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EDITORIAL

Philadelphia chromosome-positive leukemia stem cells in acute lymphoblastic leukemia and tyrosine kinase inhibitor therapy

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Abstract

Leukemia stem cells (LSCs), which constitute a minority of the tumor bulk, are functionally defined on the basis of their ability to transfer leukemia into an immunodeficient recipient animal. The presence of LSCs has been demonstrated in acute lymphoblastic leukemia (ALL), of which ALL with Philadelphia chromosome-positive (Ph^+). The use of imatinib, a tyrosine kinase inhibitor (TKI), as part of front-line treatment and in combination with cytotoxic agents, has greatly improved the proportions of complete response and molecular remission and the overall outcome in adults with newly diagnosed Ph^+ ALL. New challenges have emerged with respect to induction of resistance to imatinib *via* Abelson tyrosine kinase mutations. An important recent addition to the arsenal against Ph^+ leukemias in general was the development of novel TKIs, such as nilotinib and dasatinib. However, *in vitro* experiments have suggested that TKIs have an antiproliferative but not an antiapoptotic or cytotoxic effect on the most primitive ALL stem cells. None of the TKIs in clinical use target the LSC. Second generation TKI dasatinib has been shown to have a more profound effect on the stem cell compartment but the drug was still unable to kill the most primitive LSCs. Allogeneic stem cell transplantation (SCT) remains the only curative treatment available for these patients. Several mechanisms were proposed to explain the resistance of LSCs to TKIs in addition to mutations. Hence, TKIs may be used as a bridge to SCT rather than monotherapy or combination with standard chemotherapy. Better understanding the biology of Ph^+ ALL will open new avenues for effective management. In this review, we highlight recent findings relating to the question of LSCs in Ph^+ ALL.

Key words: Acute lymphoblastic leukemia; Philadelphia chromosome; Tyrosine kinase inhibitors; Leukemia stem cells; Prognosis

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INTRODUCTION

Over the past decades, it has been recognized that tumors contain a subpopulation of cells with biological features that are reminiscent of stem cells[1]. The modern concept of "cancer stem cell" was promoted by John Dick and colleagues, who showed that cells with the ability to transfer human acute myeloid leukemia (AML) to NOD/SCID mice are frequently found exclusively in the $CD34^+$ $CD38^-$ compartment[2,3]. Stem cells modulate tissue formation, maintenance and repair, based on a complex

interaction of cell-autonomous and cell-no autonomous regulatory mechanisms[4]. Classically, these cells could be subdivided into more or less primitive subpopulations that are organized in a hierarchy reminiscent of the normal hematopoietic system. A slow cycling fraction of cells is generating a fast cycling fraction. However, an alternative hypothesis predicts that all tumor cells have the potential to self-renew and recapitulate the tumor but with a low probability that any tumor cell enters the cell cycle and finds a permissive environment[5]. Acute lymphoblastic leukemia (ALL) defines a heterogeneous group of leukemias that express predominantly lymphoid cell surface markers[6]. Although proof of the existence of a stem cell-like population maintaining ALL has been elusive, subpopulations with primitive phenotypes have been reported in clinical ALL samples. Response to therapy is related to biological characteristics of the cell of origin and to the primitive stem cell-like population. Philadelphia chromosome-positive (Ph^+) ALL is regarded as a specific entity. The initial treatment of Ph^+ ALL has recently been dramatically changed by the introduction of Abl tyrosine kinase inhibitors (TKIs). However, cure depends on the eradication of the leukemia stem cell (LSC). This review will discuss current treatment and evidence for an ALL stem cell in Ph^+ ALL and its relationship with new therapeutic advances based on TKI therapy in this disease.

Ph (BREAKPOINT CLUSTER REGION-ABELSON TYROSINE KINASE)-POSITIVE B-LYMPHOBLASTIC LEUKEMIA

In 1960, Nowell and Hungerford described a small G group chromosome, the Ph[7]. The Ph^+ chromosome is the most frequent cytogenetic abnormality in human leukemia and can be detected in a range of 2% to 5% of children with ALL[8] and 20% to 40% of adults with ALL[9]. The proportion of Ph^+ ALL cases increases with age[10] but in very old persons the proportion decreases again[9]. This t(9;22) translocation leads to a head-to-tail fusion of the Abelson tyrosine kinase (*ABL*) proto-oncogene from chromosome 9 with a 5' half of the breakpoint cluster region (*BCR*) sequences on chromosome 22[11]. Transcription of *BCR-ABL* results either in a 8.5-kilobase (kb) messenger RNA (mRNA) that codes for a 210-kb protein when *ABL* moves to the major BCR (M-*BCR*) or in a 7.5-kb RNA encoding a 190-kb protein when it moves to the M-*BCR*[12]. BCR-ABL proteins demonstrate enhanced tyrosine kinase activity compared to the normal *ABL* gene product. P190 exhibits a higher transforming potential than p210 in animal models[13]. The p190 protein is usually found in 2/3 of adults with *de novo* Ph^+ ALL[14,15]. The constitutively active tyrosine kinase product BCR-ABL provides a pathogenetic explanation for the initiation of Ph^+ ALL as well as a critical molecular therapeutic target. Both possible chimeric mRNAs (p210 and p190) can be sensitively and specifically detected by the real-time polymerase chain reaction (RT-PCR)[16]. Recent reports suggest that the expression of the p190 transcript was associated with a significant increase in the risk of relapse[14]. BCR-ABL expression in hematopoietic cells is known to induce resistance to apoptosis, growth factor independence, as well as alterations in cell-cell and cell-matrix interactions[17]. Clinically, patients present with a variable white blood cell count and have an increased risk of developing meningeal leukemia during the course of treatment, although central nervous system leukemia was not significantly more frequent (5%) at diagnosis[10]. Ph^+ ALL are found almost exclusively among B-cell linage ALL ($CD10^+$ precursor B-cell ALL). Leukemic cells often present surface expression of CD34 antigen (89%), and frequent expression of myeloid markers (15% to 20%)[14]. Additional chromosome abnormalities have been observed in 70% of Ph^+ ALL patients[18], including mainly 9p abnormalities, monosomy 7 or hyperdiploid karyotypes > 50. CD117 is typically not expressed and only rarely is t(9;22) seen in T-lymphoblastic leukemia. Patients with t(9;22) classically have a poor prognosis.

