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(54) Title: MEGAKARYOCYTE AND PLATELET GROWTH, PRODUCTION AND COMPOSITION (57) Abstract Megakaryocytes and platelets are produced in culture by employing conditions encouraging megakaryopoiesis while discouraging the formation of fibroblasts. Particularly, human plasma or heparin with fetal calf serum is found to support megakaryocyte formation in containers which lack fibroblast supporting coatings. A factor associated with megakaryopoiesis is provided.		

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**MEGAKARYOCYTE AND PLATELET
GROWTH, PRODUCTION AND COMPOSITION**

CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of application serial number 593,688, filed October 5, 1990.

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INTRODUCTION

Technical Field

The field of this invention is growth and production of hematopoietic cells, more particularly megakaryocytes and platelets.

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Background

Thrombopoiesis is the differentiation and maturation of megakaryocytes and platelets. Despite enormous efforts that have been made to understand the process of thrombopoiesis and the factors involved with thrombopoiesis, there remains an extraordinary amount of confusion and uncertainty about the process. Factors that appear to have effects in vitro seem to have little or no effect in vivo. An activity called thrombopoietin has been elusive and has yet to be isolated in pure form and characterized. Efforts to provide for long-term culture of megakaryocyte progenitors providing for their maturation and formation of platelets has also been problematical.

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Thrombopoiesis is an extremely important process for the health of individuals. Platelets play a vital role in a number of protective processes, providing various factors, participating in clotting, and the like. There is, therefore, substantial interest in providing procedures which will aid in the understanding of thrombopoiesis, as well as providing for long-term production of megakaryocytes and platelets which may find application as therapeutic procedures.

10 Relevant Literature

Volume 15, number 1, 1989 of Blood Cells is dedicated to megakaryopoiesis. Hill and Levin, Blood Cells (1989) 15:141-166 describe regulators of thrombopoiesis. A discussion of the role of heparin with endothelial cells may be found in Thornton et al., Science (1983) 222:623-5. WO 90 US1725 reports a megakaryocyte growth promoting activator factor protein. Regulation of megakaryocyte differentiation by thrombin glycosaminoglycans has been reported.

20 SUMMARY OF THE INVENTION

Methods are provided for growing megakaryocytes and platelets in culture for extended periods of time, by employing media which encourage megakaryocyte progenitor and megakaryocyte growth. Various sources of megakaryocyte progenitor cells are employed. A megakaryocyte growth factor is provided for enhancing

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megakaryocyte progenitor and megakaryocyte growth.

Assay systems are provided for assaying for the effect of factors on the proliferation and maturation of megakaryocyte progenitors and megakaryocytes, as well as the formation of platelets. The megakaryocytes and platelets find use in the treatment of thrombocytopenia, identification of megakaryopoiesis activity of compounds or compositions, and for an understanding of the mechanism of megakaryopoiesis.

10 DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are provided for the growth of megakaryocyte progenitors, megakaryocytes and platelets, in culture and in vivo. Megakaryocytes can be grown in the presence of heparin and a megakaryocytic growth factor or human plasma. In culture, the megakaryocytes may be maintained for extended periods of time without replenishment of the progenitor cells from an exogenous source.

Bioassays are provided for monitoring megakaryopoiesis and factors influencing megakaryopoiesis. The megakaryocyte progenitors, megakaryocytes and platelets can be produced by growing in culture in an appropriate nutrient medium human progenitor cells under conditions which are not supportive, preferably are inhibitory, of fibroblast growth, while inactivating, as appropriate, inhibitors of megakaryopoiesis, particularly those produced by

platelets. The source of progenitor cells may be any convenient source, including bone marrow, fetal liver, non-adherent cells from bone marrow, fractionated cells, leukocytes, e.g., buffy coat free of erythrocytes, CD34+ fraction from bone marrow, low density cells, Class II HLA positive cells, or the like, so long as early progenitors are present which are capable of maintaining production of megakaryocytes.

For any long-term culture, the cell composition which is employed will desirably include hematopoietic stem cells. The cellular composition may be fetal, neonate, or adult. While the subject methodology may be used with any primate hematopoietic cellular composition comprising stem cells, the methodology desirably finds use with human cell compositions.

For many purposes, the growth of megakaryocytes, megakaryocyte progenitors, and platelets is desirable. Thus, having a system whereby one can grow cells of the megakaryocytic lineage is extremely important in providing a source of megakaryocytic cells and platelets. The following describes the method for growing cells of the megakaryocytic lineage and platelets in culture.

For the most part, containers which are used for the growth of cultures are coated to enhance fibroblast formation. In accordance with the subject invention, containers are employed which remain uncoated or have been treated to avoid encouraging fibroblast formation.

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Containers can be obtained where the containers are free of coating, either by being commercially available or by requesting such containers from sources such as Falcon, Corning, or the like. The containers may include multiple well plates, roller bottles, fermentors, Petri dishes or the like.

