

Accelerated and Safe Expansion of Human Mesenchymal Stromal Cells in Animal Serum-Free Medium for Transplantation and Regenerative Medicine

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Human bone marrow mesenchymal stromal cells (hMSC) are currently investigated for a variety of therapeutic applications. However, most expansion protocols still use fetal calf serum (FCS) as growth factor supplement which is a potential source of undesired xenogeneic pathogens. We established an expansion protocol for hMSC based on the use of GMP-produced basic medium LP02 supplemented with 5% of platelet lysate (PL) obtained from human thrombocyte concentrates. Compared to FCS-supplemented culture conditions, we found a significant increase in both colony forming unit-fibroblast (CFU-F) as well as cumulative cell numbers after expansion. This accelerated growth is optimized by pooling of at least 10 thrombocyte concentrates. A minimal requirement is the use of 5% of PL with an optimal platelet concentration of $1.5 \times 10^9/\text{ml}$, and centrifugation of thawed lysate at high speed. Cells expanded by this protocol meet all criteria for mesenchymal stromal cells (MSCs), e.g. plastic adherence, spindle-shaped morphology, surface marker expression, lack of hematopoietic markers, and differentiation capability into three mesenchymal lineages. MSC at passage 6 were cytogenetically normal and retained their immune-privileged potential by suppressing allogeneic reaction of T-cells. Additionally, gene expression profiles show increased mRNA levels of genes involved in cell cycle and DNA replication and downregulation of developmental and differentiation genes, supporting the observation of increased MSC-expansion in PL-supplemented medium. In summary, we have established a GMP-compatible protocol for safe and accelerated expansion of hMSC to be used in cell and tissue therapy.

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Multipotent mesenchymal stromal cells (MSC), also named mesenchymal stem cells, are characterized by adherence to plastic when maintained in standard cultures in vitro, by expression of surface antigens CD105 and CD90 but lack of hematopoietic markers CD34 and CD45 and by differentiation into osteoblasts, adipocytes, and chondroblasts in vitro (Dominici et al., 2006). Since their first description by Friedenstein et al. (1974), a multitude of work has been carried out to show their properties and functionality in vitro and in vivo (Pittenger et al., 1999; Deans and Moseley, 2000; Fibbe and Noort, 2003). MSC have been shown to display a considerable therapeutic potential in pre-clinical (Kopen et al., 1999; Petite et al., 2000; Mahmood et al., 2004; Jaquet et al., 2005; Tögel et al., 2005; Lange et al., 2005b) and clinical (Horwitz et al., 1999; Koc et al., 2000, 2002; Chen et al., 2004; Le Blanc et al., 2004) studies for the treatment/regeneration of neuronal degeneration, osteogenesis imperfecta, graft versus host disease, support of hematopoietic engraftment, metabolic diseases, bone and cartilage tissues as well as renal and myocardial infarction. To date, all reported clinical trials are employing human bone marrow mesenchymal stromal cells (hMSC) generated in medium supplemented with fetal calf serum (FCS). FCS, however, is an undesired source of xenogeneic antigens and bears the risk of transmitting animal viral, prion, and zoonose contaminations. Additionally, FCS has been implicated with anaphylactic or arthus-like immune reactions in patients who received cells generated in FCS-supplemented medium (Selvaggi et al., 1997), even leading to arrhythmias after cellular cardioplasty (Chachques et al., 2004). Uptake of FCS components is an active process

(Gregory et al., 2006). Although, FCS can be removed up to 99.99% by sequential cultivation of MSC first in FCS, followed by autologous or heterologous serum (Spees et al., 2004), a residual risk still remains. Several attempts have been made to omit FCS from cultures of hMSC for clinical applications and replace it by autologous or heterologous serum or plasma. In autologous serum, hMSC show faster proliferation compared to FCS at least during first passages without loss of the typical phenotype, motility, and differentiation capabilities in vitro, whereas use of allogeneic human serum resulted in hMSC growth arrest and death (Stute et al., 2004; Kobayashi et al., 2005; Shahdadfar et al., 2005; Mizuno et al., 2006). However, the amount of autologous serum necessary for sufficient expansion would exceed the amount a donor could provide.

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Thus, autologous or allogeneic serum cannot be considered as a general substitute for FCS. Another attempt was made by using serum-free medium supplemented with a serum substitute that allowed better expansion than classical α -MEM medium (Meuleman et al., 2006), however expansion of MSC is still unsatisfactory.

