Establishing a Bone Marrow Stromal Cell Transplant Program at the National Institutes of Health Clinical Center

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A repository of cryopreserved bone marrow stromal cell (BMSC) products prepared from marrow aspirates of healthy subjects has been created and is being used to treat patients with inflammatory bowel disease, cardiovascular disease, and acute graft-versus-host disease following allogeneic hematopoietic stem cell transplantation. New methods of manufacturing BMSCs are being investigated including the use of an automated bioreactor for BMSC expansion and the replacement of fetal bovine serum with human platelet lysate as a media supplement. Efforts are also being made to identify markers that can be used to assess the potency of BMSCs.

Introduction

BONE MARROW STROMAL CELLS (BMSCs), which are also known as mesenchymal stromal cells or mesenchymal stem cells, are a heterogeneous population of cells that reside in the bone marrow and have a number of potential clinical applications. Small numbers of BMSCs can be found among cells aspirated from the bone marrow, but not enough for clinical therapy. BMSCs can, however, be isolated from other cells found in the marrow by plastic adherence and can be expanded many fold in culture.

The clinical applications of BMSCs are very diverse. Multipotential skeletal stem cells found among BMSCs are capable of producing bone, cartilage, hematopoiesis-supporting stroma, and adipocytes. When BMSCs are attached to an appropriate scaffold, they form bone and hematopoietic-supporting stroma *in vivo*. They have been shown in preclinical models to be effective for repairing bone defects^{1–5} and autologous BMSCs have been used to treat bone defects in humans.^{6–8} BMSCs also have immune modulatory properties and can induce angiogenesis and tissue repair. BMSCs are being used to treat a number of immune-mediated diseases. For example, they are being tested in early phase clinical trials to treat steroid-resistant acute graft-versus-host disease (GVHD) following allogeneic bone marrow transplant. They are also being used to treat autoimmune conditions such as multiple sclerosis, systemic lupus erythematosus, and inflammatory bowel disease.^{9,10} Preclinical studies have shown that BMSCs induce increased vascularity in ischemic myocardium¹¹ and they are

being tested in patients with left ventricular failure due to ischemic vascular disease.¹²

In 2009 the National Institutes of Health (NIH) intramural program provided resources to establish a Bone Marrow Stromal Cell Transplant Program (BMSC TC). The goal of this program was to provide biologically active autologous and third party donor BMSCs to treat patients enrolled in intramural protocols, and to provide support in the preparation of the required regulatory documents for Institutional Review Boards (IRBs) and the Food and Drug Administration (FDA). The BMSC TC is a collaborative effort among laboratory investigators, clinical investigators, and the Cell Processing Section, Department of Transfusion Medicine (DTM) in the NIH Intramural Campus (Bethesda, MD). The program is led by Pamela G. Robey, PhD, Craniofacial and Skeletal Disease Branch, National Institute of Dental and Craniofacial Research (NIDCR) and Harvey G. Klein, MD, DTM, Clinical Center (CC).

One of the first tasks for the program was to identify the methods that would be used to manufacture BMSCs. Currently, stromal cells are being made from several different sources and by many different methods. We selected a manufacturing method based on the clinical applications that we expected would be needed by NIH intramural program clinical investigators. The method implemented must, of course, result in a final BMSC product that was effective for the expected clinical applications. Intramural program investigators were interested in using BMSCs for bone repair and to treat acute GVHD, inflammatory bowel disease, and left ventricular heart failure due to coronary artery

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disease. Mankani *et al.* have shown that stromal cells can be isolated from bone biopsies or marrow aspirates and expanded in flasks using media supplemented with fetal bovine serum (FBS); early passage stromal cells contain skeletal stem cells and form bone upon *in vivo* implantation in preclinical studies.^{2,3,13} Other investigators have shown that early passage BMSCs isolated from marrow aspirates and cultured in FBS-containing media were promising in treating steroid-refractory acute GVHD.^{14,15} Since the mechanism of actions of BMSCs for the treatment of acute GVHD is not well understood, validated potency assays have yet to be identified for this application. Because the culture of BMSCs in plastic flasks and with FBS has yielded cells that effectively treat steroid refractory GVHD^{14–16} and form bone in preclinical and early phase clinical trials, we elected to establish a BMSC production method that closely resembled the above methods: that is we produced BMSCs from aspirated marrow by culture on plastic surfaces using media supplemented with 20% FBS.