CURRENT THERAPEUTIC STRATEGIES IN Ph^+ ALL

TKIs

The Ph^+ chromosome has historically been the worst prognostic indicator in ALL. The initial treatment of Ph^+ ALL has been dramatically changed by the introduction of ABL TKIs. Imatinib mesylate, 2-phenylamino pyrimidine, binds to the ABL-ATP site in a competitive manner, stabilizing ABL in its inactive conformation and inhibiting its tyrosine kinase activity. Following initial studies showing that use of imatinib mesylate as a single agent in Ph^+ ALL yielded potential responses but was unlikely to be sufficient for long-term disease control, the efficacy of imatinib was explored as front-line treatment combined with chemotherapy, either concurrently (simultaneous administration) or sequentially (alternating administration)[19-23]. Imatinib was given concurrently at 400 mg/d for the first 14 d with each cycle of the hyper-CVAD regimen[19]. In this study, complete remission (CR) rate was 96%. There was no unexpected toxicity related to the addition of imatinib. Similarly, encouraging data were reported by the Japanese Adult Leukemia Study Group, in which imatinib was started after 1 wk of induction therapy and then coadministered with chemotherapy during the remainder of a standard induction[20]. The CR rate was 96% (median time to CR: 28 d) and a remarkably high molecular response rate became apparent as early as 2 mo after starting treatment. Transplant candidates had a better chance of receiving allogeneic stem cell transplantation (SCT) with imatinib-combined regimen. Alternating and concurrent imatinib-chemotherapy combinations were compared by the German Multicenter ALL (GMALL) trial in two sequential patient cohorts[24]. Efficacy analyses based on BCR-ABL transcript levels

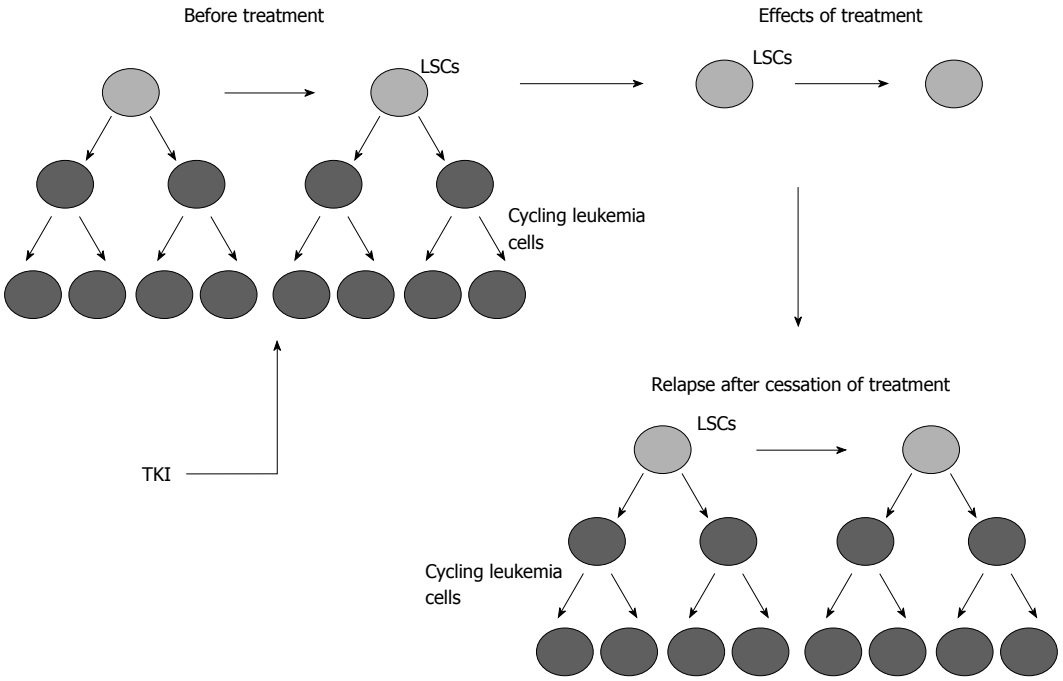

Figure 1 Effect of tyrosine kinase inhibitor on Philadelphia chromosome-positive acute lymphoblastic leukemia and their stem cell counterparts. Therapy with tyrosine kinase inhibitor (TKI) results in the depletion of cycling leukemia cells (of which progenitor cells) without eliminating the Leukemia stem cells (LSCs), then the latter can regenerate the tumor after that therapy is halted. This seems to explain why TKI treatment needs to be chronic and why future drug development needs to be focused on agents that stike at the core of tumors by destroying stem cells.

showed a clear advantage of the simultaneous over the alternating schedule, with 52% of patients achieving PCR negativity (*vs* 19%). Several approaches using imatinib-based induction therapy have been explored for elderly patients. Monotherapy with imatinib was explored in elderly patients, who had an extremely poor outcome with chemotherapy alone. Imatinib with or without corticosteroids resulted in high CR rates of 90% to 100%[22,23,25]. With relatively minimal use of imatinib (600 mg/d for phase 2 induction), the Group for Research on Adult Acute Lymphoblastic Leukemia showed a higher CR rate compared with historical controls[25]. Similar results were reported by the Italian group using continuous administration of imatinib (800 mg) only combined with prednisone[23]. The German group (GMALL) conducted a randomized study comparing induction therapy with single-agent imatinib with standard induction chemotherapy[22]. Response rate was better with single-agent imatinib (96% *vs* 50%). Achievement of molecular remission was associated with longer disease-free survival. Unfortunately, imatinib resistance developed rapidly and was quickly followed by disease progression. Disease recurrence was related to a high rate of *ABL* mutations in the tyrosine kinase domain (TKD)[26]. Data from the United Kingdom Acute Lymphoblastic Leukemia (UKALL)XII/Eastern Cooperative Oncology Group 2993 study, in which imatinib (600 mg) was started with phase 2 induction, did not initially provide clear evidence that imatinib alters the outcome of the disease[27] but finally showed an advantage for the imatinib arm[28]. Studies demonstrated that a quiescent population of LSCs with *BCR-ABL* kinase domain mutations, detectable prior to initiation of imatinib therapy, gives rise to leukemic cells that persist because they are inherently resistant to imatinib (Figure 1)[29]. Quiescent LSCs also have high BCR-ABL transcript levels.

