Combination of Early and Late Growth Factors to Enrich Transplantable Cord Blood CD34+ Cells in Short Term Cultures

Kordon Kanı CD34+ Hücrelerinin Zenginleştirilmesi İçin Kısa Süreli Kültürlerde Erken ve Geç Etkili Büyüme Faktörlerinin Kombinasyonu

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ABSTRACT Objective: Although umbilical cord blood (UCB) is a useful source of stem and progenitor cells for bone marrow transplantation, it is not possible to obtain sufficient number of transplantable cells from a single human umbilical cord for transplantation in adult patients. In this study, we combined early and late growth factors in short term cultures to enrich cord blood CD34+ hematopoietic progenitor cell count for adult patients. **Material and Methods:** Cord blood mononuclear cells (CBMC) harvested from 15 healthy volunteers, delivering in term, incubated in culture medium supplemented with interleukin-3 (IL-3), IL-6, IL-11, stem cell factor, flt3/flk2 ligand and thrombopoietin for 14 days. Starting cells and cultured cells (day 7 and 14 cells) were evaluated for; CD4, CD8, CD33, CD34, CD38 and HLA-DR by flow cytometry and seeded in semisolid methylcellulose culture for determining BFU-E, CFU-GM and CFU-GEMM colony forming abilities. **Results:** In the short-term cultures, total CBMC count significantly increased (p= 0.0001). Compared to starting cells, the expressions of CD34 (p= 0.002), CD33 (p= 0.0001), HLA-DR (p= 0.0001) and CD8 (p= 0.002) increased, but those of CD38 (p= 0.057) and CD4 (p= 0.0001) were suppressed on day 14 of cultures. There were an increase in generation of CFU-GM (p= 0.131) and CFU-GEMM (p= 0.134) colonies and a significant decrease in generation of BFU-E colonies (p= 0.0001). **Conclusion:** Ex vivo expansion of CBMC, under combination of the growth factors, contribute to CD34+ progenitor cell count and may help to reach sufficient amount of transplantable cells for adult patients. Suspension cultures may lead to elimination of graft-versus-host disease risk and protection of graft-versus-leukemia effect, through the decrease of CD4+ T cells and maintenance of CD8+ T lymphocytes, respectively.

Key Words: Cord blood stem cell transplantation; antigens, CD34; cell culture techniques

ÖZET Amaç: Göbek kordon kanı kök ve projenitör hücre açısından kemik iliği transplantasyonu için elverişli bir kaynak olmasına karşın tek bir insan göbek kordonundan erişkin hastalara nakil için yeterli sayıda hücre elde etme olanağı bulunmamaktadır. Çalışmamızda kordon kanı CD34+ hemopoetik projenitör hücreleri erişkin hastalara yetecek düzeyde çoğaltmak amacıyla erken ve geç etkili büyüme faktörlerini kısa süreli kültürlerde kombine ettik. **Gereç ve Yöntemler:** Miadında doğum yapan 15 sağlıklı gönüllüden kordon kanı mononükleer hücreleri alınarak interlökin-3 (IL-3), IL-6, IL-11, stem cell faktör, flt3/flk2 ligand and trombopoetin eklenen kültür medyumunda 14 gün süreyle inkübe edildi. Başlangıç hücreler ile kültürden alınan hücreler (7. ve 14. günler) flow sitometride CD4, CD8, CD33, CD34, CD38 ve HLA-DR yönünden analiz edildi ve aynı zamanda yarı katı metilselüloz kültürlere ekilerek BFU-E, CFU-GM ve CFU-GEMM kolonilerini meydana getirme kapasiteleri incelendi. **Bulgular:** Kısa süreli kültürlerde total kordon kanı mononükleer hücre sayısında anlamlı artış gözlendi (p= 0.0001). Başlangıç hücreleri ile karşılaştırıldığında, kültürlerin 14. gününde hücrelerde CD34 (p= 0.002), CD33 (p= 0.0001) HLA-DR (p= 0.0001) ve CD8 (p= 0.002) ekspresyonlarında artışla birlikte CD38 (p= 0.057) ve CD4 (p= 0.0001) ekspresyonunda baskılanma gözlendi. CFU-GM (p= 0.131) ve CFU-GEMM (p= 0.134) kolonilerini oluşturma kapasitelerinde artış ve BFU-E (p= 0.0001) koloni oluşturma kapasitelerinde anlamlı düşüş gözlendi. **Sonuç:** Büyüme faktörlerinin kombinasyonu altında kordon kanı mononükleer hücrelerinin vücut dışı ortamda çoğaltılması, erişkin hastalara yetecek miktarda nakledilebilir CD34+ projenitör hücre sayısına ulaşmaya yardımcı olabilecektir. Süspansiyon kültürü sonrası CD4+ T hücre sayısındaki azalma graft-versus-host hastalığı riskinin ortadan kaldırılmasına yardımcı olabilecek ve CD8+ T lenfositlerinin yaşamlarını sürdürmeleri ile de graftın anti-lösemik etkisi korunabilecektir.

