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(54) Titre : PLAQUETTES DE CELLULES SOUCHES
(54) Title: PLATELETS FROM STEM CELLS

(57) Abrégé/Abstract:
Human embryonic stem cells are induced to differentiate first into the hematopoietic lineage and then into megakaryocytes, the cells which generate platelets. The proper in vitroculture of megakaryocytes results in the production and shed of platelets. This makes possible, for the first time, the in vitro production of a human blood factor needed by many patients.

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(54) Title: PLATELETS FROM STEM CELLS

(57) Abstract: Human embryonic stem cells are induced to differentiate first into the hematopoietic lineage and then into megakaryocytes, the cells which generate platelets. The proper in vitro culture of megakaryocytes results in the production and shed of platelets. This makes possible, for the first time, the *in vitro* production of a human blood factor needed by many patients.

PLATELETS FROM STEM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. provisional patent applications Ser. No. 60/623,922 filed November 1, 2004 and Ser. No. 60/714,578 filed September 7, 2005.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] To be determined.

BACKGROUND OF THE INVENTION

[0003] Stem cells are defined as cells that are capable of a differentiation into many other differentiated cell types. Embryonic stem cells are stem cells from embryos which are capable of differentiation into most, if not all, of the differentiated cell types of a mature body. Stem cells are referred to as pluripotent, which describes the capability of these cells to differentiate into many cell types. A type of pluripotent stem cell of high interest to the research community is the human embryonic stem cell, sometimes abbreviated here as hES or human ES cell, which is an embryonic stem cell derived from a human embryonic source. Human embryonic stem cells are of great scientific and research interest because these cells are capable of indefinite proliferation in culture as well as differentiation into other cell types, and are thus capable, at least in principle, of supplying cells and tissues for replacement of failing or defective human tissue. The existence in culture of human embryonic stem cells offers the potential for unlimited amounts of genetically stable human cells and tissues for use in scientific research and a variety of therapeutic protocols to assist in human health. It is envisioned in the future human embryonic stem cells will be proliferated and directed to differentiate into specific lineages so as to develop differentiated cells or tissues that can be transplanted or transfused into human bodies for therapeutic purposes.

[0004] Platelets are an essential blood component for blood clotting. Platelets are a sub-cellular blood constituent, having no nucleus but hosting cell membranes, receptors, enzymes, granules and other cellular processes, so that platelets are capable of responding to several factors in the blood to initiate blood clot formation. Platelet transfusions are indicated when patients suffer large traumatic blood loss, are exposed to chemical agents or high dose radiation exposure in the battlefield and in a variety of other medical circumstances, such as thrombocytopenia, especial after bone marrow ablation to treat patients with leukemia. The short life span of

platelets in storage (typically only 5 days by FDA and AABB regulation) causes recurring shortages of platelets on the battlefield and in civilian healthcare systems.

[0005] Of all of the cellular components of blood currently stocked for medical purposes, platelets are among the most fragile. There is currently no clinically applicable method for the long term storage of platelets. For modern healthcare institutions, a shelf life of five days for platelets translates to the clinic shelf life of three to four days, after allowing time for testing and shipping. Many blood banks constantly have logistical difficulties keeping platelets fresh and in stock. Reliably supplying platelets to military field hospitals presents even greater difficulties.

[0006] In the body, platelets arise from processes, or proplatelets, formed on cells known as megakaryocytes. The differentiation of megakaryocytes from mouse and human adult hematopoietic stem cells has been studied, but the molecular mechanisms of this differentiation are, as yet, unknown. Long term culture of both adult hematopoietic stem cells and megakaryocytes is difficult, which makes the purification and genetic manipulation of these cells almost impossible. No native human megakaryocyte cDNA library exists and no genetic profiles of normal megakaryocytes are available. The *in vitro* differentiation of mouse embryonic stem cells has been demonstrated to produce platelets, but the biological function of those platelets is yet unproven. Human and mouse platelets differ significantly. Mouse platelets are smaller and exhibit more significant granule heterogeneity as compared to human platelets. The mechanisms of human and mouse release of platelets from megakaryocytes appears to be significantly different.

[0007] There is still significant lack of clarity in the understanding of the process of formation of platelets and the budding of platelets from megakaryocytes. The accepted thesis is that a combination of factors, including plasma and endothelial bound membrane factors, megakaryocyte cytoskeletal or organelle rearrangement, and shearing forces from the blood stream, combine to cause final separation of the mature platelets from the proplatelet structure formed on the megakaryocytes. However, this thesis is largely unproven and the formation and separation of platelets from megakaryocytes is still an area of research where much remains to be uncovered.

BRIEF SUMMARY OF THE INVENTION

[0008] The present invention is summarized as a method for the generation of human platelets includes the steps of culturing human embryonic stem cells under conditions which favor the differentiation of the cells into the hematopoietic lineage; culturing the cells of the

hematopoietic lineage into megakaryocytes; culturing the megakaryocytes to produce platelets; and recovering the platelets.