New strategies using second generation TKIs are being developed to overcome resistance to imatinib. A recent phase Ⅱ study combining the hyperCVAD regimen with dasatinib (50 mg *bid*) for the first 14 d of each cycle showed CR achievement in 93% of newly diagnosed Ph+ ALL, with molecular remissions observed even after the first cycle[30]. In a series combining dasatinib (70 mg *bid*) with only steroids, CR was achieved in all cases with a very marked clearance of blasts already at day 22[31]. Nilotinib as monotherapy also appeared to have promising activity and a favorable safety profile[32]. Its use in combination with chemotherapy is currently tested. Even 20-fold more potent BCR-ABL inhibition with nilotinib did not induce apoptosis of quiescent CD34+ cells nor did inhibition with a dual SRC-ABL kinase inhibitor[33].

The appearance of mutations which are most probably but not exclusively related to resistance led to avoiding induction drugs, such as anthracyclines or alkylating agents, which can cause mutational resistance and preference for methotrexate, cytarabine and asparaginase. Such a trial in older adult patients led to high CR rate and improved survival[34].

The role of hematopoietic SCT in Ph+ ALL

Allogeneic SCT from a related or unrelated donor has historically been the standard form of consolidation in Ph+ ALL, with 27% to 65% of long-term survival in

patients grafted in first CR[28,35,36]. The aggressivity of the disease often resulted in a rapid relapse prior to transplant and only 30% to 50% of patients in first remission eventually underwent allogeneic SCT. The relapse-free survival advantage of the patient developing acute or chronic graft-*vs*-host disease (GVHD) and the description of PCR conversion after developing GVHD have suggested that Ph+ ALL was particularly sensitive to the graft-*vs*-leukemia effect[37]. The recent incorporation of TKIs into standard ALL therapy has resulted in improved remission induction rates of 95%, with 50% to 70% of patients achieving molecular negativity. More patients were able to receive SCT in first CR, resulting in improved overall survival rates of 43% to 78% with 1-3 years of follow-up[20,21,24,38,39]. The combination of imatinib and multiagent chemotherapy did not result in increased toxicity and did not unfavorably affect allogeneic SCT. The molecular detection of the BCR-ABL fusion mRNA by PCR has been shown to be an effective method for monitoring disease. BCR-ABL levels before transplantation are prognostic. Patients who are in remission morphologically and molecularly have the best outcome, whereas those who are molecularly positive, although still in conventional remission, suffer a relatively higher relapse rate[40]. Results after allogeneic SCT may be improved by the use of imatinib as maintenance. Patients remaining *BCR-ABL* positive after allogeneic SCT can achieve molecular remission with imatinib and survive long-term. Thus, post-transplant imatinib can reduce the relapse rate. However, imatinib should not be given before 6 to 8 wk after transplantation to avoid cumulative toxicity. Duration of TKI maintenance remains open, either for 2 years or stopped after repeated minimal residual disease negativity.

RESISTANCE TO TKIs

The effectiveness of imatinib alone is limited by the relatively frequent development of resistance. Mechanisms that have been implicated in resistance include rapid drug efflux[41], amplification of the *BCR-ABL* gene[42], reduced binding affinity of imatinib to the ATP-binding site due to genetic changes[43], and *BCR-ABL* independence resulting from secondary transforming events[44]. As in chronic phase myeloid leukemia (CML), secondary resistance in Ph+ ALL is frequently associated with point mutations in the TKD of *BCR-ABL*[43,45]. Among these resistance mechanisms, dose escalation of imatinib or the use of more potent ABL inhibitors could resolve the first events, while only the use of multitargeted inhibitors could restore sensitivity in the other mechanisms. *ABL* TKD mutations generally are comprised of two categories: mutations that directly impede contact between imatinib and Bcr-Abl, such as the gatekeeper mutations *T315I* and *F317L*[46], and mutations that alter the spacial conformation of the BCR-ABL protein by affecting the P-loop containing the ATP-binding pocket and/or the activating loop[45]. To date, more than 50 *ABL* TKD mutations have been identified. Data on the frequency and incidence of mutations in Ph+ ALL have been relatively sparse. However, the German group showed that *ABL* mutations could be detected in nearly 40% of patients at the time of diagnosis in imatinib-naïve patients (P-loop mutations in 80% and *T314I* mutation in 17%)[26]. At the time of recurrence, *ABL* mutations were found in 84% of cases (P-loop mutations in 57% and *T315I* mutation in 19%). The mutated clone then consistently represented the dominant population. Almost all patients with mutant *BCR-ABL* detected before imatinib therapy had the same mutation at relapse. Remission duration did not differ significantly between patients with or without a detectable early mutation. Imatinib initially suppressed the dominant, unmutated leukemic population, without simultaneous outgrowth of the pre-existing mutant subclones. However, these pre-existing mutant clones almost invariably give rise to eventual relapse. To significantly improve outcome, it would be necessary to eliminate clones harboring mutations during the early phase of treatment, before they have acquired additional resistance mechanisms. The cause for relapse has been related to TKI resistance of the LSCs and/or immune tolerance of leukemic cells. *In vitro* experiments have suggested that TKIs have an antiproliferative, but not a proapoptotic or cytotoxic effect on the most primitive Ph+ stem cells (CD34+ CD38- cells)[47,48]. Second generation TKI dasatinib has been shown to have a more profound effect on the stem cell compartment when compared to imatinib or nilotinib, but the drug was still unable to kill the most primitive CD34+ CD38- LSCs *in vitro*[48]. However, recent analyses in CML with successful TKI therapy have demonstrated the eradication of most Ph+ CD34+ CD38- cells from the central bone marrow[49]. Selective chemical inhibition of Src family kinases decreases growth and expression of stem cell genes, including *Oct3/4* and *Nanog*, involved in self renewal and survival of LSCs[50].

