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Isolation of hematopoietic progenitors. An approach to two different immunomagnetic methods at the lab scale

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Abstract The hematopoietic progenitors are a pool of cells which share the expression of a membrane glycoprotein: CD34. These cells are present in low frequencies in umbilical cord blood, adult peripheral blood and bone marrow (respectively, 0.1-0.6%, 0.01-0.1% and 0.8-4% of nucleated cells). The suitability of high-affinity monoclonal antibodies against the CD34 molecule, the necessity of rapid, sterile isolation methods and the chance of CD34-negative cell tumor purging in autologous stem cell transplantation or T-cell depletion in allogeneic transplantation has favoured a rapid development of different technical approaches for CD34+ cell selection.
We can distinguish two different approaches to the CD34+ cell selection techniques: a) immunomagnetic which include a direct or indirect linkage between CD34+ cells and immunomagnetic particles, and b) immunoadsorption of CD34+ cells to an antibody-precoated plastic or avidinized poliacrilamide surfaces.
All these techniques achieve variable yields between 30-70% and median purities ranging from 50-95%. An approach to the positive selection of CD34+ cells by two different indirect immunomagnetic methods is given.

Introduction:

Hematopoietic progenitor cells cells are included in a heterogenous cell group from the most mature colony forming units in semisolid cultures until the most primitive hematopoietic stem cell with autorenewal capability. Part of these cells can be studied by functional studies according to their growth in semisolid cultures: colony-forming-units for granulocytes and monocytes (CFU-GM), burst forming units for erythroid cells (BFU-E) and colony forming units granulocyte-erythroid-monocyte-megakaryocyte (CFU-GEMM) for more immature progenitor cells capable to produce white, red and megakaryocyte lineage cells (1). Some of these functional assays for committed progenitor cells were already developed 25 years ago and they are still useful (2).
However, during the 80's, Andrews, Civin and others (3,4), showed the importance of a glycoprotein present in the pool of hematopoietic progenitor cells and some leukemic cells. This antigen was designed later as CD34 (5).
The antigen CD34 is a heavily glycosylated protein (mainly, by syalic and neuraminic acid). This glycoprotein can be divided into three enzyme-sensitive epytopic regions according to Sutherland (6). These regions come from the different susceptibility of epytopic regions to neuraminidase, glycoprotease and chymopapain and different monoclonal antibodies are able to recognize these different regions.
Why such a high interest in the isolation of hematopoietic progenitor cells in last years?. The study of hematopoietic progenitor cells has become a very interesting topic mainly due to its practical application to the transplantation in the allogeneic and autologous setting. Biochemical, functional, cytological and cryopreservation have been clearly favoured by the development of fast, reliable purification methods. Besides, the use of the isolation of hematopoietic progenitor cells can indirectly produce a purging effect for the graft in bone marrow or peripheral blood transplantation. It is an interesting tool for producing expansion of these selected cells and for gene marking and gene therapy on isolated progenitor cells.

There are two approaches for this isolation. Firstable, the oldest one, the fluorescence activated cell sorting (FACS) based on the separation of positive cells for the presence of a fluorochrome linked by a monoclonal antibody to the marker antigen. Secondly, and more recently developed, those methods based in the attachment and later release of the target cells to different surfaces.
These immunoadsorption methods have been developed due to the necessity of a fast, reliable, reproducible, sterile isolation system for further long-lasting culture studies and clinical purposes. These methods can be based in the use of avidinized polyacrylamide where cells linked to the biotinylated CD34 MoAb will be retained or based on the use of plastic flasks which contain an anti-CD34 MoAb covalently linked to their surfaces and where positive cells can be retained or can be based on the use of magnetic beads or particles which are coated by an antibody targetted against the the CD34+ cells. In this last case, positive cells will be retained after passing them along a magnetic field. This work will be referred to our experience in some of these last immunomagnetic methods.