[0009] The present invention is also summarized by quantities of human platelets produced *in vitro* on demand and in therapeutically significant quantities.

[00010] It is a feature of the present invention that platelets produced *in vitro* do not have bound to them factors encountered in the human bloodstream.

[00011] Other objects, features and advantages of the present invention will become apparent from the following specification.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[00012] FIG 1 shows a flow diagram of platelet production from human embryonic stem (hES) cells.

FIG 2 (A) shows a time course analysis of megakaryocyte colony formation; and (B) shows the effect of growth factors on megakaryocyte production.

FIG 3 (A-B) shows images of proplatelets at different magnifications.

DETAILED DESCRIPTION OF THE INVENTION

[00013] What is contemplated here is the production of platelets by a process of *in vitro* culture and differentiation beginning with human embryonic stem cells. Human embryonic stem cells (hES cells) are induced to produce megakaryocytes in culture *in vitro*, and these megakaryocytes are cultured to produce biologically functional human platelets. This process may be thought of as being done by a three major step process. The first major step is the directed differentiation of human ES cells to hematopoietic cells, a differentiation process that, in turn, can be done several ways. Two methods of differentiating hES cells to hematopoietic lineages are described in detail here. In one technique for the hematopoietic differentiation process, human embryonic stem cells (ES cells) are cultivated to form embryoid bodies (EBs), using a previously known technique. The embryoid bodies are cultured so that differentiation of various differentiated cell types can begin, after which the embryoid bodies are disaggregated into a cell suspension in a medium selective for megakaryocyte precursors. With the help of a time course cDNA microarray analysis, we have identified the most optimal time to harvest definitive hematopoietic cells that have the highest hematopoietic potential. The other technique, already demonstrated to be sufficient for the creation of hematopoietic cells, calls for exposure of the human ES cells to stromal cells, an exposure that causes the ES cells to differentiate preferentially to cells of the hematopoietic lineage. The result of either of these processes is a culture of cells that are, to some degree of purity, predominantly ES cell derived hematopoietic cells. We particularly favor the embryoid body approach not only because it does not have contamination from feeder cells of different species, but also it can be performed with a defined serum free media. In the second major part of the process, these hematopoietic cells are then

exposed to a selective megakaryocyte formation medium containing growth factors that specifically encourage the formation of megakaryocytes and promote maturation of these cells. Finally these mature megakaryocytes are exposed to platelet formation media to promote *in vitro* platelet production. During all these processes animal or human serum and plasma can be avoided.

[00014] Platelets are an exemplary target for the production of biological products for human use from hES cells, because platelets carry no chromosomal genetic material. Platelets may be thought of as cytoplasmic fragments of the parental megakaryocytes. Importantly, platelets exhibit both cell surface factors that can carry out adhesion, aggregation and granule secretion. Since the process of platelet maturation and the process of platelet shed from megakaryocytes are both processes that are poorly understood, it was not known if biologically functional platelets could be recovered from *in vitro* cell cultures derived from human ES cells. Here it is disclosed that platelets can be recovered in useful quantities from such cell cultures.

[00015] Importantly, it is also demonstrated here that platelets are capable of being formed and shed from human megakaryocytes in an *in vitro* cell culture. Given the uncertainty surrounding knowledge of the detailed biology of this process, it was not known previously if this would or could occur in culture. The results here demonstrate that it can and does.

[00016] The present process begins with hES cells, which are by definition undifferentiated cells in culture. It has been previously demonstrated that hES cells can be induced to differentiate into a culture of cells in which cells of hematopoietic lineage predominate. Two different techniques are so far known in the literature for achieving this directed differentiation, and it is envisioned that other techniques will work as well. One known technique calls for the development of embryoid bodies, which are aggregates of hES cells which acquire a three dimensional structure, and that structure seems to encourage differentiation of stem cells into committed progeny lineages. From such embryoid bodies, which produce differentiated cells of a variety of lineages, selective protocols can then be used to isolate cells of the lineage sought, such as cells of hematopoietic lineage. A detailed time course analysis of the hematopoiesis done by us has provided us a genetic profile of these hematopoietic precursors and at the same time we have optimized our protocol to produce the highest yield. The other documented technique involves the co-culture of hES cells with human or non-human stromal cells. Such a co-culture with stromal cells also seems to induce hES cells to produce predominantly hematopoietic cells, but current techniques are based on culture conditions some might seek to avoid.

[00017] An intermediate step in the EB method, which has been found to increase the yield of cell of the various hematopoietic lineages, is to fragment the EBs. One of the characteristics of EBs is that the EBs can grow so large as to exceed the ability of the medium to provide oxygen and nutrients to the cells in the center by diffusion. The result can be a necrotic area in the center of the EB, which also causes growth of the EB to stall. It has now been found that fragmenting the EBs, i.e. by physically chopping the EBs into pieces, one can restart the growth of the EBs which result in more differentiated cells. In our hands, using this technique has resulted in a dramatic increase in the numbers of blood cells recovered from the overall process. Various mechanical devices and systems can be used to perform this fragmenting or chopping process of the EBs.