Resistance attributable to kinase domain mutations can lead to relapse despite the development of second-generation compounds, including dasatinib and nilotinib. Despite these therapeutic options, the cross-resistant *BCR-ABL* ^{T315I} mutation remains a major clinical challenge. The first evaluations of AP24534 present this drug as a potent multi-targeted kinase inhibitor active against T315I and all other BCR-ABL mutants[51-54]. AP24534 could be the next treatment of choice in hematological malignancies with Ph+ chromosome, particularly Ph+ ALL known for its frequent occurrence of *T315I* mutation. However, its potential action on LSCs is still unknown.

LSCs IN ALL

ALL defines a heterogeneous group of leukemias. Reports assessing the cell of origin have been contradictory. Discrepancies may be related to the heterogeneity of the disease. In ALL and other malignancies, compelling research suggests that a population of cancer stem cells is able to regenerate or self-renew, resulting in therapeutic

resistance and disease progression. A number of studies indicate that quiescent LSCs are resistant to therapies that target rapidly dividing cells. However, the marked differences in response to therapy could be related to the different characteristics of the cell of origin, and to the existence and relative importance of a primitive LSC population for a given ALL subtype. Rearrangements of the T-cell receptor or the immunoglobulin heavy chain genes support the theory that T- and B-lineage ALL originate in cells already committed to the T- or B-cell lineages. *In vivo* xenotransplantation model showed that $CD34^+$ $CD38^+$ $CD19^+$ and $CD34^+$ $CD38^-$ $CD19^+$ cells from pediatric patients with B-ALL initiate B-ALL in primary recipients, whereas the recipients of $CD34^+$ $CD38^-$ $CD10^-$ $CD19^-$ cells showed normal human hematopoietic repopulation[55]. Furthermore, transplantation of $CD34^+$ $CD38^+$ $CD19^+$ cells resulted in the development of B-ALL in secondary recipients, demonstrating self-renewal capacity. In T-ALL, cells capable of long-term proliferation have been demonstrated in the $CD34^+$ $CD4^-$ and $CD34^+$ $CD7^-$ cell subfractions[56]. In *TEL/AML1* rearranged ALL, the earliest population that consistently conferred leukemia to immunocompromised mice has been defined as $CD34^+$ $CD38^-$ $CD19^+$[57,58]. Engraftment was also reported from a more differentiated $CD34^-$ $CD19^+$ subpopulation[59], questioning the existence of a strict hierarchy in this type of ALL. Normal individuals may have B cells harboring a *TEL/AML1* fusion that never undergo leukemic transformation, suggesting a 'multi-hit' model of leukemogenesis, in which *TEL/AML1*-positive precursor cells constitute a pre-leukemic pool[60]. In hyperdiploid ALL, it appears that some cases have leukemia-initiating activity in populations consistent with later mid-stage B-cell development[61]. Only $CD34^+$ $CD10^-$ or $CD34^+$ $CD19^-$ cells resulted in reliable leukemic engraftment in ALL samples with normal karyotype[62]. *MLL-AF4*-positive cells were reported in high frequency in the $CD34^+$ $CD19^-$ compartment. These cells, carrying an immature phenotype, suggest a developmental stage prior to commitment to the lymphoid lineage[59]. The microenvironment may play an important role in determining lineage fate in *MLL*-rearranged leukemias[63,64].

LSCs IN *BCR-ABL*-POSITIVE ALL

$CD34^+$ stem cells cannot be effectively killed by BCR-ABL kinase inhibitor imatinib treatment both *in vitro* and *in vivo*[47,65]. BCR-ABL transcripts are still detectable in $CD34^+$ cells after a long-term treatment with imatinib[65], suggesting that these LSCs cannot be eradicated through inhibiting BCR-ABL kinase activity. Similarly, imatinib does not eradicate LSCs in mice[66]. These results indicate that some unknown pathways contribute to the maintenance of survival and self-renewal of LSCs. The LSCs seem to be biologically distinct from their more differentiated progeny. Therefore, the agents acting against the more mature blasts will not be as efficient in eradicating the LSCs. Divisional asymmetry and environmental asymmetry are two mechanisms responsible for asymmetric cell division. In the first case, specific cell-fate determinants redistribute unequally among daughter cells, of which one receives these determinants, while the other proceeds to differentiation. In the other case, a LSC would first undergo a symmetric division. However, only one cell remains in the bone marrow niche and conserves stem cell fate, while the other cell enters a different microenvironment and subsequently produces signals initiating differentiation. Identification of novel genes that play critical role in regulating the function of LSCs can contribute to the development of new therapeutic strategies through targeting LSCs.

