Review Article

Methods for Isolation of Bone Marrow Stem Cells: Comparative Analysis

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Received: 2/Jan/2010, Accepted: 24/Jun/2010

Abstract

During the past decade, regenerative medicine has emerged as a key technology in the next generation of medical care, and cell therapy and organ repair using stem cells have become very attractive options for regenerative medicine. The application of stem cells in regenerative medicine has required modified methods for isolation. Furthermore, the process of cell separation plays an important role in cell therapy and regenerative medicine using stem cells. So, in this review, we compare different methods for the separation of cells from bone marrow for transplantation to humans, with emphasis on the advantages and disadvantages of each method.

Keywords: Stem Cells, Cell Separation, Bone Marrow

Cell Journal(Yakhteh), Vol 12, No 4, Winter 2011, Pages: 438-445

Introduction

Advances in medicine and medical technology have resulted in a tremendous improvement in health and welfare. However, we are still faced with various diseases that are difficult to treat using contemporary medicine. For organ failure (heart, renal & liver failure) and neurodegenerative diseases (Parkinson's and Alzheimer's disease), there is, at present, no effective treatment other than the transplantation of organs from human donors or cells from a fetus (1). The many problems associated with transplantation, such as immunological rejection, infectious diseases, and a lack of donors have prompted the search for a novel treatment method. During the past decade, regenerative medicine has emerged as a key technology in the next generation of medical care, and cell therapy and organ repair using stem cells have become very attractive options for regenerative medicine.

Various multipotent stem and progenitor cells exist in adult tissues and organs to replace lost or injured cells and are almost comparable to embryonic stem (ES) cells with respect to their ability to differentiate into various tissues *in vitro* and *in vivo* (2-4). Thus, there has been tremendous progress in understanding the mechanism of tissue regeneration. Application of these cells in regenerative medicine has required modified methods for isolation. The process of cell separation holds an important role in cell therapy and regenerative medicine using stem cells. Stem cells are usually present in only small quantities in adult tissues and organs, and an effective separation procedure for stem cells is always required. The separation of cells is different from the separation of other materials. Since cells are living and variable, depending on the surrounding environment, the separation processes are limited by the medium and operation. The speed of the operation and viability during separation are important operating factors. During the last decade, several technologies have been proposed to purify hematopoietic cells for clinical use (2, 3).

In this article we focus on different methods used for the separation of stem cells from bone marrow for transplantation and compare these methods, pointing out their advantages and limitations.

Procedures for Cell Separation

The ability and efficiency of various techniques to purify adult stem cells from a heterogeneous cell population is an important factor in the successful characterization and application of stem cells. Existing cell separation methods can be classified into two main groups. The first is based on physical criteria like size, shape, and density differences and includes filtration and centrifugation

techniques (4). These methods are commonly used for debulking heterogeneous samples. The second group comprises affinity methods such as capture on affinity solidmatrix (beads, plates, fibers) (5, 6), fluorescence-activated cell sorting (FACS) (7, 8) and magnetic cell sorting (9, 10), which are based on biochemical cell surface characteristics and biophysical criteria (in FACS).

Here, we are going to review development of methods applied to the isolation of cells from bone marrow (BM).

Enrichment of mononuclear cells

Several techniques for the enrichment of mononuclear cells (MNC) from original BM have been developed. These techniques include sedimentation with agents such as hydroxyl ethyl starch (HES) (11, 12), density gradients using Buffy coat (13, 14), and blood cell separators (15-18). The latest blood cell separators, with fully automated methods, allow BM processing in a closed system, which simplifies separation procedures and improves the quality of the collected cells in terms of cell recovery, collection selectivity, and microbiologic safety (19).

