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Isolation and characterisation of mesenchymal stem cells derived from human placenta tissue

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Abstract

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AIM: To explore the feasibility of placenta tissue as a reliable and efficient source for generating mesenchymal stem cells (MSC).

METHODS: MSC were generated from human placenta tissue by enzymatic digestion and mechanical dissociation. The placenta MSC (PLC-MSC) were characterized for expression of cell surface markers, embryonic stem cell (ECS) gene expression and their differentiation ability into adipocytes and osteocytes. The immunosuppressive properties of PLC-MSC on resting and phytohemagglutinin (PHA) stimulated allogenic T cells were assessed by means of cell proliferation *via* incorporation of tritium thymidine (^{3}H-TdR).

RESULTS: The generated PLC-MSC appeared as spindle-shaped cells, expressed common MSC surface markers and ESC transcriptional factors. They also differentiated into adipogenic and osteogenic lineages when induced. However, continuous cultivation up to passage 15 caused changes in morphological appearance and cellular senescence, although the stem cell nature of their protein expression was unchanged. In terms of their immunosuppressive properties, PLC-MSC were unable to stimulate resting T cell proliferation; they inhibited the PHA stimulated T cells in a dose dependent manner through cell to cell contact. In our study, MSC generated from human placenta exhibited similar mesenchymal cell surface markers; MSC-like gene expression pattern and MSC-like differentiation potential were comparable to other sources of MSC.

CONCLUSION: We suggest that placenta tissues can serve as an alternative source of MSC for future experimental and clinical studies.

Keywords: Mesenchymal Stem Cell, Placenta, Immunophenotyping, Immunomodulation, Growth Kinetics

INTRODUCTION

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A substantial amount of research over the past two decades has resulted in greater understanding of human adult stem cell biology not only in the basic sciences but also in relation to therapeutic usage[1,2]. Among stem cells, mesenchymal stem cells (MSC) have become an important component in stem cell-based neo-therapies for tissue regeneration and transplantation. MSC are widely distributed in a variety of adult tissues such as adipose tissue, bone, lung, peripheral blood and are either constantly present or their pool is replenished due to migration from the bone marrow[3-5]. It was recently demonstrated that MSC are also present in umbilical cord blood, placenta and foetal tissues[4,6].

Unlike other stem cells, MSC derived from bone marrow have been investigated extensively for their immunosuppressive activity and have been exploited in treating autoimmune diseases as well as graft *vs* host disease (GVHD)[7]. Current literature indicates MSC-exerted immunosuppression is an important modulator in the allogenic immune response that involves mainly lymphocytes[8-10] and antigen presenting cells[11,12]. Some of these effects have been well exploited in therapeutics, such as in induction of tolerogenic response in GVHD[7,13] and enhanced antitumor therapy[14,15]. In addition, studies in animal models have shown that transplanted MSC have the potential to migrate to sites of injury, differentiate into appropriate phenotype and regenerate the injured tissue[16-20].

Bone marrow is the are the most extensively studied source of MSC (BM-MSC). However, BM-MSC have to be aspirated using an invasive procedure that can cause discomfort to the donor. This limited accessibility is couple with relatively low cell yield (0.001%-0.01%), with the numbers of stem cells significantly decreasing with age[21,22]. MSC derived from embryonic and aborted foetal tissues can overcome the volumetric problem but their usage in clinical application and research is still hindered by ethical issues and remains controversial. To overcome these problems, an alternative source of MSC which avoids ethical issues and is easily accessible at low cost is recommended.

In this study, we assessed the presence of MSC in human delivery waste tissues such as placenta which are more readily available for research at low cost[23]. We have showed that the MSC generated from human placenta tissues were able to expand and express similar characteristics as of BM-MSC in terms of mesenchymal and functional properties. We have also documented that placental MSC undergo cellular senescence as detected by morphological changes and thereby impose limitations on the culture expansion.