Manufacturing BMSCs

We first developed a repository of cryopreserved BMSCs prepared from healthy subjects or third party donors.¹⁷ Currently, we are using BMSCs from this repository to treat patients with acute GVHD, marrow failure, and tissue injury after hematopoietic stem cell transplantation and inflammatory bowel disease. This repository is needed because subjects with acute GVHD or inflammatory bowel disease require treatment prior to the ~4 weeks required to isolate and expand BMSCs from a marrow aspirate. The use of third party donor BMSC products is likely to be effective since preliminary clinical studies suggest that for the treatment of steroid refractory acute GVHD, BMSCs do not need to persist *in vivo* long term and no matching of histocompatibility antigens between the BMSC donor and infusion recipient is required. In addition, cryopreserved BMSCs appear to be as effective as fresh BMSCs for this and many other applications.

The methods used to culture BMSC for the third party donor repository] were described by Sabatino *et al.*¹⁷ Marrow was aspirated from the posterior iliac crest of healthy subjects under an IRB approved protocol. A single cell suspension of marrow was prepared; without red blood cell lysis or density gradient separation. The marrow was suspended in alpha-minimum essential media supplemented with 20% lot-selected U.S. origin defined FBS and cultured in T-75 flasks (75 cm² flask, canted neck, nonpyrogenic, sterile polystyrene; Corning, Inc., Corning, NY). After 24 h the nonadherent cells were removed. When the BMSC colonies become >70% confluent, they were harvested and re-plated into 2-layer multiple level flasks (Cell Factory, easy fill 2-trays; Nunc A/S, Roskilde, Denmark) to reduce the number of containers required for cell growth. The use of cell factories also reduced the labor involved with BMSC culture and reduced the chance of microbial contamination. The BMSCs harvested from the cell factories were re-plated twice more in 10-layer multiple level flasks (Cell Factory, easy fill 10-trays; Nunc A/S). After the final harvest the BMSCs were washed, cryopreserved in “units” of 100×10⁶ cells, and stored in the vapor phase of a liquid nitrogen freezer. The manufacturing methods have been reported to

the FDA in a drug master file. BMSC units are thawed and used as needed and administered to patients intravenously. Patients receiving BMSC products from the repository must be enrolled in an NIH Intramural Program IRB-approved treatment protocol.

Several commercial groups are manufacturing BMSCs and related products from third party donors and are testing them in clinical trials involving immune modulation and tissue repair. The BMSCs produced by the NIH and other academic health centers are typically expanded over three or four passages and are relatively early in their replicative lifespan.^{14,18} Commercial laboratories produce BMSCs over five or more passages.^{19,20} They typically produce a master cell bank by isolating and expanding BMSCs over two to three passages. The BMSCs from aliquots from the master cell bank are then expanded over several more passages. As a result, commercial BMSC products have undergone many more population doublings and are likely much closer to senescence. The difference in the degree of expansion of BMSC products produced at the NIH and commercial laboratories, likely results in differences in function. Several groups have found that prolonged culture of BMSCs is associated with changes in morphology, reduced proliferation ability, and loss of the ability to differentiate into bone, cartilage, and adipose tissue.^{21–26} The manufacture of third party donor BMSCs at the NIH and other academic centers allows early passage BMSCs to be tested in clinical trials.