Manual MNC isolation

Early attempts to process BM were carried out using manual techniques. A simple technique to separate red blood cells (RBC) from BM by sedimentation after addition of HES was developed specifically for ABO-incompatible allogeneic bone marrow transplant (BMT) (12, 20). Although effective, manual methods are, by nature of the excessive handling and exposure to the environment, more likely to result in bacterial contamination of the BM. Inclusion of unwanted components, including neutrophils and platelets, is also problematic when using manual buffy coat preparation, as there is no difference in the differential count of BM nucleated cells before and after processing (12, 21).

(22) (This ref should be moved) Density gradient separation using Ficoll-Hypaque as described by Wells et al. (23) presumably (are you uncertain whether it did or didn't?) yielded a nearly RBC-free BM cell suspension. Ficoll-Hypaque gradient centrifugation allows rapid and efficient isolation of mononuclear cells from human peripheral blood (24) and also bone marrow. Using these kinds of techniques MNC yields generally exceed 50% of original cell numbers (13, 16, 22, 23, 25-27), but such techniques are associated with potential cell injury from the reagents used, as demonstrated by a significant reduction in tritiated thymidine incorporation and loss of lymphocyte viability following culture with Ficoll-Hypaque (28). The method is also inefficient for large volumes and requires considerable technical expertise. As such, this technique is the starting point for most studies of human lymphoid cells. In general, Ficoll-Hypaque centrifugation does not change either the phenotype or the function of the isolated mononuclear cell population. However, it may be best to verify this in studies of cells from patients with various diseases (29). Schwella et al. (1996) noted that BM products obtained by manual means are comparable to those processed by a blood cell separator (30) and that separating cells by Ficoll-Hypaque centrifugation also often decreases the cytometry time for acquisition and removal of nonviable cells. The majority of groups prefer applying unfractionated autologous BM MNC, manually isolated by Ficoll density gradient-based separation (31-37). This conventional method is a time-consuming process involving at least two washing steps that make the system 'open'. With operator-dependent results, manual Ficoll density gradient, based MNC separation, is neither standardized nor reproducible and thus not optimally suitable for clinical implementation, although groups have tried to standardize it to good manufacturing practices (GMP) grade (38).

Automated MNC isolation

Although sedimentation techniques are highly efficient methods for removing RBC (11, 12, 20), newer automated techniques have apparent advantages in terms of preparation of more pure populations of MNC without the need to expose patients to reagents like HES. Automated nondensity gradient separation techniques are currently available and produce BM fractions with minimal RBC and plasma contamination while preserving a high percentage of preprocessed MNC (18, 21, 27, 39-42).

BM MNC concentration using a cell separator was first described in 1977 using the Haemonetics system (43). In the following years, investigations were performed using other devices, for example, COBE 2991, Fenwal CS3000, Fresenius AS104, Terumo SteriCell, Du Pont Stericell, Dideco T90, and COBE Spectra (13, 15, 18, 21, 26, 27, 40-42). Davis et al. (18) compared results of BM processing using two techniques, COBE Spectra MNC concentration and light-density cell fraction isolation using the COBE 2991. The two procedures provided similar recovery of nucleated cells (22% versus 21%, respectively) and gave progenitor recoveries of 132% and 100% (CFU-GM) and 101% and 104% ($CD34^+$ cells), respectively. The Spectra recovered a larger percentage

of MNC and had less contamination with mature granulocytes than did the density gradient technique. Moreover, the Spectra MNC concentrate was prepared with reagents approved for injection by the U.S. Food and Drug Administration. The mean recovery rates reported in this study were 35.8% (marrow nucleated cells), 78.8% (MNC), 90.2% (lymphocytes), 153.3% (CFU-GM), and 77.2% ($CD34^+$ cells). Other investigators (15, 26, 27, 39, 41) have shown similar results for cell recovery and RBC reduction. However, comparisons with results from other institutions may not be valid because of the lack of standardization of cell counting techniques, differential cell count analysis, $CD34^+$ cell flowcytometric enumeration, and progenitor cell culture assays. Many centers have started to transfer the responsibility for BM and peripheral blood stem cell (PBSC) harvest and processing to smaller but trained and experienced staff. Collection facility staff are highly skilled in the use of apheresis equipment for apheresis and, therefore, should easily adapt to similar techniques for processing BM components. It seems that automated separation of the mononuclear fraction of BM can be performed both rapidly and simply in a closed, sterile and approved system without use of density gradient materials using the COBE Spectra. A large percentage of viable progenitor cells can be collected, with little RBC and granulocyte contamination. In other hands, recovery of MNC, CFU-GM, and $CD34^+$ cells was comparable to that reported using other automated methods (15, 18, 27, 39, 41). In summary, the COBE Spectra, an instrument originally designed for clinical cell separation and plasmapheresis, which is found in many transplant centers, can be used successfully for preparation of a concentrated BM component that is rich in MNC.

