Efficacy of novel culture environments on the *ex vivo* expansion kinetics of cord blood progenitor cells

We demonstrate the efficacy of two cytokine-rich supernatants, one from peripheral blood cell cultures stimulated with anti-CD3 and the other from monocyte-conditioned media, in enhancing the rapid (within 6 days) expansion and clonogenicity of CD34+ progenitor cells. We, thereby, increased the generation of mostly myeloid precursors able to support stem cell transplants.

The efficacy of a stem cell culture protocol lies in its capacity to promote *ex vivo* expansion with conserved self-renewal and effective reconstitution potential in order to replace inadequate grafts or to stimulate production of committed myeloid precursors able to support transplants.[1] Although the optimal combination of factors capable of maximizing multi-lineage expansion and increased self-renewal of stem cells is still unknown,[2,3] factors traditionally included in cytokine cocktails are stem cell factor (SCF), interleukin-6 (IL-6), erythropoietin (EPO), thrombopoietin (TPO), granuylocyte colony-stimulating factor (G-CSF), granuylocyte-macrophage colony-stimulating factor (GM-CSF) and FLt3 ligand (FL).[4-7] We studied proliferation and colony-forming unit (CFU) profiles of cord blood (CB) CD34+ cells selected by MACS beads (Miltenyi Biotech, Germany) in media conditioned by supernatants from peripheral blood (PB) cultures stimulated with anti-CD3 (ACD3S)[8] or from monocyte cultures (MCM)[9] or by a cytokine cocktail containing FL (100 ng/mL) (Biosource, CA, USA), SCF (40 ng/mL), TPO (40 ng/mL), IL-3 and IL-6 (40 ng/mL) (R&D Systems, MN, USA), EPO (10 U/mL) (Eprex, Switzerland), and GM-CSF (20 ng/mL) (Schering Plough, Switzerland). The ACD3S-conditioned medium consisted of 1:10 v/v ratio of supernatant to Myelocult 5100 medium (Stem Cell Technologies, Canada) and the cytokines: SCF at 40 ng/mL and FL at 100 ng/mL (ACD3S/FL/SCF). The MCM culture medium (MCM/FL/SCF) was prepared likewise. Suspension cultures were established at concentrations of 1.0 $\times 10^5$ cells/mL and supplemented every 2 days with fresh medium, supernatants and/or

