



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) **EP 1 085 842 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention
of the grant of the patent:
08.03.2006 Bulletin 2006/10

(51) Int Cl.:
A61F 2/28 (2006.01) **A61F 2/46** (2006.01)
A61L 27/32 (2006.01) **A61L 27/36** (2006.01)
A61L 27/38 (2006.01) **A61L 27/48** (2006.01)
A61L 27/58 (2006.01)

(21) Application number: **99925776.9**

(22) Date of filing: **21.05.1999**

(86) International application number:
PCT/US1999/011413

(87) International publication number:
WO 1999/059500 (25.11.1999 Gazette 1999/47)

(54) **APPARATUS AND METHODS FOR PREPARING AN IMPLANTABLE GRAFT**

VORRICHTUNG UND VERFAHREN ZUR HERSTELLUNG EINES IMPLANTIERBAREN
GEFÄSSTRANSPLANTATS
APPAREIL ET PROCÉDES POUR LA PRÉPARATION D'UNE GREFFE IMPLANTABLE

(84) Designated Contracting States:
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE**

(30) Priority: **21.05.1998 US 82984**

(43) Date of publication of application:
28.03.2001 Bulletin 2001/13

(73) Proprietor: **THE CLEVELAND CLINIC
FOUNDATION
Cleveland, OH 44195 (US)**

(72) Inventor: **MUSCHLER, George, Frederick
Cleveland Heights, OH 44106 (US)**

(74) Representative: **Brasnett, Adrian Hugh et al
Mewburn Ellis LLP
York House
23 Kingsway
London WC2B 6HP (GB)**

(56) References cited:
**WO-A-98/00174 US-A- 5 645 729
US-A- 5 700 289 US-A- 5 718 899**

Remarks:

The file contains technical information submitted after
the application was filed and not included in this
specification

EP 1 085 842 B1

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description**Background of the Invention**

5 **[0001]** Bone grafting is widely used to treat fractures, non-unions and to induce arthrodeses. Autogenous cancellous bone, which is taken from one site in the graftee and implanted in another site in the graftee, is currently the most effective bone graft. Autogenous cancellous bone provides the scaffolding to support the distribution of the bone healing response. Autogenous cancellous bone also provides the connective tissue progenitor cells which form new cartilage or bone. However, the harvest of autogenous bone results in significant cost and morbidity, including scars, blood loss, pain, prolonged operative and rehabilitation time and risk of infection. Furthermore, in some clinical settings, the volume of the graft site can exceed the volume of the available autograft. Accordingly, alternatives to autografts have been developed in an attempt to reduce the morbidity and cost of bone grafting procedures.

10 **[0002]** Several purified or synthetic materials, including ceramics, biopolymers, processed allograft bone and collagen-based matrices have been investigated or developed to serve as substitutes for autografts. The FDA has approved a porous coral derived synthetic hydroxyapatite ceramic for use in contained bone defects. A purified collagen/ceramic composite material is also approved for use in acute long bone fractures. Although these materials avoid the morbidity involved in harvesting autografts from the graftee and eliminate problems associated with a limited amount of available autograft, the clinical effectiveness of the synthetic materials remains generally inferior to autografts.

15 **[0003]** The synthetic graft materials have also been used as carriers for bone marrow cells. When such composite materials have been implanted into skeletal defects, the connective tissue progenitor cells differentiated into skeletal tissue. In some instances, the composite implants were made by soaking the synthetic graft material in a cell suspension obtained from a bone marrow plug. However, the connective tissue progenitor cells, which have the capacity to differentiate into cartilage, bone and other connective tissue such as fat, muscle, and fibrous tissue are present in the bone marrow in very minute amounts. The numbers of such cells present in 1 ml of bone marrow varies widely from subject to subject from about 100 cells to 20,000 cells. This represents a mean of about one in 20,000 to one in 40,000 of the nucleated cells in bone marrow. Thus, a composite implant made by soaking a given volume of synthetic carrier graft material in a comparable volume of fresh bone marrow contains relatively few connective tissue progenitor cells.

20 **[0004]** Accordingly, a technique has been previously developed to increase the relative concentration of connective tissue progenitor cells in composite implants. This technique involves plating a suspension of bone marrow cells onto tissue culture dishes, culturing the cells in a select medium for one or more days until the number of connective tissue progenitor cells in the culture increases, and then detaching the cells from the tissue culture dishes to provide a cell suspension containing a culturally-expanded population of connective tissue progenitor cells. Composite implants are then made by soaking synthetic ceramic carriers in this suspension of culturally-expanded cells. Unfortunately, this method of preparing composite implants is very time consuming. Moreover, if the culturally-expanded cells used in this method are derived from bone marrow aspirates obtained from the graftee, the graftee must undergo multiple invasive procedures, one to remove his or her bone marrow and one at a later date to implant the composite implant. In addition, the graftee may be exposed to anaesthesia more than once.

25 **[0005]** Accordingly it is desirable to have a new method of preparing a composite bone marrow graft which can be performed intraoperatively, i.e., at the same time bone marrow is being taken from the graftee. An intraoperative method of preparing a composite bone marrow graft which uses bone marrow aspirate as the source of the connective tissue progenitor cells and which results in the formation of a composite bone graft containing an enriched population of connective tissue progenitor cells is especially desirable.

30 **[0006]** WO 98/00174 describes methods for preparing a composite bone graft comprising the steps of: (a) providing a bone marrow aspirate suspension; and (b) passing the bone marrow aspirate suspension through a porous, biocompatible, implantable substrate, as well as a kit for preparing a composite bone marrow graft from a bone marrow aspirate suspension comprising: (a) a porous, biocompatible, implantable substrate; and (b) a container for holding said substrate, said container configured to retain said substrate and to permit flow of the bone marrow aspirate suspension therethrough, said container having two ends, each of said ends defining an opening. Platelets derived from contaminating peripheral blood are mentioned as examples of nucleated cells other than connective tissue progenitor cells which are present in the bone graft (see page 19, lines 14-23 and page 6, lines 11-14 therein).

SUMMARY OF THE INVENTION

35 **[0007]** One aspect of the present invention pertains to a kit for preparing a composite bone graft from a bone marrow aspirate suspension comprising:

- 55 (a) a porous, biocompatible, implantable substrate having platelets on a surface thereof, which is obtainable by passing a platelet concentrate which contains an anti-coagulant or an isolated platelet concentrate through said

substrate; and

(b) a container for holding said substrate, said container configured to retain said substrate and to permit flow of the bone marrow aspirate suspension therethrough, said container having an inner surface and two ends, each of said ends defining an opening.

5

[0008] In one embodiment, said substrate has antibodies that bind to surface antigens expressed on the surface of connective tissue progenitor cells or platelets, wherein said antibodies are bound to an accessible surface of said substrate.

10

[0009] In one embodiment, said antibodies are selected from STRO-1, SH-2, SH-3, SH-4, SB-10, SB-20, and antibodies to alkaline phosphatase.

[0010] In one embodiment, said substrate is sterile.

[0011] In one embodiment, said kit further comprises:

15

a fluid flow regulator attachable to one end of said container for regulating the rate of flow of the bone marrow aspirate suspension through said substrate.

[0012] In one embodiment, said kit further comprises:

20

(a) a reservoir for holding the bone marrow aspirate suspension; and

(b) a fluid flow regulator attachable to said reservoir for regulating flow of the bone marrow aspirate suspension from said reservoir into said container.

[0013] In one embodiment, said kit further comprises:

25

an effluent receiver for receiving an effluent of the bone marrow aspirate suspension from said container.

[0014] In one embodiment, said substrate has external dimensions and a total accessible surface area at least five times greater than the surface area of a solid object having the same external dimensions.

30

[0015] In one embodiment, said substrate is formed from a ceramic comprising calcium phosphate or bioglass.

[0016] In one embodiment, said substrate is formed from a material selected from: collagen, mineralized bone, and demineralized bone.

[0017] In one embodiment, said substrate is formed from hyaluronic acid or a synthetic biopolymer.

[0018] In one embodiment, said substrate is formed from a synthetic biopolymer.

[0019] In one embodiment, said substrate is formed from hyaluronic acid.

35

[0020] In one embodiment, said substrate has cell adhesion molecules bound to a surface thereof.

[0021] In one embodiment, said substrate has growth factors bound to a surface thereof.

[0022] In one embodiment, said substrate has pores or passageways having a diameter greater than 40 μm .

[0023] In one embodiment, the container further comprises a porous member for retaining the substrate within the container.

40

[0024] In one embodiment, the container is made of a material that is biocompatible.

