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(71) Applicants: THE UNITED STATES GOVERNMENT, represented by THE SECRETARY OF THE NAVY [US/US]; Counsel for Intellectual Property, Naval Medical Research and Development Command, National Naval Medical Center, 8901 Wisconsin Avenue, Bethesda, MD 20889-5606 (US). CELLCO, INC. [US/US]; 12321 Middlebrook Road, Germantown, MD 20874 (US).

(72) Inventors: DAVIS, Thomas, A.; 13161 Ladybank Lane, Herndon, VA 22071 (US). LEE, Kelvin, P.; 4628 Woodfield Road, Bethesda, MD 20814 (US). KIDWELL, William, R.; 10505 Lowell Court, Ijamsville, MD 21754 (US).

(74) Agents: GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

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(57) Abstract

The application concerns methods for stimulating expansion of hematopoietic cells. Compositions derived from the supernatant of porcine microvascular endothelial cells are also provided. The application further concerns methods for producing these compositions in serum-free medium.

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Compositions and Methods for Stimulating Hematopoietic Cell Expansion

Field of the Invention

The invention relates to methods for stimulating expansion of hematopoietic cells. Compositions derived from the supernatant of porcine microvascular endothelial cells are also provided. The invention further relates to methods for producing these compositions in serum-free medium.

Background of the Invention

Mature blood cells are derived from pluripotent hematopoietic stem and progenitor cells which are typically present at very low frequencies (<1.0%) in hematopoietic tissues. During ontogeny, hematopoiesis, the production of mature blood cells, moves from yolk sac to liver/spleen and then to the bone marrow (Tavassoli, M., *Blood Cells 17*:269 (1991)). During early fetal life, hematopoiesis occurs within the liver and spleen. In the latter part of gestation, bone marrow spaces begin to develop and expand. Hematopoietic stem cells then migrate from liver/spleen to the bone marrow occupying "niches" in the developing marrow (Zanjani *et al., J. Clin. Invest. 89*:1178 (1992)). Hematopoiesis subsequently primarily occurs in the bone marrow (Gordon *et al., Bone Marrow Transplant 4*:335 (1989)). The defining characteristics of hematopoietic stem cells are the capacity for extensive self-renewal, the ability to rescue animals from lethal irradiation and the retention of multilineage differentiation potential. Hematopoietic stem cells proliferate and differentiate to produce progenitor cells, which in turn form precursor cells, which differentiate to form mature blood cells.

There has been much interest in the *ex vivo* expansion of hematopoietic stem cells (Edgington S.M., *Biotechnology 10*:1099 (1992)). Successful *ex vivo* expansion of primitive stem and progenitor cells would allow transplantations in situations where, using currently available technology, adequate amounts of bone marrow cannot be harvested from the patient. It

has been demonstrated that proliferation and differentiation of hematopoietic stem cells are regulated by a group of glycoproteins known as hematopoietic cytokines. Numerous investigations have focused on the ability of different combinations of these hematopoietic growth factors to stimulate hematopoietic cell expansion (Moore *et al.*, *Proc. Natl. Acad. Sci. USA 84*:7134 (1987); Leary *et al.*, *Blood 71*:1759 (1988); Brandt *et al.*, *J. Clin. Invest. 86*:932 (1990); Kobayashi *et al.*, *Blood 78*:1947 (1991); Meunch *et al.*, *Blood 81*:3463 (1993); Bernstein *et al.*, *Blood 77*:2316 (1991); and Bodine *et al.*, *Blood 79*:913 (1992)).

Generally speaking, two types of hematopoietic culture systems have been developed: liquid suspension cultures which include hematopoietic cells and cytokines without stromal support cells and stromal/hematopoietic cell co-culture systems (e.g., Dexter-type cultures). Liquid suspension cultures have resulted in a large degree of nonadherent cell expansion (Haylock *et al.*, *Blood 80*:1405 (1992); Brandt *et al.*, *Blood 79*:634 (1992)). However, cellular differentiation and depletion of primitive cells (e.g., stem cells) often occurs in these systems. For example, after culture of enriched primitive hematopoietic cells, measurements of long-term culture-initiating cells (LTC-IC) were reported as being below the input value (Sutherland *et al.*, *Blood 78*:666 (1991); Verfaillie, C.M., *Blood 79*:2821 (1992)).

Dexter-type long term bone marrow cultures (LTBMCs) were developed in the 1970s. LTBMCs provided stable *ex vivo* hematopoietic systems for several months (Dexter *et al.*, *J. Cell Physiol. 91*:335 (1977); Dexter *et al.*, in: Wright, D.G., Greenberger, J.S. (Eds.): Long-term Bone Marrow Culture. New York, NY, Liss, p. 57 (1984)). However, LTBMCs exhibit exponentially decreasing numbers of total and progenitor cells with time, rendering the cultures unsuitable for cell expansion for clinical use (Eaves *et al.*, *J. Tissue Cult. Methods 13*:55 (1991)). Koller *et al.* co-cultured mononuclear cells (MNC) on monolayers of irradiated marrow stroma--a heterogenous cell mixture including adipocytes, macrophages, fibroblasts and endothelial cells. This resulted in the expansion of progenitor cells (Koller

et al., Blood 82:378 (1993); Koller *et al., Biotechnology 11*:358 (1993); Palsson *et al., Biotechnology 11*:368 (1993)). However, it is difficult to analyze factors and conditions affecting hematopoiesis in these co-culture systems due to the heterogeneity of both the hematopoietic cells (when MNC are used) and the supporting stromal cell layer. Thus, there is a continuing need for compositions capable of expanding hematopoietic stem cells in a defined liquid suspension culture system without loss of long-term reconstitution potential.

Summary of the Invention

The invention provides compositions and methods for stimulating the expansion of hematopoietic cells. The inventors have discovered that one or more hematopoietic growth factors are secreted into the supernatant during culture of porcine microvascular endothelial cells (PMVEC). An initial step to obtaining these compositions involves concentrating the PMVEC supernatant by filtration. The inventors have discovered that compositions containing the concentrated PMVEC supernatant are capable of stimulating expansion of hematopoietic cells.

The invention further provides the partial purification of the hematopoietic growth factor(s) present in the PMVEC supernatant. The inventors have discovered that approximately 95% of the hematopoietic growth promoting activity in the PMVEC supernatant resides in proteinaceous fractions having a molecular weight of about 60-95 kD. Thus, the invention provides a composition comprising a partially pure proteinaceous fraction of PMVEC supernatant having a molecular weight of about 60-95 kD which is capable of stimulating expansion of hematopoietic cells. Preferably, the proteinaceous fraction has a molecular weight of about 60-70 kD. More preferably, the proteinaceous fraction has a molecular weight of about 65-70 kD. Most preferably, the proteinaceous fraction has a molecular weight of about 68-70 kD.

The compositions of the present invention can be used to stimulate expansion of a variety of hematopoietic cells, including stem, progenitor and precursor cells. Preferably, the hematopoietic cells are enriched for $CD34^+$ cells prior to culture. In another preferred embodiment, the compositions of
5 the invention are used in conjunction with one or more cytokines to further stimulate hematopoietic cell expansion.

The compositions of the present invention are useful for either the *ex vivo* or *in vivo* expansion of hematopoietic cells. *Ex vivo* culturing systems that can be used include cell-free liquid suspension cultures and perfusion
10 culturing systems such as hollow fiber bioreactors. *In vivo* administration of the compositions of the present invention can be by any suitable method, including intravenous, subcutaneous, or intramuscular injection.

The invention further provides methods for producing PMVEC supernatant capable of stimulating expansion of hematopoietic cells. The
15 method involves culturing the PMVEC in culture medium capable of supporting PMVEC growth, harvesting the supernatant secreted into the medium during culture, and concentrating the harvested supernatant by filtration. Preferably, the harvested supernatant is filtered to concentrate proteins having a molecular weight of greater than 30 kD. In another
20 preferred embodiment, the PMVEC are cultured in serum-free medium.

Brief Description of the Drawings

Figure 1: CFU-C growth of purified $CD34^+$ bone marrow cells with either 0.5%, 1.0%, 2.5%, 5.0%, or 10% PMVEC CM treatment or with GM-CSF+ IL-3+SCF+IL-6 treatment. The Y-axis represents the number of
25 CFU-C per 2000 $CD34^+$ cells plated. Data represent mean $\pm$ SD from triplicate cultures.

Figure 2: Total number of CFU-GM colonies per 5×10^4 low density mononuclear bone marrow cells plated, grown in 14 day methylcellulose

cultures in the presence of hematopoietic growth factors with and without PMVEC CM. Data represent mean ± SD from triplicate cultures.

Figure 3: Total number of CFU-Mix colonies per 5 X 10^4 low density mononuclear bone marrow cells plated, grown in 14 day methylcellulose cultures in the presence of hematopoietic growth factors with and without PMVEC CM. Data represent mean ± SD from triplicate cultures.

Figure 4: Total number of BFU-E colonies per 5 X 10^4 low density mononuclear bone marrow cells plated, grown in 14 day methylcellulose cultures in the presence of hematopoietic growth factors with and without PMVEC CM. Data represent mean ± SD from triplicate cultures.

Figure 5: Enhancement of $CD34^+$ cell proliferation by PMVEC CM (5% final concentration) in combination with GM-CSF+IL-3+SCF+IL-6. Data represent three independent experiments using purified $CD34^+$ cells from different healthy bone marrow donors.

Figure 6: SDS-polyacrylamide gel of unfractionated 70X PMVEC CM and various protein fractions eluted from a preparative PAGE gel. Lane (1), molecular weight markers (row a: 200 kD, row b: 97 kD; row c: 68 kD; row d: 43 kD; row e: 31 kD); lane (2), unfractionated PMVEC CM (70X); lane (3), protein eluted from a 68-70 kD PAGE gel slice, reduced, denatured and then electrophoresed on SDS-PAGE gel; lane (4), protein eluted from a 72-78 kD PAGE gel slice, reduced, denatured and then electrophoresed on SDS-PAGE gel; lane (5), protein eluted from a 79-95 kD PAGE gel slice, reduced, denatured and then electrophoresed on SDS-PAGE gel; lane (6), protein eluted from a 100-130 kD PAGE gel slice, reduced, denatured and then electrophoresed on SDS-PAGE gel; lane (7), protein eluted from a >130 kD PAGE gel slice, reduced, denatured and then electrophoresed on SDS-PAGE gel.

Figure 7: Profile of the isoelectric focusing fractionation and refractionation of 6.6X PMVEC CM. The pH of every fraction produced from isoelectric focusing (4 hours until a constant voltage of 500 V was attained) 6.6X PMVEC CM was measured. Fractions with a pH of 3.58 -

6.00 were pooled and refractionated by operating the apparatus until a constant voltage of 1300 V was attained. The pH of every fraction was again measured. Results are shown graphically. Note that the uppermost refractionation curve should be read against the secondary (right-hand side) Y-axis.

Figure 8: Samples from an isoelectric focusing run of PMVEC conditioned medium were loaded on a Tris-Glycine SDS 8-16% gel. The gel was developed for 2 hours at 125 Volts, 50 Watts, and 31 mAmps/gel and stained either with Coomassie Blue or silver stain to visualize the protein bands. Lane 1 - fraction 11, pI 4.8; lane 2 - fraction 12, pI 4.9; lane 3 - fraction 13, pI 4.94; lane 4 - fraction 14, pI 5.00; lane 5 - fraction 15, pI 5.1; lane 6 - molecular weight standard; lane 7 - fraction 16, pI 5.2; lane 8 fraction 17, pI 5.36; lane 9 - fraction 18, pI 5.44; lane 10 - unfractionated PMVEC CM.

Figure 9: Samples from an isoelectric focusing run of PMVEC conditioned medium were loaded on a Tris-Glycine SDS 8-16% gel. The gel was developed for 2 hours at 125 Volts, 50 Watts, and 31 mAmps/gel and stained either with Coomassie Blue or silver stain to visualize the protein bands. Lane 1 - fraction 3, pI 4.01; lane 2 - fraction 4, pI 4.10; lane 3 - fraction 5, pI 4.16; lane 4 - unfractionated PMVEC CM; lane 5 - fraction 6, pI 4.20; lane 6 - fraction 7, pI 4.25, lane 7 - molecular weight standard, lane 8 - fraction 8, pI 4.45; lane 9 - fraction 9, pI 4.55; lane 10 - fraction 10, pI 4.65.

Figure 10: The effect of different fractions purified from PMVEC conditioned medium by isoelectric focusing on $CD34^+$ cell expansion is shown graphically. The fractions are described in terms of pI.

Figure 11: The effect of different fractions purified from PMVEC conditioned medium by isoelectric focusing on $CD34^+$ cell expansion is shown graphically. The fractions are described in terms of fraction number.

Figure 12: 40,000 isolated $CD34^+$ cells (70% pure) in culture medium were added to a culture dish. Either cytokines alone (GM-CSF, SCF, IL-3,

IL-6) (control) or the cytokines plus 50 μl of 40X concentrated PMVEC conditioned medium (m.w. >10 kD and $<$ 30kD) was added to the culture. The effect on $CD34^+$ cell expansion is shown graphically.

Detailed Description of the Invention

5 The invention provides compositions and methods for stimulating expansion of hematopoietic cells. By the invention, hematopoietic cells are contacted with a composition derived from the supernatant of porcine microvascular endothelial cells (PMVEC) and cultured in the presence of the composition to achieve hematopoietic cell expansion.

10 A previously reported culture system utilizing PMVEC to stimulate expansion of enriched $CD34^+$ cells is described in United States Patent Application No. 08/142,569 ('569). However, the '569 application indicates that the $CD34^+$ cells must be cultured while in direct contact with the PMVEC cells in order to obtain a significant expansion of hematopoietic cells, 15 especially $CD34^+CD38^-$ stem cells. In contrast to the '569 application, the present inventors have discovered that PMVEC supernatant, which has been concentrated by filtration, is capable of stimulating significant hematopoietic cell expansion (including $CD34^+CD38^-$ cells) in liquid suspension culture. Thus, the present inventors have shown, for the first time, that one or more 20 soluble hematopoietic growth factors are secreted into the supernatant during culture of PMVEC in serum-free medium and that compositions containing the growth factor(s) are capable of stimulating hematopoietic stem cell expansion in the absence of PMVEC support cells. Moreover, in addition to hematopoietic stem cells, the compositions of the present invention also 25 stimulate the expansion of hematopoietic progenitor and precursor cells.

As the results in Table 9 of Example 1 show, a monoclonal antibody against the c-kit (SCF receptor) completely blocks the proliferative effects of human stem cell factor (SCF) on $CD34^+$ bone marrow cells. In contrast, this monoclonal antibody had no inhibitory effect on the ability of the

hematopoietic growth factor(s) present in the PMVEC supernatant to support CD34^{+} cell proliferation. Thus, these data indicate that the hematopoietic activity contained within the PMVEC supernatant is not porcine SCF.

By "PMVEC supernatant" is intended PMVEC cellular products which are secreted into the surrounding culture medium during PMVEC culture. Such cellular products include one or more hematopoietic growth factors which are capable of stimulating expansion of hematopoietic cells.

PMVEC can be isolated from the central nervous system of pigs. Preferably, the PMVEC are isolated from the brains of pigs using the procedure described in Robinson *et al.*, *In Vitro Cell. Dev. Biol. 26:*169 (1990). After isolation, the cells can be grown to confluency in culture flasks thereby providing a readily accessible source of PMVEC supernatant. PMVEC supernatant can be generated according to conventional culturing techniques. For example, PMVEC can be plated at approximately 1-2 X 10^6 cells and grown in a suitable culture medium such as endothelial cell culture medium (ECCM) which contains M199 media supplemented with 10% fetal calf serum, 50 μg/ml preservative-free heparin, 100 mM L-glutamine, 50 μg/ml gentamicin and 100 U/ml penicillin/streptomycin. After culturing the cells for approximately 1-3 weeks, the PMVEC supernatants are harvested and filtered to remove cell debris. For example, a 0.2 μm membrane can be used to filter the PMVEC supernatants.

In a recent study of lymphocyte transplantation, the investigators reported that recipient patients experienced Type III hypersensitivity reactions due to bovine serum proteins adherent to the infused cells. Thus, it is preferable that the bovine serum proteins be removed prior to using the PMVEC supernatant to stimulate expansion of hematopoietic cells. This will reduce the possibility of an adverse immune reaction in the event that hematopoietic stem or progenitor cells expanded according to the present invention are infused into a patient. Thus, after culturing the PMVEC for approximately 1-2 weeks, the cells can be washed and the ECCM replaced with a suitable serum-free medium (i.e., a medium which does not contain

bovine serum) which is capable of maintaining PMVEC growth. For example, the cells can be washed with a buffer solution such as PBS and the ECCM replaced with a serum-free medium consisting of Iscoves modified Dulbecco's media (IMDM) supplemented with 100 mM L-glutamine and 100 U/ml penicillin/ streptomycin. After an additional period of growth (e.g., one to two weeks), the PMVEC supernatants are harvested and filtered to remove cell debris as described above.

After filtration to remove cellular debris, the PMVEC supernatant can be either stored at 4°C for future processing or processed immediately by concentrating the proteins present in the supernatant by filtration. Membranes having a molecular weight cut-off of 30 kD are readily available. Filtering the supernatant with such a membrane concentrates proteins having a molecular weight of greater than about 30 kD. Membranes having different molecular weight cut-offs can also be used. As Example 1 shows, filtering PMVEC supernatant through a commercially available 30 kDa cut-off membrane results in an approximately 70-fold concentration of the remaining supernatant. The concentrated composition can then be stored at -20°C or used immediately to support hematopoietic cell proliferation. Thus, in one aspect, the present invention is directed to a composition comprising PMVEC supernatant which has been concentrated by filtration wherein the composition is capable of stimulating expansion of hematopoietic cells, including hematopoietic stem, progenitor, and precursor cells. Preferably, the composition of PMVEC supernatant is filtered to concentrate proteins having a molecular weight of greater than 30 kD.