The medium which is employed may be any convenient medium providing the required salts, minerals and nutritional supplements, such as amino acids, glucose and vitamins. Also, a small amount of a physiologically acceptable reductant is used such as 2-mercaptoethanol. The mercaptoethanol will generally be present in from about 10^{-4} - 10^{-6} M, preferably about 10^{-5} M.

Various additives may be employed to discourage or inhibit fibroblast formation and proliferation or preferentially encourage megakaryopoiesis.

Additives which may be employed to discourage fibroblast formation in the presence of fetal calf serum include heparin, citrate, or other additives that prevent aggregation and degranulation of platelets. The amount of heparin will be at least about 50 μ g/ml, preferably at least about 100 μ g/ml and usually not exceed 4 mg/ml, more usually not exceed 2 mg/ml, preferably being in the range of about 0.5 to 1.5 mg/ml. The amount of citrate will vary, when present, generally in the range of about 0.1 to 1%. Other additives will be used in accordance with their activity and any adverse effects on the system. The medium generally

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will also have a low level of plasma or serum, human plasma or serum, fetal calf serum or fetal bovine serum, preferably being about 10% or less, more preferably being about 5%, there generally being at least about 1% in the absence of specific cytokines in a defined medium. The amount of serum, as well as the other additives may be optimized, where cells of the megakaryocytic lineage are encouraged to grow, while fibroblast growth and proliferation may be inhibited.

Any complex eukaryotic growth media may be used, which includes MCDB107, IMDM, RPMI, EX-CEL, etc., normally enhanced with a low level of serum.

Desirably, factors which may be produced by platelets and inhibit megakaryopoiesis will be maintained at a low level. Factors of particular concern include platelet factor 4 (PF-4) and its degradation product thrombospondin. It is found that heparin is able to bind to PF-4 and diminish its inhibitory activity against megakaryopoiesis. Thus, when heparin is maintained in the medium, the heparin prevents the PF-4 and its degradation product from exerting their inhibitory influence. In the absence of heparin, other methods may be used for minimizing the level of PF-4. This can be achieved by continuous changing of the medium and removing PF-4 and its degradation products from the medium, the addition of antibodies to PF-4, dialysis for removal of PF-4, and the like.

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One may control the calcium level and the calcium/phosphate ratio to enhance the preservation of platelets which are formed in the culture. Generally the calcium level should be in the range of about 0 to 10mM, while the phosphate concentration will be in the range of about 0 to 10mM. The level of ADP may also be controlled by providing for a system to convert ADP to ATP. A convenient system includes creatinine phosphate and creatinine phosphate kinase, although other systems may find use. The concentration of the creatinine kinase and creatinine kinase phosphate will be maintained in a range to provide the desired ADP/ATP levels. These concentrations are not critical and are intended to be primarily of convenience, enhancing the productivity of the system. Platelet preservation may also be achieved with dextrose acid citrate.

Megakaryocytic cell cultures may also be grown in the substantial absence of serum ($\leq 1\%$) by employing at least one growth factor in combination with the subject megakaryocytic growth factor. Growth factors include the interleukins IL-1, -3, -6 and erythropoietin, particularly IL-6 and erythropoietin at relatively high concentrations, usually greater than about 25 ng/ml, preferably greater than about 50 ng/ml, and may be 100 ng/ml or higher. A rich defined medium should be employed, having high levels of cofactors, e.g., vitamins, and other components, e.g., β -carotene,

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insulin, etc., such as X-VIVO and EX-CEL (J.R. Scientific).

The cell composition obtained in the medium is highly enriched for megakaryocyte progenitors, megakaryocytes and platelets, greater than 10% by number, usually greater than 50%, of the liquid culture medium being in the megakaryocyte/platelet dedicated lineage. Cell compositions in liquid culture can be obtained having at least 90 weight percent of megakaryoblastoid cells and megakaryocytes (excluding platelets in the weight count), particularly megakaryocytes. Thus, a culture is obtained comprising cells committed to the megakaryocyte/platelet lineage, an adherent population of cells which may provide factors and a conditioned medium supportive of megakaryopoiesis by virtue of comprising the appropriate growth factors, agent(s) for stabilizing platelets and, as appropriate, agent(s) for inhibiting fibroblast growth and proliferation.

The cells of interest may be isolated from the culture medium or bone marrow. Cells of interest may also be available from buffy coat isolated from blood by using separation techniques associated with surface membrane protein markers. Cells of interest will fractionate as CD34+, -41+, -14-, and usually -33+. These cells will be diploid, as distinguished from the polyploid mature megakaryocytes. Thus, one can obtain a highly enriched cellular composition of megakaryocyte

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progenitors by separation using magnetic beads, employing positive and negative selection, FACS, affinity columns, and the like. By employing an enriched population of megakaryocyte progenitors, more rapid maturation and production of platelets can be achieved.

Factor(s) present in the conditioned medium associated with megakaryopoiesis are found to be stable for greater than two weeks at 41°C, usually at ambient temperatures or higher.