[0025] Another aspect of the present invention pertains to a composite bone marrow graft comprising:

45

(a) a porous, biocompatible, implantable substrate having platelets on a surface thereof, which is obtainable by passing a platelet concentrate which contains an anti-coagulant or an isolated platelet concentrate through said substrate;

(b) a heterogenous population of nucleated bone marrow cells; and

(c) an enriched population of connective tissue progenitor cells.

50

[0026] The present invention provides a new and improved method for preparing an implantable graft, particularly a composite bone graft. As used hereinafter the term "bone graft" refers to a graft which comprises connective tissue progenitor cells and is, therefore, capable of differentiating into cartilage or bone. The method comprises providing a bone marrow aspirate suspension and passing the bone marrow aspirate suspension through a porous, biocompatible, implantable substrate to provide a composite bone graft having an enriched population of connective tissue progenitor cells. Because the method is preferably performed intraoperatively using a bone marrow aspirate from the grafter, it reduces the time and expense required for graft preparation and also the number of times the grafter must return to the operating room to undergo invasive procedures. The improved composite bone graft prepared by the present method contains an enriched population of connective tissue progenitor cells and a greater number of connective tissue progenitor cells per unit volume than that found in the original bone marrow aspirate.

55

[0027] The present invention also relates to a composite bone marrow graft prepared according to the present method.

[0028] The present invention also provides a kit comprising the apparatus for preparing an implantable graft, particularly a composite bone graft. The kit comprises a porous, biocompatible substrate and a container configured to retain said substrate and to permit flow of a cell suspension, particularly a bone marrow aspirate suspension therethrough. Preferably, the substrate is sterile.

Brief Description of the Figures

[0029]

Figure 1 is a representation, somewhat schematic, of an apparatus used to prepare a composite bone graft in accordance with the present invention.

Figure 2a is a graph showing the effect of increasing the concentration of nucleated cells in the bone marrow aspirate suspension on the number of nucleated cells retained on a composite bone graft comprising a hydroxyapatite substrate. Data summarizes results from nine human subjects using identical conditions.

Figure 2b is a graph showing the effect of increasing the concentration of nucleated cells in the bone marrow aspirate suspension on the number of connective tissue progenitor cells retained on a composite bone graft comprising a hydroxyapatite substrate. Data summarizes results from nine human subjects using identical conditions.

Figure 3a is a graph showing the effect of increasing the concentration of nucleated cells in the bone marrow aspirate suspension on the concentration of nucleated cells retained on a composite bone graft comprising a demineralized human cancellous bone matrix substrate.

Figure 3b is a graph showing the effect of increasing the concentration of nucleated cells in the bone marrow aspirate suspension on the number of connective progenitor cells retained on a composite bone graft comprising a demineralized human cancellous bone matrix substrate. Data summarizes results from three human subjects using identical conditions.

Detailed Description Of The Invention

[0030] The present invention provides a new and improved method for preparing a composite bone graft. The method comprises collecting a bone marrow aspirate from a donor, preferably in the presence of an anti-coagulant to provide a bone marrow aspirate suspension, and passing the bone marrow aspirate suspension through a porous, biocompatible, implantable substrate. Preferably, the substrate is sterile. Preferably, the method is performed intraoperatively using a bone marrow aspirate preferably from the graftee.

[0031] The present invention also provides a method for preparing an implantable graft having platelets on the surface thereof. The method comprises passing a suspension of platelets through a porous, biocompatible, implantable substrate. Suitable suspensions include, by way of example, bone marrow, isolated platelet concentrate, and blood which contains an anticoagulant.

Preparing A Bone Marrow Aspirate Suspension

[0032] Bone marrow aspirate contains plasma, nucleated connective tissue progenitor cells, nucleated hematopoietic cells, endothelial cells, and cells derived from contaminating peripheral blood, including red cells and platelets. Since bone marrow aspirate also contains peripheral blood, it is preferred that the bone marrow be collected in a syringe containing an anti-coagulant. Suitable anti-coagulants include, for example, heparin, sodium citrate, and EDTA. Preferably, the bone marrow aspirate is mixed with a sterile isotonic solution to provide a concentration in the range of from about 10 million to about 300 million nucleated cells/ml, preferably from about 20 million to about 250 million nucleated cells/ml, more preferably from about 50 million to about 200 million nucleated cells/ml. Suitable isotonic solutions include, for example, isotonic buffered salt solutions, such as Hank's Balanced Salt Solution and phosphate buffered saline, and tissue culture medium such as minimal essential medium. As used herein, the term "bone marrow aspirate suspension" refers to a bone marrow aspirate that has not been mixed with an isotonic solution and to a bone marrow aspirate that has been mixed with an isotonic solution.

Substrate

[0033] The substrate is made from a biocompatible, implantable graft material. Preferably, the material has a charged surface. Examples of biocompatible, implantable graft materials having a charged surface include ceramics comprising calcium phosphate such as, for example, hydroxyapatite or tri-calcium phosphate; as well as demineralized bone matrix; or mineralized bone matrix. Other suitable graft materials include biopolymers such as, for example, polylactic acid,

polyglycolic acid, polygalactic acid, polycaprolactone, polyethylene oxide, polypropylene oxide, polysulfone, polyethylene, and polypropylene. Other suitable graft materials are hyaluronic acid, which may be purified with or without crosslinking, bioglass and collagen.

[0034] More preferably, cell adhesion molecules are bound to the surface of the substrate. The term "cell adhesion molecules" refers collectively to laminins, fibronectin, vitronectin, vascular cell adhesion molecules (V-CAM), intercellular adhesion molecules (I-CAM), tenascin, thrombospondin, osteonectin, osteopontin, bone sialoprotein, and collagens.

[0035] Optionally, the substrate has growth factors bound to the surface thereof. As used herein, the term "growth factors" encompasses any cellular product that modulates the growth or differentiation of other cells, particularly connective tissue progenitor cells. Growth factors include, but are not limited to, isoforms of platelet derived growth factors (PDGF), fibroblast growth factors, epithelial growth factors, isoforms of transforming growth factor Beta, insulin-like growth factors, and bone morphogenic proteins.

[0036] Optionally, the substrate has antibodies which have affinity for connective tissue progenitor stem cells bound to the surface thereof. Suitable antibodies, include by way of example, STRO-1, SH-2, SH-3, SH-4, SB-10, SB-20, and antibodies to alkaline phosphatase. Such antibodies are described in Haynesworth et al., Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. *Bone* 13:69-80,1992a; Bruder, S. et al. Identification and characterization of a cell surface differentiation antigen on human osteoprogenitor cells. *Trans Ortho Res Soc* 21:574; 1996; Haynesworth, S. E., et al. Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. *Bone* 13:69-80; 1992; Stewart, K., et al, Co-expression of the STRO-1 antigen and alkaline phosphatase in cultures of human bone and marrow cells. *J Bone Miner Res* 11 (Suppl.):S142;1996; Flemming JE, et al., Monoclonal Antibody Against Adult Marrow-Derived Mesenchymal Stem Cells Recognizes Developing Vasculature in Embryonic Human Skin. *Developmental Dynamics* 212:119-132, 1998, and Bruder SP, et al, Monoclonal Antibodies Reactive With Human Osteogenic Cell Surface Antigens. *Bone* 21(3): 225-235, 1997.

[0037] Preferably, the substrate has a sufficient number of pores or passageways so that the total accessible surface area of the substrate is at least five times greater than a solid object having the same external dimensions. Thus, the preferred total surface area can be achieved by using a substrate which comprises a mass of powder, a mass of granules, a mass of fibers, or a highly porous block of substrate material. Preferably, the size of the pores in the substrate is greater than 20 μm , more preferably greater than 40 μm , most preferably greater than 100 μm .

[0038] Particularly suitable graft materials include, for example, isolated mineralized cancellous bone sections, powders or granules of mineralized bone, demineralized cancellous bone sections, powders or granules of demineralized bone, guanidine-HCl extracted demineralized bone matrix, sintered cortical or cancellous bone, coralline hydroxyapatite sold by Interpore under the trade name Interpore 500, or Interpore 200. and granular ceramics such as that incorporated into the bone graft substitute Collagraft sold by Zimmer, or filamentous sponges such as those made from collagen by Orquest.

Substrate Container

[0039] Preferably, the substrate is disposed in a container configured to retain the substrate in the container and to allow fluid and bone marrow cells to flow through the container. This is accomplished by using a container having two openings at either end thereof and comprising a member having one or more pores disposed between the substrate and one of the openings. Preferably, the pores of the member have a diameter of sufficient size to allow fluid and cells of the bone marrow aspirate suspension to flow therethrough and to retain the substrate in the container. Preferably, the length of the container is greater than the width of the container to increase residence time of the suspension in the substrate.