Given the central role that LSCs play in leukemia maintenance, studies have focused on identifying pathways of proliferation, self-renewal and survival that are differentially active in LSCs rather than normal hematopoetic stem cells. The expression of p190 or p210 BCR-ABL fusion forms is sufficient to cause leukemia in animal models[67]. The JAK-STAT, Ras-Raf-MEK-ERK, PI3K-AKT, c-Myc, SAPK-JNK and NF-κB pathways are among the pathways activated by BCR-ABL. Those pathways are involved in cell proliferation and inhibition of apoptosis. Ph^+ ALL is also characterized by a high degree of genomic instability that is induced by the BCR-ABL protein, as demonstrated by the increased frequency of DNA insertions and deletions present in BCR-ABL pre-leukemic mice[68]. Ph^+ ALL often presents aberrant splicing of key genes in lymphoid development, such as *BTK* and *SLP-65*, and deleterious mutations of *IKZF1*, a gene that encodes the zinc-finger transcription factor Ikaros[69,70]. The aberrant splicing of *SLP-65* and *BTK* in B-cell precursors results in shorter transcripts that halt lymphoid maturation[71]. The deletion of exons of *IKZF1* results in a dominant negative form of Ikaros that lacks the DNA-binding domain. This mutated form halts B-cell differentiation and contributes to the expression of some myeloid specific genes[70]. Its overexpression may also contribute to resistance to TKIs[72]. A common additional mutation in Ph^+ ALL is the deletion of 9p21, compromising the *INK4A-ARF* gene[73]. The activation of $p14^{ARF}$ induces cell cycle arrest and apoptosis through p53 activation and Arf-null BCR-ABL^+ cells induce more severe leukemia in irradiated mice recipients. Another particularity of Ph^+ ALL is the dependence of BCR-ABL transformation on Src kinases that do not appear to be required for the induction of CML[74]. However, CML progression to lymphoid blast crisis may also depend on Src kinases as lymphoid blast crisis cells are also dependent on Lyn for survival, to a higher extent than myeloid blast crisis CML cells, suggesting a lineage-specific signaling pathway or mechanism[75].

In contrast with CML, if Ph^+ ALL is lineage restricted, one should find the Ph^+ only in lymphoid cells. However, original studies in patients did not yield such a clear cut distinction. Involvement of the myeloid compartment has been reported for Ph^+ ALL[76]. The Ph could be detected in both mature myeloid cells and

myeloid colony-forming units, suggesting that at least in some patients the leukemia-initiating event occurs in a primitive cell that has not already undergone lineage commitment[77]. An alternative explanation would be that *BCR-ABL* ALL blasts reacquire specific lineage promiscuity. Recent functional studies conclude that LSC in Ph^+ ALL is a primitive cell lymphoid that is restricted as it will not originate from myeloid or erythroid colonies, although the studies differ on the characterization of this LSC. The $CD34^+$ $CD38^-$ $CD19^-$ cell compartment has been shown to be involved in patients with p210 *BCR-ABL* ALL but not in those with p190 *BCR-ABL* ALL[58]. The p210 *BCR-ABL* transcript could also be identified in $CD34^+$ $CD33^+$ and $CD34^-$ $CD33^+$ myeloid precursors, which was not the case for the p190 transcript. However, $CD34^+$ $CD38^-$ $CD19^-$ p210-positive cells did not induce leukemia in NOD/SCID mice. This contrasts with other results showing NOD/SCID engrafting leukemic cells only in the $CD34^+$ $CD38^-$ subfraction and not in the $CD34^+$ $CD38^+$ cells[78]. The $CD34^+$ $CD19^-$ cells have also been shown as the most undifferentiated leukemia progenitors in patients with Ph^+ ALL but they did not differentiate into myeloid colonies[79].

CONCLUSION

By definition, cure of leukemia requires eradication or transcriptional control of LSCs. To date, little is known regarding the efficacy of TKIs in the longer term. Although reduction in the number of LSCs by TKI therapy is feasible, quiescent LSCs are likely to survive to TKIs combined with chemotherapy. Allogeneic transplantation remains therefore the treatment of choice, preferably in first CR. However, a more complete understanding of the biology of Ph^+ chromosome is needed in order to cure patients who cannot receive allogeneic SCT. Patients with Ph^+ ALL may be heterogeneous. The determination of residual populations of quiescent Ph^+ cells is important for evaluating response in treated patients. Specific inhibition of LSCs in Ph^+ ALL is a suitable approach to developing a cure for this disease in the future. Several gene products required by LSCs have been shown to be potential targets for inhibiting LSCs. The mechanisms by which involved pathways regulate the function of LSCs need to be further studied.

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ORIGINAL ARTICLE

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

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Abstract

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.

Key words: Mesenchymal Stem Cell; Placenta; Immunophenotyping; Immunomodulation; Growth Kinetics

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INTRODUCTION

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

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 ℃ 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.

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 Strepto-

Table 1 Primers for transcription factors for indicated genes

Gene	Forward primer	Reverse primer
Nanog	AGTCCCAAAGGCAAACAACCCACTTC	ATCTGCTGGAGGCTGAGGTATTTCTGTCTC
Sox2	ATGCACCGCTACGACGTGA	CTTTTGCACCCCTCCCATTT
Rex-1	CAGATCCTAAACAGCTCGCAGAAT	GCGTACGCAAATTAAAGTCCAGA
4-Oct	CGACCATCTGCCGCTTTGAG	CCCCCTGTCCCCCATTCCTA
Osteopontin	GAAGGACAGTTATGAAACGAGT	AACATAGACATAACCCTGAAGC
Osteocalcin	ATGAGAGCCCTCACACTCCT	CAAGGGGAAGAGGAAAGAAG
GAPDH	TTGCAACTGTTTTAGGACTTT	AGCATTGGGAAATGTTCAAGG

mycin 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 ℃ 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

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).

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.