MATERIALS AND METHODS

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Generation of MSC from human placenta tissue

Placenta samples (n = 5) were collected upon delivery from normal full term pregnancies with the assistance of gynaecologists from Britannia Women and Children Specialist Centre. All samples were obtained with written, informed consent in accordance with the ethical committee requirements of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. The human placenta tissues were processed using our established mechanical disassociation and enzymatic digestion method[15]. Briefly, placenta tissue was minced into a paste-like consistency and digested in enzymatic mixtures containing 0.4% type II collagenase (Worthington, New Jersey, USA) and 0.01% DNAse (Worthington, New Jersey, USA). Following this, tissues were mechanically dissociated using a hand held cell homogenizer (Hassen Wagger). The single cell suspension was resuspended in MSC complete media containing Dulbecco's Modified Eagle's medium with nutrient mixtures F-12 (HAM) (1:1) with GLUTAMAX-I (Gibco, Invitrogen, USA), 10% foetal bovine serum (Stem Cell Technology Inc., London, UK), 1% Penicillin and Streptomycin (Gibco, Invitrogen), 0.5% Fungizone (Gibco, Invitrogen), 0.1% Gentamicin (Gibco, Invitrogen) and 40 ng/mL basic fibroblastic growth factor (bFGF) (Promega). The single nucleated cells (30×10^6 cells/T25 culture flask) were cultured in MSC complete media. Primary cultures were incubated for at least a week in a 37 °C humidified 5% CO_2 incubator and non-adherent cells were removed by replacing the media. Upon reaching 70% to 80% confluence, adherent MSC were harvested *via* trypsinisation (0.05% trypsin-EDTA, Invitrogen, BRL, Canada) for use in downstream experiments.

Immunophenotyping of PLC-MSC

Placenta MSC (PLC-MSC) were stained with a panel of MSC specific monoclonal antibodies: CD73-PE, CD29-PE, CD90-PE, MHC I-PE-Cy5, MHC II-FITC, CD45-FITC, CD34-FITC, CD80-PE, CD86-APC, (Becton Dickinson, Biosciences Pharmingen) and CD105-FITC, STRO-1-FITC (RandD System). 7-amino-actinomycin-D (7-AAD) (BD Pharmingen) was added for dead cell discrimination. Immunophenotyping was performed on the same cells aliquoted equally into different tubes according to experimental design. Stained cells were re-suspended in PBS, analysed using FACSCalibur flow cytometer (Becton Dickinson). The computed data were analysed using CellQuestPro software provided by the manufacturer.

RT-PCR of PLC-MSC

Total RNA was extracted from PLC-MSC, differentiated adipocytes and osteocytes using TRIzol® Reagent (Invitrogen, USA). RT-PCR was performed using the ImPromII™ Reverse Transcription System (Promega, USA) and cDNA and Taq DNA Polymerase kit (Qiagen). Genes of interest were obtained using primers synthesized from EUROGENTEC AIT as shown in Table 1.

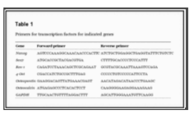

Table 1

Primers for transcription factors for indicated genes

Differentiation assay

PLC-MSC at 100% confluence were induced to differentiate into adipocytes, and osteocytes using MSC Adipogenesis Kit and MSC Osteogenesis Kit (CHEMICON). Adipogenesis induction medium contained 10% FBS, 1 μmol/L dexamethasone, 0.5 mmol/L IBMX, 10 μg/mL insulin, 100 μmol/L indomethacin, 1% Penicillin and Streptomycin and 90% DMEM/F12. Osteogenesis induction medium consisted of 10% FBS, 0.1 μmol/L dexamethasone solution, 0.2 mmol/L ascorbic acid 2-phosphate solution, 10 mmol/L glycerol 2-phosphate, 1% Penicillin and Streptomycin and 87% DMEM/F12. After differentiation, the adipocytes and osteocytes were fixed and stained with Oil Red O Solution and Alizarin Red Solution respectively. Chondrogenesis was induced using a Chondrocyte Differentiation Kit (STEMPRO®). Micromass cultures were generated by seeding 5 μL (1.6×10^7 cells/mL) droplets and were cultured in Chondrogenesis differentiation medium contained fresh 90% STEMPRO® Chondrocyte Differentiation Basal Medium and 10% STEMPRO® Chondrogenesis Supplements. After 21 d of cultivation, the chondrocytes were fixed and stained with 1% Alcian Blue solution and visualized under light microscope.