[0040] Preferably, the container is made of a material which is biocompatible and pyrogen-free. Suitable container materials include for example glass, plastic or metal. Although the container may comprise two fluid flow restrictors blocking the openings at either end of the container, preferably, a fluid flow regulator is attached to at least one end of the container to regulate flow of the bone marrow aspirate suspension through the substrate.

Conditions

[0041] To allow for implantation of the graft into a subject, it is preferred that the substrate be sterile and that the inner surface of the container which holds the substrate also be sterile. Preferably, the bone marrow aspirate suspension is permitted to flow through the sterile substrate under hydrostatic pressure which may be generated by external forces or by the force of gravity. Preferably, the linear elution rate of the suspension through the substrate is between 2 and 500 mm/minute, more preferably between 5 and 200 mm/minute, most preferably between 10 and 100 mm/minute.

[0042] Optionally, the effluent is collected sterilely in an effluent collector and recycled through the substrate one or more times to increase the number of connective tissue progenitor cells in the composite bone graft.

[0043] Optionally, a wash solution is passed through the substrate after the original bone marrow aspirate suspension

and any effluents have been passed through the substrate. Preferably, the wash solution comprises a sterile, isotonic, buffered solution having a pH range of 7.3 to 7.5. Suitable wash solutions include, for example, phosphate-buffered saline, Hank's balanced salt solution, and minimal essential medium.

5 [0044] Optionally, growth factors or additional cells which secrete or present (i.e., express on their surface) growth factors are added to the composite bone graft prior to use, i.e. before, during or after the time the bone marrow aspirate suspension is passed through the substrate. Growth factors which may be added include for example, isoforms of platelet derived growth factors, fibroblast growth factors, epithelial growth factors, transforming growth factor Beta, insulin-like growth factor(s), parathyroid hormone (PTH) or PTH related peptide, and bone morphogenic proteins. Preferably, growth factors are added by passing a solution containing the growth factors through the substrate after all previous suspensions and solutions have been passed through the substrate. Alternatively, growth factors are added by incorporation into the wash solution. Platelets, which are known to secrete growth factors and to adhere to negatively charged surfaces, are added to the graft by passing a suspension of platelets, such as blood or platelet concentrate which contains an anti-coagulant, through the substrate.

10 [0045] The following examples of methods of preparing a composite bone graft are intended to illustrate but not to limit the present invention:

Example 1

20 [0046] The present method for preparing a composite bone graft may be more readily understood by reference to Figure 1 which depicts a preferred embodiment of the apparatus for performing the method. The apparatus, shown generally as 10, comprises a porous, biocompatible, implantable substrate 12, a container 14, for holding substrate 12, a reservoir 16 for holding the bone marrow aspirate suspension, a first fluid flow regulator 18, a second fluid flow regulator 20, and an effluent collector 22. Prior to preparation of the composite bone graft, all of the components of the apparatus are sterilized.

25 [0047] Following removal of top 23, the bone marrow aspirate suspension is introduced into reservoir 16. Then fluid flow regulator 18 is opened to allow the bone marrow aspirate suspension to flow out of reservoir 16 and into opening 30 in removable top 24 of container 14 and onto substrate 12.

30 [0048] As the suspension enters substrate 12, fluid flow regulator 20 which is attached to tip 34 of container 14 is opened to permit the bone marrow aspirate suspension to flow through porous member 32, through opening 36 of container 14 and into effluent collector 22.

35 [0049] Reservoir 16 and removable top 24 are then detached from container 14 and the improved composite bone marrow graft is then removed from container 14. The improved composite bone graft, which comprises substrate 12, an enriched population of connective tissue progenitor cells and a heterogeneous population of other nucleated bone marrow cells, blood cells, and adherent growth factors and adhesion molecules derived from marrow and blood, is ready to use as an implant or *in vitro*.

Example 2

40 [0050] Nine cylindrical disks of coralline hydroxyapatite (HA) measuring 13 mm in diameter and 5 mm in thickness were obtained from Interpore, Inc., Irvine, California. Each disk was placed in the tip of a vertically mounted 10 cc syringe barrel fitted with a stopcock. Marrow samples were taken from the anterior iliac crest of nine volunteer human subjects by aspiration. Samples were collected using a Lee-Lok bone marrow aspiration needle and a 10 cc syringe containing 1 ml of normal saline and 1000 units of Sodium-Heparin. Two ml of bone marrow were aspirated from each site. Marrow samples were suspended in α -MEM to prepare a suspension of marrow cells containing 50 million nucleated cells per ml. 2 ml of the marrow cell suspension were introduced into the top of the syringe and the stopcock was adjusted to allow the marrow cell suspension to elute through the disk at 2 ml/minute. Each sample of effluent was recycled through the disk three times. After the effluent was collected, the disk was washed with 6 ml phosphate buffered saline at an elution rate of 2 ml/min, to remove loosely adherent cells.

45 [0051] The number of nucleated cells in the initial suspension, the effluents, and the washes were counted using a hemocytometer to determine the number of nucleated cells retained in the resulting composite bone grafts. To determine the number of connective tissue progenitors retained in the resulting composite bone grafts, the number of connective tissue progenitors in the initial suspensions, the effluent, and the washes were assayed by colony counting on tissue culture plastic. For colony counting, 500,000 nucleated cells from the original suspension, the effluents and the wash were plated in separate 35 mm diameter tissue culture wells and cultured in α -MEM containing dexamethasone (10^{-8} M) and ascorbate (50 mg/ml) for 9 days. The cultured cells were then stained for alkaline phosphatase activity using N', N', dimethyl naphthol M-X phosphate as a substrate and Texas Fast Red as a stain. Alkaline phosphatase activity is a marker of osteoblastic differentiation. Thus, the number of colonies which stain positively for alkaline phosphatase activity reflect the number of connective tissue progenitors present in the original suspension, the effluents and the wash.

EP 1 085 842 B1

[0052] The number of nucleated cells and connective tissue progenitor cells which were retained on the substrate following each step were calculated by subtracting the number of nucleated cells and connective progenitor cells found in the effluents or wash from the number of nucleated cells and connective tissue progenitor cells in the initial suspension. The average number of nucleated cells and connective tissue progenitor cells retained in the nine composite bone grafts and the percentage of nucleated cells and connective tissue progenitor cells retained in the composite bone grafts are shown in Table 1.

Example 3

[0053] Composite bone grafts were prepared as described in Example 2 except that bone marrow samples were taken from the anterior iliac crest of three different volunteer human subjects and the substrates used were cylindrical disks of demineralized human cancellous bone matrix obtained from Life Net, Virginia Beach, Virginia.

[0054] The number of nucleated cells and connective tissue progenitor cells retained in the composite grafts were determined as described above in Example 2. The average number of nucleated cells and connective tissue progenitor cells retained in the composite bone grafts and the percentage of nucleated cells and connective tissue progenitor cells retained in the composite bone grafts are shown in Table 1.

TABLE 1 Retention of Cells in Composite Bone Grafts made using disks of hydroxyapatite or demineralized human cancellous bone

	HA Disks	Cancellous Bone
Nucleated Cells in Original Suspension	100 x 10 ⁶	100 x 10 ⁶
Nucleated Cells Retained before Wash	56.45 x 10 ⁶	40.00 x 10 ⁶
Nucleated Cells Removed with Wash	9.12 x 10 ⁶	15.78 x 10 ⁶
Nucleated Cells Retained after Wash	47.33 x 10 ⁶	24.22 x 10 ⁶
CTPC in Original Suspension	7800	11100
CTPC Retained After Wash	5162	4950
Percent of all Nucleated Cells Retained	47%	24%
Percent of all CTPC Retained	66%	44%
Ratio of CTPC to Nucleated cells	1.4	1.8
Concentration of CTPC in Composite Bone Graft vs Concentration of CTPC in Original Suspension	2.8	1.3
CTPC = Connective Tissue Progenitor Cells		

[0055] As shown in Table 1, composite grafts made with a substrate of hydroxyapatite or demineralized human cancellous bone retained a significant percentage of the nucleated cells (47% and 24%, respectively) and an even greater percentage of the connective tissue progenitor cells (66% and 44%, respectively) in the original suspension. As also shown in Table 1, washing substrates of cancellous bone or coralline hydroxyapatite resulted in removal of a mean of 16.2% (range 10% - 33%) of the nucleated cells which are initially retained in a coralline HA substrates and 39.45% (range 33 -86%) of the cells retained in a demineralized cancellous bone matrix substrates.

[0056] As shown in Table 1, the composite grafts made with either the hydroxyapatite or the demineralized human cancellous bones selectively retained the connective tissue progenitor cells as compared to other marrow derived nucleated cells. This selective retention is illustrated by the ratio (>1) of % connective tissue progenitor cells retained vs % nucleated cells retained on the substrate. Thus, the composite bone grafts prepared with either the hydroxyapatite disks or the demineralized human cancellous bone disks comprise an enriched population of connective progenitor cells.