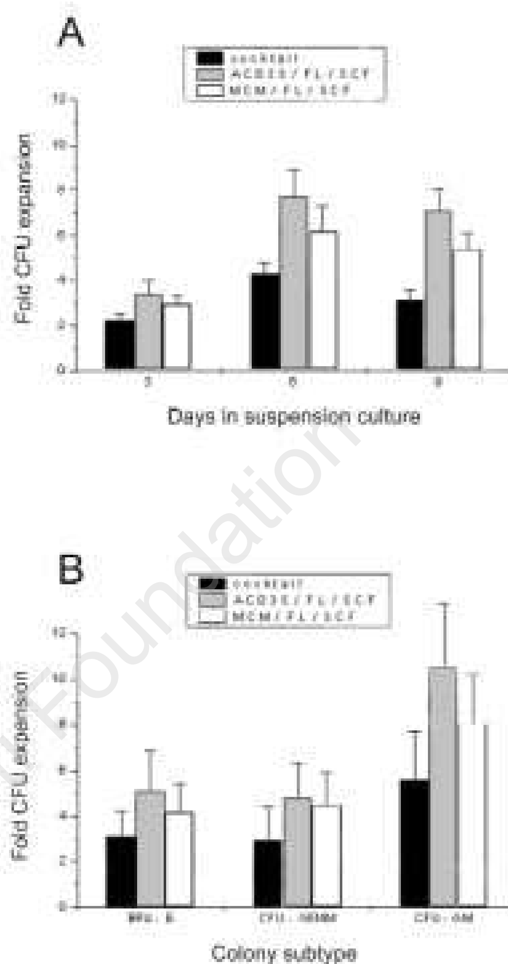

Figure 2 [above]. A. Effect of the cocktail and supernatant-conditioned media on total CFU expansion. CD34+ cells were cultured in suspension for 3, 6 and 9 days. Uncultured (day 0) and cultured cells (day 3, 6, or 9) were then seeded into clonogenic media to assay hematopoietic potential. Data represent average fold increases in total recovered colony numbers over input CFU observed in quintuplet cultures ± SEM from five independent CB samples. B. CFU classification into multipotent and committed progenitor subtypes. CD34+ cells were cultured for 6 days in each medium and then underwent clonogenicity assays. Number and incidence of colony subtype (CFU-GEMM, BFU-E, or CFU-GM) were scored after 14 days. Data represent average fold increases in number of colonies of a particular subtype on day 6 over input CFU in quintuplet cultures ± SEM from five separate samples.

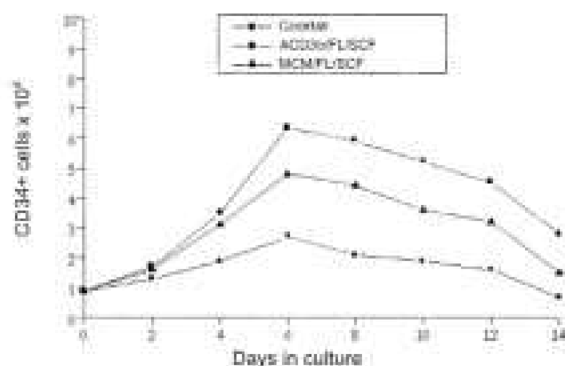

Figure 1 [left]. Degree of retention of the CD34+ phenotype over time by progenitor cells cultured in the presence of the cytokine cocktail or supernatant-conditioned media. Freshly-isolated CD34+ cells were grown in the indicated suspension media for two weeks. Total cell proliferation counts as well as the presence of the CD34 antigen were determined every two days. The data are from a representative experiment depicting absolute numbers of CD34+ cells calculated by taking into account the percentage of cells expressing CD34 and total cell counts on a given day.

©Ferrata Storti Foundation

cytokines. Clonogenicity assays were performed with 5×10^3 freshly-isolated CD34$^+$ or cultured cells in the presence of GM-CSF (Myelogen) at 20 ng/mL, IL-3, IL-6 and SCF at 40 ng/mL (R & D Systems), and EPO at 10 U/mL (Eprex). Colonies were scored after 14 days and classified as myeloid, CFU-granulocyte macrophage (CFU-GM) or burst-forming units-erythroid (BFU-E), or multilineage, CFU-granulocyte erythrocyte monocyte megakaryocyte (CFU-GEMM). The CD34$^+$ profile of freshly-isolated and cultured cells was obtained by FACS analysis with CD34-FITC (Caltag, CA, USA) antibodies.

The ACD3S/FL/SCF and MCM/FL/SCF media generated 140 - and 150-fold respective amplifications in total progenitor cell numbers over input cells as compared to 70-fold mediated by the cytokine cocktail during 14 days of culture ($p<0.005$). They also achieved increased cell proliferation within just 6 days of culture (data not shown). The ability of each medium to maintain the primitive CD34$^+$ status of progenitor cells was assessed by determining cell conservation of the antigen over time in culture. Figure 1 depicts absolute numbers of CD34$^+$ cells after considering total proliferation counts as well as percentages of cells retaining the CD34$^+$ phenotype during culture. Both ACD3S/FL/SCF and MCM/FL/SCF promoted higher preservation of immature progenitors than the cocktail, with values peaking following 6 days of expansion primarily in the ACD3S/FL/SCF medium (7.0- versus 5.4- and 3.0-fold increases, respectively) ($p<0.005$).