Methods:

Two indirect immunomagnetic methods for positive selection of CD34+ cells have been extensively used in our lab. The first of them based on the use of indirect binding to antibody-coated 4.5 μm paramagnetic microspheres (Dynabeads; Dynal, Oslo, Norway) followed by release of cells from the beads by chymopapain treatment. The second one is the magnetic activated cell sorter (MACS) developed by Miltenyi et al (7) which consists of indirect binding to antibody-coated 50 nm superparamagnetic particles.
Bone marrow aspirates, umbilical cord bloods and leukapheresis aliquots from mobilized peripheral blood were obtained from consenting adult volunteers. Low-density (density < 1.077 g/cm^3) leukocytes were obtained from bone marrow aspirates and umbilical cord bloods. Leukapheresis samples were simply washed twice at low speed centrifugation (150 x g) and platelet-rich plasma was discarded.
Dynabeads binding method: Isolated mononuclear cells or nucleated cells from mobilized peripheral blood were first incubated for 30 minutes at 4°C with CD34 monoclonal antibody My10 0.25 μg/10^6 cells (obtained from a murine hybridoma, clone 28/8/8/14/4 from the American Type Culture Collection, Rockville, MD) in a medium composed by phosphate-buffered-saline pH 7,2 (PBS), human serum albumin 1% (HSA) (Behring, Barcelona, Spain) and human gamma globulin 0.5% (Flebogamma, Instituto Grifols, Barcelona, Spain). After two washes, the antibody-treated cells were resuspended at the same concentration and mixed with sheep antimouse IgG-coated magnetic microspheres (Dynabeads M-450, Oslo, Norway) at a ratio of 0.5 beads per cell. This mixture was incubated at 4°C for 30 minutes on a rotator at 4 rpm. After incubation, cells were separated using a strong magnet (Isolex-50, Baxter Healthcare Corporation, Santa Ana, CA). CD34 negative cells were removed by washing the rosetted cells five times with PBS/HSA medium. Detachment of CD34+ cells from beads was performed by incubation with chymopapain (250 pKat for 15 minutes at room temperature). The reaction was stopped by the addition of cold PBS/HSA medium and followed by removal of CD34+ cells from the magnetic field by two washes. The viability was routinely higher than 90% by trypan blue exclusion.
Magnetic activated cell sorting. Mononuclear cells were incubated with the mouse anti-CD34 MoAb QBEND-10 (Miltenyi Biotec Inc; Bergisch Gladbach, Germany) linked to a hapten for 15 minutes at 4°C in a medium containing PBS/Human immunoglobulin and etilendiamintetracetic acid (EDTA) 5 mM or citrate/citric acid solution 2% (ACD-A). Cells are washed once, and thereafter, they are incubated with 50 nm-paramagnetic colloidal particles coated with a mouse anti-hapten MoAb (Miltenyi Biotec Inc, Bergisch Gladbach, Germany) for 15 minutes at 4°C. Clumps were removed by passing the cells through a nylon mesh. Later, cells were applied to a MiniMACS column (type MS) (Miltenyi Biotec Inc, Bergisch Gladbach, Germany) and the column was washed four times. The CD34 negative cells were removed at this point. Finally, CD34+ cells were released by flushing down the column with a plunger.
Anti-CD34 immunofluorescence assays were performed by using PE-8G12 (Becton-Dickinson, San José, CA) in an EPICS Profile II or Elite cytometer (Coulter, Miami, FL). Clonogenic cultures were performed in 1.12% methylcellulose, supplemented with 25% fetal calf serum, 1% bovine serum albumin (Sigma, St. Louis, MO), 2 U/ml rh-erythropoietin (Epopen, Pensa, Barcelona, Spain), 50