Very recently, human platelet lysates (PL) have been shown to serve as a safe substitute for animal serum for hMSC expansion (Yamada et al., 2004; Doucet et al., 2005; Müller et al., 2006), but the resulting hMSC are still not fully characterized. In this study, we have extensively analyzed animal-serum free culture conditions for hMSC expansion using PL as a substitute for FCS. Compared to FCS-supplemented culture conditions, we found a significant increase of both colony forming unit-fibroblast (CFU-F) as well as cumulative cell numbers after expansion. Our optimized protocol includes pooling of at least 10 thrombocyte concentrates for the preparation of PL. The optimal platelet concentrations for PL-preparation appeared to be 1.5×10^9 /ml when 5% of PL were added to the medium. Additionally, centrifugation of thawed lysate at high speed showed to be superior over low speed centrifugation. Cells obtained by this protocol meet all criteria for MSCs, e.g. plastic adherence, spindle-shaped morphology, surface marker expression, lack of hematopoietic markers, and differentiation capability into three mesenchymal lineages. MSC after passage 6 were cytogenetically normal and retained their immune-privileged potential by suppressing allogeneic reaction of T-cells. Additionally, gene expression profiles showed increased mRNA levels of genes involved in cell cycle and DNA replication, and downregulation of developmental and differentiation genes, supporting the observation of accelerated MSC-expansion in PL-supplemented medium without loss of multipotency. Taken together, our GMP-compatible protocol allows for safe and accelerated expansion of hMSC which could be of interest for cell and tissue therapies.

Materials and Methods

Isolation of bone marrow mononuclear cells and culture of human MSCs

Bone marrow samples (BM) from healthy donors were collected after informed consent according to the hospital's guidelines approved by the Hamburg Ethics Committee and processed as described (Lange et al., 2005a). Briefly, BM was diluted with PBS (Gibco, Karlsruhe, Germany) and layered onto Percoll (Biochrom, Berlin, Germany; density 1.124 g/ml) diluted to density of 1.068 g/ml. Percoll-gradient was spun with 800g for 20 min at room temperature (RT). Mononuclear cells (MNC) of interphase were collected, washed twice with PBS and plated at 1×10^7 /25 cm² in 5 ml of respective media described below. Cells were incubated at 37°C and 5% CO₂. After 2–3 days, nonadherent cells were washed off with PBS. Cells were fed twice a week with fresh medium and incubated until reaching confluence of 90–95%. At this time point, the cultures were designed as P0. Cells were detached with 0.05% Trypsin-EDTA (Gibco), counted, seeded at 500 cells/cm² and designed as P1. Passaging was continued until MSCs reached senescence.

Media preparations

The standard cultivation-medium consisted of D-MEM/LG (Dulbeccos's Modified Eagle Medium/low glucose, Gibco) + 1% Penicillin/Streptomycin (P/S; Gibco) + 10% pre-selected FCS (BioWhittaker, Apen, Germany) and is designed throughout the text as M1. The medium M2 consisted of α -MEM instead of D-MEM supplemented with 1% glutamine (Gibco) and the components described above. For medium M3, α -MEM was used as basic medium supplemented with 1% glutamine, 1% P/S, 10 IU Heparin (Roche, Grenzach-Wyhlen, Germany; 5,000 IU/ml) per 5 ml medium, and 5% freshly thawed PL. Medium M4 contained LP02 as basic medium (low protein medium for adherent cells; MacoPharma, Langen, Germany) supplemented with 10 IU Heparin per 5 ml medium and 5% PL.

PL was prepared either from pooled thrombocyte concentrates designed for human use (produced as TKSF from the blood bank at the University Hospital Hamburg-Eppendorf, pooled from five donors) or from 7 to 13 pooled buffy coats after centrifugation with 200g for 20 min. The platelet rich plasma (PRP) was aliquoted into small portions, frozen at -80°C , thus obtaining PL and thawed immediately before use. PL-containing medium was prepared freshly for each cell feeding.

Colony forming unit-fibroblast assay

The CFU-F activity of each BM in all 4 media was tested according to the method of Castro-Malaspina (Castro-Malaspina et al., 1980). Cells were seeded into six-well plates at 5×10^4 to 1×10^6 MNC in 3 ml medium per well and incubated at 37°C and 5% CO₂. After 2–3 days nonadherent cells were washed off with PBS. Cells were fed twice a week with fresh medium. Colonies were washed with PBS after an incubation period of 14 days, fixed for 5 min with methanol, stained with 1% aqueous crystal violet and counted under a microscope.

Platelet lysate: Comparisons of different preparations and platelet concentrations

MNC from a randomly chosen donor were expanded in LP02 medium supplemented with three different PL-preparations up to passage 6. Each PL-preparation was derived from differing numbers of buffy coats (7–10) with distinct platelet concentrations (1.2 – 1.9×10^9 /ml).

To optimize and standardize the PL-preparations for MSC expansion, PRP was either used undiluted or diluted to platelet concentrations of 1.5, 1.0, 0.75, and 0.5×10^9 /ml, frozen down at -80°C and used at 5% in LP02 medium. Cells from three different donors were expanded in LP02 medium supplemented with PL from three different PRP-preparations.

The use of PL in combination with LP02 resulted in a layer of fine granular deposits in the culture vessels. In order to decrease the precipitate, PL were centrifuged with 900g or 8,000g before being added to the medium. The influence of PL-centrifugation on precipitate formation and cell expansion over an expansion period of five passages was compared.