In carrying out the production of megakaryocytes and platelets, usually from about 1×10^5 to 1×10^7 human bone marrow cells per ml will be seeded, preferably 1×10^6 cells per ml, or a unit of leukocytes (buffy coat) from an adult, containing about 2×10^8 white cells and freed of erythrocytes. Conventional physiologic conditions will be employed for growing human cells in culture, namely about 37°C, in a humidified chamber, with air having about 5% carbon dioxide, where the oxygen will be at least about 5% usually at least about 20%. Since higher oxygen levels are reported to inhibit fibroblast growth, high oxygen levels, usually not exceeding 90% may be employed, depending on the other conditions used for the growth of the cells. Conveniently, wells of 6 - 10 cm or 96 well plates may be employed, which allows for the growth of the adherent cells to the surface. Within about 7 days, megakaryocytic cells can be observed and the culture may be continued for six months

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or more. Unless dialysis is used, the media is changed on a regular basis, generally at least about twice weekly, preferably at least about every 2 days. One observes islands of cells, with adherent cells set down, which differentiate to mature megakaryocytes, observed as multi-lobular cells. An adherent layer is observed which is a combination of megakaryocytes and adherent (stromal) cells. Islands of cells appear on the adherent layer which have the characteristic CD41 marker for megakaryocytes but are mononucleated, supporting the cells being megakaryocyte progenitors (megakaryoblasts). The progenitor cells will also carry the CD34 marker. In the medium and associated with the adherent layer will be platelets. The megakaryocyte cells are shown to have the surface protein markers CD41 (IIb/IIIa) (integrin) and CD42 (Ib), and when the cells are stained with Wright-Giemsa, show the multi-lobular or polyploid characteristic of megakaryocytes. The progenitor cells, by comparison, will carry the CD41 and CD34 markers and be mononucleated.

Megakaryocytic cells may be further distinguished by staining with Megacolor (Cytocolor, Inc., Hinckley, OH). In megakaryocytes, the cytoplasm stains intensely purple. Also, as evidenced by scanning electron microscope, a majority of the cells in the culture are actively budding platelets.

Alternatively, the level of src protein expression and its kinase activity may be used as a measure of

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megakaryocytic cell growth, measuring megakaryocyte progenitors, megakaryocytes and platelets. The assay can be carried out with cellular lysates of relatively homogeneous or heterogeneous phenotype or populations selected by one or more markers, isolating the src kinase, e.g. by means of specific antibodies, and then measuring src kinase activity under conditions supportive of src kinase catalyzed phosphorylation. To remove non-specific proteins from the isolated src kinase, one or more washes may be employed. By using a labeled nucleotide triphosphate, e.g. radioisotope labeled, one can determine the level of phosphorylation as indicative of the presence of cells associated with megakaryopoiesis. Alternatively, Western blotting or SRC ELISA can be used to quantitate src protein.

As the cultures mature, the concentration of platelets continues to increase, so that after about one week, the concentration of platelets is $\sim 10^6$ platelets per ml, and may be maintained at about 10^6 - 10^7 platelets/ml. The level of platelet production may be maintained by the continuous growth of the culture and maintenance of the medium at the appropriate component composition, and may be increased by concentration of the platelets using various techniques for removing fluid.

The cells may be harvested by separating the adherent layer mechanically, using chelating agents, e.g., EDTA, sonication, enzymes, e.g., proteases, such

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as trypsin, collagenase, plasmin, etc., or the like.

The particular manner employed is not critical to this invention. The megakaryoblasts and megakaryocytes may then be isolated using various techniques, such as

5 cytopsin, FACS, affinity separation, density separation, magnetic bead separation, and the like. As a result, a substantially pure cellular composition can be obtained of cells dedicated to megakaryopoiesis, namely dedicated megakaryocyte progenitors, megakaryocytes and platelets.

10 The composition will be free of components of peripheral blood, which might be associated with isolation of such cellular composition from bone marrow or blood. In addition, each of the types of cells may be isolated in substantially pure form, namely, megakaryoblasts, 15 megakaryocytes and platelets, generally comprising at least 75% by number of the same cellular type, usually at least about 90% of the same cellular type and up to and including about 100%.

The conditioned medium, or human plasma, may be 20 used for isolation of the factors associated with megakaryopoiesis. Fractionating ammonium sulfate precipitation is used to enrich protein fractions for growth factors. Further enrichment is achieved using gel permeation to separate different size fractions, 25 followed by assaying for the enriched fractions. The enriched fractions are then further enriched using rp-HPLC, eluting with an aqueous acidic medium against n-propanol or acetonitrile, e.g., 0.1% trifluoroacetic

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acid, or 1M acetic acid.

Purification of the megakaryocyte activity may be further achieved by HPLC using a cationic column, where the column buffer is at a Ph of about 7.5 to 9, and the sample is eluted with a high ionic strength eluent, e.g. 2 M inorganic salt, e.g. NaCl, using a linear gradient in the column buffer. The activity is observed in the fractions of the tail end of the highest peak when using a linear gradient, generally eluting in the fractions of about 1.25 to 1.6 M salt.