Growth Kinetics and doubling time

PLC-MSC (4×10^3 cell/well) were plated into 6-well plates and incubated at 37 °C in a 5% CO_2 humidified incubator. Media was changed twice weekly. Triplicates of PLC-MSC were harvested every 2 d until day 14 using 0.05% trypsin-EDTA and the growth curve of PLC-MSC was determined by performing trypan blue exclusion cell counts. About 0.3×10^6 of MSC from every passages were cultured in 100 mm Petri dishes and a trypan blue cell count was performed when the cells had attained full confluency. The initial seeding, days in culture and cell yield were recorded and the doubling time determined using the Patterson Formula [Td = Tlg2/lg (Nt/N0)], Td is the doubling time (h), T is the time taken for cells to proliferate from N0 to Nt (hour), and N is the cell count.

T cell proliferation assay

PLC-MSC was co-cultured with T cells at 1:5, 1:10, 1:50 and 1:100 ratios in a 96 well plate and stimulated with phytohemagglutinin (PHA) (Roche). Cultures were incubated for 72 h and pulsed with Tritium thymidine (^{3}H-TdR) [0.037 MBq/well (0.5 µCi/well) (Perkin Elmer)] for the final 18 h of incubation. At 72 h, cells were harvested onto glass fiber filter mats A (Perkin Elmer) using a 96 well plate manual cell harvester (MACH IIIM-FM, Tomtec, Inc. Hamden, CT USA). Scintillation cocktail was added and thymidine incorporation was measured by liquid scintillation spectroscopy using the Microbeta Trilux β counter (Pelkin Elmer). For transwell assays, T cells were physically separated from PLC-MSC by transwell chambers with 1 µm pore size membrane (Becton Dickinson).

Statistical analysis

The data were expressed as mean ± SE. The Student t-test was performed to compare the values of two means. Significance level was determined as $P < 0.05$.

RESULTS

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PLC-MSC exhibit mesenchymal morphological features

Formation of heterogeneous monolayer, adherent and spindle shaped fibroblast-like cells were observed for PLC-MSC and an average of 14 d was required for PLC-MSC to attain confluence (Figure 1A). The initial growth of PLC-MSC cultures at passage 0 (P0) consisted of two different heterogeneous populations; one with fibroblast-like morphology and the other with epithelial-like morphology. Upon trypsinisation and sub-cultivation, the epithelial-like population disappeared from the culture and could no longer be found in subsequent passages (Figure 1B). PLC-MSC were successfully cultured and expanded till P15. At early passages, MSC were obtained with well-defined smaller sized spindle shaped cells. However, these features gradually changed at later passages (P15 onwards). PLC-MSC at later passages appeared less defined, larger in size, less adherent and produced more debris in the culture supernatant (Figure 1C).

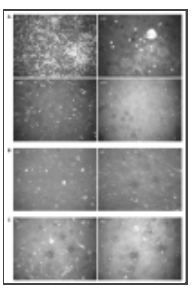


Figure 1

Morphology of placenta mesenchymal stem cells primary cultures. A: The formation of heterogeneous populations of placenta mesenchymal stem cells (PLC-MSC) with fibroblastic and epithelioid morphologies at passage 0, P0 until confluence at day 14; B: Homogenous **...**

Expression profile of PLC-MSC

Immunophenotyping of PLC-MSC was performed from P2 to P15. At early passage (P2), more than 90% of PLC-MSC were positive for integrin markers (CD29), mesenchymal markers (CD105 and CD73), CD90 and major histocompatibility class I antigen (MHC I) (Figure 2). All samples showed negative expression for hematopoietic cell markers (CD45 and CD34), co-stimulatory molecules (CD80 and CD86) and major histocompatibility class II antigen (MHC II). RT-PCR showed that PLC-MSC express embryonic stem cell (ESC) transcriptional factors such as Nanog, Sox2, Rex1and Oct4 (Figure 3). Expression of these markers was consistent for the subsequent passages.