Phenotypic analysis of MSCs

In passage 6, the expression of the antigens CD34, CD59 (BD Bioscience, Heidelberg, Germany), CD45, CD90 (Coulter, Krefeld, Germany), and CD105 (Serotec, Eching, Germany) was measured using a FACS-Calibur (BD Bioscience) equipped with the CellQuest software. Appropriate isotype-matched controls were included.

Differentiation assays

Differentiation assays into adipo-, osteo-, and chondrogenic lineages were carried out as described (Lange et al., 2005a). After induction of differentiation, calcium deposits in osteoblasts were detected with silver nitrate, fat accumulation in adipocytes with sudan red and proteoglycans secreted from chondroblasts with alcian blue. To assure direct comparability, the induction time for adipo-, osteo- and chondrogenic differentiations was kept constant for all cell preparations.

Mixed lymphocyte culture (MLC)

We used Ki-67-antigen staining to estimate T-cell alloreactivity in MLC in the presence of hMSC as third party (Fang et al., 2006). MLC were carried out with human peripheral blood mononuclear cells (hPBMC) used as effectors and allogeneic hPBMC as stimulators mixed at the ratio 1:1. Unrelated hMSC added at the ratio 1:1 as third party to the MLC were expanded in PL-supplemented LP02 medium. After an incubation period of 72 h, surface expression of CD3, CD56 (Coulter), and intracellular expression of Ki-67 (Dako Cytomation, Hamburg, Germany) were determined using a FACS-Calibur. For estimation of proliferating CD3+ T-cells, CD56+ NK cells were excluded from cells in the lymphocyte gate.

Cytogenetic monitoring

Three cell preparations after six passages in LP02 supplemented with PL were exposed to colchicine for 16 h, subjected to 0.075 M KCl for 20 min, fixed in methanol-acetic acid (3:1) and subjected to GTG-banding and Multicolor karyotyping by FISH. GTG-banding of

metaphase chromosome spreads was obtained according to standard protocols. A minimum of 25 cells was analyzed at approximately 350–450 band level. Karyotypes were described by the recommendations of the International System for Human Cytogenetic Nomenclature 1995 (ISCN).

Multicolor karyotyping by FISH (M-FISH) allows the fluorescence labeling of each individual chromosome. Hereby, numerical and structural chromosomal aberrations, even those which are cryptic or not further specified by conventional cytogenetic techniques, can be precisely detected and described. M-FISH was performed using the 24Xyte color kit for human chromosomes (MetaSystems, Altussheim, Germany) according to the supplier's recommendations. Briefly, metaphases were hybridized with chromosome paints for all chromosomes simultaneously. Each paint was labeled with one of five different fluorochromes (DEAC, FITC, Spectrum Orange, Texas Red, Cy5) or a unique combination of them. DNA was counterstained with 4,6-diamino-2-phenylindole. After hybridization, gray-scale images of the fluorochromes were acquired using an epifluorescence microscope equipped with a high-resolution cooled CCD camera. A 24-pseudocolor image was built up by overlay of the gray-scale images and analysis by the MetaSystems Isis software package (MetaSystems).

Gene expression profiling

Two MSC preparations expanded for three passages in medium M1 and M4, respectively, were used for gene expression analysis. High quality RNA was isolated using the Invisorb spin cell RNA mini kit (Invitex, Berlin, Germany) according to the supplier's recommendations. RNA was amplified to cRNA and labeled with biotin-UTP according to the CodeLink UniSet Human 20K I Bioarray protocol (GE Healthcare/Amersham, Freiburg, Germany). cRNA (10 µg) were hybridized overnight to CodeLink 20K chips, stringently washed and stained with Cy5-streptavidin. Microarrays were scanned on an Affymetrix 428 scanner and quantified with the CodeLink Expression Software v4.1. Data analysis was conducted with the statistical open-source software R.

Statistics

For statistical analysis, the Student's *t*-test was applied. *P*-values <0.02 were designated as significant.

Results

Isolation of MSC

First, we compared the isolation of MSC under different medium conditions. As shown in Figure 1, addition of supplements into basic media, e.g. FCS or PL, already yielded significantly different numbers of MSC from BM. MSC colonies had a bigger size but even resulted in higher numbers of colonies in PL-containing media (Table 1). Accordingly, the addition of PL to α -MEM instead of FCS increased the CFU-F number by 2.8. Comparison of LP02 + PL with DMEM + FCS resulted in an increase of colony numbers of 6.9. Remarkably, in PL, colonies are obtained at cell densities of only 5×10^4 /well, whereas at this density in standard medium (M1) no colonies were found.

Accelerated growth and improved morphology of PL-expanded MSC

Already in the primary cultures, the cells showed an more elongated morphology in cultures supplemented with PL. This morphology of cells was even more evident in cultures with LP02 as basic medium and is preserved in cultures of later passages. Figure 2 shows MSC-cultures of primary and late passages grown in the different media used. Notably, cultures supplemented with FCS did not reach the high passage number of PL-supplemented culture media. Cells grown in media + FCS grew linearly up to three to four passages and reduced growth speed afterwards, whereas cells in media + PL went through six to seven passages within the same time. Cells grown in α -MEM always showed a flattened morphology compared to DMEM or LP02. Interestingly, cells grown in LP02 + PL preserved their spindle like morphology even at passages as late as P11 until senescence (not shown).

