ORIGINAL ARTICLE

Induction of stem cell-like plasticity in mononuclear cells derived from unmobilised adult human peripheral blood

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SUMMARY

Undifferentiated pluripotent stem cells with flexible developmental potentials are not normally found in peripheral blood. However, such cells have recently been reported to reside in the bone marrow. Herein are reported methods of inducing pluripotency in cells derived from unmobilised adult human peripheral blood. In response to the inclusion of purified CR3/43 monoclonal antibody (mAb) to well-established culture conditions, mononuclear cells (MNC) obtained from a single blood donor are converted into pluripotent haematopoietic, neuronal and cardiomyogenic progenitor stem cells or undifferentia ted stem cells. The haematopoiet ic stem cells are CD34+, clonogenic and have been shown to repopulate non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The neuronal precursors transcribe the primitive stem cell markers OCT-4 and nestin, and on maturation,

differentially stain positive for neuronal, glial or oligodendrocyte-specific antigens. The cardiomyogenic progenitor stem cells form large bodies of asynchronously beating cells and differentiate into mature cardiomyocytes which transcribe GATA-4. The undifferentiated stem cells do not express haematopoietic-associat ed markers, are negative for major histocompatibility complex (MHC) class I and II antigens, transcribe high levels of OCT-4 and form embryoid body (EB)-like structures. This induction of stem celllike plasticity in MNC may have proceeded by a process of retrodifferent iation but, in any case, could have profound clinical and pharmacological implications. Finally, the flexibility and the speed by which a variety of stem cell classes can be generated ex vivo from donor blood could potentially transfer this novel process into a less invasive automated clinical procedure.

Introduction

Somatic cell plasticity is an emerging field in stem cell biology. In this respect, a variety of more committed cells have been shown to re-switch their developmental potential in response to either nuclear transfer into an enucleated oocyte¹, or to more remote microenvironmental cues or pressures, as is the case with stem cells *in vivo*²⁻⁵. On the other hand, the phenomenon of cell transdifferentiation, during which more committed cells traverse the differentiation barrier and adopt a new specialisation fate *in vitro*, is well documented in the literature.

Transdifferentiation from one specialised fate to another has been demonstrated for many cell types: retinal pigmented epithelial cells into lens tissue or retinal neuronal cells^{6,7}; keratinocytes into mesenchymal myogenic-like cells⁸; dermal papilla cells into hair follicle epidermal cells⁹; squamous vaginal epithelial cells into cuboidal mucinous cells¹⁰; human pancreatic islet cells to pancreatic ductal cells¹¹; fat storing cells into myofibroblasts¹²; chondrocytes into osteocytes¹³; oligodendrocyte precursors into multipotential CNS stem cells¹⁴; blood cells into brain⁴ and vice versa⁵ and pro-B cells into a variety of leukocyte subsets¹⁵. In most cases, the newly generated cells have been noted to redifferentiate into cells of the original lineage and stage.

While transdifferentiation is well documented, the underlying mechanisms remain poorly understood. The loss of differentiation markers, commonly termed dedifferentiation¹⁶, has been demonstrated in multinucleated heterokaryons¹⁷, a transfected cell line¹⁸, gut cells¹⁹ and mammary epithelial cells¹. However, explanations for dedifferentiation provided to date are ambiguous because they do not elucidate what is behind the loss of the differentiated state. Analyses of *de novo* gene activation in such cells suggest some sort of reprogramming.

Another mechanism proposed to explain the partial loss of the differentiated state of a cell is retrodifferentiation²⁰⁻²⁵ which is defined in terms of what occurs during forward differentiation of a cell. The precursor principle underlying retrodifferentiation is the inversion of the differentiation programme to generate a cell at a progenitor or stem cell stage. In this form of retrograde development, a committed cell reverts to an earlier ontogenic stage. In contrast to transdifferentiation, evidence supporting retrodifferentiation is meagre, coming primarily from studies of cell lines such as myelomonocytic²⁶ and erythroid²⁷ leukaemias. regenerating liver cells²⁸ and neoplastic colon cells²⁹. Such studies previously reported loss of differentiation markers, however, not to a stem cell stage.

Pluripotent stem cells with multi-developmental potentials are not found circulating in unmobilised adult human peripheral blood. These types of precursors have been reported recently to reside in the bone marrow³⁰. The wide clinical indications where stem cell therapies are believed to benefit many patients with leukaemia, lymphoma, some solid tumours or degenerative diseases puts an added constraint on their current sources. Unmobilised adult peripheral blood contains insufficient quantities of stem cells to be used to treat such a wide spectrum of clinical indications. Herein, I report the in vitro production of a variety of stem cell classes derived from mononuclear cells (MNC) obtained from human adult peripheral blood in response to the addition of purified CR3/43 monoclonal antibody (mAb) to well-established culture conditions.

