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(54) Title: METHODS FOR OBTAINING COMPOSITIONS ENRICHED FOR HEMATOPOIETIC STEM CELLS AND ANTIBODIES FOR USE THEREIN

(57) Abstract

A method for obtaining human hematopoietic stem cells is provided by enrichment for stem cells using a novel stem cell marker. Compositions obtained thereby and reagents for use therein are also provided.

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Methods for obtaining compositions enriched for hematopoietic stem cells and antibodies for use therein

The field of this invention is the isolation of a population of cells enriched for human hematopoietic stem cells.

Mammalian hematopoietic cells provide a diverse range of physiological activities. These cells are divided into lymphoid, myeloid and erythroid lineages. The lymphoid lineage, comprising B cells and T cells, provides for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes as well as other cells, monitors for the presence of foreign bodies, provides protection against neoplastic cells, scavenges foreign materials, produces platelets, and the like. The erythroid lineage provides the red blood cells, which act as oxygen carriers.

Despite the diversity of the nature, morphology, characteristics and function of hematopoietic cells, it is presently believed that these cells are derived from a single precursor cell population, termed "stem cells." Stem cells are capable of self-regeneration and can become lineage committed progenitors which are dedicated to differentiation and expansion into a specific lineage. As used herein, "stem cells" refers to hematopoietic cells and not stem cells of other cell types.

A pluripotent stem cell can be defined as follows: (1) gives rise to progeny in all defined hematolymphoid lineages; and (2) limiting numbers of cells are capable of

fully reconstituting a seriously immunocompromised host in all blood cell types and their progenitors, including the pluripotent hematopoietic stem cell, by self-renewal.

A highly purified population of stem cells is necessary for a variety of *in vitro* experiments and *in vivo* indications. For instance, a purified population of stem cells will allow for identification of growth factors associated with their self-regeneration. In addition, there can be as yet undiscovered growth factors associated with: (1) the early steps of dedication of the stem cell to a particular lineage; (2) the prevention of such dedication; and (3) the negative control of stem cell proliferation.

Stem cells find use in: (1) regenerating the hematopoietic system of a host deficient in any class of hematopoietic cells; (2) a host that is diseased and can be treated by removal of bone marrow, isolation of stem cells and treatment with drugs or irradiation prior to re-engraftment of stem cells; (3) producing various hematopoietic cells; (4) detecting and evaluating growth factors relevant to stem cell self-regeneration; and (5) the development of hematopoietic cell lineages and assaying for factors associated with hematopoietic development.

Stem cells are important targets for gene therapy, where the inserted genes promote the health of the individual into whom the stem cells are transplanted. In addition, the ability to isolate stem cells can serve in the treatment of lymphomas and leukemias, as well as other neoplastic conditions where the stem cells are purified from tumor cells in the bone marrow or peripheral blood, and reinfused into a patient after myelosuppressive or myeloablative chemotherapy. Thus, there have been world-wide efforts toward isolating stem cells in substantially pure or pure form.

Stem cells constitute only a small percentage of the total number of hematopoietic cells. Hematopoietic cells are

identifiable by the presence of a variety of cell surface "markers." Such markers can be either specific to a particular lineage or progenitor cell or be present on more than one cell type. Currently, it is not known how many of the markers associated with differentiated cells are also present on stem cells. One marker, which was previously indicated as present solely on stem cells, CD34, is also found on a significant number of lineage committed progenitors. U.S. Pat. No. 4,714,680 describes a population of cells expressing the CD34 marker.

In view of the small proportion of the total number of cells in the bone marrow or peripheral blood which are stem cells, the uncertainty of the markers associated with the stem cell as distinct from more differentiated cells, and the general difficulty in assaying for stem cells biologically, the identification and purification of stem cells has been elusive. Characterizations and isolation of stem cells are reported in: Baum et al. (1992) Proc. Natl. Acad. Sci. USA 89:2804-2808; and Tsukamoto et al. U.S. Patent No. 5,061,620.

Decreased rhodamine 123 (rho123) staining of hematopoietic cells appears to correlate to stem cell potential. This so-called "rho^{lo}" marker is determined not by the initial dye accumulation but by an efflux process sensitive to P-glycoprotein (P-gp) inhibitors. Retention of several P-gp-transported fluorescent dyes, including rho123, in human bone marrow cells was inversely correlated with the expression of P-gp. Bone marrow cells expressing physical and antigenic characteristics of pluripotent stem cells show high levels of P-gp expression and fluorescent dye efflux. Fractions of human bone marrow cells isolated on the basis of either increased rho123 efflux or P-gp expression contain practically all the primitive progenitor cells of human bone marrow, including long-term culture-initiating cells (LTC-IC). Chaudhary and Roninson (1991) Cell 66:85-94.

Recently, the mouse stem cell has been obtained in at

least highly concentrated, if not purified form, where fewer than about 30 cells obtained from bone marrow were able to reconstitute all of the lineages of the hematopoietic system of a lethally irradiated mouse. Each assayed cell is multipotent for all hematopoietic lineages, while self-renewal is variable amongst these cells. Spangrude et al. (1988) Science 241:58-62; Smith et al. (1991) Proc. Natl. Acad. Sci. USA 88:2788-2792; Uchida (1992) Ph.D. Thesis Stanford U.; and see also, EPA 89 304651.6 and the references cited therein which describe the isolation of mouse stem cells.