According to the observed morphology, the cumulative cell numbers in PL-supplemented media calculated until senescence reached much higher values compared to FCS-containing media. During each passage, at least twice as much cells were harvested from one flask grown under the influence of PL compared to FCS-containing media (not shown). In Figure 3, the cumulative cell numbers of one characteristic example are shown. Each symbol represents one time point of trypsinization. Notably, already during the first passages the PL-supplemented cultures produced significantly more MSC. At the end of the cultures, these differences could reached up to 10 logs. Although, each donor showed differences in the growth profile, this trend was observed within each experiment. Table 1 supplementary online material shows the data for all 11 BM-donors including expansion time until senescence and the lowest (italic) and highest (bold) cell numbers reached within the donor group with a defined medium. The difference of MSC grown from 10^7 MNC in standard medium M1 compared to clinical grade medium M4 could be as high as 10^9 cells.

The faster growth of MSC in PL-supplemented media was reflected in the number of population doublings (Fig. 4). Whereas the population doubling number of MSC in FCS-containing media reached approximately 30, cells in PL-supplemented media showed population doubling as high as approximately 60.

Phenotype of MSC

MSC grown in the different media were analyzed for their surface antigen expression at P6. At this stage, cultures grown with FCS already had reduced their proliferation rates. However, the majority of MSC expanded in all investigated media still were clearly distinguishable from hematopoietic cells by the lack of the hematopoietic markers CD45 and CD34 and expression of the panel CD90, CD105, and CD59 typically expressed by MSC (Table 2). In contrast to early passages,

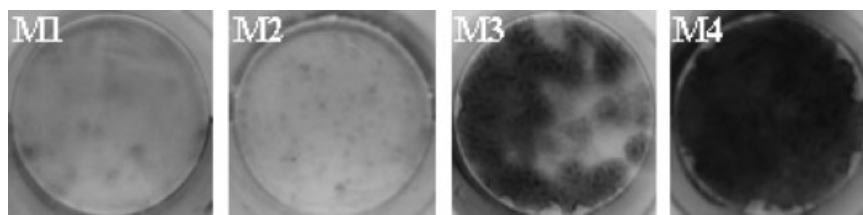


Fig. 1. Isolation of hMSC in FCS and PL containing media. MSC seeded at 5×10^5 in six-well plates in medium 1–4 as indicated in Materials and Methods were stained with crystal violet after 14 days. Results illustrate those of one example repeated 11 times.

TABLE 1. Improved isolation of hMSC in PL-containing media

	M1	M2	M3	M4
Mean $\pm$ SE	233 $\pm$ 77	415 $\pm$ 97	1181 $\pm$ 244*	1604 $\pm$ 441**

Statistical analysis of CFU-F grown in medium M1–M4 as indicated in Materials and Methods from 11 donors. Mean $\pm$ SE of CFU-F were calculated for 10^7 plated cells.

* $P < 0.02$ for M2/M3.

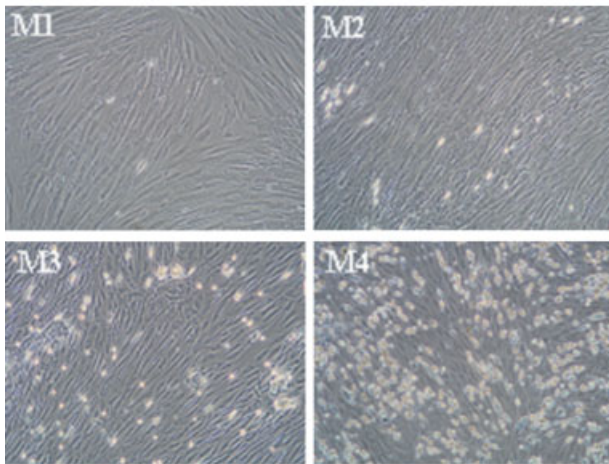
** $P < 0.02$ for M1/M4.

where 95–98% of all cells express CD90, CD105, and CD59 (not shown), at this late time point a smaller proportion of cells (77–92%) has been shown positive for these antigens in all media.

Differentiation capability of expanded MSC

MSC at P6 were investigated for their potential to differentiate into adipo-, osteo-, and chondrogenic lineages. All expanded MSC were able to differentiate into these three lineages.