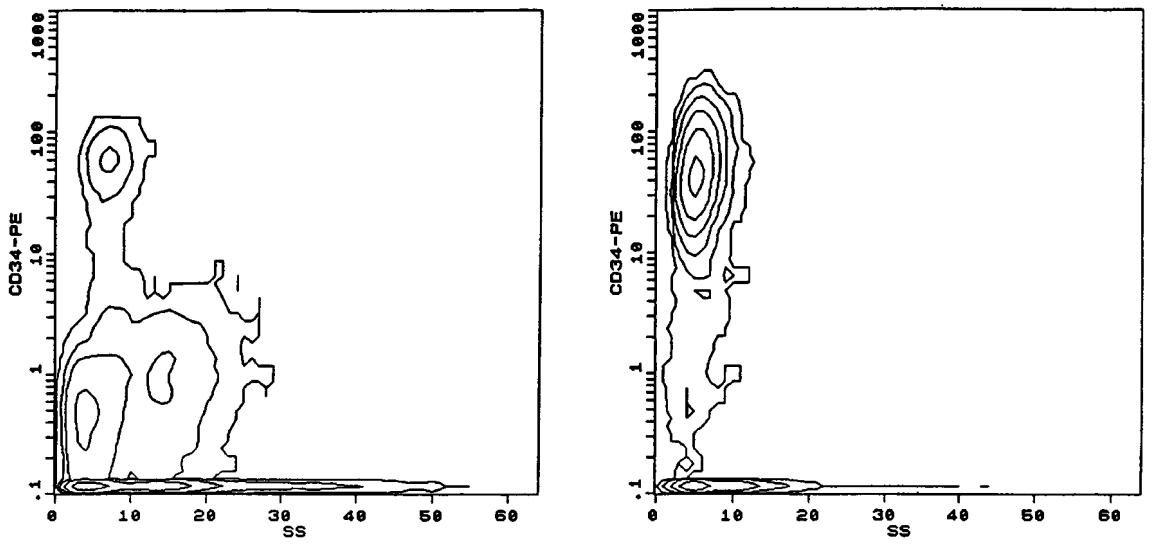

Fig. 1. Representative example of the CD34+ cell content in mobilized peripheral blood before (A, 2,5%) and after the selection (B, 92%).

ng/ml rh-SCF (kindly provided by Amgen, Thousand Oaks, CA), 10 ng/ml rh-IL-3 and 20 ng/ml rh-GM-CSF (kindly provided by Sandoz, Vienna, Austria). Colonies were scored according to Coutinho et al (8) after 14 days in culture. Twenty eight-day cobblestone area forming cell enumeration was performed according to Breems et al (9) on 15 Gy-irradiated human bone marrow stroma on a basis of limiting dilution analysis.

Data presentation: Results are shown as median, range and coefficient of variation (CV). The Mann-Whitney U test was used for statistical comparisons. The yield is shown as a percentage and calculated as the final number of CD34+ cells divided into the starting number of nucleated cells for mobilized peripheral blood or mononucleated cells after low density separation for cord blood and bone marrow. Capture efficiency (CE) was calculated as CE(%)=100-100x{(number of CD34+ cells in the negative fraction)/preselection number of CD34+ cells} and indicates the proportion of positive cells rosetted with the immunomagnetic spheres.

Results:

Dynabeads immunomagnetic method: A median final purity of 87% (range 32.7%-99.7%) was achieved with no statistical differences according to the source of hematopoietic progenitors used. A median yield of 44.8% (range 15%-83.5%) was observed. The median immunomagnetic capture efficiency was 65% (range 25-100%). In fig. 1, it can be observed a representative example of flow cytometry analysis of a selection of CD34+. In fig. 2, it is expressed the results of clonogenicity before and after 5 different selections of hematopoietic progenitors of cord blood and mobilized peripheral blood.

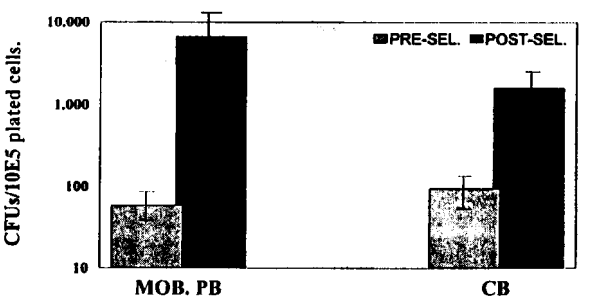

Fig. 2. Clonogenicity of pre-selected (PRE-SEL.) and post-selected (POST-SEL.) cells from mobilized peripheral blood (MOB. PB) and cord blood (CB). Results by 10^5 plated cells are given.

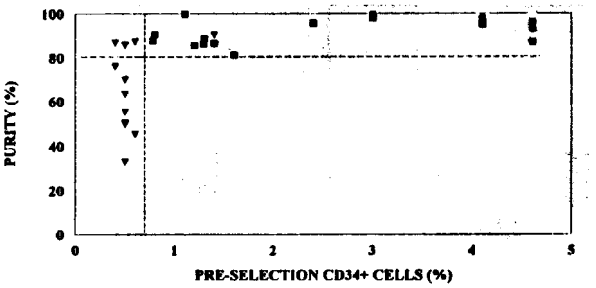

Fig. 3. Dot-plot representing the final purity with respect to the starting percentage of CD34+ cells in mobilized peripheral blood. Black squares are referred to samples obtained from patients primed with chemotherapy with or without rhG-CSF. Black triangles indicate samples obtained from patients primed with rhG-CSF alone.