The NIH BMSC TC is also collecting autologous marrow to produce BMSCs to treat heart failure due to coronary artery disease. Marrow is aspirated from the posterior iliac crests of these patients and cultured in the same way as the third party donor BMSCs except that the cells undergo one less passage and as a result they spend ~21 days in culture rather than 28 days. More importantly, these cells are not cryopreserved. Rather, immediately after the final harvest the BMSCs are given by intracardiac injection at the time of cardiac revascularization surgery. Although third party donor BMSCs are being commercially manufactured for early phase clinical trials autologous BMSCs are not. The small lot sizes of BMSCs used for autologous therapy and logistical difficulties associated with personalized therapies make the production of autologous BMSCs more expensive and more difficult to commercialize.

The BMSC manufacturing methods being used at the BMSC TC were validated using several assays. The final BMSC product was shown to contain skeletal stem cells by demonstrating that they form bone and support host hematopoiesis in an immune-deficient mouse transplant model.²⁷ We also showed that the BMSC express CD73, CD90, CD105, and CD146 but not CD34, CD45, CD14, and CD11b; they inhibited mixed lymphocyte reactions and produce the cytokines IL-6, IL-10, CXCL12, basic fibroblast growth factor, hepatocyte growth factor, platelet-derived growth factor bb, vascular endothelial growth factor, transforming growth factor beta 1 (TGFB1), and TGFB2.

Improving the BMSC Manufacturing Process

While the manufacturing process developed at the BMSC TC is reliable and the preliminary results of clinical trials have been promising, we are investigating methods to improve it. We have completed a preliminary analysis of

BMSCs cultured in an automated hollow fiber cell culture system, the Quantum Cell Expansion System (Terumo BCT, Lakewood, CO). This system has the advantage of requiring less labor and reducing the risk of microbial contamination since it is a closed system. The cells are cultured in a closed hollow fiber bioreactor system and cell loading, feeding, and harvesting processes are automated. Consequently, these processes require less time with the bioreactor than with the conventional T-flasks and cell factory culture system. Since the Quantum bioreactor is a closed system, microbial contamination is much less likely than with open T-flasks and cell factories. In addition, manufacturing cells in flasks and cell factories requires ISO 7 tissue culture rooms and biosafety cabinets to minimize the risk of microbial contamination, but this is not required for the closed bioreactor system. The disadvantage of the bioreactor is the need to invest capital to purchase the reactors and the relatively high cost of the disposable cartridges used in the bioreactors. An initial comparison using BMSCs grown in T-flasks and cell factories with those grown in the bioreactor using global gene expression analysis and an *in vivo* transplant model found that the BMSCs expanded in the bioreactor are similar to those expanded using T-flasks and cell factories, but further validation is needed.

A goal of our program is to develop xeno-free reagents for manufacturing BMSCs. We have been investigating the use of human platelet lysate as a BMSC culture media supplement that could be used in place of FBS. While BMSCs grown in FBS are effective in preclinical studies and in early phase clinical trials, FBS use is associated with several potential risks to the recipient of the cells. For instance, FBS could carry a pathogen that is transmitted with the BMSCs to the recipient. In addition, some recipients of cellular therapies manufactured with FBS have developed hypersensitivity reactions to FBS.²⁸ Lysed platelets are being used by some centers in place of FBS to manufacture BMSCs.^{29,30} Many groups have shown that platelet lysate supports the growth and expansion of BMSCs and that the BMSC grown in platelet lysate express the BMSC markers CD73, CD90, and CD105. However, BMSCs grown in platelet lysate have not been shown to be comparable to those grown in FBS for all clinical applications. The platelet lysate used by many cell processing laboratories has been manufactured by freezing and thawing²⁹ or sonicating³⁰ out-dated platelets acquired from blood centers. We elected to evaluate a commercially prepared platelet-derived media supplement, human platelet growth factor C18, GwoWei Technology Co. Ltd. (Taipei, Taiwan). Each lot of the product that we have been evaluating is made from ~40 apheresis platelet components that have been pooled. This is a larger pool of platelets than would be practical for us to prepare if we made platelet lysate ourselves from out-dated platelets. This larger pool of platelets will better compensate for biological inter-donor variability among the platelet products and result in a more uniform final product. In addition, the product that we are testing has been solvent detergent treated, which inactivates encapsulated viruses and bacteria, and as a result, reduces the risk of transmitting a pathogen with the media supplement.³¹