A



B

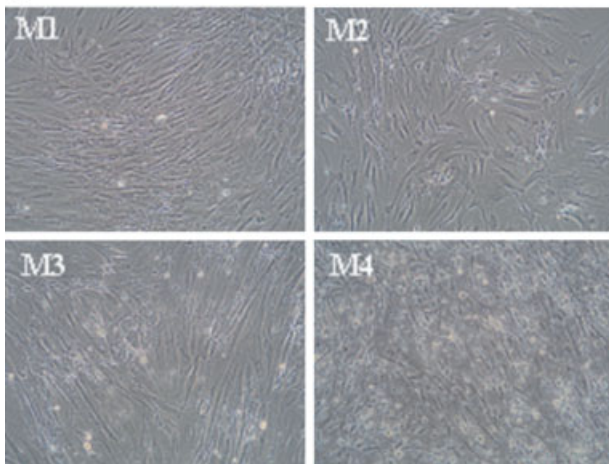


Fig. 2. Preserved spindle shaped morphology of hMSC in PL containing media. Pictures of cells plated in media as indicated in Materials and Methods were taken at confluency of primary (A) and late (B) passages. In late passages, pictures of FCS-containing media M1 and M2 were taken at P3 whereas M3 and M4 show pictures of P6 or P7, respectively, grown for the same time. Results illustrate those of one example repeated 11 times. Original magnification 100 $\times$.

Figure 5 shows one example for adipocytes (Fig. 5A), osteoblasts (Fig. 5B), and chondroblasts (Fig. 5C) derived from MSC of one donor. Induction of adipogenic differentiation of cells grown in FCS-containing media was more efficient compared to growth conditions with PL. However, adipocytes were clearly detected. Additionally, we tested the differentiation capability of MSC at P10 from cultures still growing for this long period which mainly was reached only by PL-supplemented cultures. Interestingly, the differentiation potential of this MSC did not decrease but rather was comparable as at P6, particularly for chondrogenesis (not shown).

Platelet lysate: Standardization of different preparations and platelet concentrations

For future clinical application, standardized medium preparation will be necessary. Therefore, we tested the optimal number of thrombocyte concentrates to be pooled and the concentration of containing platelets for PL preparation. Figure 6A shows the influence of the number of thrombocyte concentrates used for pooling on the expansion of hMSC over six passages. The thrombocyte concentrations were 1.46×10^9 /ml for PL1, 1.2×10^9 /ml for PL2, and 1.9×10^9 /ml for PL3. Our results show a more pronounced expansion by addition of PL obtained by pooling higher numbers of PRP preparations, particularly by pooling of 10 PRP.

Furthermore, we determined the optimal number of thrombocytes needed for PL-preparation with the best expansion-supporting properties. In Figure 6B, the influence of different platelet concentrations on population doublings are shown on MSC from different donors. In all but one cases the expansion rates with PL of 1.5×10^9 /ml were similar or even higher compared to the starting platelet concentration ranging between 1.6 and 1.9×10^9 /ml. Further dilution of platelets resulted in decreased expansion of MSC within the investigated six passages.

The formation of granular deposits, particularly in MSC cultures maintained with LP02 + PL, makes the microscopic observation difficult and could be of concern (compare M4 in Fig. 2) for long-term expansion, although in no case we have observed any growth inhibition by these precipitates. Therefore, an attempt was made to decrease the particle formation by enforced centrifugation of PL after thawing. Using 8,000g instead of 900g to eliminate remaining membrane compounds, a slight but nonsignificant decrease of deposit formation has been observed (not shown). Comparative cell numbers over an expansion period of five passages did not show any significant differences leading us to define centrifugation of PL with 8,000g as the most suitable method.

MSC retain their immunomodulatory properties

MLC were carried out with different combinations of allogeneic hPBMC used as effectors (E) and irradiated stimulators/activators (A) at ratios of 1:1. Human MSC (M) added to the MLC were from unrelated healthy donors. In all 6 experiments, hMSC added at effector/stimulator/MS ratio of 1:1:1 suppressed T-cell proliferation efficiently ($P = 0.000004$). The average inhibition at this ratio was $84.8 \pm 9.7\%$ (Fig. 7). At an effector/stimulator/MS ratio of 1:1:0.5 and 1:1:0.2, the proportion of proliferating T-cells in the culture was increasing to $46.1 \pm 34.7\%$ and $31.7 \pm 10.7\%$. Despite the high variance between the experiments, the trend could be clearly reproduced and gave highly significant results with P -values < 0.02 .

MSC display a stable karyotype

Expansion of hMSC in the FCS-free medium M4 for six passages resulted in cells with normal karyotypes in all examples carried