The fractions are bioassayed and enriched fractions may be further purified by gel electrophoresis or other separation technique.

A megakaryocytic growth factor can be obtained by fractionating medium from the culture or human plasma through a heparin column. The factor is characterized by binding to heparin, capable of being transferred from heparin-Sepharose to heparin in a conventional complex eukaryotic growth media, with heparin at a concentration of at least about 1 mg/ml, preferably at least about 3 mg/ml, and at 4°C. The ratio of heparin-Sepharose beads to medium will generally be at least about 1 g:2 ml, preferably 5 ml, and not more than about 1 g:10 ml. The factor is further characterized by supporting cell growth and proliferation of cells of the megakaryocytic lineage in the culture system as described above, while its absence results in the substantial absence of

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proliferation and maturation of cells of the megakaryocytic lineage.

As measured in solution, the molecular weight of the factor appears to be greater than about 40kD. It also is stable at 56° C. in whole plasma. It also appears to be species specific, not being effective with mouse cells under comparable conditions to being effective with human cells. Finally, it does not bind Affi-gel blue CM.

The subject factor may be further purified by conventional techniques as described above to provide for 1,000-fold or greater enhancement in concentration and specific activity.

In order to evaluate the response of megakaryocyte progenitors to various factors, the factors may be evaluated using a standard semi-solid medium assay composition, e.g., methylcellulose assay composition comprising factors which support megakaryocyte growth. The methylcellulose which is employed will generally be at least about 1.5% and not more than about 3% v/v in water. The cellular source may be the same as that used for the culture. Also included in the culture will be at least about 15% fetal calf serum, fetal bovine serum, or human plasma, preferably about 20%. A convenient volume for carrying out the assay is to employ about 200 μ l in a 24-well plate.

To support growth of the megakaryocytes, high concentrations of the cytokines IL-1, -3 and -6, as well

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as erythropoietin are included, generally at least at about 0.5 times saturation or higher. Other factors may be included such as sl or steel factor at comparable concentration. By saturation it is intended that a
5 further increase in concentration does not have a significant effect on the number of colonies of cells which are produced. Conveniently, about 100 ng/ml of each of the factors may be employed.

The cells which are seeded will include CD41+,
10 CD34+ cells, which cells serve as megakaryocyte progenitors. The cells may be whole bone marrow or an enriched fraction comprising CD41+, CD34+ cells. The mature cells in the colonies which form will, for the most part, be free of the CD34 marker and will normally
15 have the CD33 marker, as well as having the morphology of the mature megakaryocyte.

A factor or composition of interest may be studied by adding such factor or composition to the medium with all of the above-indicated factors or where one or more
20 of the above-indicated factors are lacking. Thus, one may determine the inherent capability of a composition in conjunction with fetal calf serum to stimulate megakaryocyte growth or its ability to interact with other factors or cytokines to provide for megakaryocyte
25 growth. The assay is carried out for at least about 5, usually at least 7 days, usually not more than about 21 days, preferably from about 7 to 14 days. The conditions for the assay are that the liquid medium or

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methylcellulose is maintained at 37°C, 5% CO₂ in a humidified chamber.

By employing various dosages of a composition or factor, one can determine the activity of such composition or factor. In this manner, rapid screening can be carried out to determine whether a particular composition or factor is useful for the growth of megakaryocytes or the production of platelets.

The megakaryocyte progenitors and/or megakaryocytes may be used for the treatment of thrombocytopenia by themselves or in conjunction with the infusion of platelets. In many instances, such as surgery, chemotherapy, etc., autologous megakaryocytic cells may be expanded in culture and returned to the host to minimize bleeding or other effect of the treatment which megakaryocytes and/or platelets may address. The cells may be infused in a physiologically acceptable medium, e.g., saline, generally being present at a concentration of at least about 10⁵ cells/ml, usually in the range of about 10⁶ - 10⁸ cells/ml. The dosage will generally vary in the range of about 10⁶ - 10⁸ cells/kg of host.

Usually, the composition will be at least 25%, more usually at least 50% and frequently at least 90% by weight of megakaryocyte progenitors and megakaryocytes. One or more infusions may be necessary, as required. Platelets may be harvested and administered in accordance with conventional ways.

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The megakaryocytic cells may be used for gene therapy, where the cells may serve to maintain function under conditions where normal megakaryocytic cells may be adversely affected or to provide a function normally unavailable from megakaryocytic cells. For example, the multiple drug resistance (mdr) phenotype may be introduced into the cells, which would make them resistant to chemotherapeutic agents, allowing for the continued normal megakaryocytic response during the chemotherapy treatment. Genes encoding growth factors may be introduced, which would provide an enhanced source of growth factors in the bone marrow, where a deficiency might exist during a particular episode or one wished to enhance the growth of megakaryocytes or other hematopoietic cells. Various methods are known for providing transformation of cells, providing for stable extrachromosomal elements or homologous or random integration. Viral vectors incapable of replication have been described in the literature, which can be used for infection, transfection and integration. By being able to grow megakaryocytic cells in culture, one has the opportunity to transform such cells and expand and clone desirable transformants.