In order to maintain high standards and ensure the clinical safety required for cellular therapeutics, the automated Sepax cell-separation system was brought into routine BM processing in 2006. The functionally closed Sepax system provides a reliable and reproducible MNC isolation method. The principle of both manual and automated MNC isolation by Sepax lies in a Ficoll density gradient-based separation on lymphocyte separation medium.

Usage of specific cell types

An emerging clinical strategy is therapy based on the use of specific cell types to provide selective therapeutic benefit. The development of cellular therapy is one of the newest and most exciting concepts currently being investigated for the treatment of cancer, autoimmune disease, and the correction of certain genetic abnormalities.

Affinity separation methods for isolation of rare cell populations most commonly are based on the use of antibodies against differentially expressed cell-surface antigens. Enrichment of hematopoietic stem cells (HSC) is based on the use of antibodies against surface CD34 and CD133 antigens. During the last decade, several technologies based on the recognition of CD34 antigen have been proposed to purify hematopoietic cells for clinical use.

The CD34 antigen is a transmembrane glycoprotein that is expressed on human hematopoietic progenitor cells and most endothelial cells, but is not found on mature blood cells and is not expressed by most solid tumors (44). Several techniques based on the recognition of CD34 antigen have been used to purify HSC from human bone marrow, umbilical cord, and peripheral blood.

The percentage of $CD34^+$ cells differs between the different sources of hemopoietic cells. They comprise less than 0.5% of peripheral blood cells but may increase from 1 to 5% after mobilization, whereas marrow mononuclear cells contain an average 1.5% of $CD34^+$ cells. In cord blood, there are 0.5 - 2.5% $CD34^+$ mononuclear cells.

In the autologous setting, the use of separation techniques employing antibodies against CD34 antigen allows for effective purging of malignant cells that do not co-express this antigen (45). However, some malignancies, such as acute lymphoblastic leukemia, co-express CD34 (46). Some studies have used these kinds of cells for treatment of patients with cirrhosis (47, 48). In such cases, separation techniques are based on the recognition of the CD133 antigen, which is not co-expressed by the malignant cells. CD133, a 120 kDa transmembrane glycoprotein is a unique stem cell marker and, in contrast to the CD34 antigen, is not expressed by late progenitors (49). Highly purified $CD133^+$ mobilized peripheral stem cells have already been successfully used for autologous and allogeneic transplantation in acute lymphoid leukemia (50, 51). Enrichment of $CD133^+$ cells from bone marrow have been successfully used for patients with heart disease (52, 53).

Selection technologies

Antibody Panning Selection

The panning technique is a simple approach which does not require special material or equipment. The method is based on the use of sterile polystyrene flasks that contain soybean agglutinin (SBA), a cell binding lectin, or the class II anti-CD34 monoclonal antibody ICH3, covalently immobilized to the inner surface of the device (CD34 Cellec-

tor flask AIS). The first step is negative selection, and the second, positive selection. A final purity of 50-70% of $CD34^+$ cells is obtained. Moreover, the purified $CD34^+$ population is functional in both short and long-term hematopoietic progenitor assays in vitro. Technical improvements with reduction of procedural time have been proposed (54) to enhance the purity of the final suspension. The procedure is rapid and simple. However, because of the difficulties in reproducibly removing bound cells and obtaining high final purity, clinical applications have been limited (55) .