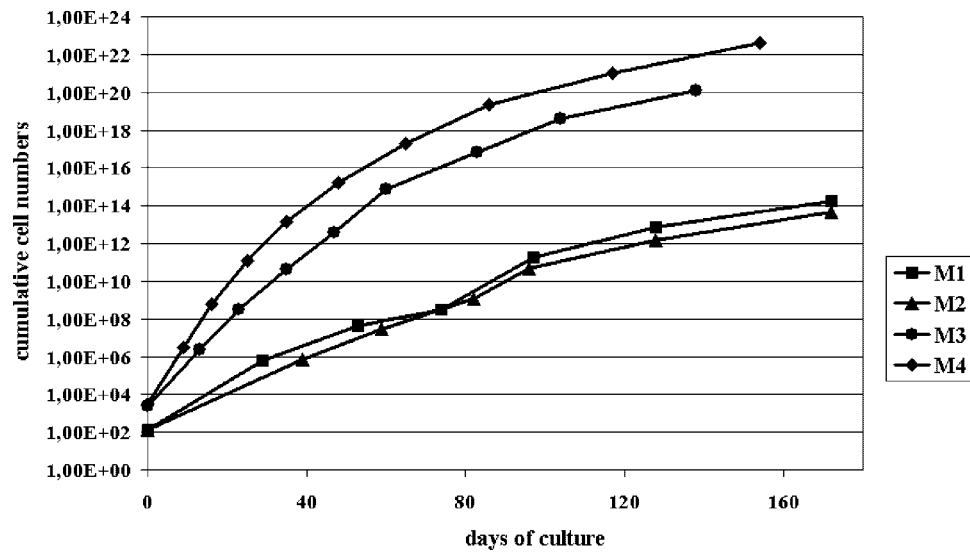


Fig. 3. Growth kinetics of hMSC in different media. Cumulative cell numbers illustrate those of one example repeated 11 times grown in media until senescence as indicated in Materials and Methods. Each symbol represents a single time point of trypsinization.

out. Neither by M-FISH (Fig. 1A supplementary online material) nor by GTG-banding (Fig. 1B supplementary online material) any genomic aberrations were detected suggesting the PL-supplemented medium for hMSC expansion as a safe approach.

The gene expression profile supports the observation of accelerated MSC expansion in PL-supplemented medium

The hierarchical clustering using all 11,046 genes with significant signal above background from hMSC cultured in either medium M1 or M4 using *manhattan* metric and average distance according to Shimodaira (2004) revealed a cluster stability >95%. This suggests significant differences in gene expression in dependence of the used expansion medium (not shown). The top 500 genes with high variability without prior group definition throughout all four samples (Fig. 8) were hierarchically clustered in three distinct groups (C1–C3)

using the deviation from the first principle component as a figure-of-merit as described by Feig et al. (2006). The evaluation with the databases for Gene Ontology terms (GO, functional gene classification), BioCarta (signal transduction gene classification), and Kyoto Encyclopedia of Genes and Genoms pathways (KEGG, metabolic gene classification) revealed interesting results in differential gene expression of nonoverlapping gene groups (Fig. 8): (I) genes from gene-families involved in differentiation/development (Fig. 2A supplementary online material), cell adhesion/extracellular matrix–receptor interaction (not shown), TGF- β signaling (Fig. 2B supplementary online material), and trombospondin-1 (TSP-1) induced apoptosis (Fig. 2C supplementary online material) were downregulated, whereas (II) genes from gene-families involved in cell cycle (Fig. 2D supplementary online material), DNA replication, and purine metabolism (not shown) were upregulated. Interestingly, genes involved in fatty acid metabolism (Fig. 2E supplementary online material) and major histocompatibility complex (MHC) class I and II responsible for antigen-dependent T-cell activation (Fig. 2F supplementary online material) could be shown to be downregulated as well.

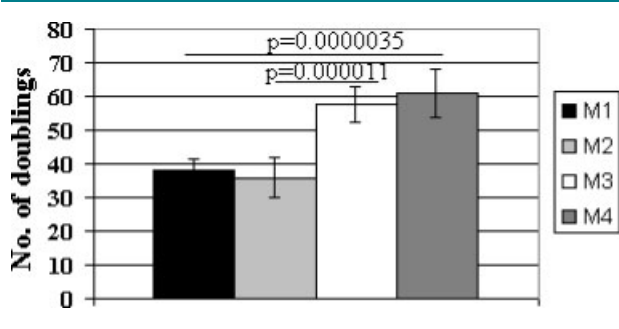


Fig. 4. Increased population doublings of hMSC in PL-containing media. Results illustrate summarized population doublings of 11 experiments during the expansion time in media M1–M4 as indicated in Materials and Methods. Highly significant differences are shown by P-values for PL- (M3 and M4) compared to FCS-containing media (M1 and M2).

Discussion

MSCs are extensively investigated for their potential use as cellular therapeutics in regenerative medicine. It has been shown that both autologous and allogeneic MSC can be used

TABLE 2. Phenotype of hMSC is preserved after cultivation in FCS-free medium

	CD59	CD90	CD105	CD45	CD34
M1	92.3 ± 3.9	79.3 ± 9.2	78.3 ± 7.5	0.2 ± 0.3	0.2 ± 0.3
M2	88.7 ± 9.0	85.4 ± 5.4	80.1 ± 8.5	0.3 ± 0.2	0.2 ± 0.3
M3	83.8 ± 8.5	85.3 ± 8.6	76.9 ± 8.6	0.1 ± 0.2	0.2 ± 0.3
M4	88.5 ± 5.3	88.6 ± 6.7	78.2 ± 8.1	0.2 ± 0.3	0 ± 0.1

FACS analysis was carried out at P6 for hMSC grown in media M1–M4 as indicated in Materials and Methods. Shown are the mean $\pm$ SD expressions of analyzed surface antigens of 11 experiments.