[00018] Once cells of the hematopoietic lineage are produced, the cells are then cultured to preferentially produce megakaryocytes. This process does not have to be absolute, but culture conditions preferential for megakaryocytes will increase the proportion of megakaryocytes in relation to other blood product precursor cells in the culture. Conditions favorable for the production of immature and mature megakaryocytes include culture of precursor cells cultures with thrombopoeitin (TPO), interleukin 3 (IL3), interleukin 6 (IL6) and stem cell factor. Immature megakaryocytes can be further expanded if bFGF (basic fibroblastic growth factor) is present. The megakaryocytes obtained by this method are positive for CD41, CD42a, CD42b, CD61, CD62P, CD38, weak CD45, but negative for HLA-DR, CD34, CD117. This immunophenotypic profile is constant with normal mature megakaryocytes. There is no significant CD45+ population suggesting that the leukocytic contamination is very minimal if present at all.

[00019] Platelet formation and release by megakaryocytes then can be made to occur in culture. While the exact mechanism responsible for release of platelets *in vivo* is not completely characterized, platelets in cell culture can be made to release from their parental megakaryocytes. We think that four factors that could be potentially crucial. These four factors are shearing force, megakaryocyte-endothelial cell interaction, plasma factors and finally molecular mechanisms in megakaryocytes. Shearing force of the blood can be simulated by physical manipulation of the culture container, as by shaking, rotating or similar process. The role in release actuated by plasma proteins and platelet receptors can be actuated by the megakaryocytes themselves, or factors can be individually added, as needed. Large platelets have been described in certain congenital platelet abnormalities such as Bernard Soulier disease and von Willebrand factor (VWF) disease. We have observed some similarly large platelets in some of our embryoid body derived platelets. If this phenomenon is observed, due to inefficient pinching of platelets from

proplatelets caused by the lack of plasma factors such as VWF, this problem can be addressed by the addition of VWF alone or of VWF included in plasma. It has been hypothesized that cGMP can promote platelet formation from neoplastic megakaryocytes. cGMP can be activated by nitric oxide. We have found that the addition of GNSO, a nitric oxide releasing compound, can quickly fragment megakaryocytes into small platelet-like particles in 2 hours. Finally, a by-product of this process is the relatively pure endothelial cells. We are testing to determine if endothelial cells can also help platelet shedding. By all these techniques, we can significantly increase the efficiency and regularity of platelet formation. To understand the biological mechanism of platelets, we have set up 3D real time fluorescent microscopy to record the platelet release with and without the presence of plasma. We have been able to record the 3D image of proplatelets and we are currently are in the process of doing the time lapse to better monitor this process.

[00020] Platelets will then be gathered and packaged. At the final stage of megakaryocyte differentiation on day 12, non-cohesive megakaryocytes will be transferred into the upper well of a multi-well plate with 3μM pore size filter. Incubation will be carried out in an incubator with gentle shaking and GNSO. Platelets will be collected in the lower chamber, if necessary in the presence of human plasma, or VWF and fibrinogen at physiological concentrations. Platelets isolated from this *in vitro* system will be purified by sequential centrifugation and re-suspended in citrate buffer as donor platelets. The collected platelets will be further centrifuged at low speed (3000g for 30 min) to separate the other debris and then filtered through an appropriately sized filter to rid the preparation of any nucleated cells. The platelet containing product thus produced can feature the platelets concentrated to any desirable concentration. The *in vitro* produced platelets can be further purified as serum or plasma free products to fit particular clinical needs. All containers can and should be sterilized to decrease the bacterial contamination, a common problem with donor platelets from conventional sources.

[00021] The platelets thus produced from *in vitro* cell culture will be different from those that have previously been available to science or medicine, in that these platelets will not have been exposed to the bloodstream. Platelets produced *in vivo* in an organism can not completely separated from plasma. As a result, the packaged platelets in current medical use today also carry small quantities of leukocytes and plasma contaminants that can cause transfusion reactions in some patients. Platelets produced from this *in vitro* system by differentiation from human ES cells will be free of leukocytes and will never have been exposed to serum or plasma. Platelets produced by this *in vitro* system will only carry fibrinogen or VWF if those factors were added in the growth or separation process.

[00022] A related problem is that some immunoglobulins spontaneously adhere to platelets. Thus platelets isolated from human donors inevitably carry immunoglobulin molecules from the donor, another possible contributor to adverse reactions. Platelets produced *in vitro* from ES cells will not have been exposed to IgGs and will thus be free of them. The "ABO" blood typing antigens also appear on platelets, although weakly. It is unclear if the occasional ABO-type reactions from platelet transfusions are from the platelets or from serum contaminants. The Rh factor is not present in platelets. Platelets produced from this process will thus be medically and scientifically more adaptable as well as readily distinguishable from platelets produced by conventional separation techniques.