[0057] Concentration of connective tissue progenitor cells above that found in the original bone marrow sample is illustrated by dividing the number of connective tissue progenitor cells retained by the volume of the disks (.63 cm³). As shown in Table 1, the mean concentration of connective tissue progenitor cells retained in the composite bone grafts comprising HA disks was 2.8 times greater than the concentration in the original marrow sample. Similarly, the mean concentration of connective tissue progenitor cells retained in the composite bone grafts comprising demineralized cancellous bone matrix was 1.3 times greater than in the original marrow sample.

Example 4

5 [0058] Forty-five composite bone grafts were prepared as described in Example 2 except that the concentration of nucleated cells in the marrow suspension was varied between 5, 10, 20, 40, and 50 million cells/ml from each of the nine human donors. The number of nucleated cells and connective tissue progenitor cells retained on each of the resulting composite bone grafts were determined as described in Example 2. The results are shown in Figures 2a and 2b.

10 [0059] As shown in Figures 2a and b, the number of nucleated cells and the number of connective tissue progenitor cells retained in the composite bone grafts increased in an essentially linear fashion as the number of marrow cells passed through the hydroxyapatite substrate was increased, indicating that saturation of the hydroxyapatite substrate with marrow derived cells did not occur over the range of cells to substrate volume evaluated.

Example 5

15 [0060] Fifteen composite bone grafts were prepared using disks of demineralized cancellous bone matrix as described in Example 2 except that the concentration of nucleated cells in the marrow suspension was varied between 5, 10, 20, 40, and 50 million cells/ml from each of the three human donors. Data reflecting the number of nucleated cells and the number of connective tissue progenitor colonies retained in the resulting composite bone grafts is presented in Figure 3a and 3b.

20 [0061] As shown in Figures 3a and 3b, the number of nucleated cells and the number of connective tissue progenitor cells retained in the composite bone grafts increased in an essentially linear fashion as the number of marrow cells passed through the demineralized cancellous bone matrix substrate increased, indicating that saturation of the substrate with marrow derived cells did not occur over the range cells to substrate volume evaluated.

Example 6

25 [0062] A composite bone graft was prepared as described in Example 2 using a 2 cc marrow suspension containing 5 million nucleated cells/ml except that the substrate was not washed with 6 ml of phosphate buffered saline after loading. Compared to an identical disk loaded in an identical manner which was washed as in Example 2, the unwashed disk retained the same number of connective tissue progenitors (1000 in the case shown) and a greater number of marrow derived nucleated cells (2.2 million vs 1.2 million in the washed example). After culture for 24 days *in vitro*, the presence of these additional cells resulted in greater proliferation and differentiation of the connective tissue progenitors. This was manifest by a greater surface area covered by cells that expressed alkaline phosphatase activity, which is a marker of osteoblastic differentiation.

Example 7

35 [0063] A composite bone graft was prepared as in Example 2 except that the bone marrow suspension was recycled over the hydroxyapatite disk only once, rather than three times. This reduced the number of cells and connective tissue progenitors which remained attached to the disk of coralline hydroxyapatite.

Example 8

40 [0064] Three composite bone grafts were prepared as in Example 2 except that the concentration of nucleated cells in the marrow suspension was increased from 100 to 150 million nucleated cells per ml. This increase in the number of cells passed through the hydroxyapatite disks increased the number of nucleated cells and connective tissue progenitor cells retained in the composite bone grafts by a mean of 66.84% and 52.0%, respectively. These highly cellular suspensions exhibited increased viscosity and slower elution flow rates.

Example 9

50 [0065] Heparinized human bone marrow samples were harvested with informed consent during elective Orthopaedic procedures by repeated aspiration of 2 cc samples from separate sites along the anterior iliac crest. Sterile samples of human allograft bone matrix powder (425-850 m dia, 0.1 ml volume) (Musculoskeletal Transplant Foundation, USA) were loaded into 1 ml syringes. A screen retained the particles in the syringe. Samples of bone marrow cell suspensions were passed through the allograft powder at defined concentrations and flow rates. Samples of marrow were assayed for cell number and CFU-Os using established techniques before and after passage through the matrix. The number of cells and CFU-Os retained in the matrix were calculated. A selection ratio for CFU-Os vs nucleated cells was also calculated (CFU-Os retained/CFU-Os loaded, cells retained/cells loaded). A selection ratio greater than 1.0 indicating

that CFU-Os were positively selected over other marrow derived nucleated cells.

[0066] The results indicated that cell and CFU-O retention on demineralized allograft powder increased as the number of cells loaded increased from 25 million cells to 200 million cells, where saturation occurred (flow rate 30 mm/min). Saturated matrices retained a mean of 80×10^6 nucleated cells (range 20 to 140×10^6) and 3800 CFU-Os (range 500 to 6600). This mean concentration of CFU-Os of 38,000 / ml was 19 fold greater than the mean concentration of CFU-Os in the original aspirate. The mean selection ratio at a loading density of 25 million cells was 2.4, but fell to 1.4 at a loading density of 200 million cells, indicating that CFU-Os appear to bind selectively to the matrix surface, but once the matrix surface is saturated with cells, further accumulation of cells in the matrix void spaces is much less selective. Comparison of flow rates from 15 mm/min to 60 mm/min revealed no influence of flow rate in this range on the number of cells or CFU-Os retained. Recycling samples through the matrix up to three times did not increase the retention of cells or CFU-Os. As shown in this example, human bone marrow derived osteoblastic progenitors harvested by aspiration can be concentrated in allograft matrix 19 fold by use of a rapid method suitable for intra-operative use.

[0067] These methods of preparing composite bone marrow grafts typically required less than sixty minutes to complete. Thus, these methods can be performed while the bone marrow donor/graftee is in the operating room. Accordingly, the number of occasions the graftee must undergo invasive procedures to receive a composite bone graft can be reduced by using these methods.

[0068] The improved composite bone grafts prepared according to these methods comprised a biocompatible, implantable substrate and an enriched population of connective tissue progenitor cells. As used herein the term "enriched population of connective tissue progenitor cells" means that the percentage of connective tissue progenitor cells as compared to all nucleated bone marrow cells is greater in the composite bone marrow graft than in the original bone marrow aspirate. In addition, the concentration of the connective tissue progenitor cells in the improved composite bone marrow grafts was about two times greater than the concentration of these cells in the original aspirate.

[0069] The improved composite bone grafts also comprised a population of nucleated cells other than connective tissue progenitor cells, including endothelial cells and hematopoietic cells derived from bone marrow, and a population of platelets derived from peripheral blood. The red blood cells and liquid plasma in the bone marrow aspirate suspension are not selectively retained in the composite bone grafts and, thus, the improved composite bone grafts typically contain less than five % of the red blood cells in the original suspension. Proteins and adhesion molecules present in plasma are also concentrated on the surface of the substrate as a combined function of their concentration in the plasma and relative affinity for the substrate surface.

[0070] The improved composite bone graft is suitable for implantation into the bone marrow aspirate donor or into an immunologically compatible host. The improved composite bone graft is also useful for assessing the effect of exogenous cytokines, hormones and other bioactive molecules on the proliferation and differentiation of connective tissue progenitor cells *in vitro*.

Claims

1. A kit for preparing a composite bone graft from a bone marrow aspirate suspension comprising:

(a) a porous, biocompatible, implantable substrate having platelets on a surface thereof, which is obtainable by passing a platelet concentrate which contains an anti-coagulant or an isolated platelet concentrate through said substrate; and

(b) a container for holding said substrate, said container configured to retain said substrate and to permit flow of the bone marrow aspirate suspension therethrough, said container having an inner surface and two ends, each of said ends defining an opening.

2. A kit according to claim 1 for preparing a composite bone graft from a bone marrow aspirate suspension comprising:

(a) a porous, biocompatible, implantable substrate having platelets on a surface thereof, which is obtainable by passing a platelet concentrate which contains an anti-coagulant through said substrate; and

(b) a container for holding said substrate, said container configured to retain said substrate and to permit flow of the bone marrow aspirate suspension therethrough, said container having an inner surface and two ends, each of said ends defining an opening.

3. A kit according to claim 1 for preparing a composite bone graft from a bone marrow aspirate suspension comprising:

(a) a porous, biocompatible, implantable substrate having platelets on a surface thereof, which is obtainable by passing an isolated platelet concentrate through said substrate; and

EP 1 085 842 B1

(b) a container for holding said substrate, said container configured to retain said substrate and to permit flow of the bone marrow aspirate suspension therethrough, said container having an inner surface and two ends, each of said ends defining an opening.