The following examples are offered by way of illustration and not by way of limitation.

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EXPERIMENTAL

Example 1: Heparin containing fetal bone marrow cultures.

A. Cells: K265 fetal bone marrow, 2x.

5 Media: IMDM + 5% Gibco FCS + 10^{-5} M

2-mercaptoethanol. The FCS and 2-mercaptoethanol were added fresh each media change.

Heparin: Sigma 178 U/ng, stock solution 200 mg/ml.

Plate: 100 mm Falcon plate.

10 Plate 1: No added heparin.

Plate 2: 1000 μ g/ml heparin sigma concentration.

Both plates were tended to by removal of 10 ml medium and addition of media plus heparin approximately every day, occasionally every other day. By day 10 a
15 noticeable difference in the predominant cell morphology was evident. Photomicrographs were taken evidencing the morphology. Plate 1 had sheets of fibroblast-like cells which would form large balls of cells often dislodging from the plate. Plate 2 had confluent layers of rounder
20 cells with a different morphology. No sheets of fibroblasts were observed.

B. The above study was repeated except that 5% normal human plasma was used in place of FCS. In both plates egg-shaped morphology indicative of megakaryocyte type
25 cells was observed, indicating that normal human plasma could be used in place of FCS and that the effect of the heparin was related to the FCS.

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Example 2: High heparin concentration effect on fetal bone marrow cultures.

Bones: K266 and K267 samples were stripped of protein by cutting along the long axis in one half, incubating at 37°C with collagenase or trypsin and every 0.5 hr, mixing for 3 hours followed by three washes with media. Cell concentration was 2×10^6 per ml.

Plates: 100 mm Falcon plate.

Medium: IMDM plus 5% FCS plus 10^{-5} M 2-ME.

Heparin: When used, 1000 µg/ml (Sigma) diluted in sterile water.

Four plates were prepared, two each of K266 and K267, each pair including a plate with heparin and a control.

As observed in the previous experiment, the plates without heparin became overgrown in ten days to two weeks with elongated spindle-shaped cells. The plates with added heparin show the growth of the "egg-shaped" cells seen previously. These cultures were tended as previously described with change of media approximately QOD.

Example 3: The effect of different plates on megakaryocyte and fibroblast growth.

The procedure described in Examples 1 and 2 was employed, using the cell preparation K275. Four plates were prepared, two plates from Corning and two plates from Falcon, where each pair included a control and a heparin-containing medium.

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The cultures were tended in the same way as previously described.

The Corning control plate showed a rapid growth of adherent cells with a fibroblast morphology. Within 7 to 10 days, the culture was overgrown in areas and the cells peeled away from the plate. Often, balls of these cells were found floating in the medium. The medium became yellow rapidly despite frequent changes. In the Corning heparin-containing medium, a mixed pattern of growth was observed with both fibroblast-like cells and cells with the "egg-shaped" morphology. The growth of the fibroblast-like cells was very significantly reduced and the overall cellularity of the culture was reduced compared to the control plate. At about 2-3 weeks, the fibroblast-like cells predominated in the culture and the number of egg-shaped cells was reduced. Overall, the non-adherent cell number was minimal.

In the Falcon plates, in the control plate, there was a rapid overgrowth of fibroblast-like cells. These cells did not come to confluence as rapidly as in the Corning control plate and there was a greater number of "egg-shaped" cells than in either of the Corning plates. These cells form clusters in an area which seemed to exclude the fibroblast-like cells. Finally, in the Falcon plate with heparin-containing medium, growth to confluence of the egg-shaped cells was observed with eventual formation of multinucleated cells. An adherent

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layer of megakaryocytes and stromal cells formed supporting colonies with megablastoid cells.

Example 4: Fluorescence-activated cell sorter scan.

5 Cells from the K266 and K267 cultures were collected by gentle pipetting, containing about 10^5 cells/ml. The antibodies used were anti-CD7 and anti-CD42 in 0.1 PBS plus 2% FCS. The cells were incubated for 20 min on ice with 20 μ l of antibody and then washed
10 through FCS. FITC labeled goat anti-mouse Ig diluted 1:100 (20 μ l/sample) was incubated on ice with the mixture described above for 20 min, followed by washing the cells through FCS. The cells were then analyzed on a FACScan. The scan was ungated, standard threshold 52
15 (arbitrary unites) and the amplification was 1. Culture K266 showed 2 sharp peaks where the CD7 peak was substantially displaced from the CD42 peak. A similar result was obtained with K267. By comparison, a control showed no significant increase in fluorescence intensity
20 obtained over no added antibody (autofluorescence).

Example 5: Methylcellulose assay.

In the next study, a methylcellulose assay was carried out with conventional conditions, employing 20% fetal calf serum and 100 ng/ml each of IL-1, -3 and -6
25 and erythropoietin. Whole bone marrow was employed at different seeding levels as well as selected cells based on CD34 and CD41. The CD34+ CD41+ is between 0.5 and 0.9% of the whole bone marrow population.

