut separation on Ficoll-Hypaque gradients (1.070 $g/cm^3$). A MACS kit (Miltenyi Biotech, Germany) was used to isolate the $CD34^+$ cells from the cell suspension (Kim, 2003). $CD34^+$ cells were incubated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 30% fetal bovine serum (Gibco-Life), 1% BSA (Gibco), 0.1 mM 2-mercaptoethanol (Sigma), and 2 mM L-glutamine (Sigma) with either recombinant human GM-CSF (rhGM-CSF 50 ng/ml; Stem Cell Technologies, Vancouver, Canada), and rhIL-3 (10 ng/ml; Stem Cell Technologies) for CFU-GM differentiation, or rhEPO (1 U/ml; Stem Cell Technologies) for erythroid differentiation. Cells were plated in 35-mm culture dishes.

### Colony forming assay

To monitor the clonogenic growth of differentiated cell lineages, cells were plated in triplicate and incubated in IMDM at 37°C under 5% $CO_2$. Colonies were counted under an inverted microscope as previously described (Han *et al.*, 2003).

### Western blot analysis

Protein samples were boiled in a 1 × SDS-sample loading buffer (50 mM Tris-HCl pH6.8, 10% glycerol, 1% SDS, 0.02% bromphenol blue, and 5% β-mercaptoethanol), fractionated by 12% SDS-PAGE, and electroblotted (10 mM CAPS, 10% methanol, pH11) onto a nitrocellulose membrane. After blocking the nonspecific binding (blocking buffer; 20 mM Tris, pH 7.8, 145 mM NaCl, 0.1% Tween-20, 3% nonfat dried milk), the membrane was incubated with different antisera (cyclin A, cyclin D1, cyclin D3, cyclin E, p27, p53, and β-actin). The immunoblot was detected by ECL techniques (Amersham Phamacia Biotech).

## Results and Discussion

### CKβ8-1 did not alter G1 cell cycle protein expression

Chemokines have a biological activity to inhibit proliferation of hematopoietic progenitor cells. Although previous studies have reported that several chemokines suppressed the proliferation of progenitor cells, the underlying molecular mechanisms of these chemokines still remain to be established (Broxmeyer *et al.*, 1997; Su *et al.*, 1997; Crow *et al.*, 2001; Han *et al.*, 2003). We also reported that chemokine CKβ8-1 and Lkn-1 suppressed the proliferation of myeloid progenitor cells from human CB (Han *et al.*, 2003; Kim *et al.*, 2003) without clarification of functional mechanism of suppression by them.

To investigate whether CKβ8-1 was effective in the regulation of a cell cycle for its suppression activity, we monitored the expression of cell cycle regulating proteins. As shown in Figure 1, we did not observe any significant activity of CKβ8-1 on the expression of the G1/S cycle regulating proteins (cyclin D1, D3, and E), CDK inhibitors, p27and Rb, and other cell proliferation regulating protein, p53 in CB $CD34^+$ cells. The cyclin D1 protein that was absent in the freshly harvested myeloid progenitor cells, appeared and was maintained during a 5 days culture. The cyclin D3 protein was very low at all stages of the culture. It is likely that cyclin D1 plays an important role rather than cyclin D3 in colony formation of

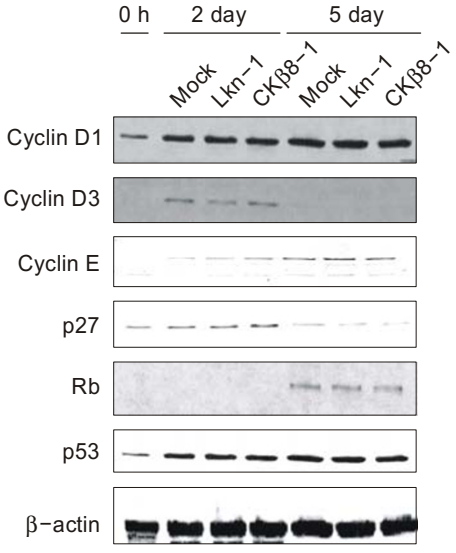


**Figure 1.** Immunoblotting detection of cell cycle related proteins in myeloid progenitor cells of human CB. $CD34^+$ cells from human CB were allowed to myeloid lineage culture in the absence or presence of CKβ8-1 or Lkn-1. About 50 g protein were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with the antibodies to the indicated proteins.

myeloid progenitor cells from human CB $CD34^+$ cells. A previous study has indicated that the action of IFN-γ to reduce CFU-GM and BFU-E colony formation from $CD34^+$ cells may be linked to effects on the cell cycle (Selleri *et al.*, 1996). Recently, Lkn-1 affected cell cycle the signal progression by cyclin D3 induction (Kim *et al.*, 2003). We showed that cyclin E protein appeared at day 5, whereas p27 protein was down-regulated in these cells. Regarding the role of p27 protein, it should be noted that p27 protein is an inhibitory protein specific to cyclin E in CB hematopoietic cell cycle progression. In this set of experiments, the p53 protein was up-regulated at day 2 and day 5 compared to cells at day 0. Signaling pathway involving p53 is the main pathway utilized by $CD34^+$ cells to arrest cell cycle progression at multiple checkpoints to halt proliferation (Tao *et al.*, 2003). These results together can be explained by two different hypotheses; one is that heterogeneous myeloid progenitor cell population may not be arrested by a single mechanism during cell cycle, the other is that cell cycle is not involved in myelosuppresive action of CKβ8-1 on human CB $CD34^+$ cells. To further investigate the inhibitory mechanism of CKβ8-1, we focused on CFU-GM cells from human CB $CD34^+$ cells because of the importance of CFU-GM for granulocyte recovery in bone marrow transplantations (Jansen *et al.*, 2002).