EXAMPLES

[00023] **Hematopoietic precursors from embryoid bodies**

[00024] Embryoid body (EB) formation is a method that has been used to study both hematopoietic differentiation of mouse and human ES cells. However, unlike mouse ES cells, human ES cells in a single cell suspension fail to efficiently form embryoid bodies. Instead, to form embryoid bodies from human ES cells, intact colonies of human ES cells cultured on mouse embryonic fibroblasts (MEFs) were digested for 5 min by 0.5mg/ml dispase to form small cell clusters. These cell clusters were then allowed to further aggregate in serum-containing stem cell cultivation medium (20%FCS). Easily distinguishable cell masses, embryoid bodies start to form after 6 days of culture with 50% single cells that fail to participate into the cell mass and undergo apoptosis. After 12 days of culture, the embryoid bodies resembled the early embryonic structure of the yolk sac. Taking sections of the embryoid bodies and then subsequent immuno-labeling the sections by a CD34 antibody revealed the histological features of yolk sac. Non-adhesive hematopoietic precursor cells were found to be present in the lumen of small vessels and the endothelial lining, as revealed by the cells being CD34 positive. The embryoid bodies were then treated by trypsin digestion (.05% Trypsin/0.53 mM EDTA) at 37°C. Approximately 10^5 embryoid body-derived cells containing primary hematopoietic precursor cells were plated in methylcellulose cultures (Stem Cells Inc. Canada) and cultured for 10-12 days. Erythrocytes and megakaryocytes colony forming units (CFUs) were then detected by their native red color or immunolabeling with monoclonal anti-CD41 or CD61 antibodies. Definitive hematopoietic precursor cells that can give rise to macrophage and granulocyte colonies formed at day 12. This activity represents the first wave of primary and definitive hematopoiesis. We now have established a serum free embryoid body culture system free of both animal and human serum.

[00025] ***In vitro*** **expansion of embryoid body-derived megakaryocyte precursors**

[00026] After 12 days of embryoid body formation and culture, a single cell suspension culture was made by a 30-minute collagenase (1 mg/ml) and 5 minute trypsin digestion (0.05% Trypsin/0.53 mM EDTA) at 37°C of the resulting cell cultures. CD34+ cells were separated from other EB cells since they can interfere with hematopoiesis. CD34+ cells were cultured on a poly-HEME surface in the presence of thrombopoietin (TPO), interleukin 3 (IL3), interleukin 6 (IL6), and stem cell factor (SCF), all factors chosen to specifically promote megakaryocyte differentiation and proliferation. A yield of 10^6 CD41+ megakaryocytes per 10^6 starting ES cells was obtained (n=6). Interestingly, when plated in collagen–based semisolid matrix, these megakaryocytes formed extremely long processes with bead-like structures representing proplatelets. Such long structures have not previously been reported when human adult hematopoietic stem cells or mouse ES cells were used in attempts to generate megakaryocytes. Small CD41 positive cell fragments, identified as released platelets, were detected to be present close to the megakaryocytes. By flow cytometry, we found these megakaryocytes are positive for CD41, CD42a, CD42b, CD61, CD38, CD45 (weak) and CD62P, but negative for CD34, CD117, and HLA-DR. This phenotypic profile is consistent with normal human mature megakaryocytes.

[00027] **Differentiation of Megakaryocytes from Human ES Cells on Stromal Layers**

[00028] The OP9 stromal cell line is a cell line established from newborn calvaria op/op deficient mice that has been used to support mouse hematopoiesis. The op/op mouse carries a mutation in the coding region of the macrophage colony-stimulating factor (M-CSF) gene. Results of differentiation of human ES cells to hematopoietic lineage using the OP9 system were similar to the method of differentiation of human ES cells by embryoid body formation, but the stromal cell method usually gave a higher yield of more mature precursor cells. Briefly, human ES cells were seeded on confluent OP9 stromal cells and then cultured in alpha-MEM medium supplemented with 20% fetal bovine serum (FBS). Differentiation was started with 10^5 ES cells per well of a six-well plate or 8 x 10^5 cells in a 10 cm^2 culture dish. After 6 days of culture, the ES cells differentiated into hematopoietic progenitors, as indicated by the emergence of CD34+ cell surface markers on the cells. For differentiation into megakaryocytes, the cells were trypsinized on day 6 (.05% Trypsin/0.53 mM EDTA at 37°C/5% CO_2) for 5 minutes and passed onto fresh confluent OP9 cells in the same culture medium containing 10ng/ml TPO. After an additional 8 days of culture, megakaryocytes could start to be seen by visual inspection. About 30% of cells in the supernatant of the culture were megakaryocytes, as confirmed by CD41 immuno-staining. These megakaryocytes are multinucleated but without the significant long processes that were seen in embryoid body-derived megakaryocytes. These megakaryocytes are

believed to be definitive megakaryocytes that closely resemble the adult megakaryocytes. Interestingly, during the culture, there was no sign of platelet formation which is quite different from the murine system. It is likely that OP9 cells can promote and support megakaryocyte differentiation and proliferation, but can not support platelet formation. This is another indication that the mechanisms of platelet formation in mouse and human are different, even though some of the mechanisms of megakaryocyte differentiation and proliferation are similar.