In another aspect, the present invention is directed to the partial purification of the hematopoietic growth factor(s) present in the concentrated PMVEC supernatant. The inventors have discovered that greater than about 95% of the hematopoietic growth promoting activity in the PMVEC supernatant resides in proteinaceous fractions having a molecular weight of about 60-95 kD. The partially pure 60-95 kD proteinaceous fractions can be isolated from the PMVEC supernatant according to conventional

polyacrylamide gel electrophoresis (PAGE) techniques (Laemmli *et al.*, *Nature 227:*680 (1970)). For example, concentrated PMVEC supernatant can be loaded onto a 7.0% preparative PAGE gel, separated by electrophoresis, and stained with Coomassie blue to visualize gel bands which contain protein. Protein from gel slices can then be electro-eluted and the protein content quantitated using a conventional protein assay method (e.g., the Bicinchoninic Acid (BCA) assay). As indicated, using this procedure, the present inventors have discovered that greater than about 95% of the hematopoietic cell growth promoting activity in PMVEC supernatant is contained in a proteinaceous fraction which migrates at a molecular weight of about 60-95 kD. By "proteinaceous fraction of PMVEC supernatant" is intended a fraction of PMVEC supernatant which contains one or more proteins (as measured by a conventional protein assay method) capable of stimulating expansion of hematopoietic cells. Thus, the present invention is further directed to a composition comprising a proteinaceous fraction obtained from PMVEC supernatant, wherein the proteinaceous fraction has a molecular weight of about 60-95 kD and is capable of stimulating expansion of hematopoietic cells, including hematopoietic stem, progenitor, and precursor cells.

To further characterize the hematopoietic growth promoting activity in the PMVEC supernatant, eluted protein samples from the about 60-95 kD gel slices can be reduced and denatured according to conventional techniques and re-loaded on a PAGE gel for electrophoresis. For example, a 12% PAGE prepared as described in Laemmli *et al.*, *Nature 227:*680 (1970) can be used. After staining, gel slicing, and electro-elution as described above, the inventors have discovered that a proteinaceous fraction migrating at a molecular weight of about 60-70 kD contains a significant amount of hematopoietic growth promoting activity. Thus, the present invention is further directed to a composition comprising a proteinaceous fraction obtained from PMVEC supernatant, wherein the proteinaceous fraction has a molecular weight of about 60-70 kD and is capable of stimulating expansion of hematopoietic cells. As the results in Example 2 indicate (Figure 6), a single

band can be observed by PAGE migrating at a molecular weight of 68-70 kD. Thus, the invention is further directed to a composition comprising a proteinaceous fraction obtained from PMVEC supernatant, wherein the proteinaceous fraction has molecular weight of about 68-70 kD.

5 In an alternative embodiment, a proteinaceous fraction containing significant hematopoietic cell growth promoting activity can be partially purified from the PMVEC supernatant by isoelectric focusing. The purification of proteins by isoelectric focusing exploits the fact that proteins are ampholytes--that is, they contain both positively and negatively charged 10 groups. For every ampholyte, there exists a pH at which it is uncharged. This is called the isoelectric point. At the isoelectric point (pI), the ampholyte will not move in an electric field. If a protein solution is placed in a pH gradient, the molecules will move until they reach a point in the gradient at which they are uncharged; then they will cease to move. With a mixture of 15 proteins, each type of molecule will come to rest at a point in the pH gradient corresponding to its own isoelectric point. This method of separating proteins is well known in the art and is referred to as isoelectric focusing.

A pH gradient can be established by distributing a mixture of synthetic, low-molecular-weight (300-600) polyampholytes (multicharged structures) that 20 cover a wide range of isoelectric points, e.g., up to one thousand different isoelectric points per interval of one pH unit. These polyampholytes can be mixed polymers of aliphatic amino and either carboxylic or sulfonic acids. Mixtures of suitable polyampholytes are well known in the art. A pH gradient can be established with a mixture in distilled water of polyampholytes having 25 isoelectric points covering a range of, for example, three to ten pH units depending on the resolution required. When an electric field is applied, the polyampholytes will migrate. Because of their own buffering capacities, a pH gradient is gradually established.

In a typical apparatus for isoelectric focusing, the pH gradient is 30 formed in a water-cooled glass column containing a cathode tube and a cathode. Such an apparatus for isoelectric focusing is well known and

commercially available. The tube is filled with a uniform concentration of the polyampholyte solution which includes the sample to be purified. In the present invention, the tube can be filled with 50-100 mls of an aqueous solution which includes 2% w/v pH 3-10 ampholytes and PMVEC supernatant. A strong base (such as triethanolamine) is then added to the cathode tube and the main column of the apparatus is overlaid with phosphoric acid (the anode sits in this acid layer). During operation, the valve at the bottom of the tube is opened by application of sufficient voltage between the electrodes. After a sufficient period of time, the system will be at equilibrium and the proteins distributed throughout the pH gradient according to their own isoelectric points. As Example 4 indicates, the apparatus can be operated for 4 hours at a constant voltage of 500 V for an initial separation of the proteins present in the PMVEC supernatant.

The tube can then be drained through a stopcock at the bottom of the apparatus to fractionate the proteins. In a preferred embodiment, protein fractions from the separated PMVEC supernatant with pH of about 3.55-6.0 are pooled and re-introduced into the tube for an additional isoelectric focusing. After attaining a constant voltage of about 1200-1400 V, the tube is again drained and the proteins refractionated. The final protein concentration of each fraction can then be measured using a conventional assay for measuring the protein content of a sample.

To determine which of the IEF fractions contain the secreted PMVEC hematopoietic growth factor(s), a protein sample (e.g., 50-150 ng) from each fraction can be tested for its ability to stimulate growth of enriched $CD34^+$ cells *in vitro*. Using this procedure, the present inventors have discovered that the $CD34^+$ cell expansion activity correlates with protein with a pI of about 4.8-4.9 which migrates at about 60-70 kD as measured under reducing conditions by gradient (i.e., 8-16%) polyacrylamide gel electrophoresis. Thus, in another aspect, the present invention is directed to a composition comprising an isolated proteinaceous fraction of PMVEC supernatant, wherein the

proteinaceous fraction migrates at 60-70 kD and is capable of stimulating expansion of hematopoietic cells.

It has further been discovered that one or more inhibitors of hematopoietic growth promoting activity are secreted into the supernatant during the culture of the PMVEC. These inhibitory factors are present in the low molecular weight fractions (i.e., > 10 kD and < 30 kD) of PMVEC conditioned medium. Example 5 provides an experiment showing that a concentrated fraction of PMVEC conditioned medium, having a molecular weight of between about 10 kD and 30 kD, inhibits the ability of cytokines (i.e., GM-CSF + IL-3 + SCF + IL-6) to stimulate proliferation of $CD34^+$ cells. By "inhibitors of hematopoietic growth promoting activity" is intended components of the PMVEC supernatant having a molecular weight of > 10 kD and < 30 kD which at least partially inhibit the ability of certain cytokines to stimulate hematopoietic cell expansion. Preferably, the PMVEC supernatant is concentrated to eliminate or reduce the effect of such inhibitors. For example, treating PMVEC supernatant by filtration to concentrate proteins having a molecular weight of greater than about 30 kD results in a composition capable of supporting significant hematopoietic cell expansion.

As indicated, the compositions of the present invention can be used to stimulate growth of hematopoietic cells in liquid suspension culture. Thus, in contrast to prior teachings in the art, hematopoietic cells can be proliferated according to the present invention in the absence of PMVEC and stromal support cells. The compositions of the present invention can be used to stimulate expansion of various kinds of hematopoietic cells. These include hematopoietic stem, progenitor, and precursor cells. Thus, the invention contemplates contacting hematopoietic cells with a composition of the present invention for a sufficient amount of time to achieve hematopoietic cell expansion. In a preferred embodiment, the compositions of the present invention are substantially free of bovine serum. In another preferred embodiment, the hematopoietic cells are enriched for $CD34^+$ cells prior to culture. Umbilical cord blood cells, peripheral blood cells, and bone marrow

cells of mammals, including humans, nonhuman primates and mice, are mixed hematopoietic cell populations which include CD34$^+$ cells and can be obtained according to conventional techniques (Kennedy *et al., J Natl Cancer Inst 83 (13)*:921 (1991); Koller *et al., Biotechnology 11*:358 (1993)). Mononuclear cells (MNC) (which include CD34$^+$ cells) can be prepared from umbilical cord blood cells, peripheral blood cells, and bone marrow cells using conventional Ficoll-Hypaque density gradient centrifugation. For example, preparing MNC from cord blood and bone marrow using Ficoll-Hypaque density gradient centrifugation is described in Koller *et al., Blood 82*:378 (1993) and Koller *et al., Biotechnology 11*:358 (1993), respectively. The MNC themselves (without enriching for CD34$^+$ cells) can be treated with a sufficient amount of any of the compositions of the present invention to achieve the expansion of hematopoietic cells. For example, the present inventors achieved a significant *in vitro* expansion of hematopoietic cells by culturing ficoll-hypaque separated mononuclear bone marrow cells in the presence of PMVEC supernatant which had been filtered to concentrate proteins having a molecular weight of greater than about 30 kD (see Table 5 of Example 1). The starting inoculum of MNC which is sufficient to achieve hematopoietic cell expansion can easily be determined empirically. For example, a starting inoculum of 10^3-10^5 MNC/100 μl culture medium can be used.

An enriched population of CD34$^+$ cells can be prepared from umbilical cord blood cells, peripheral blood cells, and bone marrow cells from the above-described mammals according to conventional techniques. For example, the anti-CD34$^+$ monoclonal antibody cell sorting techniques described in Brandt *et al., J Clin Invest 86*:932 (1990); Edgington, S.M., *Biotechnology 10*:1099 (1992); and Srour *et al., Blood 81(3)*:661 (1993) can be used. Similarly, the avidin-biotin immunoaffinity process described in Berenson *et al., J Immunol Meth 91*: 11 (1986); Berenson *et al., Blood 67 (2)*:509 (1986); and Berenson *et al., Blood 69 (5)*:1363 (1987) can also be used for enrichment of CD34$^+$ cells. In a preferred embodiment, CD34$^+$ cells are enriched from

bone marrow cells according to the positive immunomagnetic selection technique described in Example 1.

For *in vitro* culture, the $CD34^+$ cells, as either an enriched or mixed cell population, are inoculated into a cell culturing apparatus or stored in liquid nitrogen for future use. Prior to inoculation, it is preferable to resuspend the $CD34^+$ cells in a suitable culture medium. However, if frozen, cryopreserved $CD34^+$ cells should first be thawed using standard techniques. For example, the cells can be thawed rapidly at 37°C and diluted in a suitable prewarmed (37°C) culture medium. Suitable culture mediums are described below.

The cells are resuspended in a suitable culture medium at an appropriate concentration. For example, a concentration of 1-2 x 10^3 cells/100 μl (or 1-2 x 10^4 cells/ml) of culture medium can be used. Other suitable concentrations can be determined empirically. The hematopoietic cells are then ready for inoculation. If an enriched or purified population of $CD34^+$ cells is used, as few as 10^2 - 10^3 $CD34^+$ cells/100 μl of culture medium can be inoculated for *ex vivo* expansion. Preferably, however, 2.5 x 10^3 $CD34^+$ cells/100 μl of culture medium are inoculated.

As indicated, by the invention, the hematopoietic cells are cultured in the presence of a composition obtained from PMVEC supernatant. The inventors have discovered that unconcentrated PMVEC supernatant does not demonstrate hematopoietic stimulatory activity in liquid suspension or methylcellulose clongenic cell assays. Thus, prior to use in the methods of the present invention, the hematopoietic growth factors present in the PMVEC supernatant must be concentrated. In one embodiment, the composition comprises PMVEC supernatant filtered to concentrate proteins having a molecular weight greater than about 30 kDa. In a second embodiment, the composition comprises a proteinaceous fraction of PMVEC supernatant which migrates at a molecular weight of about 60-95 kD. In a third embodiment, the composition comprises a proteinaceous fraction of PMVEC supernatant having a molecular weight of about 60-70 kD. In a fourth embodiment, the

composition comprises a proteinaceous fraction of PMVEC supernatant which migrates at 65-70 kD. In a fifth embodiment, the composition comprises a proteinaceous fraction of PMVEC supernatant which migrates at 68-70 kD.

5 The amount of the compositions of the present invention which is sufficient to stimulate significant hematopoietic cell expansion can be determined empirically. The inventors have discovered that culturing hematopoietic cells in the presence of culture medium supplemented with widely varying concentrations of PMVEC supernatant results in significant cellular proliferation. For example, as Table 3 of Example 1 shows, culturing 10 enriched $CD34^+$ cells in the presence of culture medium containing either 0.5%, 2.5%, 5.0% or 10.0% PMVEC 70X supernatant (i.e., PMVEC supernatant which has been concentrated 70-fold by filtration through a membrane having a 30 kD molecular weight cut-off) results in a significant hematopoietic cell proliferation.

15 If the compositions comprising isolated (partially pure) proteinaceous fractions are used (i.e., embodiments two through five above), the amount of protein that is sufficient to stimulate hematopoietic cell expansion can easily be determined using the $CD34^+$ cell proliferation assays described in the examples. The inventors have discovered that protein concentrations of 20 between 50-150 ng/4 ml medium (preferably 100 ng/4 ml) is sufficient to stimulate significant $CD34^+$ cell proliferation.

The hematopoietic cells should be cultured in the presence of a composition of the present invention for a sufficient amount of time to achieve hematopoietic cell expansion. The amount of time that is "sufficient" to 25 achieve expansion can easily be determined empirically. For example, the $CD34^+$ cell proliferation method described in Example 1 is useful for determining the amount of culturing time required to achieve cellular expansion. The inventors have discovered that culturing for 2 days achieves significant hematopoietic cell proliferation as determined by ^3H-thymidine 30 incorporation. Moreover, the inventors achieved a 3-5-fold expansion of $CD34^+$ cells after 7 days of culture with 50% of these cells expressing the

$CD34^+CD38^-$ phenotype. Thus, it will be recognized that different culture durations can be used depending on the exigencies of each experiment.

Any culture medium recognized in the art as appropriate can be used to support hematopoietic cell growth. In addition to one or more cytokines, the culture medium can be supplemented with serums such as fetal calf serum (FCS) and antibiotics such as Penicillin and Streptomycin. A particularly suitable medium is Isocove's Modified Dulbecco's Medium (IMDM). IMDM can be supplemented with 10% fetal bovine serum (FBS), 100 ug/mL L-glutamine, and 100 U/mL penicillin/streptomycin. This is referred to herein as complete culture medium. IMDM is readily available as are other tissue culture media.

Preferably, the compositions of the present invention are used in conjunction with one or more cytokines. A number of cytokines have been described in the literature (Moore *et al.*, *Proc. Natl. Acad. Sci. USA 84*:7134 (1987); Leary *et al.*, *Blood 71*:1759 (1988); Brandt *et al.*, *J. Clin. Invest. 86*:932 (1990); Kobayashi *et al.*, *Blood 78*:1947 (1991); Meunch *et al.*, *Blood 81*:3463 (1993); Bernstein *et al.*, *Blood 77*:2316 (1991); and Bodine *et al.*, *Blood 79*:913 (1992)). In particular, the following cytokines can be used in conjunction with the compositions of the present invention to stimulate expansion of hematopoietic cells: interleukin-1α (IL-1α), interleukin-1β (IL-1β), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-11 (IL-11), erythropoietin (EPO), leukemic inhibitory factor (LIF), PIXY-321, interleukin-7 (IL-7), thrombopoietin (TPO), FLK-2 ligand, interleukin-12 (IL-12), stem cell factor (SCF), and macrophage colony stimulating factor (M-CSF). These cytokines are readily available and can be used alone or in combinations of two or more. Preferably, GM-CSF, IL-3, SCF, and IL-6 are used.

Any suitable *in vitro* culturing method can be used to achieve the *ex vivo* expansion of hematopoietic cells according to the present invention. This

includes both cell-free static liquid suspension cultures and perfusion culturing systems (e.g., hollow fiber bioreactors).

A hollow fiber bioreactor consists of an outer shell casing that is biocompatible with the growth of mammalian cells, a plurality of semi-permeable hollow capillaries encased within the shell that are also biocompatible with the growth of mammalian cells on or near them, and the extracapillary space (ECS), which contains the cells and the ECS cell supernatant.

Tissue culture medium, which includes a composition of the present invention (e.g., concentrated PMVEC supernatant) and, if desired, additional cytokines, flows within the capillary lumens and is also included within the shell surrounding the capillaries. The tissue culture medium, which may differ in these two compartments, contains diffusible components that are capable of expanding hematopoietic cells. The operation and components of hollow fiber bioreactors are well known in the art (Knazek *et al.*, U.S. Patent Nos. 4,220,725, 4,206,015, 4,200,689, 3,883,393, and 3,821,087). United States Application No. 08/184,140 also provides a detailed description of hollow fiber bioreactors.