Materials and methods

Cell Culture

Haematopoietic-Conducive Conditions (HCC)

MNC were obtained from healthy human buffy coat samples (obtained from the National Blood Service, Brentwood, England) by density gradient centrifugation on Histopaque (Sigma) at a specific gravity of 1.077 g. After washing, MNC were resuspended at 2×10^6 per ml in Dexter's long-term culture (LTC) medium³¹ consisting of Iscove's Modified Dulbecco's Medium (IMDM) without phenol red (Invitrogen), 10% foetal calf serum (FCS), 10% horse serum (HS) (Sigma), 10^{-7} M cortisol (StemCell Technologies), and 1% penicillin/streptomycin (Sigma) supplemented with 3.5 µg per ml of purified CR3/43 (DakoCytomation). This constituted the haematopoietic-conducive condition (HCC).

The CR3/43 clone was generated by DakoCytomation in the absence of azide and antibiotics. The CR3/43 mAb is raised against human monomorphic regions of the beta chain of the major histocompatibility complex (MHC) class II antigens DP, DQ and DR. This antibody binds to B cells, monocytes, antigen-presenting cells and activated T cells. Alternatively, the animal sera, cortisol and antibiotic components of the HCC culture medium can be replaced by citrated human autologous plasma (ACDA, Baxter Inc.) supplemented with 7 µg/ml CR3/43. In order to increase the yield of CD34⁺ cells, human leukocytes enriched by dextran sedimentation or aphaeresed mononuclear fractions can be used instead of histopaque-separated MNC. Cells were plated in sixwell plates (Fisher Scientific, USA) at 2 ml per well and incubated at 37 °C and 5% CO₂ in air. The cells were fed the following day and, thereafter, every 4 days with Dexter's medium in the absence of CR3/43.

Neuronal-Conducive Conditions (NCC)

MNC were prepared and seeded as described above except that these were cultured in embryonic stem (ES) culture media³¹ consisting of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), 20% FCS (Sigma), 1% L-glutamine (Sigma), 1% MEM non-essential amino acids (Invitrogen) and 0.2% of 0.1M β -2-mercapto-ethanol (Sigma). Cells were fed the following day and thereafter every 4 days with ES medium in the absence of CR3/43.

Cardiac Conducive Conditions (CCC)

MNC were obtained as above and seeded as $20 \,\mu$ l 'hanging drops'³³ in ES³² or LTC culture media³¹, as detailed above, containing 3.5 μ g/ml purified CR3/43. Alternatively, MNC were subjected to HCC or NCC, minus cortisol and β -2-ME respectively, and seeded at

 2×10^6 per ml in six-well plates (Fisher Scientific USA) and chamber slides coated with 0.1% gelatin (StemCell Technologies). Beating areas in hanging drops³³ were observed using an inverted phase contrast microscope (Olympus CK-40) and imaged using a digital video camera.

Clonal Assays

Cells were seeded in methocult GFH4434 according to manufacturer instructions (StemCell Technologies) containing recombinant human growth factors. Differentiation into haematopoietic cell colonies was assessed and colonies were inspected and scored with time using phase contrast microscopy (Olympus CK-40).

Purification of CD34 Cells by Positive Selection

Twenty-four hours post-CR3/43 treated MNC under HCC were purified using the CD34 MultiSort Kit (Miltenyi Biotec) according to manufacturer's instructions. Briefly, prior to isolation, cells were subjected to Fc receptor blocking to prevent non-specific binding followed by direct labelling of cells with antihuman class II CD34-coated microbeads. The labelled cells were passed twice through an LD Midimacs separation column in the MACS separator and bound cells were gently flushed and collected for analysis.

Purification of Undifferentiated Cells by Negative Selection

Twenty-four hours post-incubation, an aliquot of cells incubated under NCC were subjected to co-negative selection using anti-CD45 and anti-glycophorin A-coated magnetic microbeads and applied to the LD Midimacs column in the MACS separator all according to the manufacturer's instructions (Miltenyi Biotec). The unbound cells were retained, passed through a further magnetic separation step and collected as the CD45negative/glycophorin A-negative population for analysis.

Confocal Microscopy

In order to facilitate live imaging by confocal microscopy, cells cultured in HCC were plated in organ culture dishes whereby a cover slip formed an integral part of its base. Conjugated anti-human antibodies against CD19-fluorescein isothyocyanate (FITC) and class III CD34-R-phycoerythrin-Cy5 (RPE-Cy5) (both DakoCytomation) were added at the recommended dilutions directly to the reaction/cell mixture. Cells were imaged every 3 min for up to 12 h at room temperature. Imaging of colonies in methocult culture was performed once colonies had reached maturity by directly adding the following fluorescent conjugated anti-human antibodies to the culture media: glycophorin A-FITC; CD33-RPE-Cy5; CD61-FITC (all DakoCytomation).