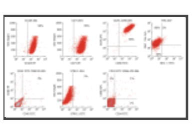


Figure 2

Immunophenotyping of placenta mesenchymal stem cells by flow cytometry. Adherent placenta mesenchymal stem cells (MSC) were harvested upon confluence and stained for MSC cell surface markers using a panel of anti human antibodies. Results represent at **...**

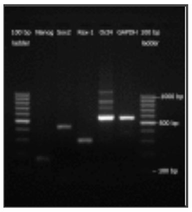


Figure 3
Gene expression by reverse transcription-polymerase chain reaction of placenta mesenchymal stem cells. Placenta mesenchymal stem cells express markers of embryonic stem cell transcriptional factors such as *Nanog*, *Sox2*, *Rex-1* and *Oct4*. *GAPDH* was used as **...**

PLC-MSC differentiate into mesodermal lineages

MSC were induced for adipogenic, osteogenic and chondrogenic differentiation along with standard culture medium as control. Histochemistry evaluation of PLC-MSC in inductive cultures showed their ability to differentiate into adipocytes, osteocytes and chondrocytes (Figure 4A). Adipogenic induction resulted in formation of lipid vacuoles which stained red with Oil-Red-O, whereas osteogenic induction resulted in deposition of calcium minerals (stained orangy-red with Alizarin Red). Chondrogenic induction resulted in formation of proteoglycans, stained blue by Alcian Blue solution. Images were captured using a phase contrast microscope. RNA analysis confirmed the osteogenic differentiation of PLC-MSC as the cells in osteogenic induction media expressed mRNA for the osteocalcin (OC) and osteopontin (OP) (Figure 4B).

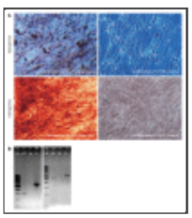


Figure 4
Differentiation potential of placenta mesenchymal stem cells into mesodermal lineages. A: Placenta MSC (PLC-MSC) after 3 wk in adipogenic or osteogenic or normal cell culture medium. Formation of lipid droplets (stained red in Oil-Red-O), calcium deposition **...**

Growth Kinetics analysis of PLC-MSC

Growth kinetics of PLC-MSC at early passage P3 (Figure 5A) showed a shorter lag-phase at day 1-6, followed by a rapid log-phase from day 6-12 until a plateau was reached. On average, the doubling time of PLC-MSC (Figure 5B) was 41 h.

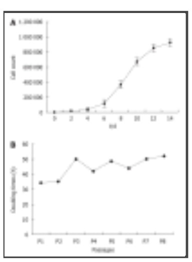
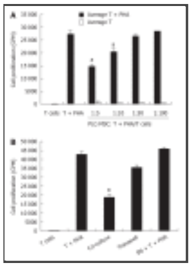


Figure 5
Growth Kinetics of placenta mesenchymal stem cells. Placenta mesenchymal stem cells (PLC-MSC) (4000 cells/well) were plated in 6 well plates and medium was changed three times a week. A: Triplicate cultures were harvested for trypan blue exclusion cell **...**

PLC-MSC inhibit stimulated PBMC via cell to cell contact

The effect of PLC-MSC on T-cell proliferation was evaluated by co-culturing MSC with resting or PHA stimulated T lymphocytes and measured by ^{3}H-TdR uptake. As shown in Figure 6, PLC-MSC were unable to stimulate the resting allogenic T cell but inhibited PHA stimulated T cells proliferation in a dose dependent manner. In order to determine the mode of inhibition, PLC-MSC were also co-cultured directly and physically separated by transwell inserts. T cells proliferation was significantly inhibited in direct co-culture whereas in the transwell system the suppression of T cells proliferation was less profound and not statistically significant. Meanwhile, PLC-MSC conditioned media (supernatant) did not suppress the activated T cell proliferation.