Methods are provided to obtain compositions enriched for hematopoietic stem cells. The methods employ a separation regimen utilizing antibodies (αEM10) specific for a unique cell surface marker (EM10) that is expressed on stem and progenitor cells ($EM10^+$ cells), while being less accessible or absent on more mature cells.

Positive selection of stem cells with antibodies that recognize EM10 can be used in combination with selection for cells expressing other stem cell markers and/or negative selection with lineage-specific (LIN^-) markers. Enriched populations of cells derived from these methods are also provided.

A hybridoma producing antibody to EM10 (SM27-1045) was deposited with the ATCC on June 2, 1995, in accordance with the Budapest Treaty, and has been assigned accession number HB 11917.

It has now been found that the cell surface marker ("EM10") recognized by antibodies specific for EM10 ("αEM10") is expressed on hematopoietic stem cells. αEM10 subdivide the $CD34^+$ cell population into approximately equal subpopulations, with the stem cell activity found predominantly in the $EM10^+$ subpopulation. As described in detail in the examples section herein, αEM10 has the following characteristics. αEM10 binds approximately 60% of $CD34^+$ cells and 1-2% of mononuclear cells

on average. αEM10 binds to a substantial subset of $CD34^+CD38^-$ cells. αEM10 binds a substantial subset of all $CD34^+Thy\text{-}1^+$ (both $rho123^{lo}$ and $rho132^{hi}$ subsets). αEM10 binds significant numbers of $CD34^+Thy\text{-}1^-$ cells. αEM10 does not bind significant numbers of $CD34^-$ cells. Exemplary of an αEM10 antibody is the monoclonal antibody SM27-1045 produced by the deposited hybridoma and further described in the examples herein.

Thus, αEM10 can be used in place of or in conjunction with antibodies to other stem cell markers e.g. CD34, to purify the majority of progenitor cells and pluripotent stem cells from heterogeneous populations of hematopoietic cells. αEM10 binds to a larger subset of $CD34^+$ cells than do mAbs to Thy-1. As described more fully in the examples section, EM10 defines a population of cells which is enriched in CFU-GM, in addition to the CAFC activity, which provides a desirable bone marrow graft composition. Further, based on SCID-hu thymus data, the $EM5^+CD34^+$ cells may include T cell progenitor activity.

The present invention thus encompasses methods of obtaining a composition substantially enriched in stem cells. The methods involve combining a mixture of hematopoietic cells with an antibody that recognizes and binds to EM10 under conditions which allow the antibody to bind to EM10 and separating the cells recognized by the antibody to obtain a population substantially enriched in $EM10^+$ cells. The methods can be used as a diagnostic assay for the number of stem cells in a sample of hematopoietic cells. The cells and antibody are combined under conditions sufficient to allow specific binding of the antibody to EM10 and the $EM10^+$ cells are then quantitated. The $EM10^+$ cells can be isolated or further purified.

The results presented herein show that the $EM10^+$ population includes the $CD34^+Thy\text{-}1^+$ population of cells which have previously been shown to be primitive pluripotential hematopoietic progenitors. Thus, EM10 is expressed on the subset of $CD34^+$ cells which contains virtually all primitive

hematopoietic stem cells and αEM10 enables the purification of this subset of hematopoietic stem cells.

The methods can include further enrichment steps for cells by positive selection for other stem cell specific markers. Suitable positive stem cell markers include, but are not limited to, $CD34^+$, $Thy\text{-}1^+$, $c\text{-}kit^+$, to obtain cellular compositions consisting essentially of cells which are, for example, positive for ED10/CD34 or ED10/Thy-1 or ED10/CD34/Thy-1. Preferably the $EM10^+$ cells are human but can be derived from any suitable animal. By appropriate selection with particular factors and the development of bioassays which allow for self-regeneration of stem cells and screening of the stem cells as to their markers, a composition enriched for viable stem cells can be produced for a variety of purposes.

Preferably, the cells are subject to negative selection against those cells that express one or more lineage specific markers, to remove those cells whose expression of such markers evidences committment to a specific lineage and retain those cells which are substantially negative in expression of such markers ("LIN^-"). Methods of negative selection are known in the art. LIN^- cells generally refer to cells which do not have significant expression of markers associated with T cells (such as CD2, 3, 4 and 8), B cells (such as CD10, 19 and 20), myeloid cells (such as CD14, 15, 16 and 33), natural killer ("NK") cells (such as CD2, 16 and 56), RBC (such as glycophorin A), megakaryocytes (CD41), mast cells, eosinophils or basophils. The absence or low expression of such lineage specific markers is identified by the lack of binding of antibodies specific to the cell specific markers, useful in so-called "negative selection". Preferably the lineage specific markers include, but are not limited to, at least one of CD2, CD14, CD15, CD16, CD19, CD20, CD38, HLA-DR and CD71; more preferably, at least CD14 and CD15. Foir example, a highly enriched composition can be obtained by selective isolation to obtain a composition consisting essentially of cells that are $CD34^+EM10^+LIN^-$.

Table 1 summarizes probable phenotypes of stem cells in fetal, adult, and mobilized peripheral blood. In Table 1, myelomonocytic stands for myelomonocytic associated markers, NK stands for natural killer cells, FBM stands for fetal bone marrow, ABM stands for adult bone marrow, and AMPB stands for adult mobilized peripheral blood. As used herein, both infra, supra and in Table 1, the negative sign or superscript negative sign ($^-$) means that the level of the specified marker is undetectable above Ig isotype controls by fluorescent activated cell sorting (FACS) analysis, and includes cells with very low expression of the specified marker.