**Table 1.** Differential lineages from human CB $CD34^+$ cells stimulated by different combinations of growth factors.

| Growth factors | BFU-E | CFU-GM | CFU-GEMM |
|---|---|---|---|
| rhEPO (1 U/ml) | 77 % | 23 % | 0 % |
| rhGM-CSF (50 ng/ml) + rhIL-3 (10 ng/ml) | 0 % | 100 % | 0 % |

## CFU-GM colonies were formed from human CB $CD34^+$ cells

Using colony assay, we monitored the lineage commitment of $CD34^+$ cells from human CB after treatment with either a combination of GM-CSF (500 ng/ml)/IL-3 (10 ng/ml) or EPO (1 U/ml) alone. In Table 1, only CFU-GM colonies formed in the presence of GM-CSF/IL-3, whereas EPO alone could obtain up to 77% of BFU-E after 7 days culture with.

## Cyclin E expression was delayed in CFU-GM cells by CKβ8-1

To investigate the underlying mechanism of CKβ8-1 on CFU-GM cells, we cultured $CD34^+$ cells from human CB in the media containing GM-CSF/IL-3. We analyzed the expression of cyclin E, which is essential for entry into the S phase in human cells (Ohtsubo *et al.*, 1995), and its inhibitory protein, p27 by immunoblot analysis. In Figure 2, the level of cyclin E increased within 24 h in control cells, whereas cyclin E protein appeared in CKβ8-1- treated cells after 24 h culture. It suggested that CKβ8-1 delays the expression of cyclin E in CFU- GM cells and consequently prevents normal entry of cells into the S phase. It is of note that the level of cyclin E was maintained at high level until day 9 but begun to decrease on day 11. However, when cells were treated with CKβ8-1, cyclin E protein completely disappeared before day 11. Colony formation had

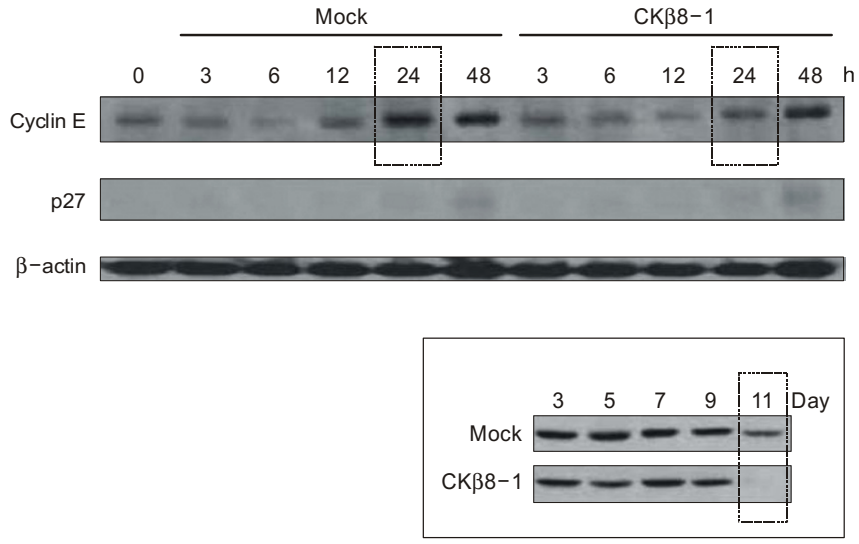


**Figure 2.** Immunoblotting detection of cyclin D, E, in CFU-GM lineage from human CB. $CD34^+$ cells from human CB were allowed to committee to CFU-GM lineage culture in the absence or presence of CKβ8-1.

been observed from approximately day 5 to day 11 in specific HSC colony culture media, suggesting that the proliferation rate of hematopoietic progenitor cells may be highly activated during this culture period. Recent work showed that cyclin A2 gene was moderately expressed, while cyclin D expression was very low in human $CD34^+$ cells under normal growth conditions (Tao *et al.*, 2003). However, our results did not show any significant difference in the level of cyclin A and D in CKβ8-1 treated cells (data not shown). Alternatively, we have shown that cyclin E was up-regulated in proliferating human CB CFU-GM cells, suggesting that cyline E may be crucial in determining S-phase transition of the CFU-GM cells. A human chemokine, PF4, suppressed colony formation of myeloid progenitor cells by interference with the cell cycle machinery (Gentilini *et al.*, 1999). PF4 treatment caused a decrease in cyclin E-CDK2 activity. This supported the notion that cyclin E may be a target molecule of CKβ8-1 in preventing colony formation of CFU-GM. Among the most important mammalian cyclin-CDK complexes known so far, cyclin E is synthesized with a peak late in G1-phase (Boonstra, 2003). Another study has reported that cyclin E had a major role in the control of the G1- and S-phase transitions of hematopoietic cells (Mazumder *et al.*, 2004). In addition, the expression of cyclin E was enhanced during Notch-induced self-renewal of murine HSCs (Satoh *et al.*, 2004). The behavior of cell cycle control genes during hematopoietic differentiation was classified into four patterns and cyclin E placed in the group having no induction or stable expression (Furukawa, 2002). Therefore, it appeared that CKβ8-1 breaks the stable expression of cyclin E and consequently prevents proliferation of CFU-GM. However, it is not known that these cell cycle arrested cells in the late G1 phase by a down-regulation of cyclin E goes through apoptotic cell death. The present study has shown that CKβ8-1 inhibits proliferation of CFU-GM from human CB $CD34^+$ cells by regulating expression of cyclin E, which is involved in the cytokine-mediated cell cycle commitment.

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