High-speed Fluorescence-activated Cell Sorting

An emerging technology utilizing immunoaffinity interactions for cell isolation is a high-speed FACS (56-58). FACS is a specific type of flow cytometry, which utilizes fluorescent affinity markers placed on the cells for the purpose of recognizing and sorting the cells. Flow cytometers measure relative fluorescence, size, and granularity of a single cell as it intersects a laser beam at a high velocity. In direct labeling of cells for FACS, a fluorochrome is chemically conjugated to a primary antibody that recognizes specifically an antigen of interest. In indirect staining, a fluorochrome-conjugated secondary antibody is used that recognizes the Fc region of a non-conjugated primary antibody. The three most common fluorochromes are fluorescein isothiocyanate (FITC), which emits green 530-nm light; phycoerythrin (PE), which emits yellow 578-nm light; and PE-Cy5 (PE conjugated to a cyanine dye), which emits light at 670 nm (56). In advanced procedures, it is possible to label cells with two, three, or even four different fluorochromes simultaneously. The average recovery from the flow sort is 60%, and the average overall recovery about 40%. Factors that affect the recovery of stem cells are the degree of contamination by red blood cells (55).

High gradient magnetic cell separation with MACS

A flexible, fast and simple magnetic cell sorting system for separation of large numbers of cells according to specific cell surface markers has been developed and tested. Cells stained sequentially with biotinylated antibodies, fluorochrome-conjugated avidin, and superparamagnetic biotinylated-microparticles (about 100 nm diameter) are separated on high gradient magnetic (HGM) columns. Unlabelled cells pass through the column, while labelled cells are retained. The retained cells can be easily eluted. More than 10^9 cells can be processed in about 15min. Enrichment rates of more than 100-fold and depletion rates of several 1,000-fold can be achieved. The simultaneous tagging of cells with fluorochromes and very small, invisible magnetic beads makes this system an ideal complement to flow cytometry. Light scatter and fluorescent parameters of the cells are not changed by the bound particles. Magnetically separated cells can be analysed by fluorescence microscopy or flow cytometry or sorted by fluorescence-activated cell sorting without further treatment. Magnetic tagging and separation does not affect cell viability and proliferation (59).

Positive selection is the most direct and specific way to isolate the target cells from a heterogeneous cell suspension and allows up to 10,000-fold enrichment of the magnetically labeled target cells. The target cells are magnetically labeled (directly or indirectly), get retained in a magnetic separator and finally are eluted after removal of the magnetic label as the enriched fraction. The drawback of this strategy is that an additional step, i.e., removal of the magnetic label, may be needed depending on the type of cells, their further application and type of magnetic particles. Miltenyi Biotech, Polysciences, and other companies have developed cell separation products based on magnetic microbeads. Larger particles usually have to be removed, while cells labeled with submicroscopic magnetic beads (e.g., with MACS microbeads by Miltenyi Biotec, which are approximately 50nm in size) may be used directly (9, 55). Besides, due to their composition of iron oxide and polysaccharide, the microbeads are biodegradable and typically disappear after a few days when the cells are cultured. The positively selected cells are virtually unaffected by MACS separation and can be used immediately for culturing or further downstream applications. Typically, MACS MicroBeads do not activate cells or influence function or viability. They have been widely used for the isolation of $CD34^+$ and $CD133^+$ cells from different sources (Table 1) on both small and clinical scales, and recently have received European Community approval for clinical use in Europe (10). Positive selection takes advantage of the high specificity of monoclonal antibodies to isolate highly pure cells that express the corresponding antigen. However, the purity and recovery in MACS typically have large variances (60). For instance, a purity of less than 50% for $CD34^+$ cells isolated using the Direct $CD34^+$ Progenitor Cell Isolation Kit was reported by Kekarainen et al. (61).