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The following table indicates the results:

	<u>WBM</u>	<u>CELLS</u> <u>CD34</u>	<u>CD41</u>	<u>CELL</u> <u>CONC.</u>	<u>COLONIES</u> <u>NO.*</u>	<u>CLUSTERS</u> <u>/10⁵</u>
5	+	-	-	1x10 ⁴ ml	0-1-3	
	-	+	-	1x10 ⁴ ml	1-2-0	
	-	+	+	1x10 ⁴ ml	10-7-19	4-5-7
	-	-	-	1x10 ⁴ ml	0-0-1	
	-	-	+	1x10 ⁴ ml	0-1-0	
10	+	-	-	2x10 ⁴ ml	0-1-5	2-3-2
	-	+	-	2x10 ⁴ ml	1-2-1	1-1-1
	-	+	+	2x10 ⁴ ml	34-40-40	9-8-12
	-	-	-	2x10 ⁴ ml	0-0-0	
	-	-	+	2x10 ⁴ ml	0-0-3	
	+	-	-	1x10 ⁵ ml	15-16-15	3-9-7

15 * These replicate colonies comprised ≥ 50 cells, while clusters comprise ≥ 20 cells.

The colonies comprised of morphologically identifiable megakaryocytes. Thus, colonies were observed at the lower concentration from the CD34+ CD41+ cells which matured to megakaryocytes. In addition, when whole bone marrow was employed at a seeding of 10⁵ per ml of cells, a significant number of colonies was observed which provided megakaryocytes. In addition, the CD34+ CD41+ population was found to be sensitive to the indicated factors, so that by removing one factor and replacing such factor with a test composition, one can determine the effect of such test factor on the formation of megakaryocytes.

30 Example 6: Growth of megakaryocytes from buffy coat.

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One unit of leukocytes (buffy coat) from an adult human contains about 2×10^8 white cells. The red cells in the unit are lysed by a 10 min incubation at 37°C in a solution of ammonium chloride buffered with Tris-HCl.

5 The leukocytes were then plated under standard heparin culture conditions as described in Example 1 at 10^7 cells/10 cm plate, 5×10^6 cells/6 cm plate, or 10^5 cells/well in a microtiter plate in a volume of 10 ml, 5 ml, or 100 μl , approximately 20 10 cm plates can be

10 made.

After 1-2 weeks following the regimen described in Example 1, a confluent monolayer of megakaryocytes and precursors is obtained. The majority of other cell types are no longer present. If one assumes that the

15 original inoculum contains 0.1% megakaryocyte precursors, then this preparation has been expanded 10^3 -fold, since a confluent 10 cm dish contains 10^7 cells.

Alternatively, aliquots of $1-2 \times 10^7$ fresh leukocytes can be frozen in liquid nitrogen in 10% DMSO

20 and 20% FCS. These vials can be thawed at a later time for use in heparin cultures as described above.

Example 7: Megakaryocyte assay by measuring src protein by ELISA.

Megakaryocyte cultures are established with 8×10^4

25 cells in 96 well plates. Each growth factor source is tested in quadruplicate (4 wells). After 10 days of growth the medium is removed from the wells and the cultures are air dried overnight.

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The src ELISA is performed by rehydrating the cells in PBS for 5 minutes. The src antibody (2-17) (mouse monoclonal) is diluted 1:800 in PBS + 1% BSA. 40 μ l are added to each well and incubated 30 minutes. The wells are washed x 3 with PBS. The second antibody, goat α mouse Ig (alkaline phosphatase conjugated) (Caltag) is diluted 1:250 in PBS + 1% BSA. 40 μ l are added to each well and incubated 30 minutes. The wells are washed x 3 with PBS. One tablet of p-nitrophenylphosphate is dissolved in 5 ml of diethanolamine buffer (Sigma) and 100 μ l are added to each well. The plate is developed for 30 minutes in the dark and read at 405 nm.

Example 8: Isolation of megakaryocyte growth factor.

5 g of heparin-Sepharose CL-6B (Pharmacia) were swollen in PBS to a final volume of 25 ml, followed by washing in 1 liter of PBS. Fresh human plasma (25 ml) pooled from three donors was added to the swollen beads in a 50 ml tube and mixed at 4°C overnight. The plasma was removed and filtered (referred to as "depleted"). Beads were then mixed for 5 h with 25 ml of IMDM containing 3 mg/ml heparin at 4°C. The medium was decanted and filtered (referred to as "eluate").

Heparin cultures were then set up as described in Examples 1 and 6 with either bone marrow or buffy coat, where the various forms of plasma: (1) plasma (10%) used without treatment; (2) depleted plasma; or (3) depleted plasma plus the eluate (30% final volume), were used in

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place of the fetal calf serum described in Example 1.

Src kinase assay was performed on cultures grown 2 wks.