PLC-MSC differentiate into mesodermal lineages

MSC were induced for adipogenic, osteogenic and chon-

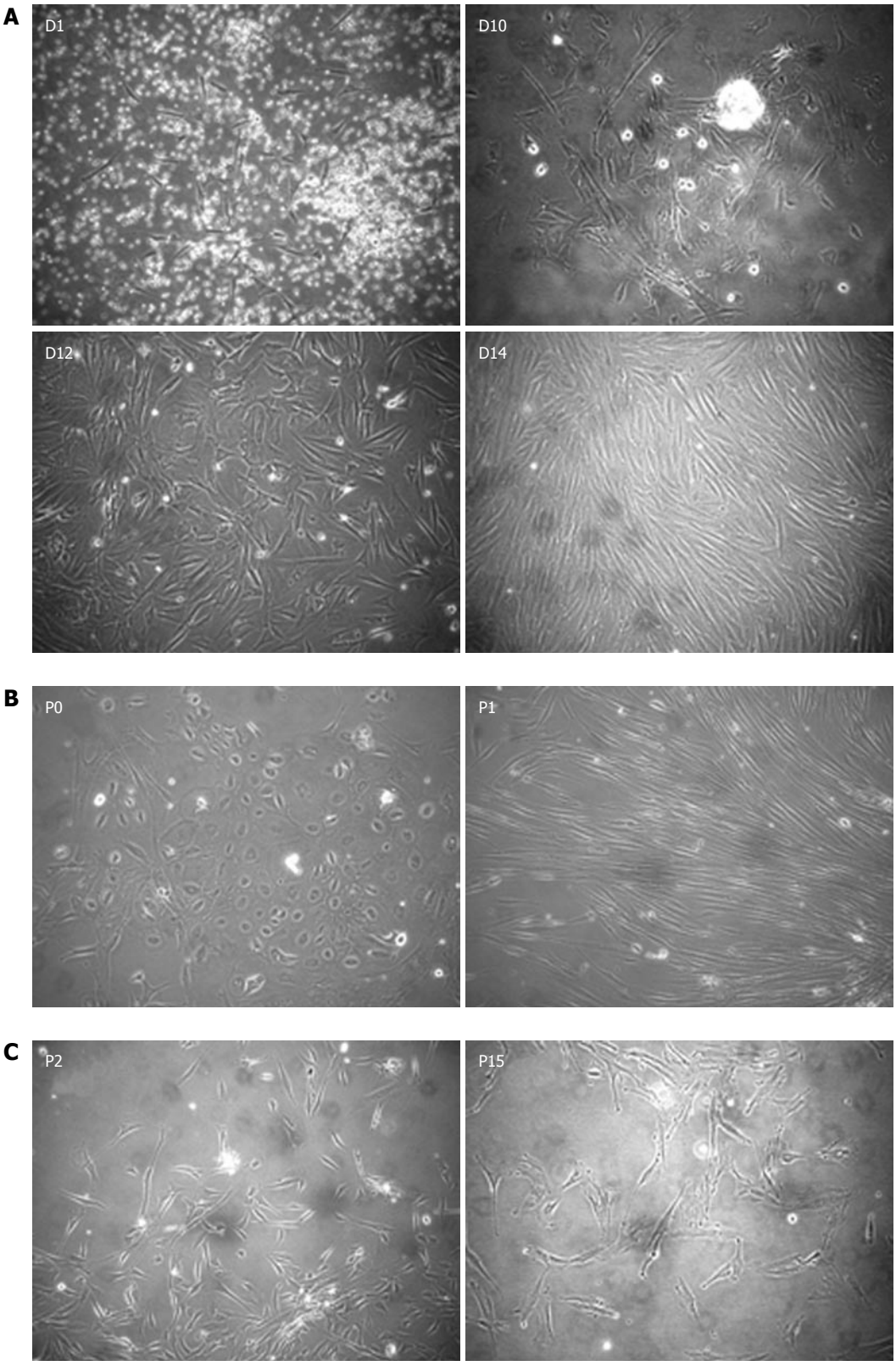

Figure 1 Morphology of placenta mesenchymal stem cells primary cultures. A: The formation of heterogeneous populations of placenta mesenchymal stem cells (PLC-MSC) with fibroblastic and epithelioid morphologies at passage 0, P0 until confluence at day 14; B: Homogenous population of PLC-MSC at P1; Disappearance of the epitheloid population and appearance of fibroblast-like cells upon trypsinisation and passaging; C: Comparison of early and late passages of primary MSC cultures (C). Photomicrographs taken using phase contrast microscope at magnification 100 ×.

drogenic 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).

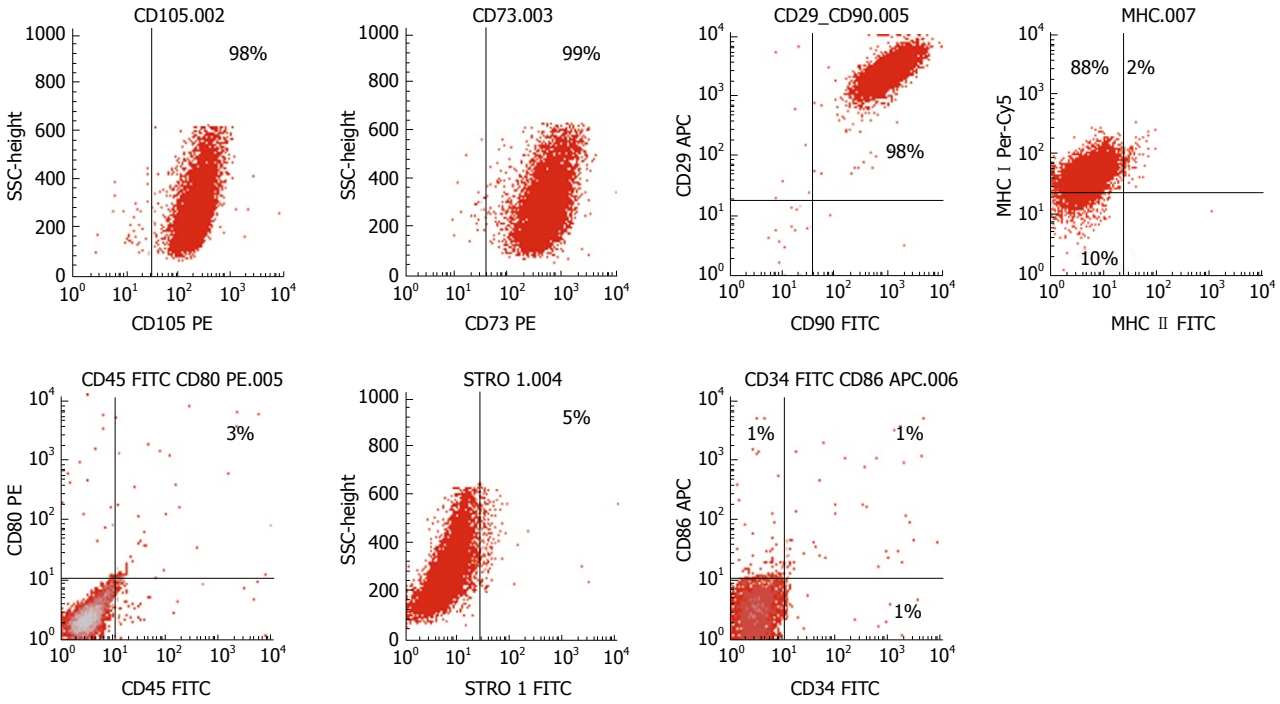

Figure 2 Immunophenotyping of placenta mesenchymal stem cells by flow cytometry. Adherent placenta mesenchymal stem cells (MSC) were harvested upon confluence and stained for MSC cell surface markers using a panel of anti human antibodies. Results represent at least 3 experiments.