Flow Cytometry

Cultured cells harvested at specified time points were washed and resuspended in PBS containing 10% human AB serum (Sigma) or an Fc blocking reagent (Miltney Biotec) to block non-specific binding. Due to homotypic aggregation and adhesion induced in response to treatment with CR3/43, single-cell suspensions were obtained by continuous scraping, stirring and pipetting of the cultured cells. According to the manufacturer's instructions, cells were labelled for 15 min at 4°C with the following directly-labelled anti-human antibodies: class III CD34-PE, CD34-FITC and CD34-RPE-Cy5, CD38-PE, CD38-FITC, CD45-FITC, c-kit-PE, CD33-PE, CD61-FITC, glycophorin A-PE, CD19-PE and CD3-FITC (all DakoCytomation) and CD133-PE (Miltenyi Biotec). Autofluorescence and negative controls were determined throughout; isotype negative controls IgG1-FITC, IgG1-PE and IgG1-RPE-Cy5 (all DakoCytomation) were used. Cells were washed with cell wash (Becton Dickinson). Events ranging from 20 to 100 000 were acquired using FACScan (Becton Dickinson) and analysed using Cellquest software version 3.3.

Formation and Immunohistochemical Staining of Embryoid Body (EB)-Like Structures

Twenty microliters containing 300 purified cells (as described above for negative selection) (i.e. 1.5×10^4 cells per ml) were resuspended as 'hanging drop' cell cultures in ES medium³³. EB-like structures were plucked from 'hanging drop' cultures and transferred to microscope slides pre-coated with poly-lysine. A coverslip was applied and the slides were incubated at -80°C for 15 min prior to fixing. In contrast, MNC cultured in NCC and CCC were directly fixed in chamber slides prior to blocking and staining. All samples were fixed for 15 min in 4% paraformaldehyde (Sigma). EB-like structures were stained using HRP-conjugated antibody for analysis by phase contrast microscopy. For HRP-conjugated staining, fixed embryoid body (EB)-like structures were incubated with peroxidase block (DakoCytomation) for 5 min and then further blocked and permeabilised using 0.15% Triton X-100/10% rabbit serum/PBS. Thereafter, EB-like structures were independently incubated with one of the following primary anti-human monoclonal antibodies: myocardial-specific actin; alpha-fetoprotein; cytokeratin-7 and -20; Desmin; S100 (all Dakocytomation) and panneurofilament (Sternberger Monoclonals) and then detected using а rabbit anti-mouse HRP (DakoCytomation), with all antibody dilutions recommended by the manufacturer. Nuclei were stained with haematoxylin (DakoCytomation). Faramount medium (DakoCytomation) was used for mounting and the stained cells visualised by inverted phase contrast microscopy (Olympus CK-40). Imaging was subsequently performed using a digital camera attached to the microscope. Cystic formation by embryoid body was performed by staining suspension of intact cell clusters with DiI-C18 (kind gift from Dr Tim McCaffery). Following copious washing, embryoid bodies were analysed using fluorescence microscopy.

Immunohistochemical Staining of Neuronal and Cardiomyogenic Development

MNC cultured in NCC and CCC were fixed as above, blocked using 0.15% Triton X-100/10% donkey serum/ PBS and then differentially co-stained for neuronal and cardiac-specific markers respectively. For neuronalspecific staining, cells were co-stained with NF/GFAP, MAP-2/Tau or oligodendrocyte/CD45-FITC primary conjugate (DakoCytomation). In this case, primary antimonoclonal antibodies human against pan-NF (Sternberger Monoclonals), MAP-2 (Sigma) and oligodendrocyte (Chemicon International) were detected donkey anti-mouse RPE-Cy5 using (Jackson Immunoresearch) whilst second-stage rabbit anti-human (DakoCytomation) and Tau (Chemicon GFAP International) were detected using donkey anti-rabbit FITC (Jackson Immunoresearch) and CD45 was directly stained with anti-human CD45-FITC conjugate (DakoCytomation).

MNC cultured in CCC were differentially co-stained for either cardiac-specific troponin I or myocardialspecific actin and human CD45. In this case, the primary anti-human monoclonal antibodies against troponin I (Fitzgerald Industries Inc.) and actin (Dakocytomation) were both detected using donkey anti-mouse RPE-Cy5 (Jackson Immunoresearch) whilst second-stage antihuman monoclonal antibody against CD45 was directly conjugated to FITC. In all cases, nuclei were stained using propidium iodide (PI) (Sigma). In addition, human NF, MAP-2 and GFAP were detected using TRITC-labelled rabbit anti-mouse conjugated antibody and nuclei stained with hoechst. Imaging was subsequently carried out by confocal microscopy.