By the invention, after culture, or at periodic intervals during culture, nonadherent cells can be harvested for enumeration and analysis. The harvested cells can be enumerated and immunophenotyped using a hemacytometer and immunofluorescence staining according to conventional techniques. For example, flow cytometric analysis of subpopulations of cells labeled with monoclonal antibodies specific for cluster determinants (CD) antigens can be used. These CD antigens can be labeled using commercially available monoclonal antibodies conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Monoclonal antibodies specific for the CD34, CD38, CD20, CD14, CD3, CD4, CD16, CD15, HLA-DR, CD33, CD11b and CD8 antigens can be obtained from Becton Dickinson Monoclonals, San Jose, CA. After labelling the harvested nonadherent cells with the above monoclonal antibodies, flow cytometric analysis can then be

performed to determine whether subpopulations of cells bearing the CD antigens are present in the sample. Thus, for example, the presence of $CD34^+$ cells and primitive $CD34^+/CD38^-$ cells are determined in the harvested nonadherent cells by labelling the cells with anti-CD34 and anti-CD38 monoclonal antibodies and performing flow cytometry.

The presence of hematopoietic progenitor subpopulations (multipotent colony forming units [CFU-MIX]; colony forming unit granulocyte/macrophage [CFU-GM]; erythroid burst-forming units [BFU-E]; blast-colony forming units [CFU-Blast]; and megakaryocyte colonies [CFU-Mk]) in the harvested nonadherent cells can be determined by conventional techniques (Meisenberg *et al.*, *Blood* *79:*2267 (1992)). For example, methylcellulose CFC (colony-forming cell) assays can be used.

The *ex vivo* culture system of the present invention has been shown to support expansion (from inoculated $CD34^+$ cells) of primitive hematopoietic stem and progenitor cells having the phenotypic markers $CD34^+/CD38^-$. $CD34^+/CD38^-$ cells include primitive pluripotent cells capable of self-renewal, multilineage differentiation and reconstitution of the hematopoietic system. Thus, contrary to previously reported attempts where liquid suspension culture of $CD34^+$ cells resulted in depletion of stem cells, the present invention provides an *ex vivo* method for expanding stem cells to substantial numbers. For example, as indicated, the inventors achieved a 3-5-fold expansion of $CD34^+$ cells after 7 days of culture with 50% of these cells expressing the $CD34^+/CD38^-$ phenotype.

The *ex vivo* expansion system of the present invention has a variety of uses. These include providing a rich source of transplantable hematopoietic stem and progenitor cells, facilitating retroviral transduction of hematopoietic stem and progenitor cells, *ex vivo* maintenance of transplantable cells during which time cells can be assayed for pathogenic contamination, and facilitating the study of factors and conditions affecting hematopoiesis.

The compositions of the present invention can also be administered to subjects *in vivo* to stimulate growth of hematopoietic cells. Suitable subjects

are mammals, including mice, humans and non-human primates. The method involves administering to the subject a sufficient amount of any of the compositions of the present invention to simulate growth of hematopoietic cells. The amount of a particular composition which is sufficient to stimulate significant hematopoietic cell expansion *in vivo* can be determined empirically using animal models. For example, the inventors have discovered that subcutaneous administration of 200 μl of a 10-fold concentrated solution of PMVEC cell conditioned medium (serum-free) (produced as described in Example 1) to mammalian subjects (mice weighing, on average, 20 g) for 7 consecutive days results in significant increases in CFU-GM progenitor cell number in peripheral blood (3.9-fold), bone marrow (2.3-fold), and spleen (10.1-fold). Moreover, the absolute number of circulating neutrophils and lymphocytes increased 3.1-fold and 1.6-fold respectively. Finally, spleen cellularity in treated mice increased 2.8-fold.

In vivo administration of the compositions of the present invention can occur by any suitable technique, including, but not limited to, intravenous, subcutaneous, or intramuscular injection.

Having generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration and are not intended to be limiting.

Example 1

The Ability of PMVEC Supernatant to Stimulate Proliferation and Expansion of Hematopoietic Cells In Vitro

Materials and Methods

5 *Endothelial Cell Culture Conditions*

Porcine brain microvascular endothelial cells (PMVEC) were isolated and grown as previously described (Robinson *et al., In Vitro Cell. Dev. Biol. 26:*169 (1990)). The phenotypic and growth properties of these cells have been extensively characterized (Robinson *et al., In Vitro Cell. Dev. Biol.* 10 *26:*169 (1990) and Robinson *et al., Blood 77:*294 (1991)).

PMVECs were isolated from the brains of 4 to 6 month old Yucatan minipigs. The brains were collected aseptically, immersed in 10% povidone-iodine (Sherwood Pharmaceutical Co., Rahway, NJ) for 2 min and washed 5-6 times with Hank's balanced salt solution (HBSS) (Gibco, Grand Island, NY) 15 to remove the residual iodine. Gray matter of the cortices was aspirated through a Pasteur pipette, centrifuged for 10 min at 500 g (room temperature), resuspended in HBSS and homogenized in a 40 ml ounce homogenizer. The homogenate was centrifuged, resuspended and subsequently sieved through sterile nylon fabric of 149, 76, and 20 micro mesh size. The retained 20 microvessels were resuspended in 6 ml HBSS and spun through discontinuous Ficoll-Paque gradients (33%-67% and 67%-75%) (Pharmacia Inc., Piscataway, NJ). The pelleted microvessels were resuspended in 2 ml HBSS and 2 ml collagenase (1 mg/ml) (Worthington Biomedical, Freehold, NJ) was added. After 2 mins, the microvessels were washed with HBSS and plated in 25 16-mm wells coated with fibronectin (Pierce Chemical, Rockport, IL) in M199 media (Quality Biological Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum, 500 micrograms/ml sodium heparin (Sigma Chemicals, St.

Louis, MO), and 2-10 uL/ml retinal-derived growth factor (see Robinson *et al., In Vitro Cell. Dev. Biol. 26:*169 (1990)). The cultures were then grown at 37°C in air with 5% CO_2. After 7 days the cells were subcultured and subcloned. After subcloning, the PMVEC cell lines were grown in
5 complete culture medium.

PMVEC were fed weekly with complete medium. When confluent, PMVEC were washed with PBS, trypsinized (0.25 mg trypsin/mL, 5mM EDTA, 37°C, 10 minutes, Sigma, St. Louis, MO) and subcultured in a ratio of 1:5 into either 75 cm^2 flasks or a cellular concentration of 1 x 10^5 cells/well
10 in gelatin-coated 6-well, tissue culture plates (Costar, Cambridge, MA) containing 3 mL of complete culture medium supplemented with an additional 10% FCS. After 48-72 hr, the adherent PMVEC monolayers (70-80% confluent) were washed twice with complete culture medium to remove nonadherent PMVEC and the culture medium was replaced with 5 mL of
15 complete cell culture medium.

A cell bank of porcine brain microvascular endothelial cells (PMVEC, passage 19) was created from a vial of passage-21 PMVECs prepared as described in Robinson *et al., In Vitro Cell. Dev. Biol. 26:*169 (1990). The cells were subcultured by a 2-min incubation in 0.5% trypsin/0.2% EDTA in
20 Hank's balanced salt solution (GIBCO, Grand Island, NY), pelleted, and resuspended in endothelial cell culture medium (ECCM) containing M199 supplemented with 10% FCS, 50 μg/ml preservative-free heparin, 200 mM L-glutamine, 50 μg/ml gentamicin and 100 U/ml penicillin/streptomycin. Passage-22 PMVECs were cryopreserved (5 x 10^6 cells/1 mL vial) and stored
25 under liquid nitrogen. All experiments were performed with confluent endothelial cell monolayers from passages 23-35.

Endothelial Cell Conditioned Medium

PMVECs were plated at 2 x 10^6 cells/175 cm^2 in 30 ml of ECCM. After 7 days of culture, confluent PMVEC monolayers were washed 2X with

PBS and the culture medium was replaced with serum-free medium consisting of Iscoves modified Dulbecco's medium (IMDM) supplemented with 100 mM L-glutamine and 100 U/ml penicillin/streptomycin. The supernatants were harvested after 7 days, filtered through a 0.2 μm membrane to remove cell debris, and stored at 4°C. The PMVEC supernatant in serum-free medium is hereinafter referred to as PMVEC conditioned medium (CM). The PMVEC CM was then concentrated 70X by ultrafiltration using a YM-30 membrane (Amicon Corporation) having a molecular weight cut-off of 30 kDa. This allowed the removal of proteins and other substances having a molecular weight of less than 30 kDa. The 70X concentrated PMVEC CM was then passed through a 0.2 μm filter, aliquoted, and stored at -20°C. All batches of PMVEC CM were tested for their ability to support $CD34^+$ hematopoietic cell proliferation.

Preparation of Human Bone Marrow Cells

Human vertebral body bone marrow was procured from cadavers as described in the Naval Medical Research Institute publication no. 90--62 (available from the Defense Technical Information Center, AD# A226 538). Marrow was obtained from the bone matrix by sterile technique and placed in sterile culture support media. In some experiments, normal bone marrow cells were obtained from informed patients with nonhematopoietic disorders. Cells were aspirated from the posterior iliac crest into a syringe containing preservative-free heparin. Low density mononuclear cells were separated over ficoll-Hypaque (specific gravity 1.077 g/mL; Pharmacia Fine Chemicals, Piscataway, NJ) density gradients at 400 g for 30 min at 22°C. Low density cells at the interfaces were harvested, washed twice by centrifugation (400 g for 10 min) and resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT) 100 μg/mL L-glutamine (Gibco, Grand Island, NY) and 100 U/mL

penicillin/streptomycin (Gibco, Grand Island, NY). This culture medium is herein referred to as complete culture medium.

CD34$^+$ bone marrow progenitor cells were further purified by positive immunomagnetic selection using a monoclonal antibody specific for the CD34 antigen (K6.1). The monoclonal antibody K6.1 was produced by fusing SP-2/0-AG14 plasmacytoma cells (American Type Culture Collection (ATCC), Rockville, MD) with splenocytes from a BALB/cByJ mouse (Jackson Laboratory, Bar Harbor, ME) which had been hyperimmunized with viable KG-1a cells (ATCC, Rockville, MD). Injections containing 10 to 20 million KG-1a cells washed in saline were performed approximately monthly for a period of 6 months; the first and last immunizations were intravenous and the other immunizations were intraperitoneal. The last injection was performed 3 days prior to fusion. Cell hybridization and selection in HAT medium were performed according to previously described techniques (Kohler *et al.*, *Nature 256:*495 (1975); Fazekas de St. Groth *et al.*, *J. Immunol. Methods 35:*1 (1980); and Lane *et al.*, *J. Immunol. Methods 72:*71 (1984)).

Culture supernatants collected approximately 2 weeks after fusion were screened for antibody activity against MY-10/CD34 antigen in KG-1a cell lysates by immunoblot (Western blot) analysis. Initially, pools of about 10 growth positive hybridoma wells were screened, and individual wells of antibody positive pools were then screened. Antibody positive wells and were subcloned by limiting dilution (Oi and Hertzenberg, in *Selected Methods in Cellular Immunology*, (1980) Mishell and Shigii, eds, p. 351), and clones were screened the same way.

KG-1a cells were solubilized at 1 x 10^8 cells /ml in Laemmli sample buffer (0.0625 M Tris-HCl, pH 6.8) containing 0.5% Triton X-100 and 2 mM PMSF, and centrifuged (30,000 x g, 30 min), and the supernatants were reduced in 50 mM DTT, 4% SDS, and 10% glycerol (60 min, 37°C). Electrophoresis was performed on 8-16% pore-gradient, SDS polyacrylamide gels according to the method of Laemmli (*Nature 227:*680, (1970)), as modified by Jones (in *Selected Methods in Cellular Immunology*, (1980)

Mishell and Shigii, eds., pp. 398-440). Proteins were then transferred to nitrocellulose membranes for immunoblot analysis (Towbin *et al., Proc. Natl. Acad. Sci. USA 76:*4350 (1979) and Burnette, *Anal. Biochem 112:*195 (1981)), using alkaline phosphatase conjugated goat anti-mouse IgG antibody (BioRad labs, Richmond, CA) for detection with BCIP/NBT as substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

The hybridoma clone K6.1 was identified as producing a monoclonal antibody of the IgG2a isotype, as determined with an isotype screening ELISA kit (Zymed Laboratories, S. San Francisco, CA) on immobilized KG-la cells (Cobbold and Waldmann, *J. Immunol. Methods 44:*125 (1981)). The hybridoma was expanded in roller bottles in IMDM containing fetal calf serum (Hyclone). After supernatant harvesting, the K6.1 antibody was purified by hydroxylapatite chromatography (Stanker *et al., J. Immunol. Methods 76:*157 (1985)), followed by pH-gradient elution from protein A-Sepharose (Ey *et al., Immunochemistry 15:*429 (1978)). The yield of antibody was 40-45 μg/liter of supernatant. This was concentrated on ultrafiltration membranes (Amicon YM-10, Danvers, MA), and dialyzed into normal saline. Analysis of antibody purity was performed on 30-40 μg reduced and unreduced samples by SDS-polyacrylamide gel electrophoresis under Laemmli conditions, followed by Coomassie blue staining.

For positive immunoselection of $CD34^+$ cells, all cell washing, incubation and selection steps were performed at 4°C (unless noted otherwise) in 0.2 μm sterile filtered "immunoselection washing buffer." The immunoselection washing buffer consisted of Hanks's balanced salt solution containing 12.5 mM HEPES buffer, 1000 units/ml DNAse 1 (Calbiochem), and 5% heat-inactivated pooled human AS serum (#34004-1, Pel, Freez Clinical Systems, Brown Deer, WI). The human serum was previously dialyzed extensively (40 volumes x 5 changes) against PBS to remove traces of biotin. This was included as a source of human IgG to saturate Fc receptors and minimize cytophilic binding of the cell specific antibody (i.e., K6.1); for therapeutic immunoselection purposes, it was assumed that

substitutes such as dialyzed serum from the marrow donor or pharmaceutically approved gamma globulins for injection would be used.

Bone marrow mononuclear cells were washed and adjusted to a concentration of 50 x 10^6/ml. Biotinylated-K6.1 antibody was prepared by mixing purified K6.1 with $NaHCO_3$ to give a solution containing 3 mg antibody/ml in 0.1 M $NaHCO_3$. Biotin-N-hydroxysuccinimide ester (Calbiochem, La Jolla, CA) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 12 mg/ml, and 5.0 μl of this was added to each ml of antibody solution. After 1 hr at room temperature, NH_4HCO_3 was added to 50 mM final concentration to stop the reaction. The mixture was then passed through a Sephadex PD-10 column (Pharmacia) equilibrated in phosphate-buffered saline (PBS, 6.7 mM Na phosphate, pH 7.2, 137 mM NaCl) to desalt and exchange the buffer. The biotinylated-K6.1 antibody was added at a ratio of 6-10μg/ml of cell suspension and incubated with occasional mixing for 30 min at 4°C. The cells were then washed by centrifugation 3-4 times, and adjusted to a concentration of 25 x 10^6/ml.

DYNABEADS M-450 (Dynal Incorporated, Great Neck, NY) were activated with goat anti-biotin antibody. The number of DYNABEADS used was proportional to the total number of bone marrow mononuclear cells, using a ratio of 1 bead/10 cells. Anti-goat IgG DYNABEADS were washed magnetically 4-5 times with a rare-earth magnet. The beads were then suspended at a concentration of 1 x 10^8/ml in washing buffer containing 2.5 μg/ml affinity purified goat anti-mouse biotin antibody (#SP-3000, Vector Laboratories, Burlingame, CA), mixed vigorously for 30 min at room temperature, and then washed magnetically twice, and resuspended to 1 x 10^8/ml. This concentration of anti-biotin was optimized in preliminary titration studies using as an endpoint the kinetics of cell dissociation from the magnetic beads. The binding of anti-goat anti-biotin does not reach equilibrium under these conditions; varying the anti-biotin provided a convenient way to compensate indirectly for different cell surface antigen densities, by controlling the amount of biotin-anti-biotin crosslinking.

The bone marrow mononuclear cells, containing biotinylated-K6.1 antibody coated target cells, were incubated with the anti-biotin DYNABEADS for 30 min on a rotator (approx. 30 rpm). The magnetic $CD34^+$ cells were then selected by attraction to a samarium-cobalt magnet, and removal of non-target cells free in suspension. Finally, the magnetic $CD34^+$ cells were suspended in medium (e.g, IMDM) containing 2.5 mg/ml biotin, put on a rotator for 1-2 hr, and free $CD34^+$ cells were recovered from magnetically immobilized DYNABEADS. The magnetic separation can be accomplished using the magnetic separation device described in U.S. Patent No. 4,710,472 issued December 1, 1987 to Saur, Reynolds and Black.

Cells isolated by this procedure showed > 99% positive reactivity with a second CD34-specific monoclonal antibody, MY10 (HPCA-2) (Becton Dickinson Immunocytometry Systems, San Jose, CA), by flow cytometric analysis indicating that a highly purified population of cells expressing the CD34 surface membrane antigen was obtained which contained highly enriched hematopoietic stem cells, progenitor cells and essentially no mature blood cells.

Isolated $CD34^+$ bone marrow cells were cryopreserved (1-5 x 10^6 cells/1 ml vial) and stored under liquid nitrogen prior to experimentation. Before use the $CD34^+$ cells were thawed using standard techniques.

As an alternative to the above protocol, $CD34^+$ cells were also isolated from mononuclear cell preparations using a commercially available $CD34^+$ cell selector and protocols provided by CellPro (Bothell, WA.).

Processing of $CD34^+$ Cells for Culture

Cryopreserved $CD34^+$ cells were thawed rapidly at 37°C, diluted in a 10 X volume of prewarmed (37°C) complete culture medium. The thawed $CD34^+$ bone marrow cells were washed twice in complete culture medium, and resuspended at 1 x 10^6 cells/ml. Cell viability was > 99% as determined

by trypan blue dye exclusion (Coligan *et al.*, *Current Protocols in Immunology* (1992), Greene Publishing and Wiley-Interscience, New York).