Anahtar Kelimeler: Kordon kanı kök hücre nakli; antijenler, CD34; hücre kültürü teknikleri

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Umbilical cord blood (UCB) is an alternative to bone marrow or mobilized peripheral blood for transplantation since it is readily available and is enriched in primitive hematopoietic cells.[1-3] Clinical results with cord blood stem cell transplantation show a high probability of engraftment and a low risk of acute graft-versus-host disease (GvHD). Other advantages of UCB use are the absence of risks for the donor and a low risk of infectious disease transmission.[4-6]

Although a number of successful uses of UCB in hematopoietic malignancies, the major goal is to achieve to enrich UCB stem cells to sufficient numbers for transplanting to adult patients.[7] Some UCB samples may contain sufficient amounts of hematopoietic stem cells (HSC) for transplanting pediatric patients but generally the number of HSC is not adequate in one UCB sample.[8,9]

The major limitations of using UCB as a source of stem / progenitor cells for clinical transplantation is the limited volume providing insufficient number of HSC to reconstitute hemopoiesis in older children and adults.[10] To overcome this limitation, ex vivo expansion of primitive cells has been attempted.[11,12]

Ex vivo expansion of human HSC is an important issue in transplantation and gene therapy.[13] Several stem cell expansion protocols have been proposed. Thus, several combinations of cytokines known to act on primitive HSC have employed in vitro in an attempt to produce culture conditions suitable for HSC expansion. The ligand for c-kit (stem cell factor; SCF) and flt3/flk2 ligand (FL), interleukin-3 (IL-3), IL-6 thrombopoietin (Tpo) are crucial to stimulate primitive HSC expansion.[14-17]

Experimental and clinical hematological studies provide us identification of mechanisms and conditions that support the expansion of transplantable HSC in vitro.[18,19] Ex vivo cultures allow a more rational approach for the development of clinical treatments involving primitive HSC.[20-23]

Identification of conditions that support the self-renewal and expansion of human HSC has stimulated considerable interest in the biology of cord blood cells' ex vivo expansion in various in vitro culture systems.[24,25]

To enhance the rapidity of engraftment and isolation and activation of selected immune cell populations for prevention or treatment of acute GvHD, infectious complications and tumor re-occurrence, several areas of ongoing research include ex vivo expansion of cord blood hematopoietic progenitor cells.[26]

In this study, we used first time a wide array of a growth factor combination such as IL-3, IL-6, IL-11, SCF, FL and Tpo and aimed to determine the effects of the early and late growth factors on $CD34^+$ hematopoietic progenitor cells, colony forming ability of the cells in short term suspension culture.

MATERIAL AND METHODS

CORD BLOOD SAMPLES

UCB samples were collected by gravity from the umbilical vein after the completion of delivery into sterile 20 ml tubes, containing 750 IU of preservative-free heparin (Sigma Chemical Co., St. Louis, MO, USA). All deliveries were normal and full term. Informed consents were obtained from all cases and the samples were processed within 4 h of collection. The procedure had the Istanbul University, Istanbul Medical Faculty Ethics Committee approval.