Table 1

Probable Stem Cell Phenotypes

	NK and T cell markers			B cell markers			Myelomonocytic			Other						P-gp Activity
	CD2	CD3	CD8	CD10	CD19	CD20	CD14	CD15	CD16	CD33	CD34	CD38	HLA-DR	C-Kit	Thy	Rho	
FBM	-	-	-	-	-	-	-	-	-	?	+	-	+	+	+	lo	+
ABM	-	-	-	-	-	-	-	-	-	-	+	-	lo/-	+	+	lo	+
AMPB	-	-	-	-	-	-	-	-	-	lo/-?	+	?	lo/-	?	+	lo	+

EM10$^+$ stem cells can be isolated from any known source of stem cells, including, but not limited to, bone marrow, both adult and fetal, mobilized peripheral blood (MPB) and umbilical cord blood. The use of umbilical cord blood is discussed, for instance, in Issaragrishi et al. (1995) N. Engl. J. Med. 332:367-369. Initially, bone marrow cells can be obtained from a source of bone marrow, including but not limited to, ilium (e.g. from the hip bone via the iliac crest), tibia, femora, spine, or other bone cavities. Other sources of stem cells include, but are not limited to, embryonic yolk sac, fetal liver, and fetal spleen.

For isolation of bone marrow, an appropriate solution can be used to flush the bone, including, but not limited to, salt solution, conveniently supplemented with fetal calf serum (FCS) or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from about 5-25 mM. Convenient buffers include, but are not limited to, HEPES, phosphate buffers and lactate buffers. Otherwise bone marrow can be aspirated from the bone in accordance with conventional techniques.

Various techniques can be employed to separate the cells by initially removing cells of dedicated lineage. Monoclonal antibodies are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation. The antibodies can be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the retention of viability of the fraction to be collected. Various techniques of different efficacy can be employed to obtain "relatively crude" separations. Such separations are where up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present not having the marker can remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Procedures for separation can include, but are not limited to, magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, e.g., plate, elutriation or any other convenient technique. Separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rho123 and DNA-binding dye Hoechst 33342).

Techniques providing accurate separation include, but are not limited to, FACS, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc.

In a first separation, typically starting with about 1 x 10^{8-9}, preferably at about 5 x 10^{8-9} cells, αEM10 can be labeled with one fluorochrome, while the antibodies for the various dedicated lineages, can be conjugated to at least one different fluorochrome. While each of the lineages can be separated in a separate step, desirably the lineages are separated at the same time as one is positively selecting for EM10 and/or other stem cell markers. The cells can be selected against dead cells, by employing dyes associated with dead cells (including but not limited to, propidium iodide (PI)). Preferably, the cells are collected in a medium comprising 2% FCS.

The purified stem cells have low side scatter and low to medium forward scatter profiles by FACS analysis. Cytospin preparations show the enriched stem cells to have a size between mature lymphoid cells and mature granulocytes. Cells can be selected based on light-scatter properties as well as their expression of various cell surface antigens.

While it is believed that the particular order of separation is not critical to this invention, the order indicated is preferred. Preferably, cells are initially separated by a coarse separation, followed by a fine separation, with positive selection with αEM10.

The foregoing separations result in cellular compositions consisting essentially of the cells desired. A "cellular composition consisting essentially of" a particular population of cells is understood to indicate a population of cells in a suitable medium which is substantially homogeneous with respect to the indicated marker, e.g., at least 80% pure, usually at least 90%, preferably at least 95% pure. In particular, compositions wherein the cellular component has at least 80%, usually at least 90%, preferably at least 95% of $EM10^+$ cells can be achieved in this manner. The desired stem cells can be further enriched by selection for one or more LIN^- markers and/or Thy-1^+ and/or rho^{lo}, or combinations of these markers as listed in Table 2, and being able to provide for cell regeneration and development of members of all of the various hematopoietic lineages. Note that the blank spaces in Table 2 do not mean that the cells are negative for the specified marker; they simply mean the marker is not used.

Table 2
Possible Combinations of Selections
for Stem Cell Populations

$EM10^+$	$CD34^+$	Thy^+	LIN^-	rho^{lo}
+	+	+	+	+
+	+	+	+	
+	+	+		
+	+			
+	+			+
+		+		+
+		+	+	+
+			+	+
+			+	
	+	+	+	
	+	+	+	+
		+	+	+
	+	+		+
		+		+
		+	+	

By separating $CD34^+EM10^+$ cells from human hematopoietic sources, the long-term culture activity is enriched in the $EM10^+$ fraction compared to $EM10^-$. Moreover, the $EM10^+$ cells will generate both B and myeloid cells in long-term cultures. In further enrichments of the $EM10^+$ cells using antibodies to Thy-1 and/or any of the combinations specified in Table 2 and/or c-kit, the stem cell frequency can be further increased.

The cells obtained as described above can be used immediately or frozen at liquid nitrogen temperatures and stored for long periods of time, being thawed and capable of being reused. The cells will usually be stored in 10% DMSO, 50% fetal calf serum (FCS), 40% RPMI 1640 medium. Once thawed, the cells can be expanded by use of growth factors and/or stromal cells associated with stem cell proliferation and differentiation.