At the start of experimentation, a sample of CD34$^+$ cells was cultured to determine the number and type of hematopoietic colony forming cells (CFC) using a methylcellulose colony forming assay (Meisenberg *et al.*, *Blood 79*:2267 (1992)). (CFU numbers were also quantitated using commercially available kits from Stem Cell Technologies, Inc., Vancouver Canada.) Briefly, purified CD34$^+$ bone marrow cells and nonadherent hematopoietic cells from harvested cultures were cultured in 35 mm Lux suspension culture dishes (Miles Laboratories, Naperville, IL) using a modification of the technique previously described (Meisenberg *et al.*, *Blood 79*:2267 (1992)). One milliliter of culture consisted of 5-500 x 10^2 bone marrow cells, IMDM medium (Quality Biologicals, Rockville, MD), 1% methylcellulose, 30% heat-inactivated fetal calf serum (FCS), 2 U/ml tissue culture grade erythropoietin (Amgen, Thousand Oaks, CA), 2 ng/ml GM-CSF, 10 ng/ml IL-3 (R&D Systems, Minneapolis, MN) and 5% conditioned medium from the bladder carcinoma cell line 5637 (ATCC, Rockville, MD). The conditioned medium from cell line 5637 was used as a source of colony stimulating activity. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. On day 14 of incubation, cultures were evaluated to determine the number of colonies (>50 cells) which had developed. At day 14, aggregates of hemoglobin containing cells were recognized as BFU-E; aggregates of granulocytes and/or macrophages and/or megakaryocytes as CFU-MIX; and aggregates containing only granulocytes and macrophages as CFU-GM.

CD34$^+$ Cell In Vitro Proliferation Assay

Incorporation of [^{3}H] thymidine (^{3}H-TdR) in liquid culture was used to assess cell growth and proliferation of purified CD34$^+$ bone marrow cells. CD34$^+$ cells (1-10 x 10^3/well) in complete culture medium were added to each well of a 96-well flat-bottom microtiter plate with the described cytokine

additions (200 ul/well final volume) and incubated at 37°C in a humidified 5% CO_2 in air atmosphere. After either 2 or 6 days of incubation liquid cultures were pulsed for 18 hr with 0.5 uCi [^{3}H]-TdR per well and then harvested onto glass fiber filters with a multiple automated sample harvester. [^{3}H]-TdR uptake was quantitated by liquid scintillation counting (LKB Pharmacia Beta Plate). All cultures were performed in quadruplicate. The results were expressed as mean cpm [^{3}H]-TdR incorporated/number of CD34$^+$ cells plated. (Cell number was also quantitated using a haemocytometer.)

Immunofluorescence Staining

Simultaneous two-color cytometric analysis of cultured nonadherent cells was performed at selected intervals of culture. Nonadherent cells were harvested, washed twice in complete culture medium, and resuspended in PBS supplemented with 2% heat-inactivated FCS, 2% (wt/vol) bovine serum albumin (BSA) and 0.1% sodium azide (staining medium). Cells were incubated for 30 minutes with saturating concentrations of either FITC-conjugated CD34 MoAb (HPCA-2, IgG1) plus PE-conjugated CD38 (Leu-17, IGg1) or FITC-conjugated CD15 (Leu-M1, IgM) (HPCA-2 FITC, Leu-17 PE, Leu-M1 FITC; Becton Dickinson Immunocytometry Systems, San Jose, CA) washed twice with staining medium and fixed with 1% paraformaldehyde. Cells stained with the appropriate conjugated isotype matched antibodies were used as controls. 10,000 events were collected in listmode on a Coulter Elite (Coulter, Hialeah, FL) flow cytometer.

Methylcellulose Clonogenic Cell Assays for Human Hematopoietic Progenitor Cells

Purified CD34$^+$ bone marrow cells and nonadherent hematopoietic cells from harvested cultures were cultured in 35 mm Lux suspension culture dishes (Miles Laboratories, Naperville, IL) using a modification of the technique as

previously described (Meisengerg *et al.*, *Blood 79:*2267 (1992)). 5-500 x 10^2 bone marrow cells were seeded into 1 mL of Iscoves IMDM medium (Gibco, Grand Island, NY), 1% methylcellulose, 30% heat-inactivated FCS, 2 U/mL tissue culture grade erythropoietin (Amgen, Thousand Oaks, CA), 2 ng/mL GM-CSF, 10 ng/mL IL-3 (R&D Systems, Minneapolis, MN) and 5% conditioned medium from the bladder carcinoma cell line 5637 (5 X concentrate) as a source of colony stimulating activity. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 14 days cultures were evaluated to determine the number of colonies (>50 cells) developed. At day 14, aggregates of hemoglobin containing cells were recognized as BFU-E, granulocyte-macrophage colonies as CFU-GM and aggregates of hemoglobin cells containing at least granulocytes and/or - macrophages and/or megakaryocytes as CFU-Mix. Morphological verification of selected colonies was determined using Wright-Giemsa stain. Triplicate assays were set up for each individual data point per experiment.

Blocking of C-Kit Receptor on $CD34^+$ Cells

MoAb SR-1 is a blocking monoclonal antibody against the human c-kit receptor. $CD34^+$ cells were preincubated with MoAb SR-1 (1:1000 dilution) for 1 hr at 37°C before incubation in liquid suspension cultures with either PMVEC CM or SCF as described above.

Results

Proliferative effects of PMVEC CM on $CD34^+$ cells

The effects of PMVEC CM, GM-CSF, IL-3, SCF, G-CSF, M-CSF, and IL-6 cytokine treatment on $CD34^+$ cell proliferation (^{3}H-TdR incorporation) were examined in liquid suspension cultures. The results showed that $CD34^+$ bone marrow cells, the purity which ranged between 85%

and 96%, are capable of extensive proliferation *in vitro* in response to PMVEC CM alone (Table 1).

Table 1. Effect of various PMVEC CM preparations on CD34$^+$ progenitor cell proliferation in liquid suspension cultures

Treatment	CPM per 10,000 CD34$^+$ cells
Experiment 1	
Media Control	2296
PMVEC CM 3/15/94	16073
PMVEC CM 3/18/94	13262
PMVEC CM 3/22/94	43964
PMVEC CM 3/30/94	10538
PMVEC CM 4/7/94	16263
PMVEC CM 4/12/94 (Cellmax Prep)	29993
Experiment 2	
Media Control	698
PMVEC CM 3/22/94	19391
PMVEC CM 5/25/94	7001
PMVEC CM 6/1/94	8797
Experiment 3	
Media Control	326
PMVEC CM 3/22/94	5877
PMVEC CM 6/7/94	5685
PMVEC CM 6/14/94	8263
PMVEC CM 6/25/94	7665
PMVEC CM 7/6/94	4356
PMVEC CM 7/27/94	11367
PMVEC CM 7/27/94 (50X) (Cellmax Prep)	5837

Purified CD34$^+$ cells (5000/200 μl of culture medium) were treated for seven days in liquid suspension cultures with either control medium or 5% PMVEC CM (70X). The dates indicate when a batch of PMVEC supernatant was harvested. Cultures were pulsed with 0.5 μCi/ml of ^{3}H-TdR during the last 18 hr of culture and harvested using a 96-well Cell harvester (MASH). Samples were analyzed using a Beta Plate scintillation counting system and the data represents mean level of ^{3}H-TdR incorporation $\pm$ 1SD from quadruplicate cultures.

Likewise, in a series of experiments, the results further showed that PMVEC CM alone as a single hematopoietic stimulant is more effective than either GM-CSF, G-CSF, M-CSF, IL-6, IL-3 or SCF alone in supporting $CD34^+$ stem cell proliferation (Table 2).

5

Table 2. Proliferative response of $CD34^+$ cells to PMVEC CM in comparison to other hematopoietic growth factors

Growth Factors	CPM per 5,000 $CD34^+$ cells
Media Control	261
PMVEC CM 3/22/94	15735
PMVEC CM 5/25/94	8785
PMVEC CM 6/20/94	14148
GM-CSF 2 ng/ml	4668
SCF 120 ng/ml	1353
IL-3 5 ng/ml	4289
IL-6 5 ng/ml	490
M-CSF 10 ng/ml	771
G-CSF 10 ng/ml	530
GM-CSF + IL-3	10051
GM-CSF + SCF	9561

10 15 20

Purified $CD34^+$ cells (5000/200 μl of culture medium) were treated for three days in liquid suspension cultures with PMVEC CM and various hematopoietic growth factors. The dates indicate when a batch of PMVEC supernatant was harvested. Cultures were pulsed with 0.5 μCi/ml of ^{3}H-TdR during the last 18 hr of culture and harvested using a 96-well Cell harvester (MASH). Samples were analyzed using a Beta Plate scintillation counting system and the data represents mean level of ^{3}H-TdR incorporation $\pm$ 1SD from quadruplicate cultures. This is a representative experiment among two repeated experiments.

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As shown in Table 3, total ^{3}H-TdR incorporation increased in a dose dependent fashion and reached a plateau at a final PMVEC CM concentration of 5%.

Table 3. Titration of hematopoietic activity in PMVEC CM

	CPM per 10,000 $CD34^+$ cells cultured			
	Final Concentration of PMVEC CM			
Treatment	**0.5%**	**2.5%**	**5.0%**	**10.0%**
PMVEC CM (3/15/94)	7401	13468	16514	16284
PMVEC CM (4/12/94)	12377	25378	28570	29031
Media Control	2296			

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Purified $CD34^+$ bone marrow cells were cultured in Iscoves IMDM supplemented with 10% FCS in the presence and absence of varying concentration of PMVEC CM for 7 days. The dates indicate when a batch of PMVEC supernatant was harvested. Cultures were pulsed with 0.5 μCi/ml of ^{3}H-TdR during the last 18 hr of culture and harvested using a 96-well Cell harvester (MASH). Samples were analyzed using a Beta Plate scintillation counting system and the data represents mean level of ^{3}H-TdR incorporation $\pm$ 1SD from quadruplicate cultures.

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Moreover, the addition of PMVEC CM to liquid suspension culture treated with GM-CSF, IL-3, GM-CSF+IL-3 and IL-3+SCF+IL-6 (Table 4) resulted in 4.6-fold, 3.0-fold and 3.2-fold increases in $CD34^+$ cell proliferation values, respectively. In contrast to these enhancing effects, proliferative responses to SCF were not significantly increased when co-cultured with PMVEC CM.

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Table 4. Proliferative response of $CD34^+$ cells to PMVEC CM in the absence and in the presence of other hematopoietic growth factors

	CPM per 5,000 $CD34^+$ cells		
Treatment	**Media Control**	**PMVEC CM (3/7/94)**	**PMVEC CM (3/15/94)**
Media Control	697	3238	6282
GM-CSF 2 ng/ml	3083	13929	17500
SCF 120 ng/ml	3246	3846	5742
GM-CSF + IL-3	6935	20806	23397
GM-CSF + SCF	14599	14197	17956
IL-3 + SCF + IL-6	8025	25557	24779
GM-CSF + IL-3 + SCF + IL-6	30388	27897	29908

Purified $CD34^+$ cells (5000/200 μl of culture medium) were treated for three days in liquid suspension cultures with various combinations of hematopoietic growth factors in the absence and presence of PMVEC CM. Cultures were pulsed with 0.5 μCi/ml of ^{3}H-TdR during the last 18 hr of culture and harvested using a 96-well Cell harvester (MASH). Samples were analyzed using a Beta Plate scintillation counting system and the data represents mean level of ^{3}H-TdR incorporation ± 1SD from quadruplicate cultures. This is a representative experiment among two repeated experiments.

PMVEC CM supports the proliferation of murine and rhesus monkey bone marrow cells

The *in vitro* hematopoietic activity of various batches of PMVEC conditioned medium was examined in liquid suspension cultures using rhesus monkey and murine ficoll-hypaque separated mononuclear bone marrow cells. The data depicted in Table 5 indicates that PMVEC CM alone as a single agent is capable of supporting the proliferation of both murine and rhesus monkey bone marrow cells in liquid suspension culture. PMVEC CM stimulation of rhesus monkey bone marrow cells was significantly greater than that detected with either human GM-CSF, IL-3 or SCF alone. In contrast, PMVEC CM was significantly less effective than murine GM-CSF on murine bone marrow cells.

Table 5. Proliferative responses of rhesus monkey and murine-hypaque separated mononuclear cells to PMVEC CM in comparison to other hematopoietic growth factors

Treatment	CPM per 10,000 $CD34^+$ cells
Rhesus monkeys bone marrow cells	
Media Control	591
PMVEC CM 3/7/94	5960
PMVEC CM 3/18/94	3130
PMVEC CM 4/12/94	3650
GM-CSF 2 ng/ml	1587
SCF 120 ng/ml	2562
IL-3 5 ng/ml	952
Murine bone marrow cells	
Media Control	356
PMVEC CM 3/7/94	7356
PMVEC CM 3/18/94	3646
PMVEC CM 4/12/94	5107
GM-CSF 2 ng/ml	11214
SCF 120 ng/ml	5342
IL-3 5 ng/ml	6904

Ficoll-hypaque separated mononuclear bone marrow cells (10,000/200 μl of culture medium) were treated for three days in liquid suspension cultures with PMVEC CM and various hematopoietic growth factors. The dates indicate when a batch of PMVEC supernatant was harvested. Cultures were pulsed with 0.5 μCi/ml of ^{3}H-TdR during the last 18 hr of culture and harvested using a 96-well Cell harvester (MASH). Samples were analyzed using a Beta Plate scintillation counting system and the data represents mean level of ^{3}H-TdR incorporation $\pm$ 1SD from quadruplicate cultures.

Testing PMVEC CM for hematopoietic cell colony-stimulating activity in methycellulose clonogenic cell assays

To determine whether concentrated PMVEC CM (70X, >30,00 Kd) contains colony-stimulating activity, various batches of serum-free PMVEC conditioned medium were screened in methycellulose clonogenic cell assays using purified human $CD34^+$ bone marrow cells. $CD34^+$ cells were cultured in 1% methycellulose supplemented with either 5% PMVEC CM+30% FCS or standard clonogenic cell medium containing potent sources of colony-stimulating activity. The results presented in Table 6 clearly show that PMVEC CM is able to stimulate CFU-C colony cell formation in cultures enriched for $CD34^+$ progenitor cells after 14 days of culture. $CD34^+$ cells cultured with PMVEC alone had a cloning efficiency of 4-17%, whereas $CD34^+$ cells treated with GM-CSF, IL3, SCF, IL-6, EPO and 5% 5637 CM had a cloning efficiency of 18%. It is important to mention that $CD34^+$ cells cultured in GM-CSF, IL-3, SCF, IL-6, EPO and 5% 5637 CM gave rise to large differentiated myeloid and erythroid colonies (>2000 cells/colony), whereas a significant proportion of the cells in cultures containing only PMVEC CM gave rise to small "blast cell like" colonies (100-500 cells/colony) with little evidence of differentiation. In addition, concentrated PMVEC CM (70X) <30,000 Kd had no significant hematopoietic cell activity. Results from titration experiments (Figure 1) demonstrate that total CFU-C increased in a dose-dependent fashion and reached a plateau at a final PMVEC CM concentration of 5%.

Table 6. Colony formation by CD34⁺ cells in the presence of PMVEC CM

Treatment	Number of CFU-C Colonies per 5,000 CD34⁺ cells
Media Control	0
PMVEC CM 3/7/94	143 ± 20
PMVEC CM 3/15/94	81 ± 21
PMVEC CM 3/22/94	344 ± 36
PMVEC CM 4/7/94	81 ± 19
PMVEC CM 4/12/94 (Cellmax Prep)	85 ± 23
PMVEC CM 3/22/93 <30,000 Kd	1 ± 1
Clonogenic Medium	353 ± 61

Purified CD34⁺ cells were assayed for CFU-C in either the presence of PMVEC CM alone as a source of hematopoietic cell activity or in the presence of clonogenic medium consisting of 2 ng/ml GM-CSF, 5 ng/ml IL-3, 120 ng/ml SCF, 4 U/ml EPO, and 5% 5637 conditioned medium. The dates indicate when a batch of PMVEC supernatant was harvested. CD34⁺ cells were cultured in methylcellulose supplemented with 30% FCS and colonies were scored on day 14. Data represents mean ± SD from quadruplicate cultures. This is a representative experiment among 3 repeated experiments.

The synergistic activity of PMVEC CM with other hematopoietic growth factors was examined using ficoll-hypaque separated human bone marrow mononuclear cells. PMVEC CM as a single agent was capable of supporting the growth of myeloid and erythroid bone marrow colony-forming cells (Figures 2-4). Furthermore, the addition of PMVEC CM to GM-CSF+IL-3 and GM-CSF+IL-3+SCF treated cultures supported significantly greater number of CFU-GM, CFU-Mix and BFU-E bone marrow progenitor cells in comparison to culture without PMVEC CM.

Replating potential of CD34⁺ bone marrow cells initially cultured in PMVEC CM versus standard hematopoietic culture medium

Purified CD34$^+$ bone marrow cells were cultured in methycellulose culture medium containing either PMVEC CM + 30% FCS or in standard clonogenic medium consisting of 30% FCS plus 2 ng/ml GM-CSF, 5 ng/ml IL-3, 120 ng/ml SCF, 5 ng/ml IL-6, 4 U/ml EPO, and 5% 5637 CM. After the first 14 days of culture, developed colonies were enumerated and cells from developed colonies were dispersed and replated over 10 dishes containing standard clonogenic medium. This replating process was continued every 14 days until no colony formation was evident. The results in Table 7 demonstrates that the initial treatment of CD34$^+$ bone marrow cells with PMVEC CM alone supports the outgrowth of primitive hematopoietic progenitor cells resulting in the formation of third and fourth generation colonies in standard clonogenic cell medium. In contrast, CD34$^+$ cells initially cultured in standard clonogenic medium generated only second-generation colonies. These results suggest that PMVEC CM hematopoietic activity is important in expansion and growth of primitive hematopoietic progenitor cells.