Potency Testing of BMSCs

To take any cellular therapy from a phase I or II clinical trial to a phase III clinical trial or licensure, it is inevitable

that some changes in the manufacturing process will be necessary. Larger lots or an increased rate of production of lots is generally required for phase III trials and licensed products since more patients will be treated. This increase in production is generally associated with changes in the manufacturing process. When manufacturing processes are changed, it is important to compare the products using the original and the new method to ensure that the critical biological properties or potency of the cell therapy is not changed by the change in the manufacturing processing. Identifying potency assays for assessing the critical biological functions of cell therapies is difficult.³² Generally, the potency of cell therapies is dependent on multiple functions and in many cases the properties of cell therapies that are responsible for the potency are not fully understood. This is the case for BMSCs. The *in vivo* transplant model developed by Kuznetsov and Robey is an excellent potency marker of skeletal stem cells and the ability for BMSC to form bone *in vivo*.²⁷ However, the use of animal models for testing the potency of cell therapies is not practical since they are expensive assays and they generally require months to complete. Consequently, it is important to develop analytic assays that can be used for potency testing since they can be completed quickly and are less expensive than assays involving animals. Some groups are using the expression on single factor such as prostaglandin E2³³ and galectin-3³⁴ to assess the immunomodulatory properties of BMSCs or a combination of factors to assess the osteogenic potential of BMSCs,³⁵ but these measures are not likely to be useful for assessing the potency of BMSCs for other clinical applications.

We have been looking for analytical assays that could be used to assess the potency of BMSCs for multiple applications. We have found that global gene expression analysis is an extremely useful tool for comparability testing of other cellular therapies. Using global gene expression analysis we have been able to show that there was no difference in dendritic cells made from fresh peripheral blood monocytes or from monocytes that had been stored for 48 h.³⁶ We have also used global gene expression analysis to show that there are important differences between BMSCs and stromal cells generated from different types of starting material.³⁷ We are now looking for sets of genes whose expression on the mRNA or protein levels are associated with the potency of BMSCs. Several studies have shown that BMSCs undergo considerable changes in phenotype and function as they are expanded serially until they reach senescence. We hypothesized that BMSC markers associated with senescence would be good markers of BMSC potency for many applications. We have initiated studies to identify markers associated with BMSC senescence. We have studied serial passages of BMSCs obtained from marrow aspirates from seven healthy subjects that had been expanded until the BMSCs became senescent. We confirmed that BMSC function changes as they become senescent as does their phenotype.²¹ However, the phenotype changes were not of great enough magnitude or reproducibility to be useful as potency markers. The data obtained from global gene expression analysis of the serial passages were analyzed using a computational biology approach to identify the minimum set of genes that can be used to predict or measure the replicative age of BMSCs in culture. Using a least angle