In another embodiment of the invention, a composition highly enriched in stem cells is provided. The results presented herein indicate that antibodies to EM10 recognize and bind with high specificity to a cell surface antigen found on human hematopoietic cells, and exposed to a high degree on stem cells. This specificity can be used to isolate and purify stem cells. Such a composition has utility in reconstituting hematopoietic systems and in studying various parameters of hematopoietic cells.

The compositions enriched for stem cells can be used in autologous hematopoietic engraftment, where the cells can be freed of neoplastic cells. Further, the use of autologous stem cells will avoid graft-versus-host disease. In addition, the cells can be modified by appropriate gene transfer, to correct genetic defects or provide genetic capabilities naturally lacking in the stem cells or their progeny, either as to the individual or as to hematopoietic cells generally. In addition, the stem cell composition can be used to isolate and define factors associated with their regeneration and differentiation.

The cells generated from $EM10^+$ cells and obtained from these cultures give rise to B cells, T cells, erythroid cells and myelomonocytic cells in the *in vivo* assays described below. In vitro analyses for hematopoietic progenitor cells have also been reported by Whitlock and Witte (1982) Proc. Natl. Acad. Sci. USA 79:3608-3612; and Whitlock et al. (1987) Cell 48:1009-1021.

In vivo demonstration of sustained hematopoietic ability of the various cell populations can be accomplished by the detection of continued myeloid, erythroid and B-lymphoid cell production in the SCID-hu bone model. Kyoizumi et al. (1992) Blood 79:1704; Chen et al. (1994) Blood 84:2497. To analyze this potential, one can isolate human fetal bone and transfer a longitudinally sliced portion of this bone into the mammary fat pad of a scid/scid animal: the bone cavity is depleted of endogenous progenitor cells by whole body irradiation of the mouse host prior to injection of the test donor population. The HLA of the population which is injected is mismatched with the HLA of the recipient bone cells.

To demonstrate differentiation to T cells, fetal thymus is isolated and cultured from 4-7 days at about 25°C, so as to deplete substantially the lymphoid population. The cells to be tested for T cell activity are then microinjected into the thymus tissue, where the HLA of the population which is injected is mismatched with the HLA of the thymus cells. The thymus tissue can then be transplanted into a scid/scid mouse as described in US Patent No. 5,147,784, particularly transplanting under the kidney capsule. Specifically, a sorted population of $EM10^+$ cells can be microinjected into HLA mismatched thymus fragments. After 6-10 weeks, assays of the thymus fragments injected with $EM10^+$ cells can be performed and assessed for donor derived T cells.

The cell compositions can find use in a variety of ways. They can be used to fully reconstitute an immunocompromised host such as an irradiated host and/or a host subject to chemotherapy; or as a source of cells for specific lineages, by providing for their maturation, proliferation and differentiation into one or more selected lineages by employing a variety of factors, including, but not limited to, erythropoietin, colony stimulating factors, e.g., GM-CSF, G-CSF, or M-CSF, interleukins, e.g., IL-1, -2, -3, -4, -5, -6, -7, -8, etc., or the like, or stromal cells associated with the stem cells becoming committed to a particular lineage, or with

their proliferation, maturation and differentiation. The EM10$^+$ cells can also be used in the isolation and evaluation of factors associated with the differentiation and maturation of hematopoietic cells. Thus, the invention encompasses the use of EM10$^+$ cells in assays to determine the activity of media, such as conditioned media, or to evaluate fluids for cell growth activity, involvement with dedication of particular lineages, or the like.

The EM10$^+$ cells can be used for the treatment of genetic diseases. Thus, the invention encompasses treatment of genetic diseases associated with hematopoietic cells by genetic modification of autologous or allogeneic stem cells to correct the genetic defect. For example, diseases including, but not limited to, β-thalassemia, sickle cell anemia, adenosine deaminase deficiency, recombinase deficiency, recombinase regulatory gene deficiency, etc. can be corrected by introduction of a wild-type gene into the EM10$^+$ cells, either by homologous or random recombination. Other indications of gene therapy are introduction of drug resistance genes to enable normal stem cells to have an advantage and be subject to selective pressure during chemotherapy. Suitable drug resistance genes include, but are not limited to, the gene encoding the multidrug resistance (MDR) protein.

Diseases other than those associated with hematopoietic cells can also be treated by genetic modification, where the disease is related to the lack of a particular secreted product including, but not limited to, hormones, enzymes, interferons, growth factors, or the like. By employing an appropriate regulatory initiation region, inducible production of the deficient protein can be achieved, so that production of the protein will parallel natural production, even though production will be in a different cell type from the cell type that normally produces such protein. It is also possible to insert a ribozyme, antisense or other message to inhibit particular gene products or susceptibility to diseases, particularly hematolymphotropic diseases.

In another embodiment, the invention encompasses antibodies which specifically recognize the cell surface marker having the epitope recognized by SM27-1045. As used herein, the term "αEM10" encompasses any antibody or fragment thereof, either native or recombinant, synthetic or naturally-derived, which retains sufficient specificity to bind specifically to the cell surface marker having the epitope recognized by SM27-1045. As used herein, the terms "antibody" or "antibodies" include the entire antibody and antibody fragments containing functional portions thereof. The term "antibody" includes any monospecific or bispecific compound comprised of a sufficient portion of the light chain variable region and/or the heavy chain variable region to effect binding to the epitope to which the whole antibody has binding specificity. The fragments can include the variable region of at least one heavy or light chain immunoglobulin polypeptide, and include, but are not limited to, Fab fragments, $F(ab')_2$ fragments, and Fv fragments.