The authors suggested an optimized scheme for the separation consisting of a two-column method and an additional labeling step, which in fact means the performance of the same purification procedure twice, implying increased time and costs. In

another $CD34^+$ cell selection procedure based on the use of Isolex magnetic beads and a fully automated device, Isolex300I, a combination of two methods, simultaneous positive selection of CD34 expressing cells and T cell depletion, resulted in 97% purity of the final HSC preparation (62).

For some experiments it may be desirable to deplet certain cell types from the cell sample in order to isolate the target cells. Depletion allows, for example, the isolation of a target cell for which no specific antibody is available, or the isolation of "untouched" cells. In a depletion strategy all unwanted cells are magnetically labeled. During magnetic separation, the labeled cells are retained in the column, while the target cells pass through into the negative, non-magnetic fraction.

If the isolation of extremely rare cells is required, it can be useful first to deplete unwanted cells from the suspension. Positive selection can then be done on the pre-enriched fraction to obtain very pure cells. This strategy is also useful if unwanted cells in the cell suspension express the same antigen required for the positive selection of the target cells.

Sometimes a combination of both methods, magnetic cell separation and FACS, is applied in order to achieve the desired level of purity in the target cells. For instance, in order to obtain highly purified HSC for highdose therapy in cancer patients, $CD34^+$ cell enrichment was carried out using Isolex immunomagnetic $CD34^+$ positive selection followed by sorting using SyStemix's fluorescence-activated high-speed clinical cell sorter, resulting in a median purity of the stem cell productof 95.3% (63). However, as mentioned earlier, despite its capability of generating pure populations of cells, FACS is not suitable when isolation of large numbers of rare cells is required.

Finally, it is also worth mentioning here that both separation procedures modify the cell membrane, thus neither technique is preferable for subsequent analysis or re-cultivation of the sorted cells (64).

Table 1: Some examples of separation technologies used for isolation of $CD34^+$ and $CD133^+$ cells

Technology	Product and Company	Antigen	Cell Source	Yield[a] %	Purity[a] %	Refs.
Immunoselection by magnetic beads	Dynabeads, Invitrogen Dynal AS	CD34	PB BM CB	5 <	30 <	(60)
	Isolex, Nexell	CD34	BM	40	93	
			PB	53	90	(65 ,55)
		CD34[b]	PB	76	97	(14)
	Isolex50,	CD34	BM	70	-	
	Baxter Health-	CD34	PB	44	92	
	care	CD34	CB	76	-	
Immunoselection by submicro- scopic colloid magnetic beads	MACS, Miltenyi Biotec	CD34	CB	52	60 41	(66, 67)
		CD34	PB	71	97	(68, 69)
				77	98	(65)
				56	97	(61)
		CD133	PB		<50	
				69	94	(70)
		CD133	CB	81	93	(69)
		CD133	BM	96	82	(10)
				23	82	(53)
High-speed fluorescence-activated cell sorting	High-speed cell sorter, SyStemix	CD34 Thy1	PB	60	88	(71)
Immuno-adsorption columns	Ceprate SC, CellPro	CD34	PB	50	-	(72)
				53	62	(73)
				35	72	(51)
		CD34	BM			
				46	64	(74)
Antibody panning selection	CD34 Cellector flask, AIS	CD34	BM	74	60	(54)
				15	33	(60)

BM bone marrow, PB; peripheral blood, CB; cord blood
[a] Median values, if not indicated otherwise
[b] Simultaneous +/– selection of CD34+ cell selection and T cell depletion

Conclusion

Selective cell separation is an essential component of many processes in biology and medicine. In the majority of cases, the aim is to achieve complete removal of the target population from a cell mixture; however, additional considerations can have a major impact on the method that is chosen to effect the separation. For some applications, a secondary aim may be to recover the selected cells for study for additional manipulation. In other cases, the purity of separation may take precedence over the yield of selected cells, or vice versa. Factors such as processing time, viability of residual cells, and exposure to nonmedically approved reagents also must be considered when selecting the separation technique.

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