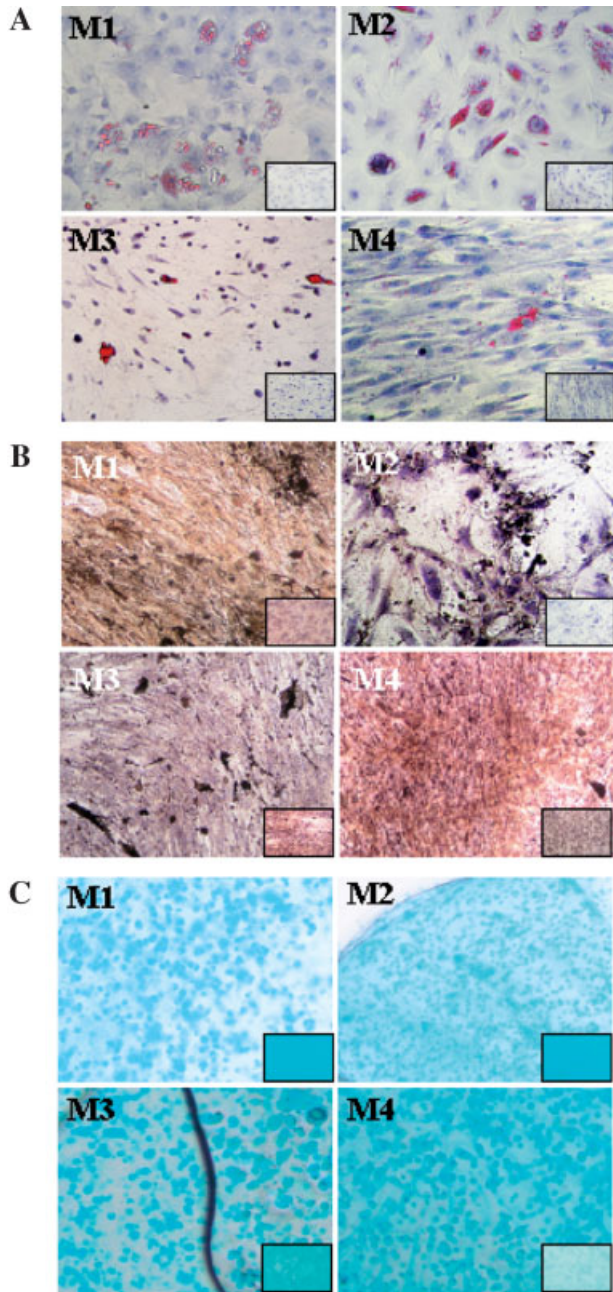


Fig. 5. Preserved differentiation potential of hMSC grown in PL-containing media. Results illustrate induced differentiations of hMSC expanded for six passages in media M1–M4 as indicated in Materials and Methods into (A) adipogenic (staining of fat droplets with sudan red), (B) osteogenic (staining of calcium precipitates with silver nitrate), and (C) chondrogenic (staining of proteoglycans with alcian blue) lineages of one representative example. Inserts show the noninduced control cultures. Original magnification 100 $\times$.

without significant immune reactions (Dai et al., 2005; Poh et al., 2006; Ringden et al., 2006). In large clinical trials where allogeneic MSC are to be used for a big cohort of recipients, huge amounts of cells from a single BM donor are needed. One preferential aim of these large scale expansions ideally should be to omit animal serum still broadly used for expansion of MSC. Autologous serum from the donor of BM is accounted to be reliable and safe at least for a small number of passages without

changing the properties of MSC (Stute et al., 2004; Kobayashi et al., 2005; Shahdadfar et al., 2005; Mizuno et al., 2006) and for small clinical studies on a single-patient basis (Dimarakis and Levicar, 2006). It is not a realistic alternative and appropriate for future “off-the-shelf” availability of stem cell therapy. From one blood donation of 500 ml, maximum 200 ml serum can be obtained, i.e. supplementing medium with 10% human serum yields 2 L expansion medium. This amount will be enough for a maximum 2–3 weeks expansion period generating MSC sufficient for one to two applications when $1\text{--}3 \times 10^6$ cells/kg body weight are infused. Allogeneic human serum is not an alternative, because MSC show retarded growth and die off after maximum 6 weeks (Spees et al., 2004; Shahdadfar et al., 2005). For all these reasons, we aimed to find a more reliable and unlimited nonanimal source for large-scale expansion of hMSC for future allogeneic clinical use.