- 5 **4.** A kit according to any one of claims 1 to 3, wherein said substrate has antibodies that bind to surface antigens expressed on the surface of connective tissue progenitor cells or platelets, wherein said antibodies are bound to an accessible surface of said substrate.
- 10 **5.** A kit according to claim 4, wherein said antibodies are selected from STRO-1, SH-2, SH-3, SH-4, SB-10, SB-20, and antibodies to alkaline phosphatase.
- 6.** A kit according to any one of claims 1 to 5, wherein said substrate is sterile.
- 15 **7.** A kit according to any one of claims 1 to 6, further comprising:
 a fluid flow regulator attachable to one end of said container for regulating the rate of flow of the bone marrow aspirate suspension through said substrate.
- 20 **8.** A kit according to any one of claims 1 to 6, further comprising:
 (a) a reservoir for holding the bone marrow aspirate suspension; and
 (b) a fluid flow regulator attachable to said reservoir for regulating flow of the bone marrow aspirate suspension from said reservoir into said container.
- 25 **9.** A kit according to any one of claims 1 to 8, further comprising:
 an effluent receiver for receiving an effluent of the bone marrow aspirate suspension from said container.
- 30 **10.** A kit according to any one of claims 1 to 9, wherein said substrate has external dimensions and a total accessible surface area at least five times greater than the surface area of a solid object having the same external dimensions.
- 11.** A kit according to any one of claims 1 to 10, wherein said substrate is formed from a ceramic comprising calcium phosphate or bioglass.
- 35 **12.** A kit according to any one of claims 1 to 10, wherein said substrate is formed from a material selected from: collagen, mineralized bone, and demineralized bone.
- 13.** A kit according to any one of claims 1 to 10, wherein said substrate is formed from hyaluronic acid or a synthetic biopolymer.
- 40 **14.** A kit according to any one of claims 1 to 10, wherein said substrate is formed from a synthetic biopolymer.
- 15.** A kit according to any one of claims 1 to 10, wherein said substrate is formed from hyaluronic acid.
- 45 **16.** A kit according to any one of claims 1 to 15, wherein said substrate has cell adhesion molecules bound to a surface thereof.
- 17.** A kit according to any one of claims 1 to 15, wherein said substrate has growth factors bound to a surface thereof.
- 50 **18.** A kit according to any one of claims 1 to 17, wherein said substrate has pores or passageways having a diameter greater than 40 μm .
- 19.** A kit according to any one of claims 1 to 18, wherein the container further comprises a porous member for retaining the substrate within the container.
- 55 **20.** A kit according to any one of claims 1 to 19, wherein the container is made of a material that is biocompatible.
- 21.** A composite bone marrow graft comprising:

(a) a porous, biocompatible, implantable substrate having platelets on a surface thereof, which is obtainable by passing a platelet concentrate which contains an anti-coagulant or an isolated platelet concentrate through said substrate;

(b) a heterogenous population of nucleated bone marrow cells; and

(c) an enriched population of connective tissue progenitor cells.

Patentansprüche

1. Set zur Herstellung eines Knochen-Verbundtransplantats aus einer Knochenmarkpunktionssuspension, umfassend:

(a) ein poröses, bioverträgliches, implantierbares Substrat mit Blutplättchen auf einer Oberfläche davon, das mittels Durchfließenlassen eines Blutplättchenkonzentrats, das ein Antikoagulans enthält, oder eines isolierten Blutplättchenkonzentrats durch das Substrat erhältlich ist; und

(b) ein Behältnis zur Aufnahme dieses Substrats, worin das Behältnis so konfiguriert ist, dass das Substrat zurückgehalten und ein Fluss der Knochenmarkpunktionssuspension durch dieses ermöglicht wird, wobei das Behältnis eine innere Oberfläche und zwei Enden aufweist, wobei jedes dieser Enden eine Öffnung definiert.

2. Set nach Anspruch 1 zur Herstellung eines Knochen-Verbundtransplantats aus einer Knochenmarkpunktionssuspension, umfassend:

(a) ein poröses, bioverträgliches, implantierbares Substrat mit Blutplättchen auf einer Oberfläche davon, das mittels Durchfließenlassen eines Blutplättchenkonzentrats, das ein Antikoagulans enthält, durch das Substrat erhältlich ist; und

(b) ein Behältnis zur Aufnahme dieses Substrats, worin das Behältnis so konfiguriert ist, dass das Substrat zurückgehalten und ein Fluss der Knochenmarkpunktionssuspension durch dieses ermöglicht wird, wobei das Behältnis eine innere Oberfläche und zwei Enden aufweist, wobei jedes dieser Enden eine Öffnung definiert.

3. Set nach Anspruch 1 zur Herstellung eines Knochen-Verbundtransplantats aus einer Knochenmarkpunktionssuspension, umfassend:

(a) ein poröses, bioverträgliches, implantierbares Substrat mit Blutplättchen auf einer Oberfläche davon, das mittels Durchfließenlassen eines isolierten Blutplättchenkonzentrats durch das Substrat erhältlich ist; und

(b) ein Behältnis zur Aufnahme dieses Substrats, worin das Behältnis so konfiguriert ist, dass das Substrat zurückgehalten und ein Fluss der Knochenmarkpunktionssuspension durch dieses ermöglicht wird, wobei das Behältnis eine innere Oberfläche und zwei Enden aufweist, wobei jedes dieser Enden eine Öffnung definiert.

4. Set nach einem der Ansprüche 1 bis 3, worin das Substrat Antikörper aufweist, die sich an Oberflächenantigene binden, die an der Oberfläche von Bindegewebe-Vorläuferzellen oder Blutplättchen exprimiert werden, worin die Antikörper an eine zugängliche Oberfläche des Substrats gebunden sind.

5. Set nach Anspruch 4, worin die Antikörper aus STRO-1, SH-2, SH-3, SH-4, SB-10, SB-20 und Antikörpern gegen alkalische Phosphatase ausgewählt sind.

6. Set nach einem der Ansprüche 1 bis 5, worin das Substrat steril ist.

7. Set nach einem der Ansprüche 1 bis 6, weiters umfassend:

einen an einem Ende des Behältnisses anbringbaren Strömungsregler zur Regulierung der Durchflussgeschwindigkeit der Knochenmarkpunktionssuspension durch das Substrat.

8. Set nach einem der Ansprüche 1 bis 6, weiters umfassend:

(a) einen Speicherbehälter zur Aufnahme der Knochenmarkpunktionssuspension; und

(b) einen am Speicherbehälter anbringbaren Strömungsregler zur Regulierung des Flusses der Knochenmarkpunktionssuspension vom Speicherbehälter in das Behältnis.

9. Set nach einem der Ansprüche 1 bis 8, weiters umfassend:

EP 1 085 842 B1

einen Ausfluss-Sammelbehälter zur Aufnahme eines Ausflusses der Knochenmarkpunktionssuspension aus dem Behältnis.

- 5
10. Set nach einem der Ansprüche 1 bis 9, worin das Substrat äußere Dimensionen und eine zugängliche Gesamtoberfläche aufweist, die zumindest fünfmal größer ist als die Oberfläche eines festen Gegenstands, der dieselben äußeren Dimensionen aufweist.
- 10
11. Set nach einem der Ansprüche 1 bis 10, worin das Substrat aus Keramik besteht, die Calciumphosphat oder Bioglas umfasst.
12. Set nach einem der Ansprüche 1 bis 10, worin das Substrat aus einem Material, ausgewählt aus Collagen, mineralisiertem Knochen und entmineralisiertem Knochen, besteht.
- 15
13. Set nach einem der Ansprüche 1 bis 10, worin das Substrat aus Hyaluronsäure oder einem synthetischen Biopolymer besteht.
14. Set nach einem der Ansprüche 1 bis 10, worin das Substrat aus einem synthetischen Biopolymer besteht.
- 20
15. Set nach einem der Ansprüche 1 bis 10, worin das Substrat aus Hyaluronsäure besteht.
16. Set nach einem der Ansprüche 1 bis 15, worin das Substrat an eine seiner Oberflächen gebundene Adhäsionsmoleküle aufweist.
- 25
17. Set nach einem der Ansprüche 1 bis 15, worin das Substrat an eine seiner Oberflächen gebundene Wachstumsfaktoren aufweist.
18. Set nach einem der Ansprüche 1 bis 17, worin das Substrat Poren oder Kanäle mit einem Durchmesser von über 40 μm aufweist.
- 30
19. Set nach einem der Ansprüche 1 bis 18, worin das Behältnis weiters einen porösen Teil zum Zurückhalten des Substrats innerhalb des Behältnisses umfasst.
20. Set nach einem der Ansprüche 1 bis 19, worin das Behältnis aus einem Material besteht, das bioverträglich ist.
- 35
21. Knochenmark-Verbundtransplantat, umfassend:
- (a) ein poröses, bioverträgliches, implantierbares Substrat mit Blutplättchen auf einer Oberfläche davon, das mittels Durchfließenlassen eines Blutplättchenkonzentrats, das ein Antikoagulans enthält, oder eines isolierten Blutplättchenkonzentrats durch das Substrat erhältlich ist;
- 40
- (b) eine heterogene Population kernhaltiger Knochenmarkzellen; und
- (c) eine angereicherte Population von Bindegewebe-Vorläuferzellen.