[00029] Megakaryocyte proliferation, maturation and purification

[00030] Precursor megakaryocytes derived by either of the above methods have demonstrated the ability to proliferate and even engraft in adult recipient mice. As a next step in the process, we used bFGF to further proliferate immature megakaryocyte and at the same time halts the megakaryocyte maturation. Estimating that each megakaryocyte can generate 2000 platelets, 10^6 human ES cells (one 6-well plate) would generate 10^6 megakaryocytes and subsequently about 2 X 10^9 platelets, which represents approximately 1/20 unit of platelets (>5.5X10^{10} platelets per unit). So, at this estimated efficiency, to make 1 unit of human platelets would require 20 T75 flasks of human ES cells. This may or may not be economically attractive at this yield, but it is clearly in the range of what a single technician can already support.

[00031] Alternative techniques to direct differentiation

[00032] The embryoid body system has a lower than desired efficiency of making hematopoietic stem cells, due to the fact that the majority of the cells are yolk sac cells. However, this system is superior to the OP9 co-culture system since the embryoid body system has no murine protein contamination. From our data, we believe that hematopoietic differentiation is still best accomplished in the EB system as opposed to the co-culture system with stromal cells. To get more definitive hematopoietic cells and make the process more efficient, we plan to prolong the EB culture. We have tried to mechanically dissect or frament the EBs into smaller fragments and continue the culture hoping that the micro-environment will continue to support blood island differentiation. Our preliminary observation suggests that dissected EBs can survive and continue to grow following this dissection. This will be the first attempt to push the differentiation further in the EB system. Even if definitive blood islands can not form, significant increase in the number of blood islands may be achieved. Addition of growth factors in the EB culture such as VEGF and SCF will also be tested.

[00033] Improved platelet release and maturation

[00034] Although we have already observed platelet formation in multiple systems including collagen matrix, OP9 and polyHEME we want to better understand the mechanism of platelet release so that the process can be optimized. In order to achieve platelet formation we

will culture 10^3~10^4 mature megakaryocytes in the presence of TPO, human plasma, human cryoprecipitate and nitric oxide. The platelets will be labeled with anti-CD41 antibody and counted by flow cytometry. The shape of the platelets will be examined by microscopy, including electron microscopy. True platelets should be discoid without processes or attachment with other platelets. Larger or linked platelets suggests the non-optimal conditions that only support proplatelet formation. Extracellular matrices such as fibrinogen and fibronectin have been shown to promote megakaryocyte proliferation and maturation. We will use fibrinogen and fibronectin coated plates to culture megakaryocytes to determine their effects on megakaryocyte proliferation and differentiation.

[00035] **Testing platelets**

[00036] Platelet aggregation in response to thrombin, ADP, and collagen. Aggregation ability in response to different stimuli of the in vitro generated platelets will be measured by an aggregometer (Chrono-log Corporation, www.chronolog.com). Platelets will be harvested from the supernatant and counted. 10^6/ml platelets will be washed with PBS and resuspended in human plasma. Different concentrations of thrombin, ADP, and collagen will be added and the aggregation kinetics will be compared to native human platelets. We have tested the produced "platelet" and mature megakaryocytes can be activated by 0.5U/ml thrombin by surface expression of CD62P a indirect marker for alpha-granule release. We are in the process of testing platelet function via the following methods:

[00037] Dense core granule release. Aliquots of 10^6 cultured human platelets will initially be labeled with [^{3}H] 5-HT (serotonin) in buffer A (120 mmol/L sodium glutamate, 5 mmol/L potassium glutamate, 20 mmol/L HEPES/NaOH, pH 7.4, 2.5 mmol/L EDTA, 2.5 mmol/L EGTA, 3.15 mmol/L $MgCl_2$, and 1 mmol/L DTT). Platelets will be washed with buffer A and then activated with 1 unit of thrombin. The reactions will be stopped by placing the samples on ice for 4 minutes, followed by centrifugation at 13, 000g for 1 minute. The supernatants will be collected and assayed as below. [^{3}H]5-HT release will be measured by scintillation counting. The kinetics of dense core granule release can also be assessed by lumi-aggregometers (Chrono-log Corporation) that simultaneously measure aggregation and ATP secretion from the dense core granules.

[00038] Alpha-granule secretion: This assay will be monitored by measuring P-selectin expression by flow cytometry using a phycoerythrin-conjugated anti-CD62 antibody AC1.2 (Becton Dickinson). Typically, 2.5 µl of fixed platelets (10^9/ml) are added to 97.5 µl of antibody solution. After 15 min the samples are diluted with 1 ml of Tyrode's buffer containing 0.35%

BSA and analyzed. The percent increase in P-selectin expression will be calculated and compared to human native platelets.