In addition, the monospecific domains can be attached by any method known in the art to another suitable molecule. The attachment can be, for instance, chemical or by genetic engineering. The αEM10 can be produced by any recombinant means known in the art. Such recombinant antibodies include, but are not limited to, fragments produced in bacteria and non-human antibodies in which the majority of the constant regions have been replaced by human antibody constant regions. In addition, such "humanized" antibodies can be obtained by host vertebrates genetically engineered to express the recombinant antibody.

As used herein, αEM10 includes monoclonal antibody SM27-1045 or any monoclonal antibody or polyclonal antibody, that binds specifically to EM10 in such a manner as to recognize, preferentially, hematopoietic progenitor and stem cells. This also includes any αEM10 having the same antigenic specificity as SM27-1045.

The αEM10 antibodies are obtained by methods known in the

art for production of antibodies or functional portions thereof. The actual methods used are described in the Examples presented herein although any method known in the art of antibody production can be used. Such methods include, but are not limited to, separating B cells with cell-surface antibodies of the desired specificity, cloning the DNA expressing the variable regions of the light and heavy chains and expressing the recombinant genes in a suitable host cell. Standard monoclonal antibody generation techniques can be used wherein the antibodies are obtained from immortalized antibody-producing hybridoma cells. These hybridomas can be produced by immunizing animals with stem cells, and fusing B lymphocytes from the immunized animals, preferably isolated from the immunized host spleen, with compatible immortalized cells, preferably a B cell myeloma.

The antibodies can be conjugated to other compounds including, but not limited to, enzymes, magnetic beads, colloidal magnetic beads, haptens, fluorochromes, metal compounds, radioactive compounds or drugs. The enzymes that can be conjugated to the antibodies include, but are not limited to, alkaline phosphatase, peroxidase, urease and β-galactosidase. The fluorochromes that can be conjugated to the antibodies include, but are not limited to, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, phycoerythrin, allophycocyanins and Texas Red. For additional fluorochromes that can be conjugated to antibodies see Haugland, R. P. Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals (1992-1994). The metal compounds that can be conjugated to the antibodies include, but are not limited to, ferritin, colloidal gold, and particularly, colloidal superparamagnetic beads. The haptens that can be conjugated to the antibodies include, but are not limited to, biotin, digoxigenin, oxazalone, and nitrophenol. The radioactive compounds that can be conjugated or incorporated into the antibodies are known to the art, and include but are not limited to technetium 99m (^{99}Tc), ^{125}I and amino acids comprising any radionuclides, including, but not limited to,

^{14}C, ^{3}H and ^{35}S. Kits, e.g., for assaying or isolating stem cells, comprising αEM10, optionally labelled or conjugated as described above, are also comprised in the scope of the invention.

EXAMPLE 1

Cell Processing and Monoclonal Antibody Production

Cadaveric Bone Marrow and Mobilized Peripheral Blood

Cadaveric bone marrow cell suspensions derived from multi-organ donor vertebral bodies are obtained from Northwest Tissue Center (Seattle, WA). Patient peripheral blood samples are obtained after informed consent and chemotherapeutic regimens designed to mobilize primitive hematopoietic cells into the periphery. Multiple myeloma patients are mobilized with a high dose of cyclophosphamide and GM-CSF according to standard techniques. Non-Hodgkins lymphoma (NHL) patients are mobilized with VP16 and G-CSF.

Cells are separated over IsoPrep (Robbins Scientific, Sunnyvale, CA) harvesting the low density mononuclear cells (∂ < 1.068 g/mL for cadaveric bone marrow, ∂ < 1.077 g/mL for peripheral blood) and are further stained with antibodies for FACS and analysis.

Production of Monoclonal Antibodies (MAbs)

C57/B1 mice are tolerized to $CD34^-$ bone marrow cells by injection with cyclophosphamide (200 mg/kg) at 24 and 48 hours after interperitoneal injection with 10^7 $CD34^-$ bone marrow cells. After 4 weeks, this tolerization regimen is repeated. Starting approximately 1 month later, mice are immunized interperitoneally with $CD34^+$ cells (e.g. $CD34^+CD38^-$ at 0.5 to 1 x 10^6 cells/mouse) at 4 week intervals for a total of three immunizations. All immunizations are in RIBI adjuvant (RIBI Adjuvant Systems). Three days after the last immunization, the spleen is harvested to create hybridomas.

Splenocytes from immunized mice are fused to the myeloma partner P3xAg8.653 using standard PEG mediated fusion according to the method described by Galfre et al. (1977) Nature 266:550-552. Hybridomas from a single spleen are plated into 19 x 96 well tissue culture plates in RPMI + 10% FCS containing HAT to kill non-hybridoma cells. Fourteen days after fusion,

supernatants from the hybridoma wells containing secreted antibodies are harvested for testing.

The hybridomas secreting SM27-1045 are subcloned two to three times by limiting dilution in the presence of Hybridoma Enhancing Supplement (Sigma) to ensure monoclonality.

SM27-1045 is found to be an IgM by an ELISA in which isotype-specific alkaline-phosphatase-labeled antibodies are used to detect SM27-1045 captured in a microtiter well with immobilized anti-mouse Ig.