TABLE 1. COEFFICIENTS AND GENES WHOSE EXPRESSION CAN BE USED TO PREDICT THE REPLICATIVE AGE OF EXPANDED BMSCs

	<i>UG cluster</i>	<i>Symbol</i>	<i>Coefficient</i>
1	Hs.559718	<i>AK5</i>	0.007
2	Hs.439145	<i>SCN9A</i>	0.007
3	Hs.535845	<i>RUNX2</i>	-0.003
4	Hs.124638	<i>TMEM90B</i>	-0.011
5	Hs.656071	<i>ADAMTS9</i>	-0.001
6	Hs.269764	<i>BACH2</i>	-0.014
7	Hs.646614	<i>KLF8</i>	-0.001
8	Hs.244940	<i>RDH10</i>	0.038
9	Hs.483238	<i>ARHGAP29</i>	-0.026
10	Hs.1407	<i>EDN2</i>	0.008
11	Hs.131933	<i>PLBD1</i>	-0.068
12	Hs.369265	<i>IRAK3</i>	-0.009
13	Hs.148741	<i>RNF144B</i>	0.005
14	Hs.525093	<i>NDFIP2</i>	0.003
15	Hs.124299	<i>FAM167A</i>	0.009
16	Hs.183114	<i>ARHGAP28</i>	0.002
17	Hs.596680	<i>EYA4</i>	-0.005
18	Hs.652230	<i>TM7SF4</i>	-0.001
19	Hs.89640	<i>TEK</i>	0.032
20	Hs.377894	<i>GCA</i>	-0.017
21	Hs.439145	<i>SCN9A</i>	0.02
22	Hs.681802	<i>FLJ00254</i> protein	0.019
23	Hs.492974	<i>WISP1</i>	-0.001
24	Hs.122055	<i>C7orf31</i>	-0.006

Adapted from Ren *et al.*²¹

regression algorithm we identified a set of 24 genes that predicts the elapsed age as a percentage of maximum lifespan of BMSCs (Table 1).²¹ The predicted age of BMSCs can be calculated using the expression of these 24 genes and the formula: $\sum c_i x_i + 0.143$ where c_i and x_i are the coeffi-

TABLE 2. COMPARISON OF THE QUALITY AS ASSESSED BY LOT RELEASE CRITERIA AND CALCULATED PERCENTAGE OF MAXIMUM LIFESPAN OF CLINICAL BMSC PRODUCTS

<i>Donor</i>	<i>Age (years)</i>	<i>Calculated percentage of maximum lifespan (%)</i>	<i>Met lot release criteria</i>
BMSC-W1002	22.9	41.47	Yes
BMSC-W1003	22.8	35.97	Yes
BMSC-W1005	21.7	33.59	Yes
BMSC-W1006	23.7	42.69	Yes
BMSC-W1007	27.2	25.40	Yes
BMSC-W1105	27.6	30.21	Yes
BMSC-W1106	22.7	35.74	Yes
BMSC-W1108	42.5	35.83	Yes
BMSC-W1004	67.9	58.56	No ^a
BMSC-W1101	56.8	47.65	Yes
BMSC-W1103	21.3	45.40	No ^b
BMSC-W1104	59.4	52.68	No ^a

The percentage of maximum lifespan was calculated based on the prediction model described in the text and the genes in Table 1. Adapted from Ren *et al.*²¹

^aPoor proliferation during primary culture.

^bHigh CD34⁺ percentage.

BMSC, bone marrow stromal cell.

cient and gene expression for the i -th gene, respectively. The gene expression values used in the formula are the (normalized) log ratios for dual-channel data. In preliminary validation studies we have found that clinical BMSC products manufactured at the BMSC TC that did not meet lot release criteria had a greater replicative age calculated with the 24 gene set (Table 2). The results indicate that this gene set maybe useful as a potency marker for clinical BMSCs.

We have saved aliquots from each BMSC lot that we manufacture and will assess them in a variety of assays including measuring the expression of the 24 replicative age predictive genes and the cytokines produced by each lot of BMSCs. We will use computational biology approaches to compare the level of cytokines from each BMSC lot with the product's clinical effectiveness. The expression of a good potency marker should be associated with clinical outcomes. We will be using BMSCs to treat more than one clinical application and maybe a different set of biomarkers will be useful for measuring the potency of BMSCs used for different applications.

Conclusions

A repository of BMSCs produced from marrow aspirates of healthy subjects has been developed and is being used to treat patients with complications related to allogeneic hematopoietic stem cell transplantation and with inflammatory bowel disease. We have also developed methods to manufacture autologous BMSCs to treat patients with left ventricular failure by intracardiac injection. We are investigating methods to automate the production of BMSCs and to replace FBS as a media supplement. We are also working to identify markers that can be used to assess the potency of BMSCs.

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Disclosure Statement

No competing financial interests exist.

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