Total protein in the dish was measured: CPM in src band was determined after excising band from the gel and

5 scintillation counting. The results are as follows:

	<u>Source*</u>	<u>CPM in src band</u>	<u>Lysate Protein mg/ml</u>	<u>Src/ protein+</u>
	1) HL-60	60	0.80	1%
	2) HEL	837	1.00	13%
10	3) HC, 10% HP	4,860	0.73	100%
	4) HC, 10% HeSE	316	0.25	19%
15	5) HC, 10% HeSe+F	3,090	0.50	93%

* HL-60 control line; myeloid leukemia

HEL human erythroleukemia line

HC heparin culture

HP human plasma

20 HeSE depleted plasma from heparin-Sepharose column

F megakaryocyte growth factor eluted from heparin-Sepharose column (eluate)

+ Expressed as percentage of culture #3.

Example 9: Purification of megakaryocyte growth factor.

25 Human plasma protein (0.65g) was placed on a 50mm x 150mm DEAE-cellofine column prepared with buffer A (10mM Tris-HCl pH 8.0, 0.1 mM EDTA) and eluted with a linear gradient of buffer A and 2 M NaCl in buffer A. The flow

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rate was 2ml/min and 5ml fractions were collected. The bioactivity was found to be in the higher salt concentration or downside of the major peak during elution with the salt gradient, the major portion of the activity being in the fractions from about 1.25 to 1.6 M NaCl.

The fractions may be assayed in a 96 titer well plate, with 4 replicates. The fractions are dialyzed against PBS. The medium comprising 5-10% of the fractions to be assayed in X-Vivo (Whittaker), 1mg/ml heparin, are seeded with 8×10^4 cells/well and fed every three days. After two weeks the cells are inspected for megakaryocyte morphology and staining with Megacolor.

It is evident from the above results, that an efficient economic procedure has been provided for producing human megakaryocytes in culture. Thus, a continuous source of platelets is provided for treatment and study of the function of platelets, as well as their formation. Also, megakaryopoiesis may be studied. In addition, a factor associated with megakaryopoiesis is provided and other factors may be similarly isolated.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent

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application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many
5 changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for producing megakaryopoiesis, said method comprising:

5 culturing human hematopoietic progenitor cells or a megakaryocyte progenitor enriched fraction thereof under megakaryocyte growth supporting conditions in a nutrient medium comprising human plasma and/or under fibroblast growth inhibiting conditions.

10 2. A method according to Claim 1, wherein said culturing is in a container lacking a coating supporting fibroblast growth.

3. A method according to Claim 1, wherein said medium comprises heparin and fetal calf serum.

15 4. A method according to Claim 1, wherein said nutrient medium comprises up to about 20% of human plasma or fetal calf serum.

5. A method according to Claim 1, wherein said medium comprises supplemental megakaryocyte growth factor.

20 6. A method according to Claim 7, wherein said human hematopoietic progenitor cells are an enriched CD34+, -41+, -14- fraction hematopoietic cell fraction.

7. An hematopoietic cellular composition enriched for cells having CD34 and 41, and lacking CD14.

25 8. A cellular composition according to Claim 8, wherein said cells are enriched for cells having CD33.

9. A cellular composition of human cells comprising at least 50% by number of cells which are

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dedicated to megakaryopoiesis and at least 10% of the cells are megakaryocyte progenitor cells.

10. A cellular composition according to Claim 9, wherein said cells are at least 90% megakaryocyte progenitor cells.

11. A cellular composition in a liquid medium wherein at least 50 weight % of the cells other than platelets are dedicated progenitors to megakaryocytes or megakaryocytes.

12. A method for assaying for factors which regulate megakaryopoiesis, said method comprising:
seeding bone marrow cells or leukocytes from buffy coat in a liquid or semi-solid nutrient medium comprising serum, the factor in interest and at least one cytokine which is Il-1, -3, -6 or erythropoietin; and

determining the number of megakaryocytic cells which grow in greater than about 5 days as compared to the number of cells that grow under the same conditions in the absence of said factor in interest.

13. A method for determining the level of megakaryopoiesis in a cell population, said method comprising:

lysing said cells to produce a supernatant:
isolating src kinase from other proteins in said supernatant; and

detecting the amount of src kinase activity as a measure of the megakaryocytic population of said cell population.

14. A factor capable of supporting
- 5 megakaryopoiesis characterized by:
- being present in plasma;
 - stable at 56°C in plasma for at least two weeks;
 - binds to heparin-Sepharose and can be eluted with heparin containing IMDM medium;
 - 10 supports megakaryopoiesis; and
 - megakaryopoiesis is diminished in the absence of said growth factor.

15. A factor according to Claim 14, wherein said factor is a human factor.

AMENDED CLAIMS

[received by the International Bureau
on 13 March 1992 (13.03.92);
new claims 16-18 added; other claims unchanged (1 page)]

detecting the amount of src kinase activity as a
measure of the megakaryocytic population of said cell
population.