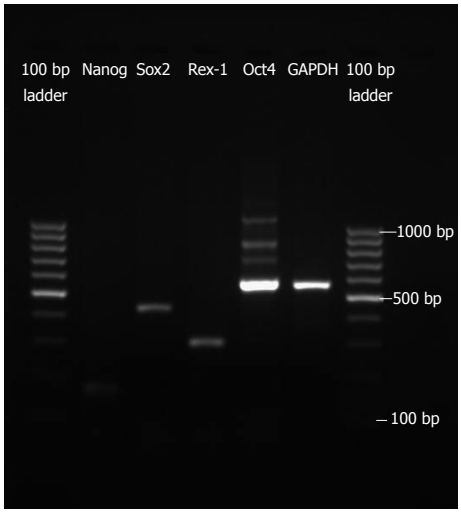

Figure 3 Gene expression by reverse transcription-polymerase chain reaction of placenta mesenchymal stem cells. Placenta mesenchymal stem cells express markers of embryonic stem cell transcriptional factors such as *Nanog*, *Sox2*, *Rex-1* and *Oct4*. *GAPDH* was used as a reference gene. 100 *bp* DNA ladder was used as a marker.

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).

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.

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.

DISCUSSION

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].

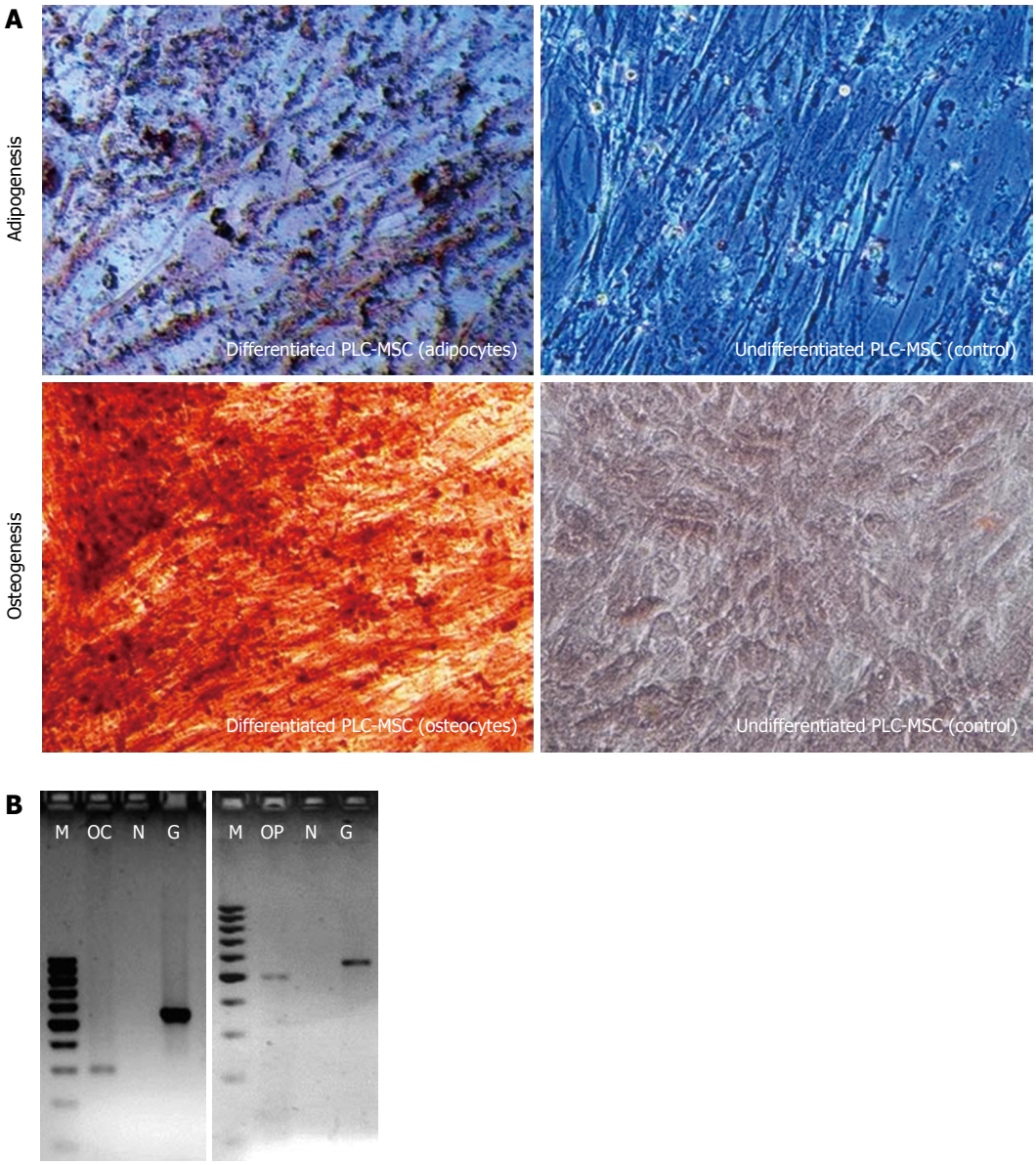

Figure 4 Differentiation potential of placenta mesenchymal stem cells into mesodermal lineages. A: Placenta MSC (PLC-MSC) after 3 wk in adipogenic or osteogenic or normal cell culture medium. Formation of lipid droplets (stained red in Oil-Red-O), calcium deposition (stained orangy-red in Alizarin Red) and formation of proteoglycans (stained blue in Alcian Blue) confirms the ability to differentiate into mesodermal lineages. The picture was taken using phase contrast microscope at 100 × magnification. B: PLC-MSC differentiated into osteocytes expresses Osteocalcin (OC) and Osteopontin (OP) (data from two independent experiments). M: 100 bp DNA ladder; N: Non template control as negative control; C: Control-undifferentiated PLC-MSC; G1: GAPDH for differentiated PLC-MSC; G2: GAPDH for control.