[00039] Lysozome release: Hexosaminidase will be measured as described by Holmsen and Dangelmaier. Five ml of citrate-phosphate buffer, pH 4.5, and 2.5 ml of 10 mmol/L substrate (P-nitrophenyl-N-acetyl--D-glucosaminide) are mixed and aliquoted (100 µL) into 96-well plates, and 5 µL of the reaction supernatant is added. After incubation at 37°C for 18 hours, 60 µL of 0.08N NaOH will be added to stop the reaction. The absorbance is read in an ELISA plate reader with a 405-nm filter.

[00040] These tests will be used to establish the biological activity of the platelets produced from the human embryonic stem cells. Platelets produced from this *in vitro* platelet production system will functionally resemble normal platelets in the human body. However, when produced by *in vitro* generation and maturation, the platelets produced will be readily distinguishable from human platelets derived from blood due to the fact that the platelets produced by this process will never have been exposed, at least as produced, to human blood. As such, the platelets will not have adhered to them the normal serum factors, such as fibrinogen, coagulation factor V and VWF, factors which platelets normally acquire from the blood after release into the bloodstream *in vivo*. This assumes that the factors were not added in significant quantity to the culture, as might be the case if VWF is added to assist in platelet separation, Of course as well, after delivery to a patient, the platelets would promptly acquire those factors from the bloodstream of the recipient.

CLAIMS

WE CLAIM:

1. A method for the production of human platelets *in vitro* comprising the steps of:

(a) culturing human embryonic stem cells under conditions which favor differentiation of the cells into hematopoietic lineage by encouraging formation of embryoid bodies and then recovering hematopoietic cells from the embryoid bodies, wherein recovered hematopoietic cells are CD34+;

(b) culturing the CD34+ hematopoietic cells recovered from the embryoid bodies in step (a) in a medium including thrombopoietin, interleukin 3, interleukin 6 and stem cell factor for 10-12 days to produce CD41+/CD61+ megakaryocytes;

(c) culturing the CD41+/CD61+ megakaryocytes of step (b) so that they produce platelets, wherein the CD41+/CD61+ megakaryocytes of step (b) are directly exposed to nitric oxide to encourage platelet budding; and

(d) recovering the platelets of step (c) apart from the CD41+/CD61+ megakaryocytes of step (c) wherein the platelets of step (c) are CD41+.

2. The method as claimed in claim 1 wherein the nitric oxide is produced by adding S-Nitroso-L-glutathione (GNSO) to the culture.

Title: PLATELETS FROM STEM CELLS
Inventor(s): James A. Thomson/Dong Chen
Application No.:
Docket Number: 960296.001 11