Figure 6



Dose dependent inhibitory effect of placenta mesenchymal stem cells on ph stimulated PBMC *via* cell to cell contact. A: Peripheral blood mononuclear cells) with or without phytohemagglutinin (PHA) stimulation were **...**

DISCUSSION

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In this study, we have successfully generated MSC from human placental tissue by using a combination of enzymatic digestion and mechanical dissociation[24]. The method yielded a high number of nucleated cells upon expansion. The MSC population was enriched by plastic adherence as the expansion capacity of MSC are dependent on initial plating densities and plastic source for adhesion[25,26].

The initial primary culture of placenta-derived single cell suspensions gives rise to a heterogeneous population of mainly fibroblast and epithelial-like morphology (Figure 1). This phenomenon, previously reported as a heterogeneous population in primary cultures, might be due to variations in cultivation method such as culture media, growth supplements and other pre-selection criteria[27,28]. However, endothelial-like cells do not contribute to the proliferation as they failed to proliferate at P0 and were unable to sustain beyond P0 in pre-optimised MSC complete media. Others have also reported that upon trypsinisation and subsequent sub-culture, the fibroblastic cells predominate the primary culture and continued to proliferate[29,30]. The growth kinetics measured for epithelial-like cell free cultures showed that the early passages of PLC-MSC had rapid growth kinetics and with an average doubling time of PLC-MSC of 41 h (Figure 5). However, the growth kinetics of PLC-MSC at later passage (P15 onwards) was sluggish; consisting of pro-longed lag and log phases; taking longer to attain confluence and showing higher doubling time in comparison to the earlier passages (data not shown).

We also evaluated the morphological changes of PLC-MSC throughout the numerous passages. In the early passages, PLC-MSC appeared to be firmly adherent, smaller in size and had a well defined shape. However, this morphology gradually changed as the passages increased. At later passages (P15 onwards), PLC-MSC appeared slightly bigger, elongated, less defined, less proliferative and eventually underwent senescence. According to Mareddy et al[31] and others, MSC cultures undergo senescence upon expansion, as indicated by the slow growth and reduced differentiation ability of MSC even though they still express normal levels of MSC surface markers[32]. In line with this, although the immunophenotype of later passage of PLC-MSC is unchanged (data not shown), continuous growth in culture and trypsinisation may be a major cause for loss of stemness as long term cultures are much inclined to spontaneous differentiation. In view of this, we have utilized PLC-MSC from early passages in our downstream experiments. However, the changes in morphology due to prolonged culture and trypsinisation need to be confirmed using karyotype analysis to determine cellular senescence.

There is no a single marker for depicting MSC. However, as per recommendation from International Society for Cellular Therapy (ISCT), a panel of antibodies was utilised as a minimal criterium to characterise human MSC. Dominici et al[33] have defined human MSC by immunophenotyping as they co-express CD105, CD73, CD90 while lacking expression for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR. Our expanded PLC-MSC cultures met most of these criteria and were non-hematopoietic and non-immunogenic as they did not express CD45, CD80, CD86 and MHC class II (HLA-DR) antigens while they were positive for typical MSC surface antigens (CD105, CD73, CD29, CD90 and MHC class I-Figure 2). Gene expression was found to be similar to that of BM-MSC and other sources of MSC[4,5,21]. PLC-MSC express the distinct surface proteins for MSC and at molecular level they also express ESC markers; Nanog, Sox2, Rex-1 and Oct4 (Figure 3). Similar expression patterns were also reported for chorion and amnion derived MSC[34]. These ESC markers are essential transcriptional factors and are usually expressed by pluripotent cells to maintain their undifferentiated state or "stemness"[35,36]. The intrinsic stemness properties of PLC-MSC, as measured by surface staining and expression of transcription factors at the molecular level, further supports the functional properties of MSC when they directly differentiated into osteoblasts, adipocytes and chondrocytes (Figure 4). These findings suggest that MSC derived from placenta tissues can give rise to mesodermal lineages, comparable to other sources of MSC[37,38]. However, the utilization of early passage of PLC-MSC is desirable as PLC-MSC are subject to cellular ageing.