EXAMPLE 2

Antibody Staining, Fluorescence Activated Cell Sorting and Analysis

The buffer used in antibody staining is Dulbecco's modified phosphate buffered saline (Ca^{++} and Mg^{++} free) supplemented with 2% fetal bovine serum, 10 mM HEPES, 10 U/mL heparin, and 1 mg/mL human gamma globulin (Gamimune™, Miles, Elkhart, IN). Cells are incubated at 10^7/mL in SM27-1045 hybridoma supernatant diluted 1/2 in buffer for 30 minutes on ice. Cells are washed and SM27-1045 binding was detected by adding PE-labelled rabbit anti-mouse IgM at 1/100 dilution (Zymed) and incubating for 30 minutes on ice. Cells are washed again and resuspended to 10^7/mL in 2% normal mouse and 2% normal goat serum and incubated for 10 minutes on ice.

FITC or sulforhodamine-conjugated anti-CD34 antibody (Tük 3, $F(ab')_2$) is added at 3 µg/mL, and to amplify the PE signal, PE-labelled goat anti-rabbit IgG is added (Zymed, 1/100) and incubated 30 minutes on ice. Cells are washed and resuspended for cell sorting or analysis in buffer containing 1 µg/ml propidium iodide to stain non-viable cells. Cells are sorted and analyzed on a FACStar^{PLUS} (Becton Dickinson, San Jose, CA) equipped with two lasers, one emitting at 488 nm and a second (CR-599, Coherent, Palo Alto, CA) tuned to 600 nm to detect sulforhodamine fluorescence.

Sort gates are established to collect cells that are sulforhodamine positive ($CD34^+$), propidium $iodide^{dim}$ (viable). These cells are further divided into subsets based upon the level of PE staining ($EM10^+$ and $EM10^-$). The sorted cells are used in the AC6.21 co-culture, methylcellulose and SCID-hu thymus assays described below.

Three color immunofluorescent labelling is performed on the FACSCAN, using Dulbecco's modified phosphate buffered saline as described above with the exception that 2% dialized BSA replaces the 2% FCS. Cells are stained with SM27-1045 and detected with the double layer of PE-labelled antibodies as described above. Biotinylated anti-CD38 (Leu-17, Becton Dickinson) is added at the same time as the PE-goat anti-rabbit IgG. After incubation for 30 minutes and washing, FITC-labelled anti-CD34 and streptavidin Red613 (Gibco, 1/50 dilution) are added and incubated for 30 more minutes. Cells are washed a final time and the cell pellet is resuspended in buffer containing propidium iodide. 2000 FITC positive, low SSC events ($CD34^+$ cells) are collected and displayed as FL2 (SM27-1045 or IgM isotype) versus FL3 ($CD38^+$ for moderately bright, or dead for extremely bright). The results show that SM27-1045 binds to a substantial subset of $CD34^+CD38^-$ cells.

For the five color analysis cells are stained but not sorted. Cells are stained with rho123 (Molecular Probes, Eugene, OR) by incubating in buffer with 1 µg/mL rho123 for 30 minutes at 37°C, washing to remove excess rho123 and then incubating cells in buffer without rho123 for 30 minutes at 37°C to allow efflux. Subsequently, cells are stained at 4°C with SM27-1045 or control IgM (detected with PE-labelled rabbit anti-mouse IgM, followed by PE-labelled goat anti-rabbit IgG, both from Zymed and diluted 1/100, FL2), anti-Thy-1 (GM201, detected with Texas Red-labelled goat anti-mouse IgG_1, FL5) and anti-CD34 (Tük 3, Cy5-labelled FL4) and propidium iodide (FL3). Rhodamine 123 staining was detected in the FL1 channel.

The results obtained in the five color assay show that

SM27-1045 binds to a substantial number of $CD34^+Thy\text{-}1^+$ cells (both $rho123^{lo}$ and $rho123^{hi}$ subsets). The FACS analysis further shows that αEM10 subdivides $CD34^+$ cells; αEM10 binds approximately 60% of $CD34^+$ cells from bone marrow and 1-2% of a mononuclear cells on average and binds nearly all $CD34^+Thy\text{-}1^+$. SM27-1045 does not bind significant numbers of $CD34^-$ cells.

EXAMPLE 3

Staining of mobilized peripheral blood

Mobilized peripheral blood is obtained from a non-Hodgkins lymphoma (NHL) patient and a multiple myeloma patient and processed as described in Example 1. Cells are then stained with antibodies according to the method described in Example 2. The results show binding of SM27-1045 to mobilized peripheral blood $CD34^+$ cells is similar to that observed in bone marrow. αEM10 binds 2-90% (avg. 39%, n=10) $CD34^+$ cells from mobilized peripheral blood. Five of the 10 samples have less than 20% of $CD34^+$ cells bound by SM27-1045. It appears that EM10 is more variably expressed in mobilized peripheral blood than in bone marrow. These findings are similar to the results obtained with mAbs to c-kit. It is unlikely that αEM10 binds to c-kit based on the cell line staining data shown in Table 3. It can be the case that the antigen bound by EM10 is involved in the process of stem/progenitor cell mobilization, such as has been proposed for c-kit. αEM10 binds a subset of $CD34^+Thy\text{-}1^+$ cells from mobilized peripheral blood.

EXAMPLE 4

Immunofluorescence Staining of Various Cell Lines by αEM10

The expression of EM10 on a variety of cultured hematopoietic cell lines is tested by indirect immunofluorescence. All cultured cell lines are obtained from the ATCC. Human umbilical vascular endothelial cells (HUVEC) is obtained from Cell Systems, Kirkland, WA. Cell lines are immunolabelled with SM27-1045 at 4°C for 30 minutes, washed and further incubated for 30 minutes and detected using PE-labelled rabbit anti-mouse IgM (Zymed). After washing twice the cells are subjected to flow cytometric analysis using a FACScan. Analysis gates are set according to a non-binding isotype matched IgM control antibody.