COLLECTION AND ISOLATION OF MONONUCLEAR CELLS

The cord blood mononuclear cells (CBMC) were separated by Histopaque-1077 (Sigma Chemical Co., St. Louis, MO, USA; d= 1077 g/ml) density centrifugation. After washing twice, cell viability was measured by trypan blue (Sigma Chemical Co., St. Louis, MO, USA) dye exclusion[27] and it was more than 96% in all samples. An aliquot of cells was used for the determination of the committed hemopoietic progenitors and CD4, CD8, CD33, CD34, CD38, HLA-DR expressions.

LIQUID SUSPENSION CULTURES

CBMC were incubated in Iscove's Modified Dulbecco's Medium (IMDM, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% foetal calf serum (FCS, Sigma Chemical Co., St. Louis, MO, USA) in the presence of 20 ng/mL IL-3, 20

ng/mL IL-6, 20 ng/mL IL-11, 20 ng/mL SCF, 20 ng/mL FL, 20 ng/mL Tpo (all from Pepro Tech EC, Ltd. NJ. USA). A total of 1×10^6/ml cells were cultured in T_{25} flasks (5 mL) for 14 days at 37 °C under 5% CO_2 humidified air. Half of the culture media were removed at day 7, while the corresponding other half were fed with fresh media and growth factors. The cells isolated from the media were resuspended in IMDM supplemented with %10 FCS. Suitable aliquots of the cell suspension were checked for viability by the trypan blue exclusion test, counted, immunophenotyped and assayed for colony forming cells.

CELL PHENOTYPING

The antigenic phenotype of UCB cells was assessed before and during ex vivo expansion by flow cytometry. Starting cells and cultured cells (day 7 and day 14 cells) at a concentration of 0.5 x 10^6 per test tube were incubated with the following monoclonal antibodies (mAb): CD4-fluorescein isothiocyanate (FITC), CD8-phycoerythrin (PE), CD33-PE CD34-FITC, CD38-FITC, HLA-DR-PE (Caltag Lab. Burlingame, CA). Appropriate isotype controls were used to determine true percentage of positive cells (Caltag Lab. Burlingame, CA). Flow cytometry was performed with FACSCalibur (Becton-Dickinson Immunocytometry System) using a total cell gate that eliminated cellular debris. At least 10.000 events were analyzed in each sample.

CLONOGENIC ASSAYS FOR COMMITTED HEMOPOIETIC PROGENITORS

CBMC from starting cells were expanded in liquid cultures and tested for the growth of committed hemopoietic progenitors using methyl cellulose media. Methyl cellulose (4.000-centipiose, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in IMDM at a concentration of 3 g per 100 ml. The media supplemented with 30% FCS, 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA), 200 mmol/L L-glutamine (Sigma Chemical Co., St. Louis, MO, USA), 10^{-5} mol/L sodium selenite (Sigma Chemical Co., St. Louis, MO, USA), 10^{-1} mol/L α-monotiyogliserol (Sigma Chemical Co., St. Louis, MO, USA), 100 µL of supernatant from 10 ng/mL phytohemagglutinin (PHA, Biochrom KG, Berlin, Germany) stimulated peripheral blood mononuclear cells[28] at a final volume of 1.1 ml in 35 mm petri dishes. Colony growth was stimulated with 3 IU/ml eritropoietin (EPO; Eprex, Cilag AG, Zug, Switzerland), 50 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF; Leucomax, Sandoz, Novartis Farma AG, Basel, Sweden) and 10 ng/ml granulocyte-colony stimulating factor (G-CSF; Neupogen, Amgen, F. Hoffmann-LA Roche Ltd. Basel, Sweden). Cultures were plated in triplicate, incubated at 37 °C in a 5% CO_2 fully humidified atmosphere for 14 days. Colonies were identified and scored under an inverted microscope according to standard criteria.[29] Burst forming units erithroid (BFUE) colonies scored as compact colonies that hemoglobin containing more than 40 cells, Colony-forming units granulocyte/macrophage (CFU-GM) colonies scored as non-hemoglobin containing areas with more than 50 cells that scatter from a dense central and Colony-forming units granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) colonies were scored as hemoglobinized areas with at least two myeloid cell series containing colonies (Figure 1).