Table 7. Replating potential of CD34^{+} cells initially cultured in methylcellulose with PMVEC alone for 14 days vs CD34^{+} cultured in standard clonogenic cell medium supplemented with GM-CSF, IL-3, SCF, IL-6, EPO, and 5% 5637 CM.

5

Experiment 1

Initial Culture Condition	Generation	No. colonies/5000 CD34+ cells 1°	2°	3°	4°	5°
a.	PMVEC CM 3/15/94	344	900	255	124	9
b.	Standard clonogenic media	783	1238	0	0	0

10

Experiment 2

Initial Culture Condition	Generation	No. colonies/5000 CD34+ cells 1°	2°	3°	4°	5°
a.	PMVEC CM 3/22/94	125	285	128	14	0
b.	Standard clonogenic media	878	36	1	0	0

15 Purified CD34^{+} bone marrow cells were cultured in methylcellulose culture medium containing either PMVEC CM + 30% FCS or in standard clonogenic medium consisting of 30% FCS plus 2 ng/ml GM-CSF, 5 ng/ml IL-3, 120 ng/ml SCF, 5 ng/ml IL-6, 4 U/ml EPO and 5% 5637 CM. The dates indicate when a batch of PMVEC supernatant was harvested. After the first 14 days of
20 culture, developed colonies were enumerated and subsequently replated into methylcellulose cultures containing standard clonogenic cell medium. This replating process was continued every 14 days until no colony formation was evident.

Proliferation of purified CD34^{+} bone marrow cells in liquid suspension
25 ***cultures with PMVEC CM in combination with other hematopoietic growth factors***

In liquid cultures supplemented with PMVEC CM plus GM-CSF, IL-3, SCF, and IL-6, there was a 40.4 fold expansion of total nonadherent cells after 7 days of culture in comparison to the 18.5 fold expansion in cytokine treated
30 cultures without PMVEC CM (Figure 5). When PMVEC CM was added to culture medium in the absence of any other exogenous cytokines the total

number of nonadherent cells present in the culture after 7 days increased 5.5-fold over that of input levels.

Expansion of $CD34^+$ cells in liquid suspension cultures with PMVEC CM in combination with other hematopoietic growth factors

5 In cultures supplemented with PMVEC CM, GM-CSF, IL-3, SCF, IL-6, and EPO, a 21.5-fold increase in the total nonadherent cell number was observed after 7 days (Table 8). $CD34^+$ cells accounted for 21.6% of the total cell number. The absolute number of harvested $CD34^+$ cells was 4.76 x 10^5, a 4.76-fold increase when compared with the initial input number of 10 $CD34^+$ cells (1 x 10^5 cells). Among these cells, $CD34^+$ $CD38^-$ and $CD34^+$ $CD38^+$ cells were increased 41.4-fold and 2.71-fold, respectively. In contrast, no significant $CD34^+$ cell expansion was measured in cytokine treated cultures in the absence of PMVEC CM.

Table 8. Expansion of $CD34^+$ bone marrow cells in cytokine treated liquid suspension cultures in combination with PMVEC CM stimulation

Growth Factors	Absolute number of $CD34^+$ cells
Input	1×10^5
No PMVEC CM	
GM-CSF + IL-3 + IL-6	3.8×10^4
GM-CSF + IL-3 + SCF + IL-6	6.8×10^4
GM-CSF + IL-3 + SCF + IL-6 + G-CSF	9.2×10^4
GM-CSF + IL-3 + IL-6 + G-CSF	1.0×10^5
GM-CSF + IL-3 + IL-6 + EPO	8.8×10^4
GM-CSF + IL-3 + SCF + IL-6 + EPO	1.4×10^5
With PMVEC CM (5%)	
GM-CSF + IL-3 + IL-6	1.7×10^5
GM-CSF + IL-3 + SCF + IL-6	1.1×10^5
GM-CSF + IL-3 + SCF + IL-6 + G-CSF	1.7×10^5
GM-CSF + IL-3 + IL-6 + G-CSF	1.4×10^5
GM-CSF + IL-3 + IL-6 + EPO	3.7×10^5
GM-CSF + IL-3 + SCF + IL-6 + EPO	5.6×10^5

Purified $CD34^+$ cells (1×10^5 cells culture condition) were treated for seven days in liquid suspension cultures with various hematopoietic growths in the presence and absence of 5% PMVEC CM.

Hematopoietic activity in PMVEC CM not blocked when $CD34^+$ cells are treated with anti-ckit receptor MoAb, however SCF stimulation is completely suppressed

The results in Table 9 show that the monoclonal antibody (SR-1) against the c-kit receptor completely blocks the proliferative effects of SCF on $CD34^+$ bone marrow cells. Strikingly, SR-1 had no affect on PMVEC CM induced $CD34^+$ cell proliferation. These data indicate that the hematopoietic activity contained within PMVEC CM is not porcine SCF.

Table 9. Effect of anti-ckit receptor blocking MoAb on PMVEC CM induced CD34$^+$ progenitor cell proliferation.

	CPM per 10,000 CD34$^+$ cells	
Treatment	Isotype control MoAb	Anti-c-kit MoAb (SR-1)
Media Control	872	899
120 ng/ml SCF	5190	628
Sample 1 PMVEC CM 1/5/94	17528	10937
Sample 2 PMVEC CM 3/15/94	10921	10448
Sample 3 PMVEC CM 3/22/94	34518	31554
Sample 4 PMVEC CM 4/12/94 Cellmax	16008	17768

Purified CD34$^+$ bone marrow cells were preincubated for 1 hr at 37°C with MoAb SR-1 against the c-kit receptor (1:1000) and then cultured for 7 days with culture medium alone, 120 ng/ml SCF alone and with various batches of PMVEC CM (5%). Cultures were pulsed with 0.5 uCi/ml of ^{3}H-TdR during the last 18 hr of culture and harvested using a 96-well Cell harvester (MASH). Samples were analyzed using a Beta Plate scintillation counting system and the data represents mean level of ^{3}H-TdR incorporation ± 1SD from quadruplicate cultures.

Clonality of single CD34$^+$ bone marrow cells cultured in PMVEC CM

Results in Table 10 demonstrate that PMVEC CM as a single agent is capable of supporting the growth of individual CD34$^+$ bone marrow cells. Individual CD34$^+$ bone marrow cells were plated into 96-well flat-bottom plates with Iscoves IMDM supplemented with 10% FCS and 5% PMVEC CM or hematopoietic growth medium consisting of GM-CSF, IL-3, SCF, IL-6, and EPO in the presence and absence of PMVEC CM. After 14 days of culture, the wells were examined and scored for cell expansion. An average of 57% of the single cells plated in PMVEC CM alone gave rise to an expansion of cells. Moreover, in 13.5% of the wells (> 100 cells observed) cells appeared

as dispersed rounded translucent cells with a blast-like cell morphology as determined by morphological examination of cytospin preparations. Although PMVEC CM when co-cultured with hematopoietic growth medium did not significantly increase the plating efficiency of the individually plated $CD34^+$ cells, a greater number of these clones generated the dispersed colony growth pattern with a increased number of progeny cells.

The ability of generated cells from individual hematopoietic cell colonies to form second-fourth generation colonies upon replating has been used to probe for "self-renewal" or primitivity (Leary and Ogawa, *Blood 69:*953 (1987); Rowley *et al., Blood 69:*804 (1987)). Huang and Terstappen (Huang and Terstappen, *Blood 83:*1515 (1994)) showed that the $CD34^+$ progenitor population that gives rise to the dispersed growth pattern has myeloid as well as lymphoid potential and has the highest replating efficiency (third and fourth generation progeny) in the presence of hematopoietic growth factors.

Table 10. Growth of single $CD34^+$ bone marrow cells in PMVEC CM

Hematopoietic Growth factors	Frequency of positive cultures			
	> 10 cells per well	> 50 cells per well	> 100 cells per well	> 1000 cells per well
PMVEC CM alone	55/96	16/96	13/96	0/96
GM-CSF+IL-3+SCF+IL-6	60/96	44/96	31/96	7/96
GM-CSF+IL-3+SCF+IL-6+PMVEC CM	68/96	44/96	29/96	15/96

Single $CD34^+$ bone marrow cells were cultured in 100 ul of Iscoves IMDM + 10% heat-inactivated FCS supplemented with either 5% PMVEC CM alone, 2 ng/ml GM-CSF + 5 ng/ml IL-3 + 5 ng/ml IL-6 + 120 ng/ml SCF or GM-CSF+IL-3+SCF+IL-6 plus PMVEC CM. After 7 days of incubation, cultures were scored and cell numbers per culture were estimated by manual microscopy using inverted phase microscope (40X magnification).

Physiochemical properties of hematopoietic activity in PMVEC CM

The hematopoietic activity contained within PMVEC CM is relatively stable (Table 11). No significant changes in hematopoietic cell activity were measured when PMVEC CM was incubated at 56°C for 30 minutes, and approximately 50% of the activity was lost at 100°C for 10 min. Repeat freezing and thawing (3 times within 72 hr) resulted in approximately 25% loss of hematopoietic cell activity (data not shown).

Table 11. Heat stability of hematopoietic activity contained in PMVEC CM.

Treatment of PMVEC CM	CPM per 5000 $CD34^+$ cells
Untreated Media Control	157
Experiment 1	
Untreated PMVEC	2079
56°C, for 30 min	2464
100°C, for 10 min	1070
Experiment 2	
Untreated PMVEC	1631
56°C, for 30 min	1410
100°C, for 10 min	714
Experiment 3	
Untreated PMVEC	1406
56°C, for 30 min	1594
100°C, for 10 min	326

$CD34^+$ bone marrow cells were cultured for three days with 5% PMVEC CM (70X) from various preparations which were untreated or treated. Cultures were pulsed with 0.5 uCi/ml of ^{3}H-TdR during the last 18 hr of culture and harvested using a 96-well Cell harvester (MASH). Samples were analyzed using a Beta Plate scintillation counting system and the data represents mean level of ^{3}H-TdR incorporation $\pm$ 1SD from quadruplicate cultures. This is a representative experiment among three repeated experiments.

Example 2

The Partial Purification of Proteinaceous Fractions Capable of Stimulating Hematopoietic Cell Expansion

Materials and Methods

5 *PAGE Fractionation and Electroelution of PMVEC CM*

The apparent molecular weight of the biological active protein(s) was determined by fractionating PMVEC CM on 7.0% PAGE gels followed by electroelution of specific bands. Briefly, concentrated PMVEC CM (5 ul of 70X per lane) sample was analyzed by preparative polyacrylamide gel
10 electrophoresis (PAGE) according to the method described by Laemmli (*Nature 227*:680 (1970). Gel slabs were 1.5 mm thick and contained 7.0% (wt/vol) acrylamide. After electrophoresis for approximately 2 h, one lane of markers and an adjacent lane of PMVEC sample was stained with Coomassie blue R-250 to reveal the protein profiles of the PMVEC CM sample. Isolated
15 bands were cut from the intact gel, sliced into 2.0 mm sections, and each section was crushed in 2.0 ml of electroelution buffer (25mM Tris + 0.2 M glycine) and placed in dialysis tubing. Protein was extracted from gel slices using electroelution for 2 h. Eluted protein samples were dialyzed overnight at 4°C against D-PBS and concentrated using Amicon C10 concentrator to a
20 final volume of 0.2 ml. Marker proteins were myosin-H chain (200,00 daltons), phosphorylase b (97,400 daltons), bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (29,000 daltons), and beta-lactoglobulin (18,400 daltons). In all assays, protein content of the samples was measured by the BCA Protein Assay method (Pierce, Rockford,
25 IL), with bovine serum albumin as a standard.

Preparation of Size Fractions on a Sizing Column

100 mls of the conditioned medium were concentrated on an Amicon C30 Centriprep concentrator to a final volume of 2.0 mls. 1.0 ml of the concentrate was passed over a Pharmacia Superdex HR 10/30 gel filtration column at 0.5 mls/min in 0.4 M $LiClO_4$ for 50 minutes. The 3 fractions that corresponded to a size range of 10-30 kDa were pooled together, and the 5 fractions that included proteins > 50 kDa were pooled. These were dialyzed overnight in the cold (4°C) against D-PBS, and concentrated down to a final volume of 1 mls on an Amicon C10 concentrator. The fractions were then used in this experiment at a dose level of 10%. The results are as shown below.

Results

Partial purification of the hematopoietic activity in PMVEC CM:

Serum-free PMVEC CM was concentrated 70-fold by Amicon ultrafiltration using a 30,000 MW cutoff membrane. Concentrated PMVEC CM was applied to a regular (7.0% polyacrylamide) preparative PAGE gel. Results in Table 12 show that the proteins eluted from the gel slices between 60,000 and 95,000 MW contain > 95% of the hematopoietic activity. Positive samples from eluted PAGE gel slices were then analyzed by SDS-PAGE. Results in Figure 6 demonstrate that only a single band was observed in lane 3, which was loaded with sample eluted from the 68-70 kD MW

PAGE gel slice. We estimate the molecular weight of this partially purified protein to be 68-70 kD.

Table 12. Fractionation of PMVEC CM on PAGE polyacrylamide gels.

Fraction	CPM per 5000 CD34$^+$ cells
Media Control	326
Unfractionated PMVEC CM	19,391
68-70 kd band	4405
79-95,000 Kd bands	7680
100,000 Kd band	530
>100,000 Kd bands	424

CD34$^+$ bone marrow cells were with PMVEC CM protein samples eluted from protein bands separated on PAGE gels and unfractionated PMVEC CM (5%). After 6 days of liquid suspension culture, cells were pulsed with 0.5 uCi/ml of ^{3}H-TdR during the last 18 hr of culture and harvested using a 96-well Cell harvester (MASH). Samples were analyzed using a Beta Plate scintillation counting system and the data represents mean level of ^{3}H-TdR incorporation ± 1SD from quadruplicate cultures.

Example 3

The Ability of PMVEC Supernatant to Stimulate Proliferation and Expansion of Hematopoietic Cells In Vivo

Materials and Methods

Mice

BALB/C, female mice were purchased from Jackson Laboratories, Bar Harbor, Maine, and housed 5 to 10 mice per cage. Animals were maintained on a 12-h light/dark cycle and were allowed food (Wayne Roden Blox) and water ad libitum. Mice 12-14 weeks old were used for these studies. Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council".

PMVEC CM infusion

Mice were injected (average weight, 20 g) with 200 ul of 10X PMVEC CM solution (>30,000 Kd), or with 200 ul of concentrated Iscoves IMDM (10X, >30,00 Kd) daily for 7 consecutive days.

5 *Peripheral blood cell counts*

Peripheral blood was obtained from metaphane-anesthetized mice by cardiac puncture using heparinized syringes fitted with 22-gauge needles. White blood cell, red blood cell, and platelet counts were enumerated using a Baker Hematology Series Cell Counter System. In addition, blood smears
10 were prepared and stained with Diff-Quick (Bayer Healthcare Corp, McGaw Park, IL). Morphological WBC differential cell counts were obtained by oil immersion microscopy. At least 200 cells per slide were analyzed.

Murine hematopoietic clonogenic cell assays

Mice were sacrificed by cervical dislocation, and the femurs and
15 spleens were excised. Cells were flushed from the femurs with 5 ml of PBS containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan Utah) and dispersed through a 25-gauge needle until a single-cell suspension was obtained. Spleens were pressed through stainless-steel mesh screens, and the cells were washed from the screens with 5 ml of medium. Cell concentrations
20 were determined by hemacytometer counts.

Bone marrow and splenic CFU-GM were assayed in 35 mm Lux dishes (Miles Laboratory, Naperville, IL). Briefly, one milliliter of culture consisted of 5-15 X 10^4 cells, Iscoves IMDM (GIBCO, Grand Island NY), 1% methylcellulose, 30% heat inactivated fetal calf serum (FCS), 2 U/ml tissue
25 culture grade erythropoietin (Amgen, Thousand Oaks, CA), 30 U/ml rmGM-CSF, and 300 U/ml rmIL-3 (Genzyme, Boston MA). Cultures were incubated

at 37°C in a humidified atmosphere of 5% CO_2 in air. On days 7 and 14 of incubation, the number of CFU-GM colonies were determined *in situ*.

Results

Hematology profile of PMVEC treated normal mice

5 BALB-C mice were injected (average weight, 20 g) with 200 ul of PMVEC CM (70X) (diluted 1:7) (>30,000 Kd), or with 200 ul of concentrated Iscoves IMDM (10X, >30,00 Kd) daily for 7 consecutive days (Table 13). PMVEC-CM treatment for 7 days was associated with a significant 2-fold increase in the number of peripheral blood white blood cell 10 count (WBC). The absolute number of circulating neutrophils and lymphocytes increased 3.1-fold and 1.6 fold respectively. Spleen cellularity in PMVEC treated mice increased 2.8-fold, whereas bone marrow cellularity remained unchanged. No significant differences in the red blood cell count, hematocrit and platelet count between the uninjected controls, carrier injected 15 control and PMVEC CM treated mice were detected.

In comparison to untreated control mice, significant increases in CFU-GM progenitor cell number were seen in the peripheral blood (3.9-fold), bone marrow (2.3-fold), and spleen (10.1-fold).