For determination of neuraminidase sensitivity of the

epitopes, HEL or bone marrow cells are incubated at 10^7 cells/mL with 0 to 500 mU/mL of neuraminidase for 1 hour at 37°C. In order to determine the glycoprotease sensitivity of the epitopes, cells are incubated at 10^7 cells/mL in HBSS and a 1/10 dilution of O-sialoglycoprotein endopeptidase (CLE100, Accurate Chemical). Cells are incubated at 37°C for 30 minutes. Cells are then washed and subsequently tested for antibody binding by indirect immunofluorescence. The results obtained are presented in Table 3 which shows the percent of EM10 positive cells for each cell line tested.

Peripheral blood mononuclear cells (PBL) and HUVEC are also tested for the expression of epitopes bound by αEM10. As a comparison, the HUVEC are first incubated with IL-1β at 10 ng/ml for 4 hrs. IL-1β activates some integrins on endothelial cells. The results are presented in Table 3. The pattern of reactivity is not identified as similar to that described for mAbs to other antigens which have been described as expressed on $CD34^+$ cells. Furthermore, no binding to PBL is observed. The level of epitope expression is modest on both bone marrow and other positively stained cells tested. The treatment of bone marrow cells with neuraminidase or glycoprotease does not diminish the binding of SM27-1045 indicating that carbohydrate components are unlikely to be major aspects of the epitope defined by this mAb. The staining of HUVEC after activation with IL-1β is no different from untreated cells.

Table 3

KG1a	KG1	Jurkat	N417	HEL	HEL' neuraminidase	Daudi	Molt4	HL60	K562	U937	TFI	MCF7	T47D	HUVEC	HUVEC' IL-1(4h)	PBL (ficoll ed)
n=3		n=2	n=4	n=4					n=2		n=3	n=2	n=2	n=2		n=6
<5	<5	<5	70	10	16	<5	<5	<5	<5	<5	38	<5	<5	<5	<5	<3

EXAMPLE 5

Characterization of EM10$^+$ Cells

Co-culture

Sorted cell populations are analyzed by limiting dilution analysis for cobblestone area forming cell frequency (AC6.21) by limiting dilution analysis according to the method described by Baum et al. (1989).

Briefly, CD34$^+$ cadaveric bone marrow cells are sorted into CD34$^+$EM10$^+$ and CD34$^+$EM10$^-$ subsets as described in Example 1. A passage of AC6, (Whitlock et al. (1987) Cell 48:1009-1021), AC6.21, is used herein and is alternatively referred to as SyS1. Confluent AC6.21 stromal cell layers are maintained for 3-7 weeks without passage by changing of the tissue culture medium every 5-7 days. The culture is supplemented with human recombinant IL-6 (10 ng/mL) and LIF (20 ng/mL) to enhance the proliferation of adult bone marrow cells. The ability to give rise to both myeloid and B lymphoid progeny is determined after 6 weeks of coculture by staining with anti-CD19-FITC, and anti-CD15-FITC and anti-CD33-PE (all from Becton Dickinson) and analyzing on the FACSCAN. The results obtained from 2 separate sets of experiments are depicted in Table 4. The results show that the long term CAFC is enriched 15- 50 fold in the double positive subset of CD34$^+$ cells compared to the single positive subset (CD34$^+$EM10$^-$). Phenotypic analyses shows CD34$^+$EM10$^+$ cells are able to produce both lymphoid and myeloid cells in the SyS1 co-culture assay.

Methylcellulose Assay

Sorted cell populations are plated into methylcellulose cultures to determine colony forming cell activity according to the method described by Brandt et al. (1992) Blood 79:634. Growth factors are kit ligand (100 ng/mL), erythropoietin (2 U/mL), GM-CSF (10 ng/mL) and IL-3 ng/mL). The results in Table 4 show that αEM10 binds about 80% of the CFU-GM and CFU-Mix and 2/3 of BFU-E.

SCID-hu thymus assay

Sorted cell populations are microinjected into depleted fetal thymic pieces and implanted under the kidney capsule of SCID-hu mice according to the method described by Galy et al. (1994) Blood 84:104-110. Six weeks after implantation, thymic pieces are recovered and analyzed for the presence of T cell progeny. Preliminary SCID-hu thymus assays do not establish whether all T cell progenitors are bound by this mAb. The results obtained are depicted in Table 4. The results obtained indicate that αEM10 subdivides T cell progenitor activity as determined by the SCID-hu thymus assay which reads out both stem cell and lymphoid progenitor potential.

Table 4
Functional Readouts of Cell Populations Subsetted With αSM27-1045

Tissue	Sort Purity (Cross-contamination)	% Double Positive	Population Assayed	AC6.21 Results Week 5	CFC/10^5 cells BFU-E/CFU-GH/CFU-Mix	SCID-hu thymus results
10362	1-3%	62	$CD34^+$ $EM10^+$	1/34	11125 / 5200 / 875	1/1
			$CD34^+$ $EM10^-$	1/499	6450 / 600 / 150	1/1
			$CD34^+$	1/72	4150 / 1925 / 275	4/4
9041	<1%	79	$CD34^+$ $EM10^+$	1/35	1600 / 225 / 74	
			$CD34^+$ $EM10^-$	1/1822	800 / 25 / 0	
			$CD34^+$	1/60	7350 / 1125 / 175	

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

WHAT IS CLAIMED IS:

1. A cellular composition consisting essentially of cells which are EM10 positive.

2. The cellular composition according to claim 1 wherein the cells express at least one additional marker associated with stem cells.