Reverse-Transcriptase PCR Analysis

Total RNA was isolated from cells at recorded time intervals using the RNAzol reagent (Biogenesis) according to the manufacturer's instructions. The RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Promega). The reverse transcriptase products served as a template for independent PCR reactions using the thermostable Taq polymerase (Promega). For PCR analysis, OCT-4³⁴, nestin³⁵, CD34³⁶, GATA4, hANP and cTnT³⁷ primers were used.

Haematopoietic Analyses

Live Image Analysis using Confocal Microscopy

Treatment of MNC obtained from adult peripheral blood with purified CR3/43 mAb increases the relative number of CD34+ cells cultured in Dexter's LTC medium³¹. The CR3/43 mAb is raised against human monomorphic regions of the beta chain of the major histocompatibility complex (MHC) class II antigens DP, DQ and DR. This antibody binds to B cells, monocytes, antigen-presenting cells and activated T cells. Under such HCC, live imaging of MNC using confocal microscopy (Figure 1A-L) reveals upregulation of the haematopoietic stem cell marker, CD34, concomitant with downregulation of the mature B lymphocyte marker CD19 (see supplementary data - real-time movie A³⁸). These phenotypic changes occur within an hour of adding CR3/43 to the MNC cultured in Dexter's medium and are accompanied by cell motility in the culture dish.

Flow Cytometry Analysis

Immunophenotypic analysis of MNC under HCC before and after 2 h and 24 h treatment with CR3/43 mAb (Figure 2) shows significant increase in the relative number of cells expressing CD34 in response to treatment. The majority of CD34⁺ cells are CD45 low and either positive or negative for CD38 antigen, typical of committed and more primitive haematopoietic progenitor cells^{39,40}, respectively. The latter type has been shown to possess more long-term SCID repopulating potentials³⁹. By 24 h in HCC, a significant proportion of CD34⁺ cells co-express c-Kit or CD133 (Figure 2A). Furthermore, significant numbers of CD34⁺ cells were purified from MNC cultured for 24h in HCC, using well-established haematopoietic progenitor purification procedures⁴¹ such as magnetic beads labelled with anti-human CD34 (Figure 2B). The purified CD34⁺ cells exhibited, as for conventional haematopoietic stem cells, CD45 at low levels with or without CD133. The CD34⁺ CD133⁺ cells were more CD34 bright⁴² than those cells that were CD34⁺CD133⁻. A significant proportion of CD34⁺ cells were either CD38 positive or negative. The latter immunophenotype have been reported to possess more long-term SCID repopulating potential³⁹ Moreover, significant levels of CD34 transcripts³⁶ were amplified from the purified CD34⁺ cells and unfractionated MNC cultured for 24 h in HCC when compared to MNC cultured in LTC alone (Figure 2C).









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С

CD34 GAPDH



А





CD34 Transcription

6 7 8 9 10

3 4 5

1 2

Following cryopreservation of the 24 h MNC cultured in HCC and subsequent purification of CD34⁺ cells, the yield of viable CD34⁺ cells per one unit of buffy coat (500 ml of donated blood) as measured by flow cytometry, and cell viability assessed by trypan blue dye exclusion assay, is approximately $100-150 \times 10^6$ cells and dependent on MNC input. For example, higher yields of MNC are obtained via aphaeresis or dextran sedimentation than when fractionated on a density gradient. Throughout culturing of MNC in HCC, cells remain viable and there is an approximate 1.6-fold increase in their absolute numbers 24 h later. On the other hand, 24 h culturing of MNC in LTC (not supplemented with CR3/43 mAb) gives rise to an increase (about 20%) in the number of erythrocytes and dead cells (Figure 2). Viability assessment of MNC cultured in HCC or LTC at 2 h and 24 h from culturing following cryopreservation, showed 30% cell death in cells cultured in LTC compared to 5% in the HCC group {data not shown}.