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

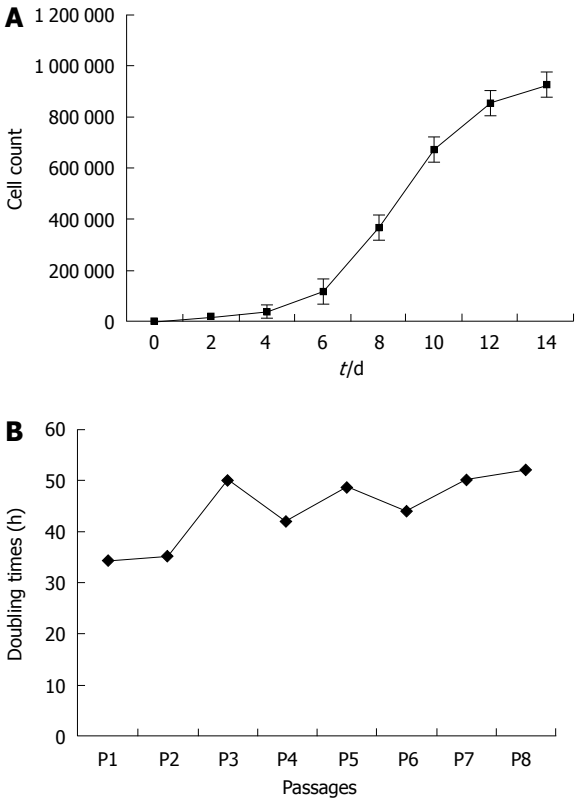

Figure 5 Growth Kinetics of placenta mesenchymal stem cells. Placenta mesenchymal stem cells (PLC-MSC) (4000 cells/well) were plated in 6 well plates and medium was changed three times a week. A: Triplicate cultures were harvested for trypan blue exclusion cell count every 2 d; B: Doubling time of PLC-MSC was measured at every passage (B). Average doubling time PLC-MSC was 41 h. Figure 5 is representative of 3 individual experiments with ± SD, results considered significant at $P < 0.05$.

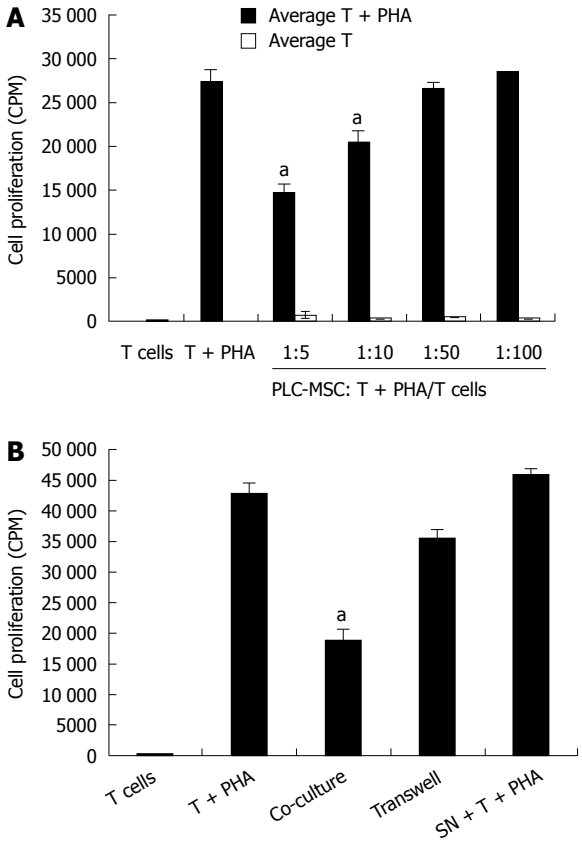

Figure 6 Dose dependent inhibitory effect of placenta mesenchymal stem cells on phytohemagglutinin stimulated PBMC *via* cell to cell contact. A: Peripheral blood mononuclear cells (5×10^5 cells) with or without phytohemagglutinin (PHA) stimulation were co-cultured with various ratios of placenta mesenchymal stem cells (PLC-MSC) and incubated for 72 h. For the final 18 h of the culture, tritium thymidine (^{3}H-TdR) was pulsed into the wells. PLC-MSC failed to stimulate proliferation of resting T cells but PLC-MSC inhibited activated T cells proliferation in a dose dependent mechanism; B: PBMC (2×10^6) stimulated with PHA were also co-cultured with 2×10^5 PLC-MSC (direct and indirect contact) and MSC conditioned media in a 12 well plate. T cells were harvested after 48 h of incubation, plated in 96-well plates and proliferation was measured by ^{3}H-TdR on day 3. This result represents the average percentage of T cell proliferation ± SD of 3 repeated experiments. $^aP < 0.05$ *vs* positive control (T + PHA).

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

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.

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London, United Kingdom

February 23, 2012
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Gordon Research Conference: Reprogramming Cell Fate
Galveston, TX, United States

March 9, 2012
Cell Culture Technology: Recent Advances, Future Prospects
London, United Kingdom

March 11-16, 2012
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March 25-30, 2012
Keystone Symposia: Advances in Islet Biology
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March 28-29, 2012
Single Cell Analysis Europe
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Aula Nuova del Sinodo
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April 29 - May 2, 2012
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Co-organized by the Society for Biological Engineering (SBE) and the ISSCR
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April 30, 2012
Stem Cells to Tissues
Boston, MA, United States

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Regenerative Biology: From Stem Cells to Tissues
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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

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