3. The cellular composition according to claim 2 wherein the additional marker or markers are selected from the group consisting of CD34, Thy-1, P-gp and c-kit.

4. The cellular composition according to claim 1, 2, or 3 wherein the cells are negative for at least one lineage specific marker.

5. The cellular composition according to claim 4 wherein the cells are negative for at least one lineage specific marker selected from CD14, CD15, CD38, HLA-DR, CD71 and CD33.

6. A cellular composition according to claim 4 or 5 wherein the cells are negative for at least one lineage specific marker selected from CD2, CD16, CD19, CD20, or glycophorin A.

7. A cellular composition consisting essentially of hematopoietic cells and enriched for stem cells wherein the cells express EM10 and are negative for at least one lineage specific marker.

8. A cellular composition according to claim 7 wherein the cells are negative for at least one lineage specific marker selected from CD14 and CD15.

9. The cellular composition according to claim 7 or 8

wherein the cells are negative for at least one lineage specific marker selected from CD2, CD16, CD19, CD20 and glycophorin A.

10. The cellular composition according to claim 7, 8, or 9 wherein the cells express at least one additional marker associated with stem cells.

11. The cellular composition according to claim 10 wherein the additional marker or markers is selected from the group consisting of $CD34^+$, $Thy\text{-}1^+$, $c\text{-}kit^+$ and $P\text{-}gp^+$.

12. A cellular composition according to any one of claims 1 through 11 inclusive comprising at least one cell which has been transduced with a non-autologous gene.

13. A cellular composition according to any of claims 1 through 12 inclusive for therapeutic use.

14. Use of a cellular composition according to any of claims 1 through 12 inclusive in the manufacture of a composition for gene therapy.

15. A method of obtaining a composition substantially enriched in hematopoietic stem cells comprising the steps of:

combining a mixture of hematopoietic cells with an antibody that recognizes and binds to EM10 under conditions which allow the antibody to specifically bind to EM10; and

isolating cells recognized by the antibody to obtain a population of cells substantially enriched in $EM10^+$ cells.

16. The method according to claim 15, further comprising the step of selecting the cells for expression of at least one additional marker associated with stem cells.

17. The method according to claim 16 wherein the additional marker is selected from the group consisting of CD34, Thy-1, P-gp and c-kit.

18. The method according to any one of claims 15 through 17 inclusive further comprising the step of selecting the cells for lack of expression of at least one lineage specific (LIN^-) marker.

19. The method according to claim 18 wherein the cells are selected for lack of expression at least one lineage specific marker selected from the group consisting of CD14, CD15, CD38, HLA-DR, CD71 and CD33.

20. The method according to claim 18 or 19 wherein the cells are selcted for lack of expression of at least one lineage specific marker selected from the group consisting of CD2, CD16, CD19, CD20 and glycophorin A.

21. A method of determining the stem cell content in a sample of hematopoietic cells comprising the steps of:

a) combining a mixture of hematopoietic cells with an antibody that recognizes and binds to EM10 under conditions which allow the antibody to specifically bind to EM10; and

b) quantitating the cells recognized by the antibody.

22. The method according to claim 21, further comprising the step of selecting the cells for expression of at least one additional marker associated with stem cells.

23. The method according to claim 19 wherein the additional marker is selected from the group consisting of CD34, Thy-1, P-gp and c-kit.

24. The method according to claim 21 further comprising the step of selecting the cells for lack of expression of at least one lineage specific marker.

25. The method according to claim 24 wherein the lineage specific marker is selected from the group consisting of CD14, CD15, CD38, HLA-DR, CD71 and CD33.

26. The method according to claim 24 wherein the lineage specific marker is selected from the group consisting of CD2, CD16, CD19, CD20 and glycophorin A.

27. Antibody recognizing EM10.

28. Antibody according to claim 27 which binds specifically to the epitope recognized by the antibody SM27-1045 produced by the hybridoma deposited with the ATCC under accession number HB 11917.

29. Antibody according to claim 28 which is SM27-1045 produced by HB 11917 or its progeny.

30. A kit for isolating or assaying hematopoietic stem cells comprising an antibody according to any of claims 27, 28, or 29.

31. Use of an antibody according to any of claims 27, 28, or 29 in a method of isolating or assaying hematopoietic stem cells.

32. A hybridoma cell line capable of producing monoclonal antibody recognising EM10.

33. The hybridoma cell line according to claim 32 which is HB 11917.

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/EP 96/02463

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08 A61K35/28 A61K35/14 A61K48/00 G01N33/577
C07K16/28 C12P21/08 C12Q1/24 C12N5/20 //(C12P21/08,
C12R1:91)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K G01N C07K C12P C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,95 03693 (SYSTEMIX, INC.) 9 February 1995 see page 7, line 1 - page 8, line 9; claims; table 1 ---	1-30
A	WO,A,95 05843 (SYSTEMIX, INC.) 2 March 1995 see page 10, line 5 - line 16; claims -----	1-30

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 September 1996	17. 10. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Ryckebosch, A

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INTERNATIONAL SEARCH REPORT

.formation on patent family members

Intern nal Application No
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