Colony Formation

By 2 h in HCC, MNC were rendered clonogenic when single-cell suspensions were seeded in methocultcontaining growth factors. They produced a variety of haematopoietic colonies such as colony-forming unitgranulocyte, erythroid, monocyte, macrophage, megakaryocyte (CFU-GEMM), colony-forming unitgranulocyte, monocyte, macrophage (CFU-GM), colonyforming unit-monocyte, macrophage (CFU-M), blastforming unit-erythroid (BFU-E) and colony-forming uniterythroid (CFU-E) (Figure 3). On the other hand, seeding MNC, cultured for 24h in HCC, at the same cell density in methocult medium (basic or growth factor-containing) supplemented with 300 µl of 24 h HCC supernatant (to basic or growth factor-containing medium) resulted in the formation of much larger and wider variety of colonies, including megakaryocytes, indicating the secretion of cytokines by cells cultured in HCC (Table 1). On the other hand, the clonogenic efficiencies of purified CD34+ cells obtained from MNC cultured for 24h in HCC is 1 in 2.5 when compared to a value of 1 in 105 MNC cultured for 24h in LTC {data not shown}. Direct immunostaining of these high proliferative potential⁴³ (HPP-CFC) haematopoietic colonies in methocult cultures with antihuman antibodies specific for myeloid and erythroid antigens using confocal microscopy showed differential expression of glycophorin A without CD33, CD33 without CD61 and CD33 with CD61 (Figure 3R-T), a staining typical of erythrocytes, pattern monocytes/granulocytes megakaryocytes, and respectively. More significantly, ploidy or cell cycle analysis of fully differentiated haematopoietic colonies grown in methocult show normal DNA content {data not shown}. Furthermore, within 24 h, MNC cultured in HCC gave rise to cobblestone areas and stromal-like cells (Figure 3K). In addition, cryopreserved 2 h and 24 h MNC in HCC were clonogenic when compared to the same MNC population cultured in LTC. Interestingly, the time at which MNC became capable of repopulating non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice44 at a relatively higher level and frequency was 3 h following the addition of purified CR3/43 mAb to MNC cultured in Dexter's medium {manuscript in preparation}.

Purification and Characterisation of Undifferentiated Cells

Apart from red blood cells, leukocytes and extremely low levels of haematopoietic precursors, mature healthy blood is not known to contain undifferentiated cells or neuronal precursors. Treatment of MNC cultured in ES culture media³² with 3.5 μ g/ml purified CR3/43 mAb resulted, by 24 h, in an increase in the number of undifferentiated cells that were both CD45 and CD34 negative (Figure 4A). These cells were purified by negative selection on a Midi Macs column using anti-human CD45

Table 1. Percentage of clonogenic progenitors within MNCcultured in HCC after 24 h (324 ± 37.9 colonies derived per 1×10^4 nucleated cells)

CFU-GEMM	CFU-GM	CFU-M	BFU-E	CFU-Meg
(%)	(%)	(%)	(%)	(%)
47.9 ± 5.8	19.8 ± 3.9	5.4 ± 2.2	9.7 ± 2.0	16.0 ± 3.1

CFU-GEMM indicates colony-forming unit (CFU)-granulocytic, erythroid, monocyte, macrophage, megakaryocytic; CFU-GM, CFU-granulocytic, monocyte, macrophage; CFU-M, CFU-monocyte, macrophage; BFU-E burst-forming unit-erythroid and CFU-Meg, CFU-megakaryocytic. The percentage of each colony type is calculated as the mean \pm standard error of mean (SEM) (n = 5). The number of phenotypically distinct progenitors were identified and measured from the use of plating conditions according to standard protocols

Figure 2 (opposite). Increase in the number of CD34⁺ cells in response to MNC cultured in HCC (A). Cells were stained as indicated with a panel of conjugated monoclonal antibodies against human CD34, CD45 and CD38, c-kit and CD133 antigens before and after (2h and 24h) culturing MNC in HCC (Dexter's medium with CR3/43 mab) and LTC (Dexter's medium alone). Cells analysed by FACScan were gated according to FSC and CD45 and in conjunction with the relevant isotype negative control. Purified CD34⁺ cells from 24-h MNC cultured in HCC using the CD34 MultiSort Kit (B). Cells were stained with a panel of conjugated monoclonal antibodies against human CD34 and CD38 and CD33. (C) RT-PCR showing CD34 transcription by purified and unfractionated 24h MNC cultured in HCC. Lanes 1 and 8, 1 KB ladder; lanes 2 and 3, GAPDH and CD34 gene transcript negative controls, respectively; lanes 4 and 5 are CD34 and GAPDH gene transcripts, respectively, in 24h MNC/LTC; lanes 6 and 7 are CD34 and GAPDH gene transcripts, respectively, in unpurified 24h MNC/HCC



Figure 3. Different types of clonogenic cells are produced in response to treatment of MNC in (A–K) HCC and (L–Q) NCC. Panels: (A–C) represent BFU-E; (D–F) CFU-GEMM; (G, H) CFU-GM; (I) CFU-M; (J) CFU-Meg; (K) cobble stone areas and stromal like cells. In contrast, MNC cultured in NCC gives rise to colonies that resemble (M) embryoid bodies or (N, O) large erythroid or (P, O) myeloid-containing colonies. Such large colonies are (L) visible to the naked eye. In addition, differential co-staining of colonies produced by MNC cultured in HCC demonstrates (R) glycophorin A-positive (green) and CD33- (red) (S) CD33 (red) and CD61 (green) co-positive and (T) CD33⁺ (red) and CD61⁻ (green) haematopoietic lineages typical of erythroid, megakaryocytic and granulocytic/monocytic cell lineages, respectively