Despite the normal expression of MHC class I, PLC-MSC failed to stimulate the proliferation of resting allogenic T cells. The hypo-immunogenicity of PLC-MSC may be due to the lack of MHC class II and co-stimulatory molecule (CD80 and CD86) expression which prevents them from presenting antigens to allogenic T cells. Nevertheless, the proliferation of PHA stimulated T cells was found to be dramatically inhibited in a dose dependent manner when co-cultured with MSC at various ratios. A similar inhibitory pattern was observed in human bone marrow derived MSC[9]. Although the transwell experiments exclude the role of soluble factors in PLC-MSC mediated immunosuppression, a noticeable yet non- significant inhibition caused by the autocrine effect of PLC-MSC secreting inflammatory cytokines in the presence of activated T cells. However, this contradicts other studies where soluble factors were also found to inhibit T cells profoundly[38,39].

In conclusion, our study has indicated that, upon successful expansion, PLC-MSC exhibit similar mesenchymal cell surface markers, an MSC-like gene expression pattern and MSC-like differentiation potential similar to other sources of MSC. This study indicates that placenta tissues have the potential to serve as an alternative to bone marrow as a source of MSC for future use experimental and clinical applications.

COMMENTS

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Background

Mesenchymal stem cells (MSC) have been widely studied for their therapeutic use in regenerative medicine and immune related disorders. Although MSC can be derived from virtually all tissues human delivery waste tissue (placenta) represents an ideal source for MSC due to its unlimited availability and freedom from ethicalconcerns. In this study, MSC from human placenta tissues were generated and characterised by immunophenotyping, early embryonic gene expression profiling and their potential for differentiation towards osteo, adipo and chondrocytes. Furthermore, placenta-derived MSC profoundly inhibited proliferation of T cells in a dose-dependent manner *via* cell-to-cell contact.

Research frontiers

MSC have attracted tremendous interest in repairing tissue injuries and dampening the inflammatory response in many disease models. It has been shown that MSC have an inherent ability to home to inflammatory sites, promising great potential for targeted tissue repair and wound healing. Thus, finding an alternative source of MSC that has less ethical concerns and a continuous supply may spur the use of MSC in therapeutic applications.

Innovations and breakthroughs

In previous studies, MSC were generated using conventional enzymatic digestion giving a low yield of single cells which took a long time to develop into adherent cells. However, in this article, placenta was processed by a mechanical disassociation method and followed by a typical enzymatic digestion. The number of single cells generated by this method was extremely high and a shorter period was required for colony formation. The authors have also characterised MSC of placental origin according to all relevant parameters (surface marker expression, mesodermal differentiation, early embryonic gene expression and immunosuppression).

Applications

This study suggests the feasibility of utilising placenta-derived MSC for clinical application in regenerative medicine. Furthermore, it also confirmed the presence of MSC in human placenta.

Terminology

PLC-MSC: MSC that derived from human placenta; T cell proliferation: Human T lymphocytes activated with mitogen and their cell division is measured by tritiated thymidine uptake.

Peer review

In this paper the authors demonstrate the feasibility of MSC derived from human placenta tissue, as an alternative to the bone marrow derived MSC. The authors show that the cells they isolated have features typical for MSCs. These features include the expression of CD105, CD73 and CD90, the deficiency of CD45 and CD34 as well as the ability to differentiate to adipocytes and osteocytes. The authors also demonstrate that, in line with the notion that MSCs act immunosuppressively, these cells have the potential to inhibit the proliferation of PHA-stimulated T-cells. The experimental design is simple and essential, and does demonstrate the feasible use of these cells as an alternative to the bone marrow derived MSCs, thus meeting its objective. Overall, these data are convincing in that the cells the authors isolated are most likely MSCs.

Footnotes

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