In order to determine the capacity of each medium to promote CFU expansion during short-term suspension cultures, we scored colonies formed by CD34$^+$ cells cultured for 3, 6 and 9 days and compared CFU levels to those of uncultured cells. Maximal stimulation of colony formation was evidenced in cells cultured for 6 days (Figure 2A). The ACD3S/FL/SCF medium induced the highest CFU generation (7.7-fold increase over input CFU, $p < 0.005$), followed by the MCM/FL/SCF medium (6.2-fold) and the cocktail (4.3-fold). We then determined effect on commitment along lineage-specific pathways by scoring increases in incidence of CFU-GEMM, BFU-E, and CFU-GM subtypes induced by 6-day cultured cells (Figure 2B). The ACD3S/FL/SCF medium mediated 4.8-, 5.1- and 10.5- fold expansions of CFU-GEMM, BFU-E and CFU-GM, respectively. Corresponding scores in MCM/FL/SCF were moderately lower, (4.5-, 4.2- and 8.0-fold) while the cocktail was the least effective (3.3-, 3.7- and 5.2- fold). We thus observed maximal production of multipotent and lineage-specific colonies from cells cultured in the supernatant media, especially in ACD3S/FL/SCF. Most expanded precursors belonged to the myeloid granulocyte-macrophage lineage.

Similar expansion profiles demonstrating the efficacy of ACD3S/FL/SCF and MCM/FL/SCF media were obtained with CD34$^+$ cells from mobilized PB as well as AC133$^+$ cells[10] from both CB and PB (*data not shown*). This preliminary study describes the functionality and efficacy of supernatant-conditioned culture media in expediting and stimulating hematopoietic precursor expansion mostly of myeloid progenitors, thereby providing a venue for accelerated engraftment.

Nike T. Cacoullos, Angelos D. Gritzapis,* Athanasios E. Tsitsilonis,° Marinos L. Tsiatas,* Constantin N. Baxevanis,* Michael Papamichail**

**Cancer Immunology and Immunotherapy Center, St. Savas Cancer Hospital, Athens, Greece; °Maternity Hospital 'Mitera', Amarousion, Greece*

Correspondence: Nike T. Cacoullos, PhD, Cancer Immunology and Immunotherapy Center, St. Savas Cancer Hospital, 171 Alexandras Ave, 115 22 Athens, Greece. Phone: international +30.1.6409468. Fax: international +30.1. 6409516. E-mail: nisiro@hotmail.com

Key words: CD34$^+$ cells, progenitor expansion, ACD3S/MCM supernatant, conditioned media.

Acknowledgments: we are grateful to Professor H. Damianou from the Dept. of Statistics at the University of Athens for statistical analysis of the data.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Carmelo Carlo Stella, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Carlo Stella and the Editors. Manuscript received October 11, 2001; accepted December 18, 2001.

Ferrata Storti Foundation

References

1. Piacibello W, Sanavio F, Garetto L, Severino A, Dane A, Gammaitoni L, et al. Differential growth factor requirement of primitive cord blood hematopoietic stem cell for self-renewal and amplification vs proliferation and differentiation. Leukemia 1998; 12:718-27.
2. von Kalle C, Glimm H, Schulz G, Mertelsmann R, Henschler R. New developments in hematopoietic stem cell expansion. Curr Opin Hematol 1998; 5:79-86.
3. Srour EF, Abonour R, Cornetta K, Traycoff CM. Ex vivo expansion of hematopoietic stem and progenitor cells: are we there yet? J Hematother 1999; 8:93-102.
4. Leary AG. Synergism between IL-6 and IL-3 in supporting proliferation of human hematopoietic stem cells: comparison with IL-1a. Blood 1998; 71:1759-63.
5. McNiece IK, Langley KE, Zsebo KM. Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and EPO to stimulate human progenitor cells of the myeloid and erythroid lineages. Exp Hematol 1991; 19:226-31.
6. Piacibello W, Sanavio F, Garetto L, Severino A, Bergandi D, Ferrario J, et al. Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. Blood 1997; 89:2644-53.
7. Lyman SD, Jacobsen SEW. C-kit ligand and flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. Blood 1998; 91:1101-34.
8. Baxevanis CN, Tsiatas ML, Cacoullos NT, Spanakos G, Liacos C, Missitzis I, et al. Induction of anti-tumour lymphocytes in cancer patients after brief exposure to supernatants from cultures of anti-CD3-stimulated allogeneic lymphocytes. Br J Cancer 1997; 76:1072-80.
9. Reddy A, Sapp M, Feldman M, Subklewe M, Bhardwaj N. A monocyte conditioned medium is more effective than defined cytokines in mediating the terminal maturation of human dendritic cells. Blood 1997; 90:3640-6.
10. Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997; 90: 5002-12.