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ban-NF, desmin and S-100 (brown stain) when compared to negative control EB-like structures stained with HRP conjugated secondary antibody alone. Nuclei were CĎ33-CĎ61-CĎÍ3-HLA-DR negative. In (C) RŤ-PĆR shows that the purified cells transcribe high levels of (lanes 3 and 5, respectively) GAPDH and OCT-4 and Figure 4. Increase in the numbers of undifferentiated CD45/lineage negative cells in response to MNC cultured in ES medium and supplemented with CR3/43 mab. For (A, B) FACScan analysis, 24-h MNC cultured in ES medium with or without CR3/43 mab were harvested and stained as indicated with a panel of conjugated CD34 and nestin, respectively. (D) Immunohistochemical analysis of EB-like structures formed 48h following culturing of the purified cells (characterised in B and monoclonal antibodies against human (A) CD34, CD45 and CD38 antigens. (B, C) Characterisation of the purified undifferentiated CD45/lineage-negative cells by flow cytometry and KT-PCR, respectively. In (B) flow cytometry shows the purified cells are HLA-ABC-CD45-CD19-CD3-CD34-CD38-CD71-glycophorinA-C) as 'hanging drops' (resuspended as 300 cells per 20 µl drop) show that they are positive for myocardial-specific actin, alpha-fetoprotein, cytokeratin 7 and 20, stained blue with haematoxylin. The stained cells were visualised by inverted phase contrast microscopy (Olympus CK-40). Imaging was subsequently performed to a lesser extent (lanes 7 and 9 respectively) CD34 and nestin. Lanes 1 and 6 are 1 KB ladder, lanes 2, 4, 8 and 10 are negative controls for GAPDH, OCT-4 using a digital camera attached to the microscope. (E) DiI-C18 labelled cystic embryoid body



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and glycophorin A-coated magnetic beads (Miltenyi Biotec), in order to deplete leukocytes and red blood cells, respectively. The unbound cells were analysed by flow cytometry using a panel of anti-human monoclonal antibody conjugates. The purified cells were negative for haematopoietic-associated markers that are typically expressed by the erythroid, lymphoid and myeloid cell lineages and include MHC class I and II antigens (Figure 4B). In addition, such purified cells transcribed high levels of the embryonic stem cell marker, OCT-4, and, to a lesser extent, nestin and CD34 (Figure 4C). Following purification, 95% of these cells were viable and, moreover, one unit of buffy coat can yield up to $90-130 \times 10^6$ of such undifferentiated cells.

Upon culturing as 'hanging drops'³³, the purified cells formed cystic EB-like structures by 48 h that expressed ectodermal, endodermal and mesodermal antigens including alpha-fetoprotein (Figure 4D-E). Taken together, these analyses demonstrated the production of undifferentiated cells 24 h following the addition of CR3/43 mAb to MNC cultured in ES medium. Similarly, 24h of culturing MNC in ES medium containing CR3/43 grown either on chamber slides (StemCell Technologies) or six-well plates resulted in the formation of EB-like structures that are loosely attached to the substratum and stain positive for ectodermal, mesodermal and endodermal antigens^{45,46}. In addition, single-cell suspensions obtained from 24 h MNC cultured in ES medium containing CR3/43 mAb seeded in methocult medium (containing recombinant growth factors and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and 20% O₂) produced uniform colonies akin to embryoid bodies (Figure 3M). In contrast the same cells, seeded in the same methocult at 37 °C containing 5% CO, and 5% O₂, formed larger colonies visible to the naked eye (Figure 3L) consisting of myelocytes and erythrocytes or myelocytes only (Figure 3, N–O and P–Q, respectively).

Neuropoietic Analyses

Notably, continued culturing of MNC in ES medium initially containing CR3/43 mAb resulted in the eventual conversion of MNC into spherical bodies and neuronal-like cells (Figure 5A). By 24 h, MNC in NCC, transcribed OCT-4 and nestin (Figure 5B)^{34,35} and on maturation stained positive for MAP-2, glial fibrillary acidic protein (GFAP) and neurofilament (NF) 200 and 70 by weeks 1 and 2 (Figure 5C). This process was always accompanied by downregulation of nestin and OCT 4.