Revendications

- 45
1. Un nécessaire pour la préparation d'une greffe osseuse composite à partir d'une suspension d'aspiration de moëlle osseuse, comprenant:
- 50
- (a) un substrat poreux, biocompatible, implantable présentant des plaquettes sur une surface de celui-ci, ce qui peut être obtenu en faisant passer, à travers ledit substrat, un concentré de plaquettes qui contient un anti-coagulant ou un concentré de plaquettes isolées; et
- (b) un récipient pour maintenir ledit substrat, ledit récipient étant configuré pour retenir ledit substrat et permettre un écoulement à travers lui de la suspension d'aspiration de moëlle osseuse, ledit récipient présentant une surface interne et deux extrémités, chacune desdites extrémités définissant une ouverture.
- 55
2. Un nécessaire selon la revendication 1 pour la préparation d'une greffe osseuse composite à partir d'une suspension d'aspiration de moëlle osseuse, comprenant:

EP 1 085 842 B1

(a) un substrat poreux , biocompatible , implantable présentant des plaquettes sur une surface de celui-ci, ce qui peut être obtenu en faisant passer, à travers ledit substrat, un concentré de plaquettes qui contient un anticoagulant; et

(b) un récipient pour maintenir ledit substrat, ledit récipient étant configuré pour retenir ledit substrat et permettre un écoulement à travers lui de la suspension d'aspiration de moëlle osseuse, ledit récipient présentant une surface interne et deux extrémités, chacune desdites extrémités définissant une ouverture.

3. Un nécessaire selon la revendication 1 pour la préparation d'une greffe osseuse composite à partir d'une suspension d'aspiration de moëlle osseuse comprenant:

(a) un substrat poreux , biocompatible , implantable présentant des plaquettes sur une surface de celui-ci, ce qui peut être obtenu en faisant passer à travers ledit substrat un concentré de plaquettes isolé; et

(b) un récipient pour maintenir ledit substrat, ledit récipient étant configuré pour retenir ledit substrat et permettre un écoulement à travers lui de la suspension d'aspiration de moëlle osseuse, ledit récipient présentant une surface interne et deux extrémités, chacune desdites extrémités définissant une ouverture.

4. Un nécessaire selon l'une quelconque des revendications 1 à 3, dans lequel ledit substrat comprend des anticorps qui se lient à des antigènes de surface exprimés sur la surface de cellules souches de tissu conjonctif ou de plaquettes, où lesdits anticorps sont liés à une surface accessible dudit substrat.

5. Un nécessaire selon la revendication 4, dans lequel lesdits anticorps sont choisis parmi STRO -1, SH-2, SH-3, SH-4, SB-10, SB-20, et des anticorps dirigés contre la phosphatase alcaline.

6. Un nécessaire selon l'une quelconque des revendications 1 à 5, dans lequel ledit substrat est stérile.

7. Un nécessaire selon l'une quelconque des revendications 1 à 6, comprenant en outre:

un régulateur d'écoulement de fluide pouvant être fixé à une extrémité dudit récipient afin de réguler le taux d'écoulement de la suspension d'aspiration de moëlle osseuse à travers ledit substrat.

8. Un nécessaire selon l'une quelconque des revendications 1 à 6, comprenant en outre:

(a) un réservoir pour maintenir la suspension d'aspiration de moëlle osseuse; et

(b) un régulateur d'écoulement de fluide pouvant être fixé audit réservoir pour réguler l'écoulement de la suspension de moëlle osseuse depuis ledit réservoir dans ledit récipient.

9. Un nécessaire selon l'une quelconque des revendications 1 à 8, comprenant en outre:

un récepteur d'effluent pour recevoir un effluent de la suspension d'aspiration de moëlle osseuse en provenance dudit récipient.

10. Un nécessaire selon l'une quelconque des revendications 1 à 9, dans lequel ledit substrat présente des dimensions externes et une aire de surface totale accessible au moins cinq fois supérieure à l'aire de surface d'un objet plein ayant les mêmes dimensions externes.

11. Un nécessaire selon l'une quelconque des revendications 1 à 10, dans lequel ledit substrat est formé à partir d'une céramique comprenant du phosphate de calcium ou d'un bioverre.

12. Un nécessaire selon l'une quelconque des revendications 1 à 10, dans lequel ledit substrat est formé d'une matière sélectionnée à partir de: collagène, os minéralisé, et os déminéralisé.

13. Un nécessaire selon l'une quelconque des revendications 1 à 10, dans lequel ledit substrat est formé à partir d'acide hyaluronique ou d'un biopolymère synthétique.

14. Un nécessaire selon l'une quelconque des revendications 1 à 10, dans lequel ledit substrat est formé à partir d'un biopolymère synthétique.

15. Un nécessaire selon l'une quelconque des revendications 1 à 10, dans lequel ledit substrat est formé à partir d'acide

hyaluronique.

5 16. Un nécessaire selon l'une quelconque des revendications 1 à 15, dans lequel ledit substrat comprend des molécules d'adhésion de cellules liées à une surface de celui-ci.

17. Un nécessaire selon l'une quelconque des revendications 1 à 15, dans lequel ledit substrat comprend des facteurs de croissance liés à une surface de celui-ci.

10 18. Un nécessaire selon l'une quelconque des revendications 1 à 17, dans lequel ledit substrat présente des pores ou des passages ayant un diamètre supérieur à 40 μm .

19. Un nécessaire selon l'une quelconque des revendications 1 à 18, dans lequel le récipient comprend en outre un élément poreux pour retenir ledit substrat à l'intérieur du récipient.

15 20. Un nécessaire selon l'une quelconque des revendications 1 à 19, dans lequel le récipient est réalisé en une matière qui est biocompatible.

21. Une greffe de moëlle osseuse composite comprenant:

20 (a) un substrat poreux, biocompatible, implantable présentant des plaquettes sur une surface de celui-ci, ce qui peut être obtenu en faisant passer, à travers ledit substrat, un concentré de plaquettes qui contient un anticoagulant ou un concentré de plaquettes isolées;

(b) une population hétérogène de cellules de moëlle osseuse nucléées; et

25 (c) une population enrichie de cellules souche de tissu conjonctif.