STATISTICAL ANALYSIS

All results are expressed as mean ± standard error of the mean (SE). Statistical significance was determined using ANOVA with Bonferroni test as post-hoc calculation. A P-value of less than 0.05 was considered statistically significant.

RESULTS

PROLIFERATIVE EFFECTS THE CYTOKINE COCKTAIL ON TOTAL CBMC AND CD34⁺ CELLS

To study the proliferative effects of the growth factors in total CBMC and $CD34^+$ cells, 15 UCB samples from healthy normal full-term deliveries were studied. In an effort to induce the short-term proliferation and amplification of $CD34^+$ hematopoietic progenitor cells, early and late growth factors known to be effective on hematopoietic progenitors were added to the liquid suspension cultures. The results are shown in Table 1. Total CBMC ($p= 0.0001$) and $CD34^+$ cells ($p= 0.002$) were significantly increased in suspension cultures.

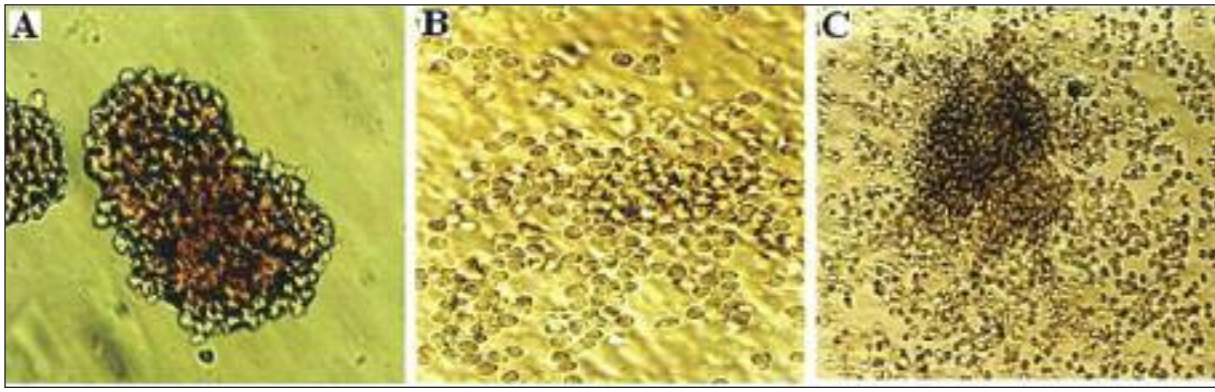

FIGURE 1: Microscopic views of BFU-E, CFU-GM and CFU-GEMM colonies form semisolid culture of CBMC.
A) BFU-E colonies scored as compact colonies that hemoglobin containing more than 40 cells. (Mag. ×200). B) CFU-GM colonies scored as non-hemoglobin containing areas with more than 50 cells that scatter from a dense core. (Mag. ×200). C) CFU-GEMM colonies were scored as hemoglobinized areas with at least two myeloid cell series containing colonies. (Mag. ×100).

EXPRESSION OF MYELOID AND LYMPHOID PROGENITOR CELLS' SURFACE MOLECULES

To determine the effects of the early and late growth factors on progenitor cells, CBMC samples were incubated in the presence of IL-3, IL-6, IL-11, SCF, FL and Tpo. Cell surface markers were detected by flow cytometry. Results are shown Table 2. CD34 (p= 0.019) expression as significantly increased at suspension culture period. In addition to an increase in CD34 expression, CD33 (p= 0.0001) and HLA-DR (p= 0.0001) expressions were significantly increased while CD38 (p= 0.057) expression as decreased after 14 day suspension cultures.