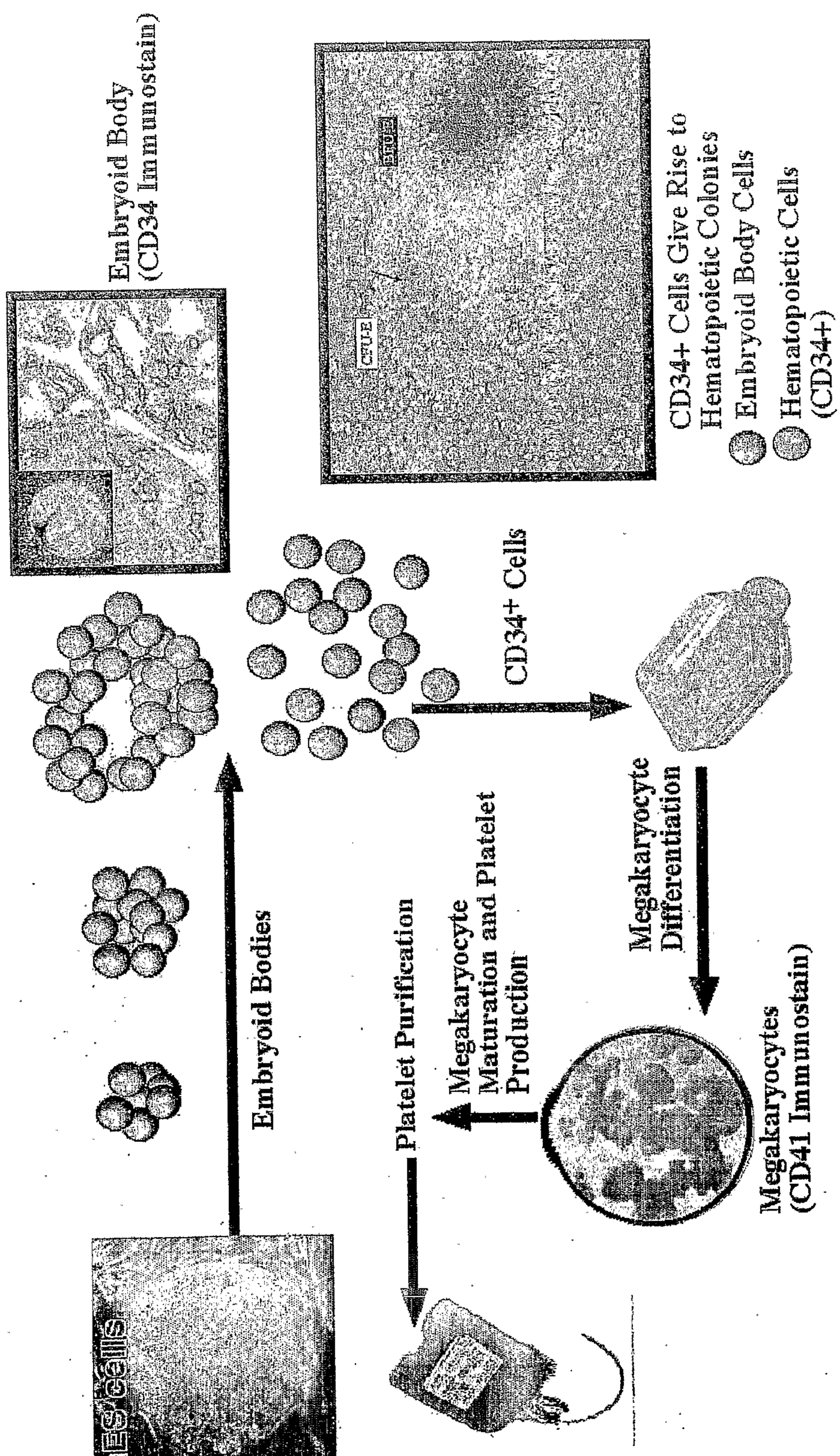

FIG 1

Title: PLATELETS FROM STEM CELLS
Inventor(s): James A. Thomson/Dong Chen
Application No.:
Docket Number: 960296.00111