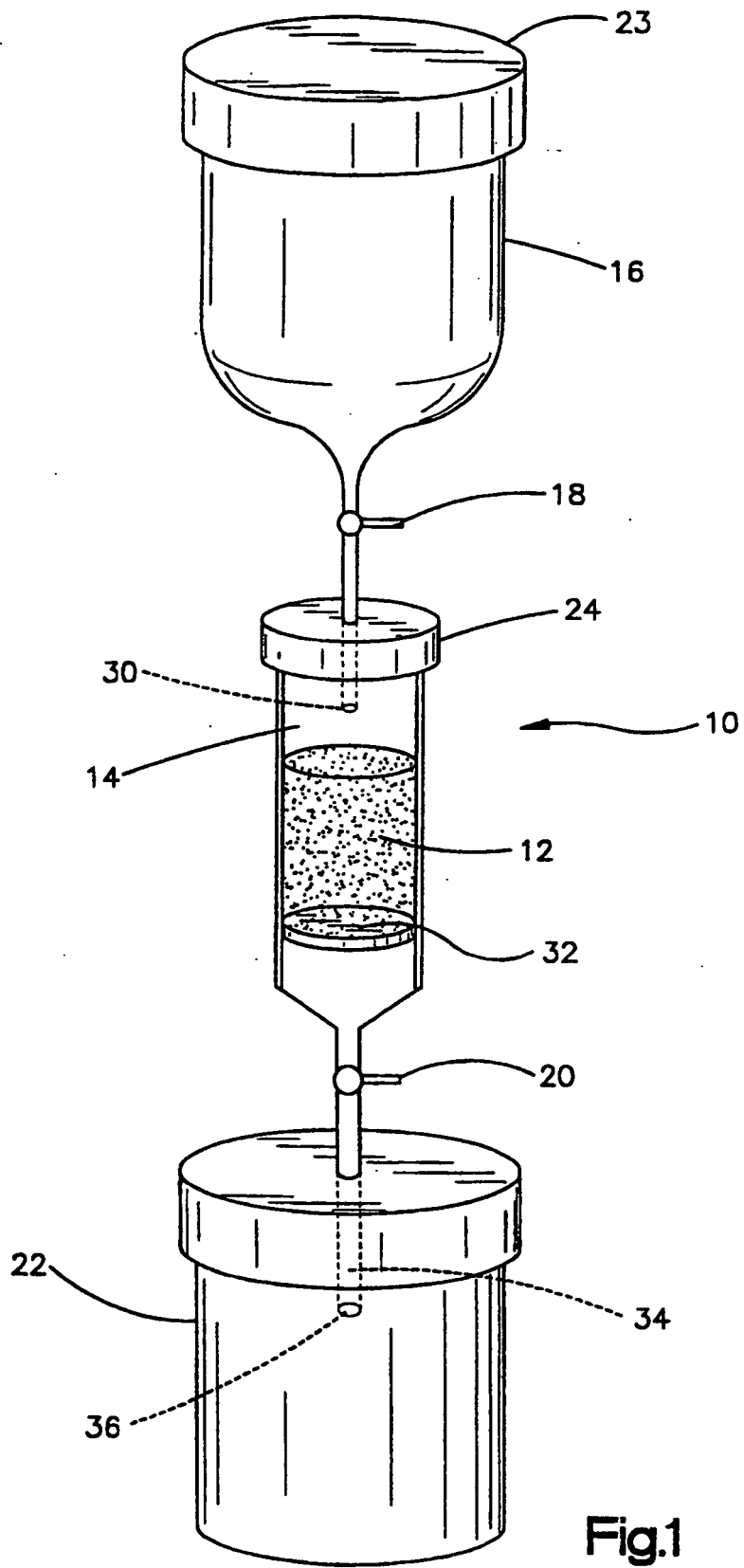


Fig.1

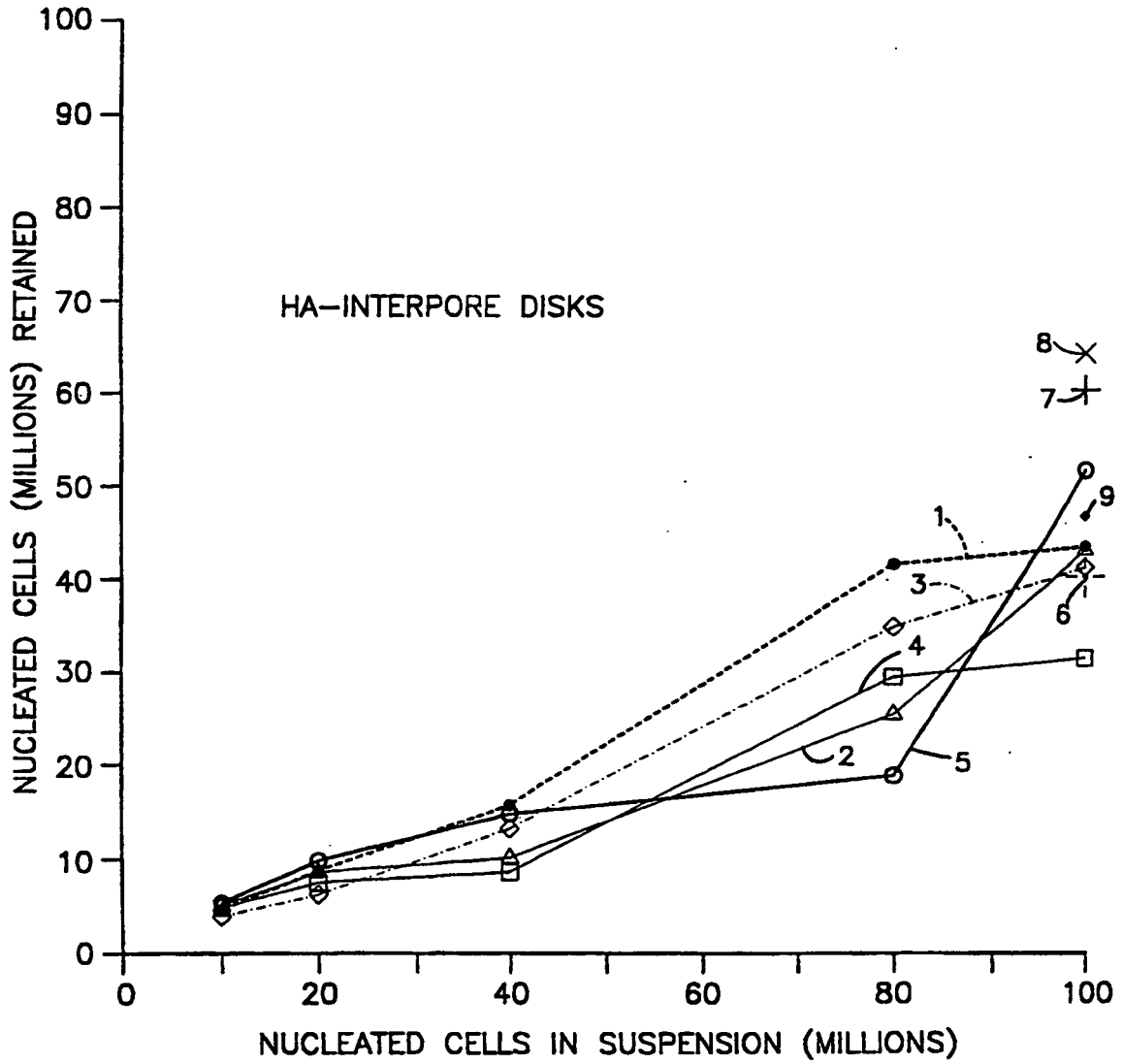


Fig.2a

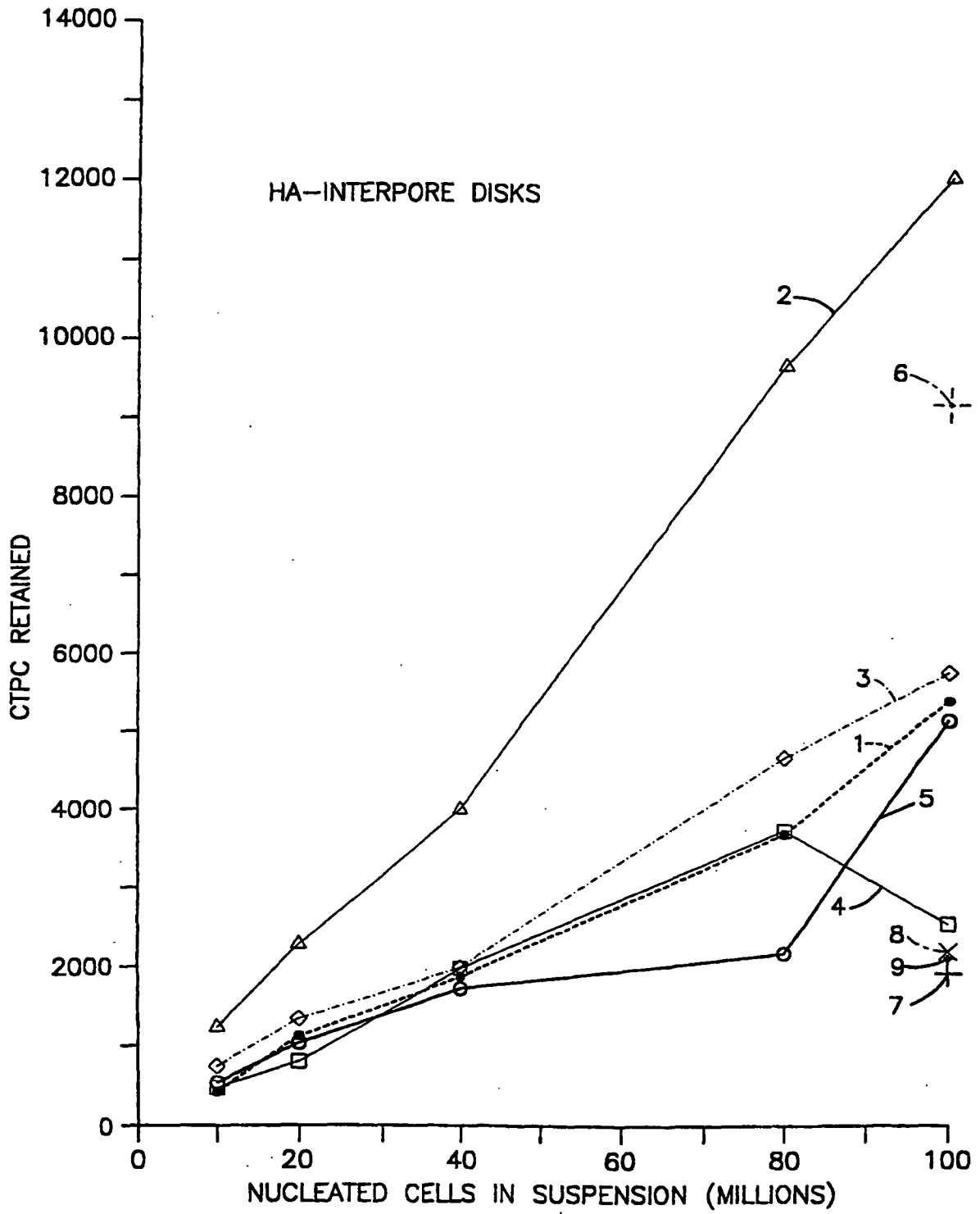