14. A factor capable of supporting megakaryopoiesis
characterized by:

being present in plasma;

stable at 56°C in plasma for at least two weeks;

binds to heparin-Sepharose and can be eluted with
heparin containing IMDM medium;

supports megakaryopoiesis; and

megakaryopoiesis is diminished in the absence of said
growth factor.

15. A factor according to Claim 14, wherein said
factor is a human factor.

16. A hematopoietic cellular composition grown in
liquid culture and enriched for cells having CD34 and 41,
and lacking CD14.

17. A cellular composition according to Claim 16,
wherein said cells are enriched for cells having CD33.

18. A cellular composition of human cells comprising
at least 50% by number of cells which are grown in liquid
culture and dedicated to megakaryopoiesis and at least 10%
of the cells are megakaryocyte progenitor cells.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07159

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12N 5/00 U.S.Cl: 435/240.2																	
II. FIELDS SEARCHED Minimum Documentation Searched ⁷ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border: 1px solid black;">Classification System</th> <th style="border: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; text-align: center;">U.S.Cl:</td> <td style="border: 1px solid black;">530/351; 435/70.3, 70.4, 240.21, 240.22</td> </tr> </table> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸			Classification System	Classification Symbols	U.S.Cl:	530/351; 435/70.3, 70.4, 240.21, 240.22											
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border: 1px solid black;">Category [*]</th> <th style="width: 70%; border: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; border: 1px solid black;">Y</td> <td style="border: 1px solid black;">Exp. Hematol., volume 14 issued 1986. J.F. Straueva et al. "Terminal Cytoplasmic Maturation of Human Megakaryocytes in vitro", pages 919-929. See entire document.</td> <td style="text-align: center; vertical-align: top; border: 1px solid black;">1-11</td> </tr> <tr> <td style="text-align: center; vertical-align: top; border: 1px solid black;">Y</td> <td style="border: 1px solid black;">Proc. Natl. Acad. Sci. USA. Volume 82, issued March 1985, K. Welte et al. "Purification and Biochemical Characterization of Human Pluripotent Hematopoietic Colony-Stimulating Factor." pages 1526-1530, see entire document.</td> <td style="text-align: center; vertical-align: top; border: 1px solid black;">12-14</td> </tr> <tr> <td style="text-align: center; vertical-align: top; border: 1px solid black;">Y</td> <td style="border: 1px solid black;">Blood, Volume 67 (3) issued March 1986, R.B. Jenkins et al. "CFU-M-Derived Human Megakaryocyte Synthesize Glycoproteins IIb and IIIa," pages 682-688. see entire document.</td> <td style="text-align: center; vertical-align: top; border: 1px solid black;">7-11, 14</td> </tr> <tr> <td style="text-align: center; vertical-align: top; border: 1px solid black;">Y</td> <td style="border: 1px solid black;">US. A. 4,350,687 (LIPTON et al) 21 September 1980, see entire document.</td> <td style="text-align: center; vertical-align: top; border: 1px solid black;">12-14</td> </tr> </table> [*] Special categories of cited documents: ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "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 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family			Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	Exp. Hematol., volume 14 issued 1986. J.F. Straueva et al. "Terminal Cytoplasmic Maturation of Human Megakaryocytes in vitro", pages 919-929. See entire document.	1-11	Y	Proc. Natl. Acad. Sci. USA. Volume 82, issued March 1985, K. Welte et al. "Purification and Biochemical Characterization of Human Pluripotent Hematopoietic Colony-Stimulating Factor." pages 1526-1530, see entire document.	12-14	Y	Blood, Volume 67 (3) issued March 1986, R.B. Jenkins et al. "CFU-M-Derived Human Megakaryocyte Synthesize Glycoproteins IIb and IIIa," pages 682-688. see entire document.	7-11, 14	Y	US. A. 4,350,687 (LIPTON et al) 21 September 1980, see entire document.	12-14
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IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; vertical-align: top;"> Date of the Actual Completion of the International Search 05 December 1991 International Searching Authority ISA/US </td> <td style="width: 50%; border: 1px solid black; vertical-align: top;"> Date of Mailing of this International Search Report 13 JAN 1992 Signature of Authorized Officer Jane Williams </td> </tr> </table>			Date of the Actual Completion of the International Search 05 December 1991 International Searching Authority ISA/US	Date of Mailing of this International Search Report 13 JAN 1992 Signature of Authorized Officer Jane Williams													
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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Journal of Biological Chemistry, volume 262, No.7, issued 05 March 1987, S.M. Greenberg et al, In vitro Stimulation of Megakaryocyte Maturation by Megakaryocyte Stimulatory Factor, pages 3296-3277, see entire document.	1-6
Y	Proc. Natl. Acad. Sci. USA, volume 82, issued May 1985, P.T. Jubinsky et al "Purification of Hemopoietin 2: A Multilineage Hemopoietic Growth Factor," pages 2764-2768, see entire document.	14
X/Y	US, A, 4,894,440 (Rosenberg) 16 January 1990 see entire document.	14 / 14

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.