Table 13. Cellularity and absolute number of CFU-GM progenitor cells in normal and PMVEC-CM-treated mice.

Tissue	Cellularity per tissue	Absolute Number of CFU-GM per tissue
Normal PB	8.6×10^6	54.3
Normal Spleen	139.4×10^6	5.37×10^3
Normal Bone Marrow	21.3×10^6	11.83×10^3
PMVEC CM PB	16.1×10^6	211.5
PMVEC CM Spleen	382.1×10^6	53.73×10^3
PMVEC CM Bone Marrow	21.4×10^6	26.50×10^3

* Peripheral blood values based on 1 ml of peripheral blood.

Example 4

Purification by Isoelectric Focusing of a 60-70 kDa Proteinaceous Fraction of PMVEC Supernatant having Hematopoietic Activity

Materials and Methods

Preparation and Concentration of PMVEC Supernatant

PMVEC CM was produced as described in Example 1 above in an Artificial Capillary Module (Asahi AMPE-M), that was washed with ethanol, followed by washes with deionized water, then coated with Pronectin™F (50 μg/ml) and sterilized by autoclaving. 500 mls of the PMVEC CM were collected. Aliquots of the PMVEC CM were concentrated either 37X or 11X using an Amicon Centriprep 10 column. 6 mls of the 37X PMVEC CM and 9 mls of the 11X PMVEC CM were combined and added to 40 mls of dI water with 2% w/v pH 3-10 ampholytes (BioRad) for a final volume and concentration of 55 mls and 6.6X PMVEC CM, respectfully. This corresponded to a final protein concentration of approximately 66 μg/ml.

Isoelectric Focusing Purification

The 55 mls of 6.6X PMVEC CM (approximately 3.58 mg of protein) were loaded onto a Rotofor™ isoelectric focusing device (BioRad). After operating the Rotofor™ for 4 hours until a constant voltage of 500 V was attained, the apparatus was harvested and the pH of every fraction was measured. Fractions with a PH of 3.58 - 6.00 were pooled and refractioned in the Rotofor™ until a constant voltage of 1300 V was attained. The pH of every fraction was again measured. All fractions were then brought up to 1M NaCl and allowed to sit for 20 min at room temperature to strip off any ampholytes bound to the proteins. The fractions from the isoelectric focusing step were then dialyzed against physiological buffer overnight in the cold (4°C). Samples were then concentrated 10X using a 10 kDa molecular weight cut-off Centricon 10 column (Amicon).

Following the concentration step, the individual fractions were assayed for biological activity in cultures of $CD34^+$ cells. Biological activity (change in number of $CD34^+$ cells) was correlated with the protein sizes and abundance in the isoelectrically focused fractions which was determined by electrophoresis on denaturing (SDS) gels as follows. The molecular weight of proteins present on the stained gels was determined using a protein molecular weight standard from Novex, Inc. (San Diego, CA), containing proteins of known molecular weights: Aprotinin (6 kDa), Lysozyme (14 kDa), Trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), Lactate dehydrogenase (36.5 kDa), glutamic dehydrogenase (45.4 kDA), bovine serum albumin (69 kDA), phosphorylase B (97.4 kDa), β-glycosidase (116.3 kDa) and myosin (200 kDa). The isoelectric focused fractions were denatured by diluting them 1:1 in sample buffer (125 μM Tris-HCl, pH 6.8, containing 2% SDS) and loaded on Tris-Glycine SDS 8-16% gradient gels.

After electrophoresis, the gels were stained with Coomassie Blue or Silver Staining (BioRad) to detect protein bands. Certain isoelectrically focused fractions were pooled together: 3 and 4; 8 and 9; 11, 12 and 13; 14;

and 16. The final protein concentration for each of the above pooled fractions was then measured using a BioRad assay ((Bio-Rad) with a bovine serum albumin standard).

$CD34^+$ Cell Expansion

5 $CD34^+$ cells were isolated to 70% purity using a Ceprate-LC-34 cell selector (CellPro). 100 ng of protein from each of the pooled fractions was added to 4 ml IMDM + 10% Fetal Calf Serum + the following cytokines: IL-3 (5 μg/ml), IL-6 (5 μg/ml), SCF (120 μg/ml), and GM-CSF (1 μg/ml) and 30,000 of the isolated $CD34^+$ cells. Culturing was performed in duplicate in 10 6 well cluster dishes (Falcon). After 7 days of culture, the total number of $CD34^+$ cells was determined using a fluorescence activated cell sorter (FACS analysis). Total cell number was quantitated using a hemocytometer.

Results

Fractionation and Refractionation Profile

15 The pH of every fraction produced from the 6.6X PMVEC CM was measured after operating the Rotofor™ for 4 hours, until a constant voltage of 500 V was attained. Fractions with a pH of 3.58 - 6.00 were pooled and refractioned by operating the Rotofor™ until a constant voltage of 1300 V was attained. The pH of every fraction was again measured. The profile of the 20 fractionation and refractionation is shown in Figure 7. Note that the uppermost refractionation curve should be read against the secondary (right-hand side) Y-axis.

IEF and SDS gradient gels

pH measurements were performed on the fractions recovered from the isoelectric focusing run in the Rotofor™. The fractions were adjusted to 1M NaCl and allowed to stand for 40 minutes to dissociate any bound ampholyte reagent. The samples were then dialyzed against phosphate buffered saline (Ca^{++}/Mg^{++} free, containing 0.1% glycerol) using 3500 molecular weight cut-off dialysis tubing (Spectra, Inc.). This operation was conducted overnight at 4°C. Each fraction was analyzed for protein content by performing SDS-gel electrophoresis (8-16% Tris-glycine SDS gradient gels, Novex Mini-Cell device) following sample concentration 10-fold using a centricon C30 column (Amicon). The gels were developed for 2 hours at 125 Volts, 50 Watts, and 31 mAmps/gel. The gels were stained either with Coomassie Blue or silver stain to visualize the protein bands (see Figs. 8 and 9).

Figure 8: lane 1 - fraction 11, pI 4.8; lane 2 - fraction 12, pI 4.9; lane 3 - fraction 13, pI 4.94; lane 4 - fraction 14, pI 5.00; lane 5 - fraction 15, pI 5.1; lane 6 - molecular weight standard; lane 7 - fraction 16, pI 5.2; lane 8 fraction 17, pI 5.36; lane 9 - fraction 18, pI 5.44; lane 10 - unfractionated PMVEC CM.

Figure 9: lane 1 - fraction 3, pI 4.01; lane 2 - fraction 4, pI 4.10; lane 3 - fraction 5, pI 4.16; lane 4 - unfractionated PMVEC CM; lane 5 - fraction 6, pI 4.20; lane 6 - fraction 7, pI 4.25, lane 7 - molecular weight standard, lane 8 - fraction 8, pI 4.45; lane 9 - fraction 9, pI 4.55; lane 10 - fraction 10, pI 4.65.

The fractions in which proteins were detected on the SDS gels (Coomassie Blue or Silver Staining) are indicated in Table 14.

Table 14. **Proteinaceous Fractions of PMVEC CM**

Fraction #	pI	Protein Size (kDa)
3, 4	4.01, 4.10	Doublet at 45
8, 9	4.45, 4.55	Doublet at 45
11, 12, 13	4.80-4.94	Doublet at 45, band at 68 and 33
14	5.00	Band at 33, 68 and >150
16	5.2	Singlet at 45, doublet at 68

$CD34^+$ Cell Expansion

30,000 isolated $CD34^+$ cells (70% pure) in culture medium (4 ml IMDM, 10% FCS) were added to a 6 - well culture dish (Falcon). Cytokines (IL-3 (5 μg/ml), IL-6 (5 μg/ml), SCF (120 μg/ml) and GM-CSF (1 μg/ml) and 100 ng of protein from one of the pooled fractions listed below were then added to the culture. This was repeated for each of the pooled fractions. The ability of the fractions to stimulate $CD34^+$ cell expansion was determined by counting the total number of $CD34^+$ cells after 7 days of liquid suspension culture using a FACS analyzer (Becton Dickinson). The results are provided in Table 15.

Table 15 **Expansion of $CD34^+$ bone marrow cells in cytokine treated liquid suspension cultures in combination with proteinaceous fractions from PMVEC CM**

Culture	Total # of $CD34^+$ Cells	Fold Expansion After 7 Days of Culture
Cytokines (IL-3, IL-6, SCF and GM-CSF) only	10,800	9.0 +/- 0.54
Cytokines + protein from fractions 3 and 4	12,360	10.3 +/- 0.4
Cytokines + protein from fractions 8 and 9	9,000	10.0 +/- 1.0
Cytokines + protein from fractions 11, 12 and 13	38,700	17.0 +/- 1.2
Cytokines + protein from fraction 14	26,130	13.0 +/- 0.9
Cytokines + protein from fraction 16	16,200	9.0 +/- 1.3

The results show a stimulation in total cell expansion and in the absolute numbers of $CD34^+$ cells. Moreover, the $CD34^+$ cell expansion activity correlated with the relative amount of the about 60 - 70 kDa protein in each of the pooled fractions. The ability of the different fractions to stimulate $CD34^+$ cell expansion is shown graphically in Figures 10 and 11. The fractions are described in terms of pI in Figure 10 and in terms of fraction number in Figure 11.

Example 5

The Presence of Inhibitory Factors in Low Molecular Weight Fractions of PMVEC Conditioned Medium

Preparation and Concentration of PMVEC Supernatant

PMVEC CM was produced as described in Example 4 above in an Artificial Capillary module (Asahi). 500 mls of the PMVEC CM were collected and concentrated 40X using an Amicon Centriprep 10 column and an Amicon Centriprep 30 column. This provided a concentrated fraction

containing proteins having molecular weights of between about 10 kD and 30 kD. 50 μl of this 40X concentrated fraction was assayed for its effect on CD34$^+$ cell expansion.

CD34$^+$ Cell Expansion

5 40,000 isolated CD34$^+$ cells (70% pure) in culture medium (4 ml IMDM, 10% FCS) were added to a 6-well culture dish (Falcon). Either cytokines alone (control) (IL-3 (5 ng/ml); IL-6 (5 ng/ml); SCF (120 ng/ml); and GM-CSF (1 ng/ml)) or the cytokines plus 50 μl of 40X concentrated PMVEC conditioned medium (m.w. $>$10 kD and $<$ 30kD) was added to the
10 culture. CD34$^+$ cell expansion was determined by counting the total number of nonadherent after 7 days of culture using a FACS analyzer (Becton Dickinson). The results shown in Figure 12.

It will be appreciated to those skilled in the art that the invention can be performed within a wide range of equivalent parameters of composition,
15 concentrations, modes of administration, and conditions without departing from the spirit or scope of the invention or any embodiment thereof.

The disclosures of all references, patent applications and patents recited herein are hereby incorporated by reference.

What Is Claimed Is:

1. A method for stimulating the expansion of hematopoietic cells, comprising:

contacting hematopoietic cells with porcine microvascular endothelial cell (PMVEC) supernatant for a sufficient amount of time to achieve hematopoietic cell expansion;

wherein said PMVEC supernatant comprises concentrated proteins having a molecular weight of greater than about 30 kD.

2. A method for stimulating the expansion of hematopoietic cells, comprising:

contacting hematopoietic cells with a proteinaceous fraction of PMVEC supernatant for a sufficient amount of time to achieve hematopoietic cell expansion;

wherein said proteinaceous fraction has a molecular weight of about 60-95 kDa.

3. The method of claim 2, wherein said proteinaceous fraction has a molecular weight of about 60-70 kDa.

4. The method of claim 3, wherein said proteinaceous fraction has a molecular weight of about 65 - 70 kDa.

5. The method of claim 4, wherein said proteinaceous fraction has a molecular weight of about 68-70 kDa.

6. The method of claim 1 or 2, wherein the hematopoietic cells comprise $CD34^+$ cells.

7. The method of claim 6, wherein $CD34^+$ cells are $CD34^+CD38^-$ cells.

8. The method of claim 1 or 2, wherein the hematopoietic cells comprise progenitor cells selected from CFU-GM, CFU-Mix, BFU-E, CFU-Blast, or CFU-Mk.

9. The method of claim 1 or 2, wherein the hematopoietic cells are isolated from a source selected from bone marrow, peripheral blood, or umbilical cord blood.

10. The method of claim 1 or 2, wherein the hematopoietic cells are cultured in the presence of at least one cytokine.

11. The method of claim 10, wherein said at least one cytokine is selected from IL-1, IL-1α, IL-1β, G-CSF, GM-CSF, IL-3, IL-6, IL-11, erythropoietic, LIF, PIXY-321, SCF, IL-7, TPO, FLK-2, IL-12, or M-CSF.

12. The method of claim 11, wherein said cytokines are GM-CSF, IL-3, SCF, and IL-6.

13. The method of claim 1 or 2, wherein said hematopoietic cell expansion occurs *ex vivo*.

14. The method of claim 1 or 2, wherein said hematopoietic cell expansion occurs *in vivo*.

15. The method of claim 13, wherein said expansion occurs in the absence of stromal or porcine support cells.

16. The method of claim 13, wherein said expansion occurs in a hollow fiber bioreactor.

17. A composition comprising porcine microvascular endothelial cell (PMVEC) supernatant, wherein:

5 (a) said supernatant comprises concentrated proteins having a molecular weight of greater than about 30 kDa; and

(b) said supernatant is capable of stimulating expansion of hematopoietic cells.

18. The composition of claim 17, wherein said PMVEC supernatant

10 is substantially free of bovine serum.

19. A composition comprising a proteinaceous fraction of PMVEC supernatant, wherein:

(a) said proteinaceous fraction has a molecular weight of about 60-95 kDa; and

15 (b) said proteinaceous fraction is capable of stimulating expansion of hematopoietic cells.

20. The composition of claim 19, wherein said proteinaceous fraction has a molecular weight of about 60-70 kDa.

21. The composition of claim 20, wherein said proteinaceous

20 fraction has a molecular weight of about 65-70 kDa.

22. The composition of claim 21, wherein said proteinaceous fraction has a molecular weight of about 68-70 kDa.

23. The composition of claim 19, wherein said proteinaceous fraction is substantially free of bovine serum.

24. A method for the production of concentrated porcine microvascular endothelial cell (PMVEC) supernatant capable of stimulating expansion of hematopoietic cells, comprising:

(a) culturing the PMVEC in culture medium capable of supporting PMVEC growth;

(b) harvesting supernatant produced during step (a); and

3. filtering said harvested supernatant to concentrate proteins having a molecular weight of greater than about 30 kD.

25. The method of claim 24, further comprising:

(d) fractionating said supernatant to produce a proteinaceous material having a molecular weight of about 60-95 kDa.

26. The method of claim 25, wherein said proteinaceous material has a molecular weight of about 60-70 kDa.

27. The method of claim 26, wherein said proteinaceous material has a molecular weight of about 65-70 kDa.

28. The method of claim 27, wherein said proteinaceous material has a molecular weight of about 68-70 kDa.

29. A method for producing serum-free PMVEC supernatant capable of stimulating expansion of hematopoietic cells, comprising:

(a) culturing PMVEC in bovine serum-free culture medium capable of supporting PMVEC growth;

(b) harvesting supernatant produced during step (a); and

(c) fractionating said harvested supernatant to produce a proteinaceous fraction capable of stimulating expansion of $CD34^+$ cells.