Fig.2b

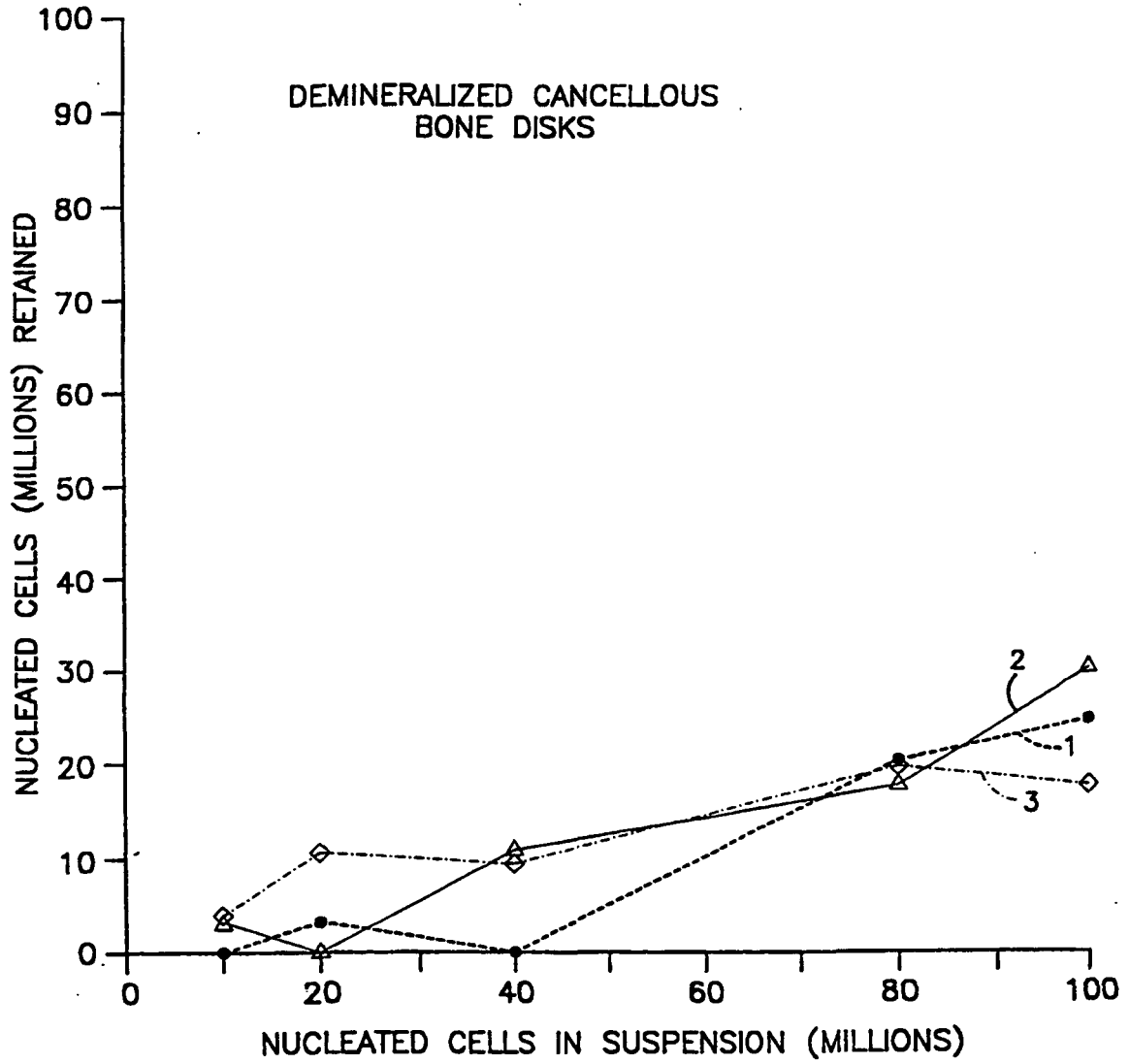


Fig.3a

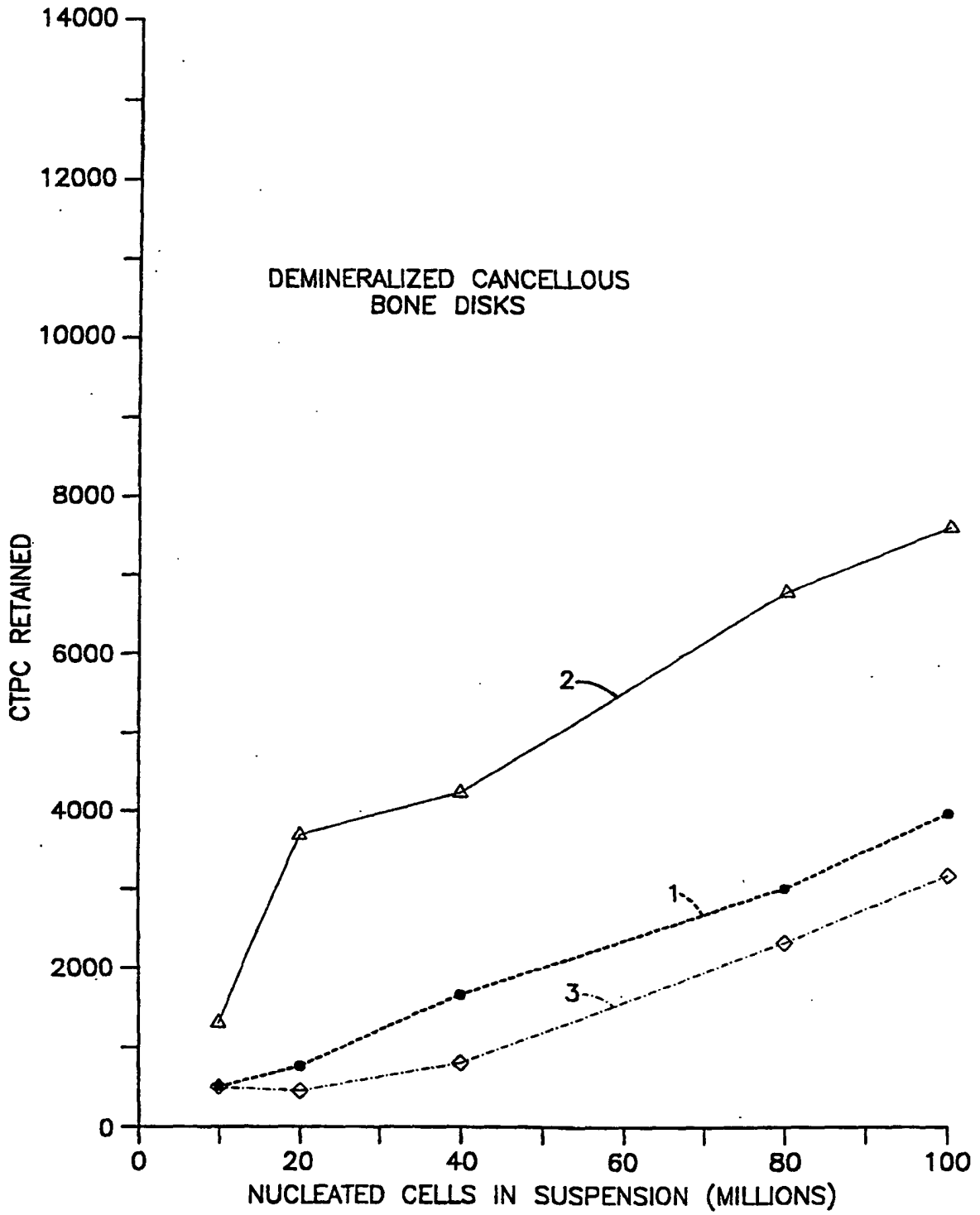


Fig.3b