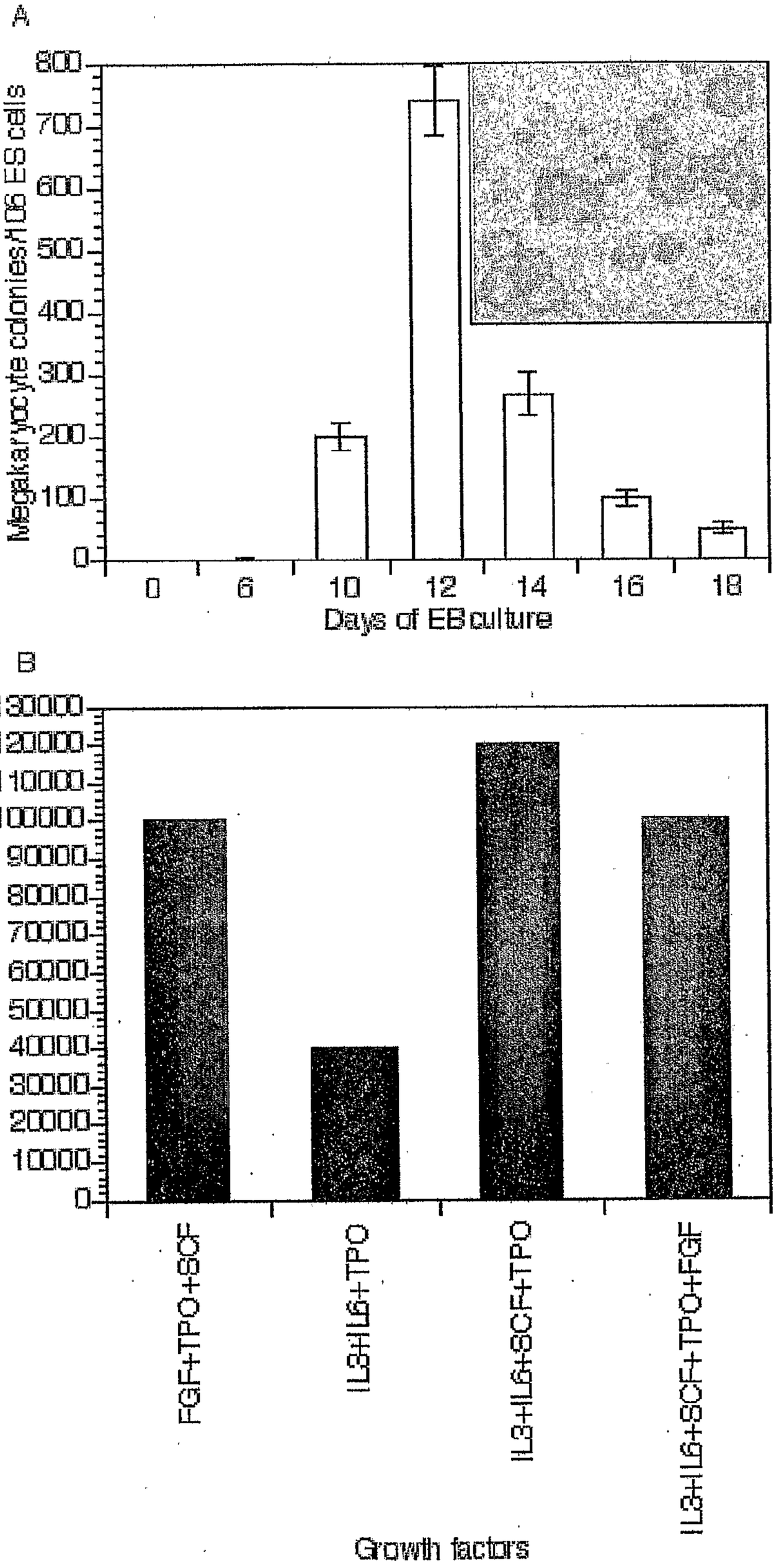

FIG 2

Title: PLATELETS FROM STEM CELLS
Inventor(s): James A. Thomson/Dong Chen
Application No.:
Docket Number: 960296.00111

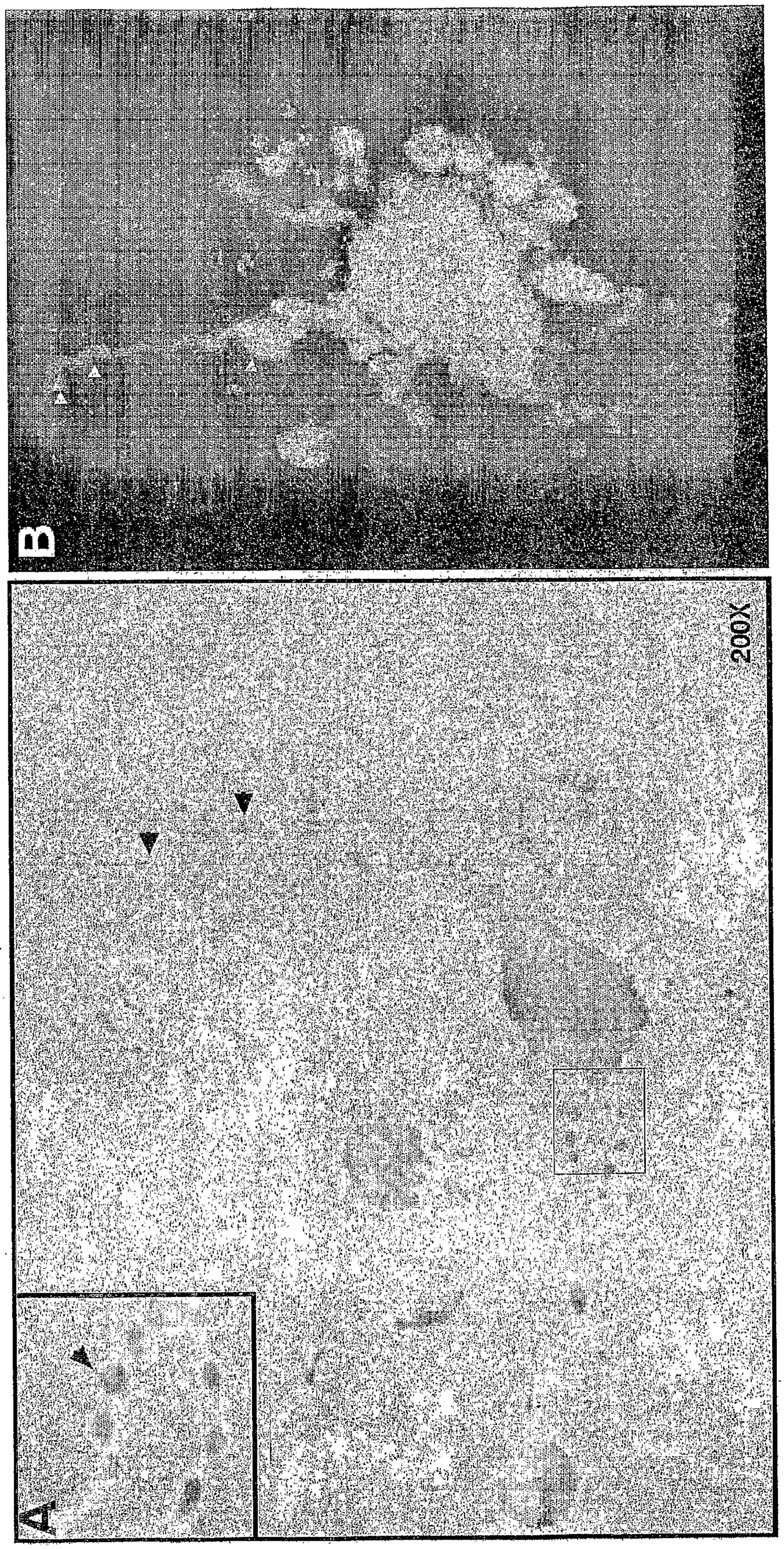

FIG 3