Dual immunohistochemical staining of MNC in NCC performed at defined time points using antibodies to MAP-2 and Tau, NF and GFAP and oligodendrocyte and CD45, and analysed by confocal microscopy, showed differential expression of neuronal, glial and oligodendrocytic antigens⁴⁷. For example, by 48 h, cellular

spheres formed that co-stained positive for MAP-2 and Tau, indicative of neurospheres containing immature neurons. One and 2 weeks later, the spheres differentiated into mature neurons, with MAP-2 being confined to cell bodies and Tau to the axons (Figure 5D). On the other hand, spheres that were analysed for NF and GFAP showed the presence of neuronal and glial precursors. However, 1 and 2 weeks later, such spheres differentiated into neurons expressing NF alone without GFAP, or astrocytic-like cells expressing GFAP without NF, the latter typical of glial cells (Figure 5E). In contrast, costaining for the pan-leukocyte marker, CD45, and oligodendrocyte, indicated the absence of CD45 expression at all time points shown, while oligodendrocyte staining was confined to the periphery of the spheres and persisted in 1- and 2-week-old cultures (Figure 5F). Moreover, time course analysis of neurotransmitters released by these neurons in response to depolarisation revealed that, as they matured, the neurons produced increasing amounts of glutamate, GABA, tyrosine, dopamine and serotonin and uptake of taurine {unpublished data}.

Cardiopoietic Analyses

The cardiomyogenic-conducive condition (CCC) involves culturing of MNC either as 'hanging drops'³³ or in HCC or NCC in six-well plates or chamber slides, coated with 0.1% gelatin. In 'hanging drops', MNC cultured in CCC formed compact embryoid-like bodies that started to beat in an asynchronous manner within 24 h from the initiation of such cultures. These large masses of cell aggregates were surrounded by novel single cells that aligned themselves along parallel axes while undergoing synchronous cyclical beating rhythms consisting of torsional contraction, elongation and rotation (see supplementary data – Real-time movie B⁴⁸). These novel cells were found in the meniscus at the centre of the hanging drops. Interestingly, the relative number of contracting cells or embryoid-like structures in the 'hanging drops' increased significantly when cortisol and mercaptoethanol were removed from HCC or NCC, respectively. Moreover, azide was noted to be a powerful inhibitor of the beating cells. Within 1 week, MNC in CCC grown on gelatinised six-well plates or chamber slides transcribed the cardiac transcription factor GATA-4, human atrial natriuretic peptide (hANP) (Figure 6B) and cTnT³⁷. Dual immuno-histochemical analysis of 1-weekold CCC-cultured MNC revealed the expression of myocardial-specific actin and cardiac-specific troponin I without CD45 expression (Figure 6A). More interestingly, 3-day and 7-day-old MNC cultured in CCC were able to differentiate into fully mature cardiomyocytes when infused into the myocardium of non-irradiated noninfarcted Rnu/Rnu nude rats⁴⁹ {unpublished data}.





Δ

usT bns 2-9AM



Figure 5. (Continued)

9A3D bns 3N



Oligo and CD45

on chamber slides or six-well plates. (A) Phase contrast microscopy of MNC before and after 24 h, 1, 2 and 3 weeks in NCC demonstrates the formation of spherical structures hoechst (blue) and either NF, GEAP or MAP2 (each red) demonstrating the progressive acquisition of neuronal-specific markers. Differential staining of neurons, glia and oligodendrocytes with time by dual staining with (D) MAP-2 (blue) and Tau (green) and lower images at 2 weeks are of the same field showing MAP-2 being confined to cell bodies Figure 5. Time course analysis of neuronal and non-neuronal differentiation. (A) morphological (B) genetic and (C) phenotypic changes in MNC cultured in NCC and grown orders for each gene transcript; lane 1, 100-bp ladder; lane 2, gene transcript-specific negative control, lane 3, 24 h MNC/NCC; lane 4, 1 week MNC/NCC; lane 5, 2 weeks MNC/NCC; lane 6, 3 weeks MNC/NCC and lane 7, MNC alone. (C) Confocal microscopy of NCC-cultured MNC at defined time points co-stained with the nuclear stain and progressive development of cells with neuron-like projections. (B) RT-PCR analysis of OCT-4, nestin and positive control GAPDH-specific transcript expression. Lane and Tau to axons, (E) Pan-NF (blue) and GFAP (green) and (F) oligodendrocyte (blue) and CD45 (green). In (D-F) nuclei were stained with propidium iodide (PI) (red)