EXPRESSION OF T CELL ASSOCIATED CELL SURFACE MOLECULES

Mature T cell surface markers were detected by flow cytometry on CBMC from fresh UCB samples, day 7 and day 14 of liquid suspension cultures. The results are shown Table 2. Despite a significant decrease in CD4 (p= 0.0001) expression, CD8 (p= 0.002) expression as increased in suspension cultures initiated by early ant late growth factors.

ASSESSMENT OF COLONY FORMING CELLS IN CBMC BEFORE AND AFTER LIQUID SUSPENSION CULTURES

In an attempt to investigate the effects of the early and late growth factors on colony generation capability, CBMC from fresh UCB samples, day 7 and day 14 of liquid suspension cultures were analyzed for in vitro colony formation (BFU-E, CFU-GM and CFU-GEMM). The results are shown in Table 3. Despite significant decrease in BFU-E (p= 0.0001) colonies, higher number of CFU-GM (p= 0.131) and CFU-GEMM (p= 0.134) colonies were detected CBMC after days 7 and 14 of liquid suspension cultures (Figure 1).

DISCUSSION

Primitive hematopoietic stem cell has self-renewal capability and differentiation into all cell lineages. This cell group is responsible for long-term engraft-

TABLE 1: Total CBMC and CD34+ cell counts of cells from fresh cord blood samples and after cultured with early and late growth factors in suspension cultures.

	Cell counts			
	Starting cells	**day 7 of suspension culture**	**day 14 of suspension culture**	**Significance**
Total CBMC	$1 \times 10^6 \pm 0.0 \times 10^6$	$1.6 \times 10^6 \pm 0.1 \times 10^6$	$2 \times 10^6 \pm 0.3 \times 10^6$	p= 0.0001
CD34+ cells	$2.8 \times 10^5 \pm 0.5 \times 10^5$	$10 \times 10^5 \pm 0.3 \times 10^5$	$25 \times 10^5 \pm 0.6 \times 10^5$	p= 0.002

Results are expressed as mean ± SE.

TABLE 2: CD34, CD33, CD38, HLA-DR, CD4 and CD8 expression of CBMC from fresh cord blood samples, day 7 and day 14 of liquid suspension cultures.

	% expression			
Cell surface antigens	**Starting cells**	**Day 7 of suspension culture**	**Day 14 of suspension culture**	**Significance**
CD34	2.8 ± 0.5	6.2 ± 2.0	12.2 ± 2.8	p= 0.019
CD33	5.5 ± 1.6	10.2 ± 2.7	27.9 ± 5.8	p= 0.0001
CD38	30.8 ± 4.1	15.6 ± 4.6	24.8 ± 5.8	p= 0.057
HLA-DR	10.0 ± 1.9	15.2 ± 2.4	34.7 ± 4.3	p= 0.0001
CD4	12.9 ± 2.0	1.9 ± 0.4	0.7 ± 0.1	p= 0.0001
CD8	10.6 ± 1.0	6.9 ± 1.1	12.5 ± 1.3	p= 0.002

Results are expressed as mean ± SE.

TABLE 3: Colony formation (BFU-E, CFU-GM and CFU-GEMM) of CBMC from fresh cord blood samples, day 7 and day 14 of liquid suspension cultures.

	Colony counts			
Colony formation	**Starting cells**	**Day 7 of suspension culture**	**Day 14 of suspension culture**	**Significance**
BFU-E/105 cells	106.6 ± 21.8	23.3 ± 6.7	13.4 ± 2.7	p= 0.0001
CFU-GM/105 cells	35.9 ± 7.5	89.0 ± 27.5	61.8 ± 10.9	p= 0.131
CFU-GEMM/105 cells	6.8 ± 2.4	9.2 ± 2.2	11.4 ± 1.9	p= 0.134