Clonogenicity of the isolated cells could be demonstrated in methylcellulose cultures showing a rough enrichment of 100-fold for mobilized peripheral blood and 30-fold for cord blood.

When we analyzed the final purity achieved respect to the starting CD34+ cell percentage (fig. 3), we could observe in mobilized peripheral blood an increase of the variability of the result when the starting CD34+ cell percentage was lower than 0.65% (median purity: 59.1%, range: 32.7-87.1%, CV=28.7%, n=11) vs when it was higher than 0.65% (median purity: 93.7%, range: 81-99.7%, CV=6%, n=18, $p<0.001$). Most cases having a lower starting percentage proceeded from patients primed with recombinant human granulocyte colony stimulating factor (rhG-CSF) alone meanwhile those ones with a higher percentage proceeded from patients primed with chemotherapy alone or plus rhG-CSF. However, no statistical diferences were observed between both groups for granulocyte, monocyte or lymphocyte relative content (10).

In order to determine the presence or absence of functionally immature progenitor cells after positive selection of CD34+ cells. We determined the 28-day cobblestone area forming cell frequency on CD34+ cells pre and postselection (fig. 4). We were able to observe at least, a maintenance of these cells after the positive selection in any source of hematopoietic progenitor cells.

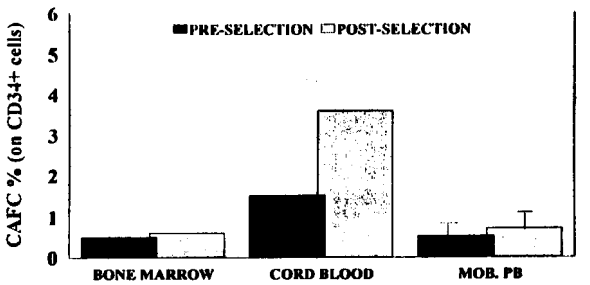

Fig. 4. 28-day cobblestone area forming cell (CAFC) frequency on CD34+ cells pre and postselection in mobilized peripheral blood (MOB. PB, n=5), bone marrow (n=2) and cord blood (n=2).

Magnetic activated cell sorting method: From a median starting percentage of CD34+ cells of 2% (0.5–4%) for bone marrow and 1% (range 0.2-3%) for mobilized peripheral blood, we could obtain a median final purity of 96.2% (range 80%-99.5%) and 94% (82-99.2%), respectively. An overall median yield of 60% (range 18%-83%) was observed with a median immunomagnetic capture efficiency of 63% (range 17-100%).

A polynomic correlation with a final plateau phase could be seen between the starting absolute number of CD34+ cells and the final purity by using this technique ($r=0.72$, $p<0.01$, fig. 5). No relation between the source of progenitor cells and the final purity was observed.

Conclusions: We describe our experience in two different indirect immunomagnetic methods for positive selection of hematopoietic progenitors. We were consistently able to get from all the sources studied (cord blood, bone marrow and mobilized peripheral blood) high-purity fractions which were able to grow in liquid and semisolid cultures, maintain their long term repopulating abilities as

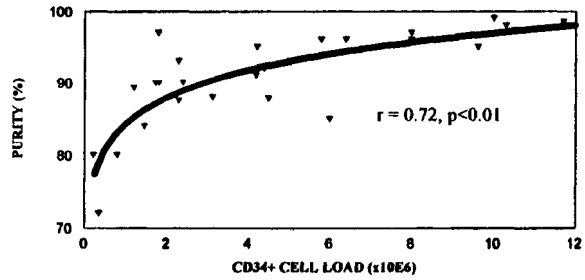

Fig. 5. Polynomic correlation plot between the starting CD34+ cell content and the final purity achieved.

determined by CAFC frequency content and so, useful for highly accurate functional studies. For, bone marrow and cord blood, after density gradient separation, no additional depletions of contaminating cells were necessary. For both methods of positive selection, it seems to appear a correlation between the either starting CD34+ cell percentage or overall CD34+ cell content and the final purity obtained.

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