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Cardiac Actin

Cardiac troponin I

Figure 6. Analysis of cardiac differentiation. (A) phenotypic and (B) time course analysis of genetic changes in MNC cultured in CCC. (A) Differential expression of either cardiac Troponin I or cardiac Actin (both blue) without CD45 (green) after 1 week in culture. Nuclei were stained (red) with Pl. (B) RT-PCR analysis of cardiac transcription factors, GATA-4 and hANP and positive control GAPDH-specific transcript expression. Lane orders for each gene transcript: lane 1, 100 bp

ladder; lane 2, gene transcript-specific negative control; lane 3, MNC alone; lane 4, 24h MNC/CCC; lane 5, 1 week MNC/CCC

Discussion

Stem cell plasticity is a newly discovered phenomenon. Recently, cells with multi-developmental potentials have been identified in the bone marrow following *ex vivo* expansion³⁰. In contrast, herein, four different types of pluripotent progenitor/stem cells were produced from MNC obtained from unmobilised peripheral adult human blood. In response to the addition of CR3/43 mAb to a variety of well-established culture conditions, HCC, NCC or CCC give rise to haematopoietic, undifferentiated and, subsequently, neuronal or cardiomyogenic progenitor cells. This occurs despite the fact that the source material can be derived from a single blood donor.

Human MNC cultured under HCC are capable of engrafting and differentiating into a variety of lymphohaematopoietic cell lineages in the NOD/SCID mouse model {manuscript in preparation}. In addition, such cells when infused into the myocardium of the Rnu/Rnu nude rat⁴⁹ engrafted and differentiated into fully mature human cardiomyocytes 1 week later {unpublished data}, thus further confirming the phenomenon of somatic cell plasticity^{2-5,50}. Moreover, a significant proportion of MNC cultured in ES medium supplemented with CR3/43 mAb were converted into cells able to transcribe embryonic stem cells antigen, OCT-4; a transcription factor that is normally restricted in its expression to pluripotent cells⁵¹. These latter undifferentiated cells can be purified and are able to form EB-like structures in 'hanging drop' cell cultures following proliferation. Continued culturing of MNC in NCC gives rise to fully mature neurons, glia and oligodendrocytes upon extinguishing of nestin and OCT-4, that become capable of secreting a variety of neurotransmitters {unpublished data} - one of the bona fide characteristics of neurons. The in vivo functional utility of such cells in the reversal of Parkinson's disease in an animal model warrants investigation. Furthermore, the cardiomyogenic progenitor cells produced in response to culturing of MNC in CCC mechanically resemble beating embryoid bodies which appear to give rise to novel cells that are capable of synchronous beating rhythms in 'hanging drop' cell cultures (see supplementary data – Real-time movie B48). As mentioned, these cells were able to engraft and differentiate in vivo into mature cardiomyocytes. The fact that the cells can follow either a neuronal or cardiomyogenic fate by either culturing against gravity as 'hanging drops' or upright in a six-well plate, with or without artificially introduced extracellular matrix, is particularly noteworthy. This implicates the physical environment in directing the specialisation fate of a group of cells spatially organised in a certain configuration, and profoundly altering their developmental destiny, a notion that may be of importance in engineering and sculpturing human tissue.

The data presented in this report shows for the first time that the phenomenon of somatic cell plasticity is not exclusive to existing stem cells in vivo or to embryonic stem cells newly formed via nuclear transfer. Optionally, differentiated cells can also exhibit pluripotency in vitro, a notion that may redefine what is a differentiated or a stem cell state. Furthermore, as documented herein, CR3/43 mAb appears to facilitate de-differentiation in MNC⁵², while the immediate physical and biochemical surroundings of the cells cause their transdifferentiation or traversal of the differentiation barrier into new and multiple specialisation fates. In this manner, examples whereby signalling through MHC class II has influenced the cell physiology and behaviour include aggregation⁵³, activation^{54,55}, proliferation^{56–59}, anergy^{53,60,61} and apoptosis62,63. As to why MHC class II cross-linking profoundly alters cell specialisation fate, may be reflected by the differential association of protein kinase C isoforms with HLA-DR β chains⁶⁴. For example, alternate protein kinase C isoforms have been implicated in controlling differentiation of murine F9 embryonal carcinoma cells: one isoform promotes differentiation into parietal endoderm. whereas another stimulates the retrodifferentiation of such endodermal cells back into multipotent progenitors⁶⁵.

The induction of stem cell-like plasticity in a heterogeneous population of leukocytes, predominantly comprising mature specialised cells, may have proceeded by a process of retrodifferentiation²⁰⁻²⁵. This mechanism behind the reprogramming of differentiation in a population of adult cells may be contentious but, nonetheless, may address the phenomena of somatic or stem cell plasticity in haematopoietic stem cells, including the recently described multipotent adult progenitor cells³⁰. Irrespective of their origins, the ultimate functional utility of all stem cell types, whatever their source, remains to be determined in diseases where normal physiological functions of a variety of degenerate tissues need to be restored, either in autologous or allogeneic settings.

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