Results are expressed as mean ⑥ SE. Colonies scored in inverted microscope after the day 14 of methylcellulose colony culture. Scoring criteria of colonies in semisolid colony culture system given at the Figure 1 with pictures of these colonies. BFU-E; burst-forming unit-erythroid, CFU-GM; colony-forming unit granulocyte / macrophage, CFU-GEMM; colony-forming unit-granulocyte / erythroid / macrophage / megakaryocyte.

ment to bone marrow and does not have CD34 surface molecules.[30-32]

Nakauchi[33] and Osawa et al[34] showed that $CD34^+$ cells have committed progenitor cell properties more than primitive stem cells, and Collins[35] implied that $CD34^+$ cells were responsible for short-term bone marrow re-population. In this study, we observed that influence of the current cytokines on CBMC caused significant increases in $CD34^+$ cells and these cells in graft has a crucial importance for short-term bone marrow repopulation. Moreover, Rubinstein et al[36] imply that $CD34^+$ count is also a good indicator for engraftment. In our study, the increase of $CD34^+$ cells by the suspension culture may contribute engraftment capability of the UCB samples as a graft in bone marrow transplantation (BMT).

FL is known as a possible modulator of early hematopoietic cell growth.[37] Dooley et al.[15] showed that FL stimulates the expansion of total cells, $CD34^+$ cells, $CD34^+CD33$, $CD34^+CD38$ and $CD34^+$HLA-DR. In this study, in addition to significant increases of CD34 cell populations, there were significant increases of CD33 and HLA-DR expressions. The results showed that there was an increase in either lymphoid or myeloid progenitors of cultured CBMC.

Sui et al[38] observed greatest synergy between IL-6 and SCF, acting through the receptors gp130 and c-Kit respectively. SCF and IL-3, FL and Tpo, and IL-3, IL-6 and Epo were combined in short term cultures by Moore and Hoskins,[39] Piacibello et al[40] and Madkaikar et al[41] respectively. In these cultures initiated by the mentioned cytokines, significant increases in total nucleated cell counts were reported. In this study, we combined these growth factors with IL-11 and observed an increase at total nucleated cell, but not as much as reported in these studies. In our opinion, this difference depends on the start condition of cultures. Likewise, they started the cultures with purified $CD34^+$ cells, but we initiated using total CBMC without any cell purification.

We observed increment in CFU-GM and GFU-GEMM colonies at semisolid culture by the cells

from suspension culture initiated by the current growth factors. The increase of CFU-GM and GFU-GEMM indicate that UCB samples improve the generation of platelet and white blood cell capacity. It is well known that neutrophil and platelet recovery are considered most important criteria for success of BMT.[42] While neutrophils originate from CFU-GM and CFU-GEMM colonies, platelets take origine from the latter ones.[43,44] We believe the increase of CFU-GM and CFU-GEMM colony generation ability by the suspension cultures may contribute platelet and neutrophil recovery after transplantation.

A significant reduction was observed in CD4 cell counts of CBMC in the suspension culture periods. The cells, having major role arising GvHD in allogenic transplantations,[45,46] were removed from the transplantation material during the suspension culture. This removal of the $CD4^+$ T cells provides a lower risk of histoincompatibility problems in transplantations such as GvHD in allotransplantations.

Harris[47] showed that $CD8^+$ cytotoxic T lymphocytes have a protective effect known as graft-versus-leukemia effects. These cells prevent the patients from relapses by killing residual leukemia cells after transplantations.[48] In this study, $CD8^+$ T cell were increased after the suspension culture period. The persistence of CD8 cytotoxic T lymphocytes in UCB grafts through the culture period may contribute to prevent relapses in BMT patients after transplantation by the graft-versus-leukemia effects provided by these cells.

As a result, hemotopoietic stem cell content of UCB samples was enriched in suspension cultures initiated by early and late growth factors. Removal of the $CD4^+$ T cells in culture period may help elimination of the GvHD risk and graft-versus-leukemia effect might be prevented by maintenance of $CD8^+$ T lymphocytes in UCB samples after the suspension cultures.

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