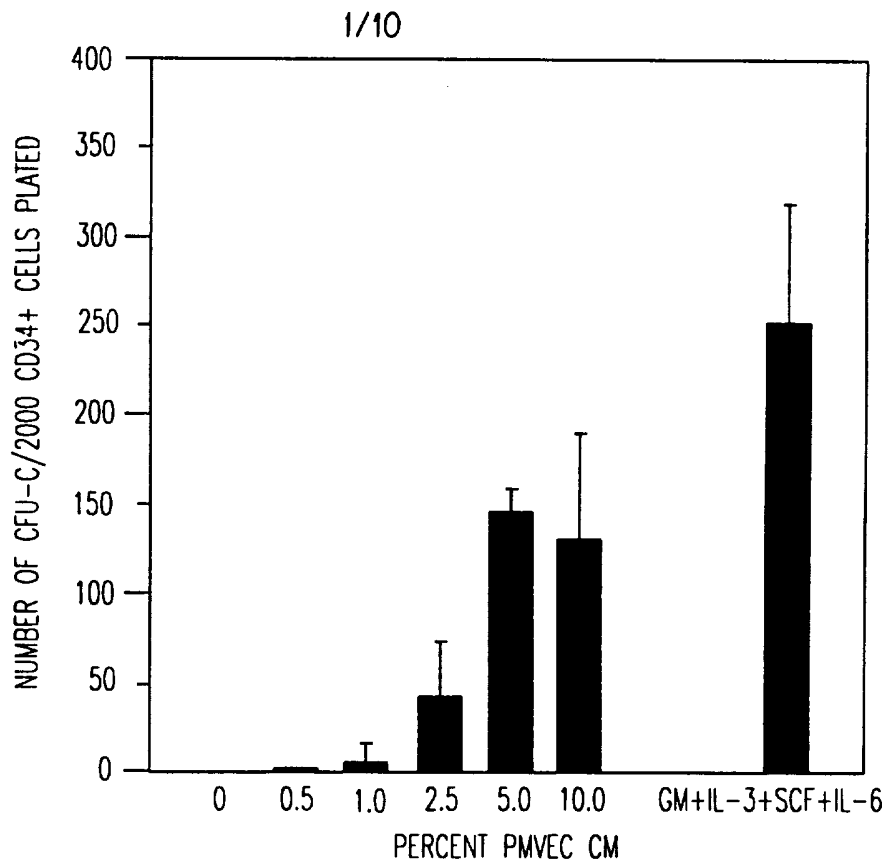


FIG.1

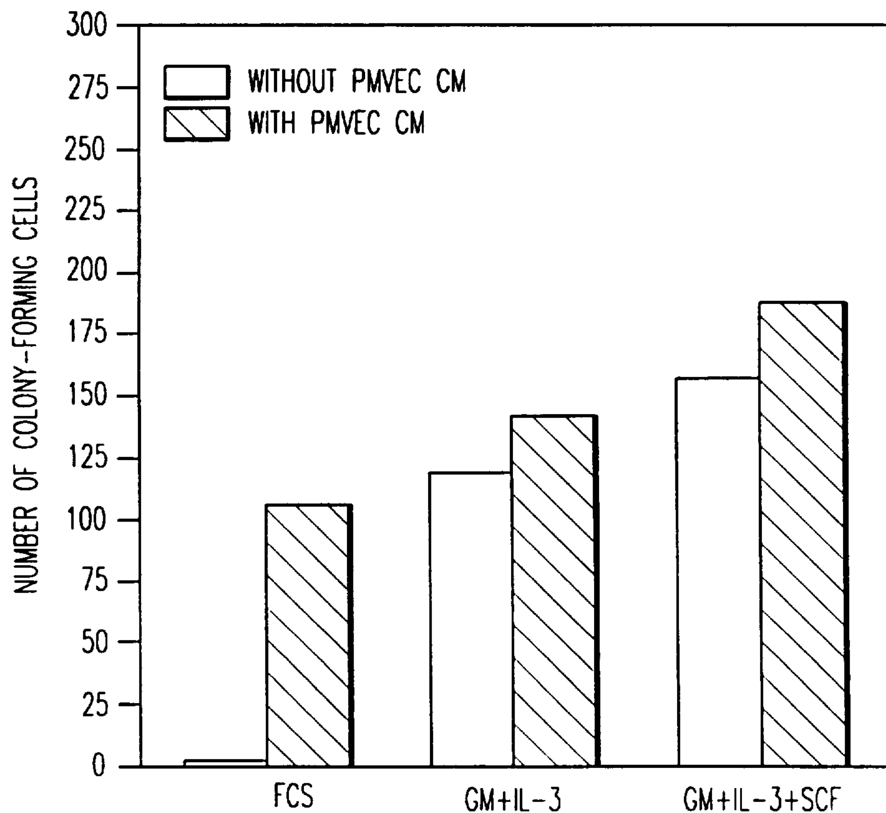


FIG.2

FIG.3

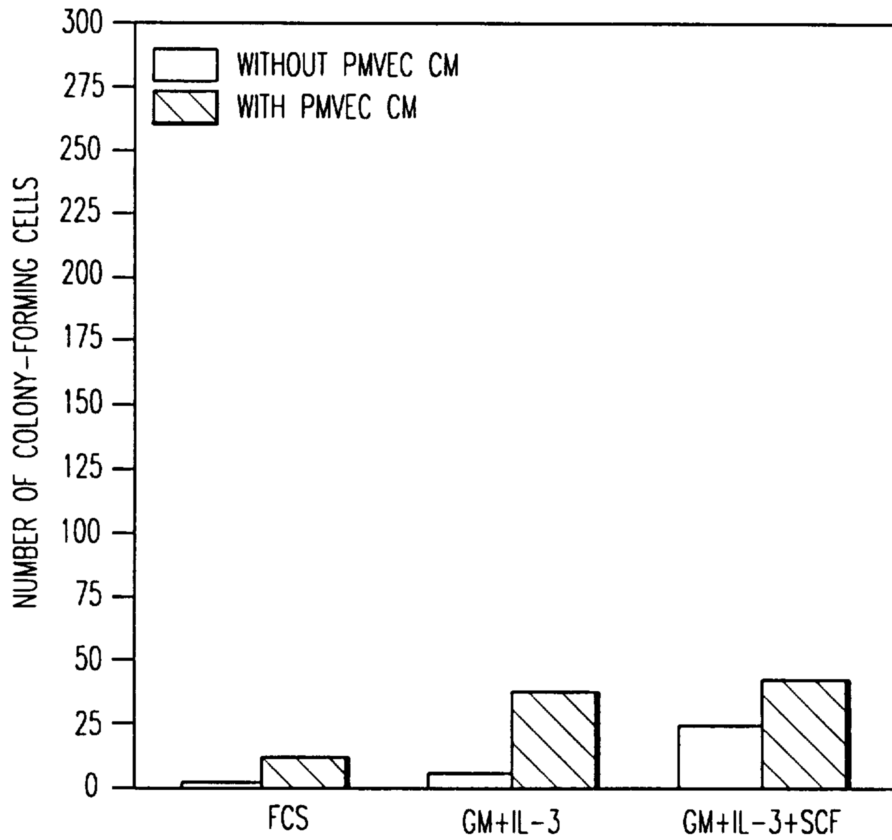
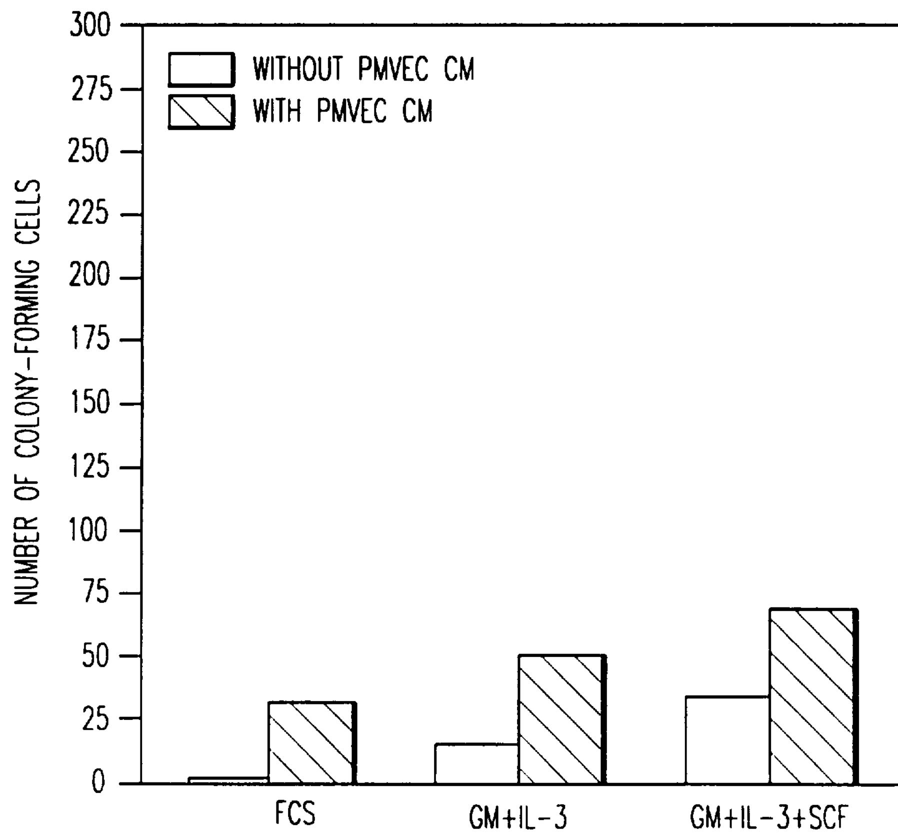


FIG.4



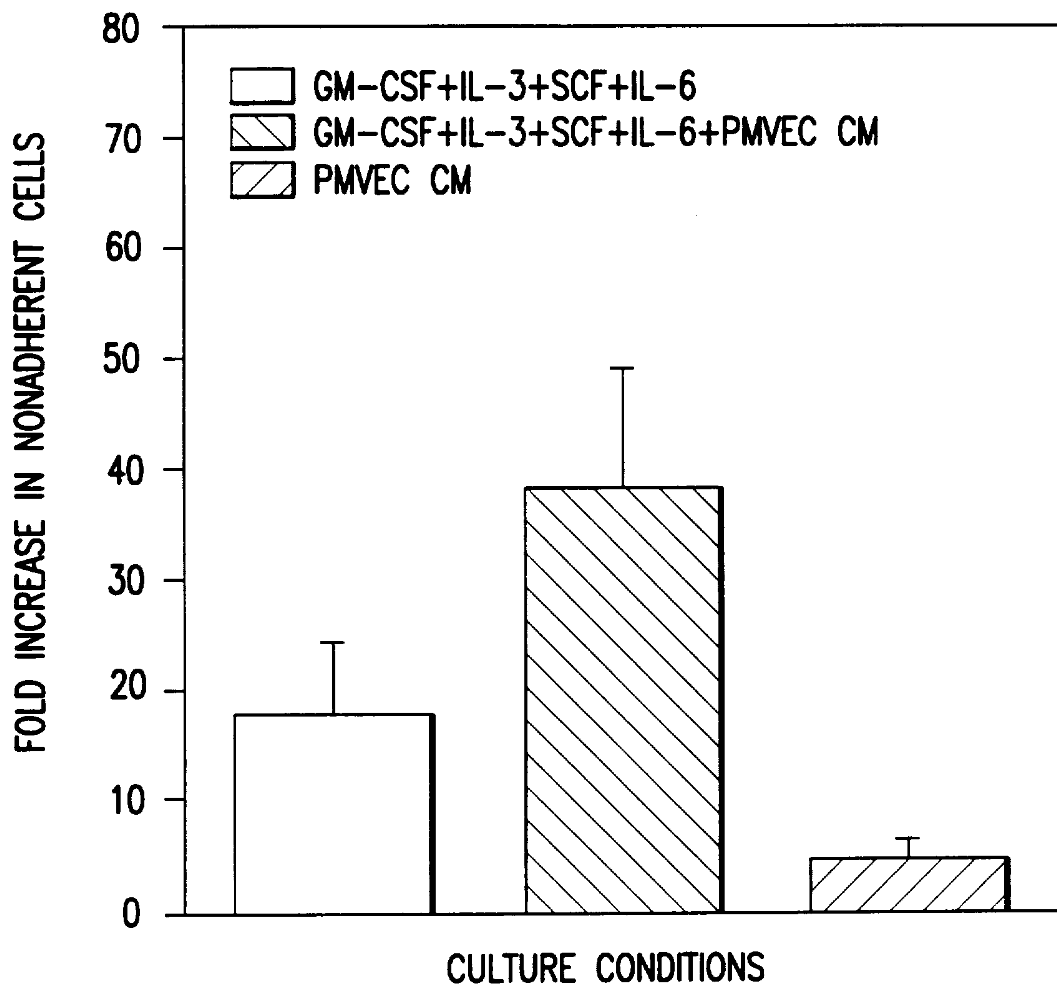


FIG.5

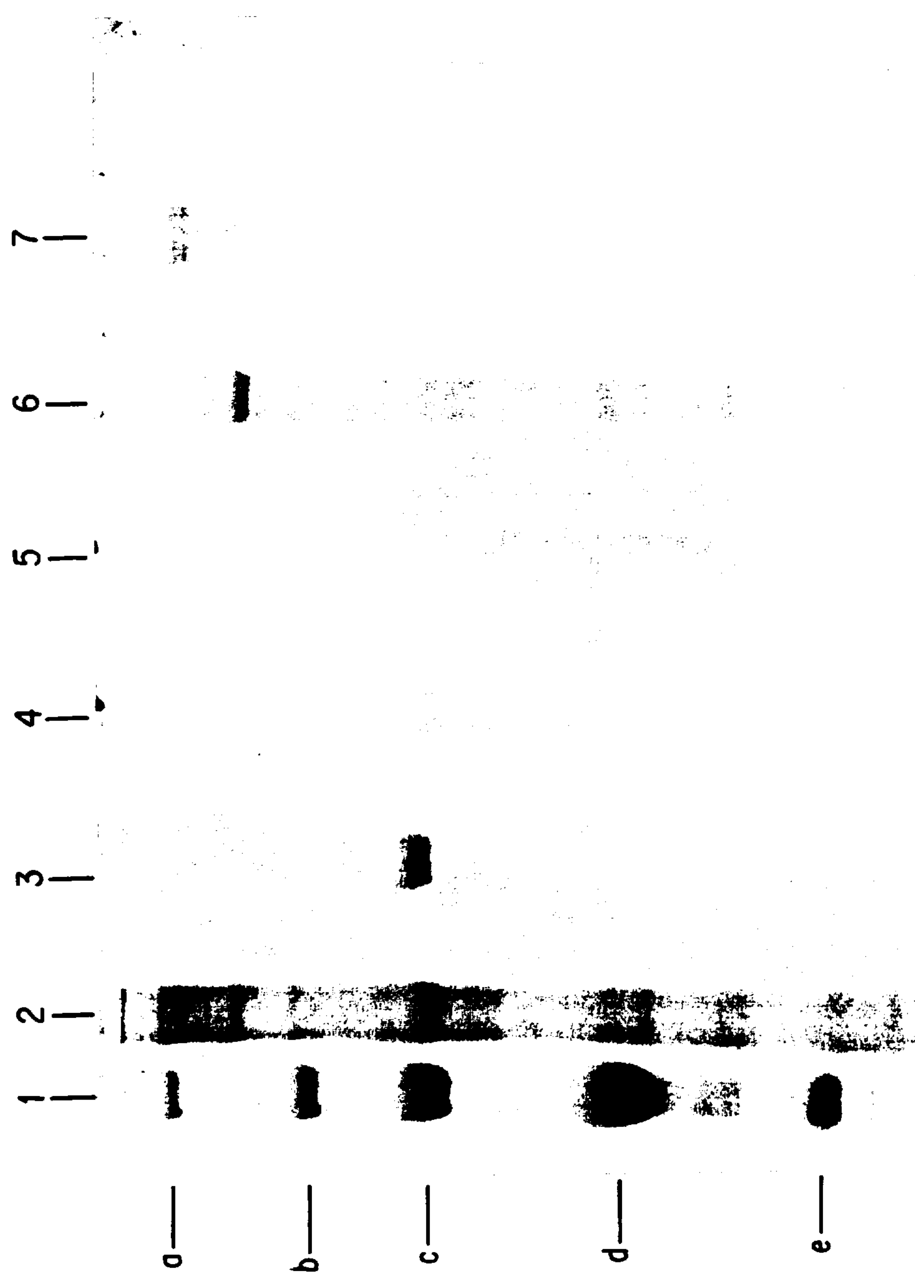


FIG.6

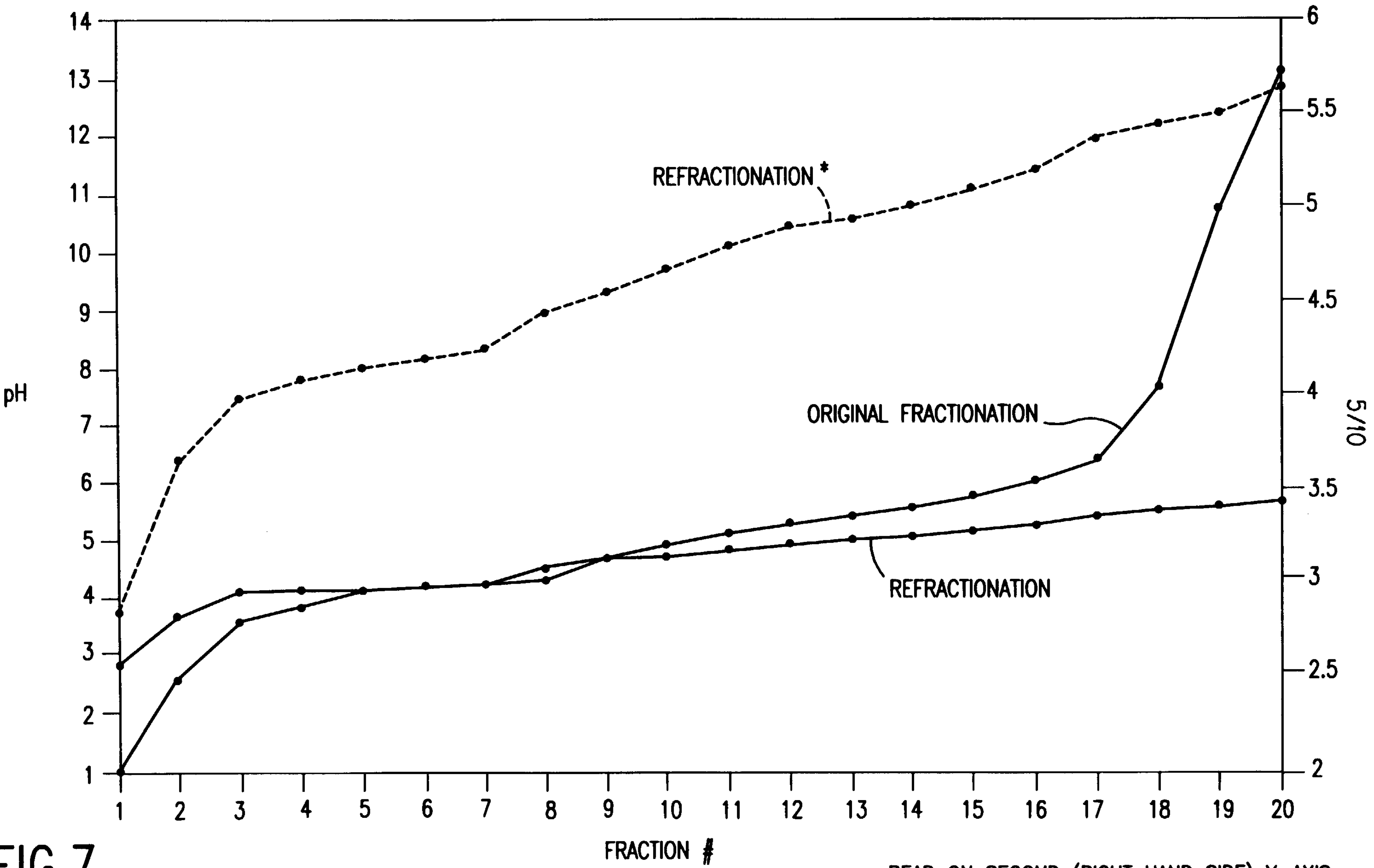


FIG.7

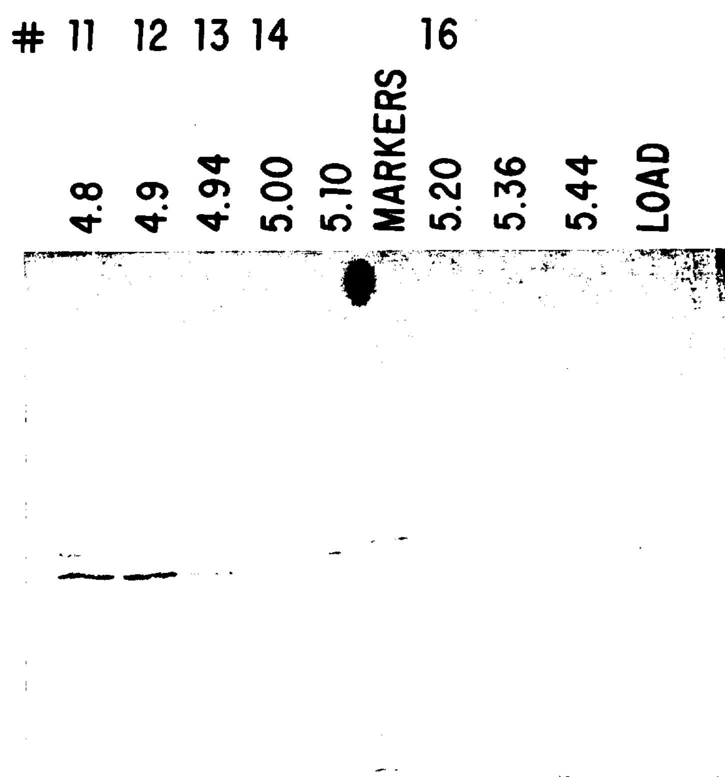


FIG. 8

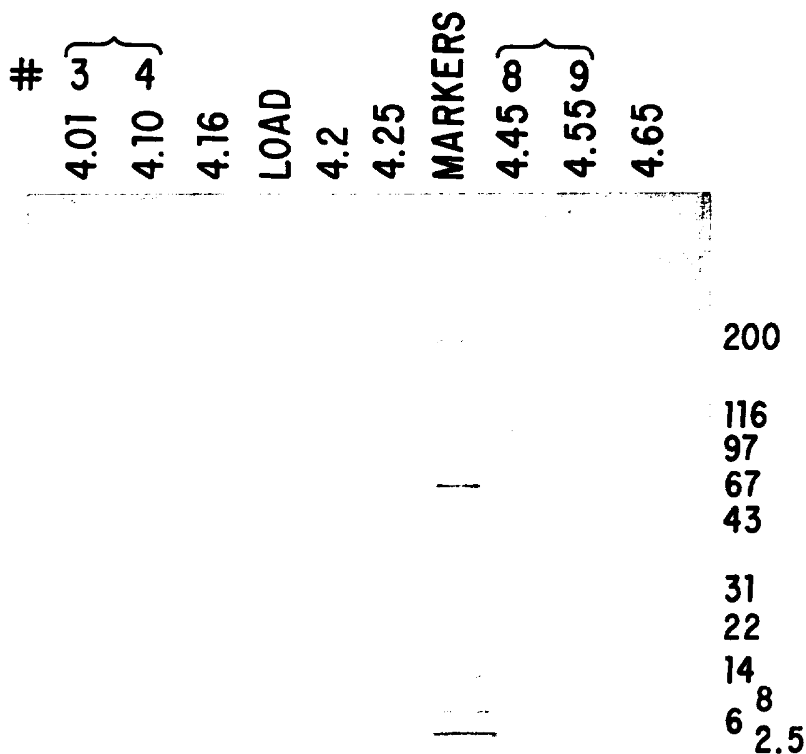


FIG. 9

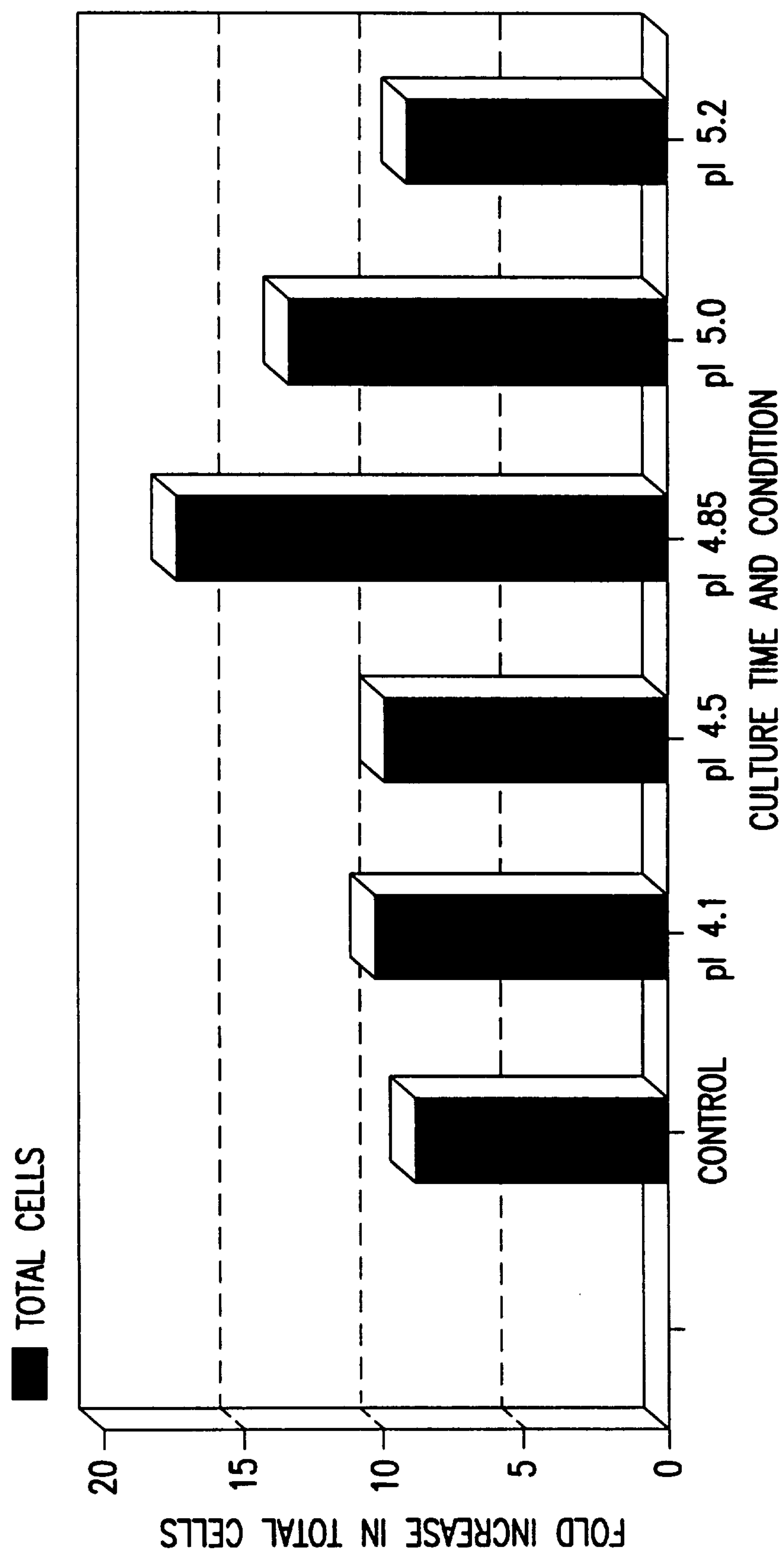


FIG.10

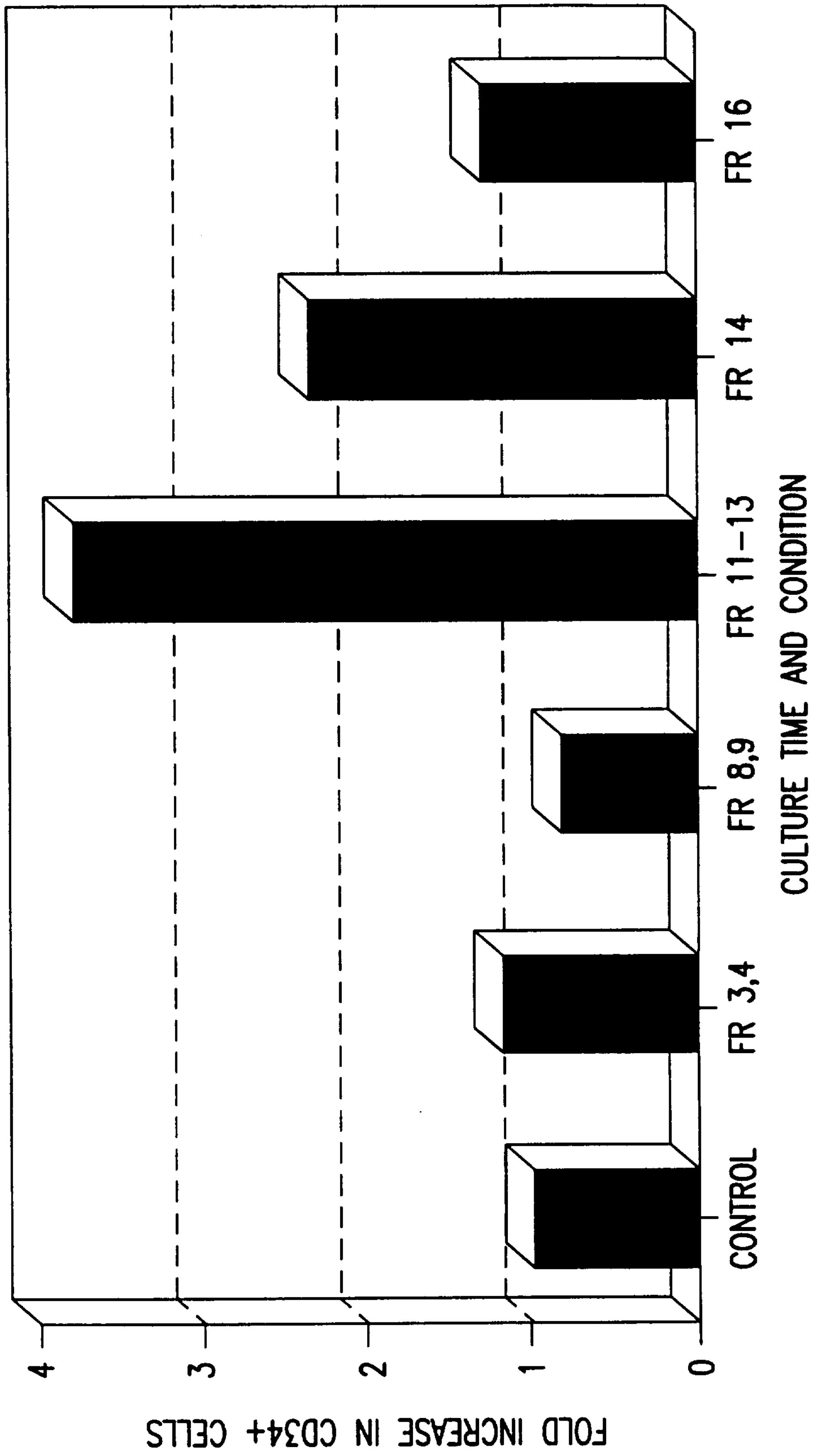


FIG.11

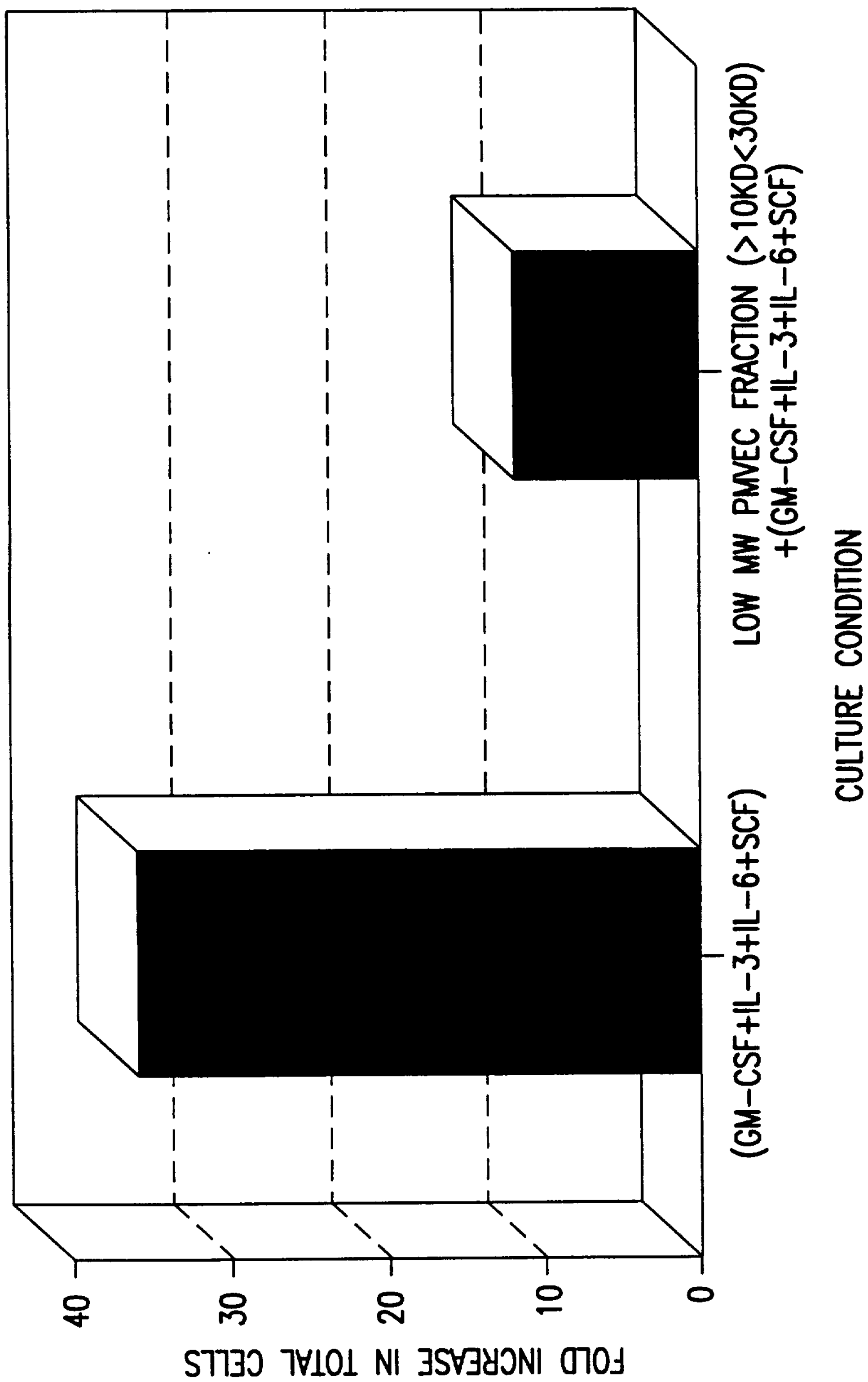


FIG.12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16350

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00; C12P 21/04; A61K 35/30, 35/12
US CL :435/240.1, 240.2, 240.3, 240.31, 70.1, 70.3; 424/570, 520
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)

U.S. : 435/240.1, 240.2, 240.3, 240.31, 70.1, 70.3; 424/570, 520

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

none

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

APS, DIALOG
search terms: porcine microvascular endothelial, hematopoietic, stroma

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Exp. Hematol., Volume 22, No. 8, issued 1994, Gupta et al., "BONE MARROW STROMA SPECIFIC PROTEOGLYCANS IN COMBINATION WITH SUBLIMINAL CONCENTRATIONS OF CYTOKINES SUPPORT THE IN VITRO MAINTENANCE OF HUMAN HEMATOPOIETIC STEM CELLS", p 767, abstract 333.	1-29
A	Clinical Research, Volume 41, No. 3, issued 1993, Gupta et al., "SOLUBLE FACTORS DERIVED FROM HUMAN HEMATOPOIETIC PROGENITORS AND PRECURSORS REGULATE CYTOKINE PRODUCTION BY HUMAN BONE MARROW STROMA", page 644A, abstract.	1-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 MARCH 1996	25 MAR 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Susan M. Dadio
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. Exp. Med., Volume 179, issued February 1994, Verfaillie et al., "Macrophage Inflammatory Protein 1α, Interleukin 3 and Diffusible Marrow Stromal Factors Maintain Human Hematopoietic Stem Cells for at least Eight Weeks In Vitro", pages 643-649, see the entire reference.	1-29
A	Blood, Volume 82, No. 7, issued 01 October 1993, Verfaillie, C. M., "Soluble Factor(s) Produced by Human Bone Marrow Stroma Increase Cytokine-Induced Proliferation and Maturation of Primitive Hematopoietic Progenitors While Preventing Their Terminal Differentiation", page 2045-2053, see the entire document.	1-29