In human clinical application, platelet gels are widespread to support bone and soft-tissue healing and have shown accelerated bone graft healing (Lucarelli et al., 2003). Consequently, the examination of PL derived from PRP highlighted that it may serve as an adjuvant for bone grafts to enhance quantity and quality of newly forming bone growth and has promoting effect on stromal cells in vitro (Lucarelli et al., 2003; Gruber et al., 2004). PRP contains seven fundamental growth factors actively secreted by platelets: platelet derived growth factor- $\alpha\alpha$ (PDGF), - $\beta\beta$, - $\alpha\beta$, transforming growth factor- $\beta 1$ (TGF) and - $\beta 2$, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) (Marx et al., 1998; Marx, 2004). The growth factors PDGF, TGF, EGF and FGF (fibroblast growth factor) have been described as mitogens for MSC (Deans and Moseley, 2000). Thrombocyte concentrates are regularly produced and applied in the everyday clinical life and meet all criteria of a safe and well controlled growth factor source. Here, we support and further improve the protocol for expansion of hMSC (Yamada et al., 2004; Doucet et al., 2005; Müller et al., 2006). However, already during the isolation of MSC we found not only enlarged colonies of CFU-F as described by Doucet et al. (2005) but also significantly higher numbers of colonies. This is particularly true for the GMP-produced medium LP02. Human MSC grown in PL-supplemented LP02 display a surprisingly long-stretched fibroblastoid morphology, leading to a considerably higher cell number at identical surface areas and cumulative cell numbers already after 5 weeks. Interestingly, the expansion rate of hMSC can be optimized by pooling a higher number of PRPs.

Previously, it has been shown that each PRP releases certain amounts of mitogenic cytokines varying e.g. for PDGF- $\alpha\beta$ between 35 and 133 ng/ml (Marx, 2004). The pooling of at least 10 PRP equalizes the preparations of PL and makes the use of the expansion medium more reliable. Likewise, titrations of platelet concentrations showed a certain threshold of cytokine amounts needed for optimal MSC expansion. Recently, it has been shown that activated platelets rapidly release stroma-derived factor 1 (SDF-1) from internal stores (Massberg et al., 2006), a cytokine involved in progenitor migration and homing which acts via its receptor CXCR-4. Using PL for MSC expansion therefore could be a good means to retain the motility of MSC for in vivo application (Kobayashi et al., 2005).

Interestingly, the observation of high-speed expansion in the PL-containing medium M4 is reflected in the differential gene expression. Numerous genes which regulate cell differentiation/development were found to be downregulated, e.g. the survival and differentiation factors nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), the activin antagonist follistatin and others. Additionally, we could demonstrate downregulation of genes involved in cell communication and extra-cellular matrix receptor (ECMR) interactions, e.g. collagens and integrin $\alpha 7$ suggesting a less dense attachment of cells to ECM proteins. Interestingly, the

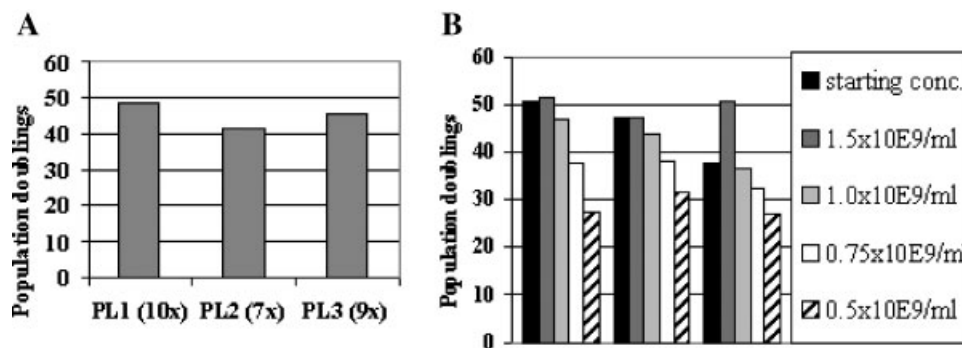


Fig. 6. Population doublings of MSC depend on the quality of PL. MSC were grown for six passages in LP02 medium supplemented with 5% PL, (A) derived by pooling different numbers of thrombocyte concentrates (in parentheses the number of pooled concentrates is shown) and (B) with PL derived from different platelet concentrations (starting concentration $1.6\text{--}1.9 \times 10^9/\text{ml}$; shown are three experiments out of five).

gene of WNT1 inducible signaling pathway protein 2 (WISP2) was also downregulated allowing cells to reenter the cell cycle faster. In addition, the thrombospondin-1 (TSP-1) induced apoptosis already at the level of the TSP receptor CD36 may lead to higher survival rates of cells. Besides, we found a strong upregulation of mRNAs coding for cell cycle promoting proteins, e.g. cyclins and cyclin-dependent kinases (Cdk) which are essential for progression through the cell cycle. Taken together, the gene expression data represent convincing evidence for an accelerated cell cycle combined with (I) less attachment of the cells to the growth surface and between the cells well known from growth retardation in stroma cultures, (II) no differentiation of MSC during the expansion period and

(III) downregulation of programmed cell death. Notably, all these effects seem to be attributed to platelet-released cytokines and GF since growth differences were related to the medium-supplements (FCS or PL) rather than to the basic medium composition.

Differentiations into osteo-, adipo-, and chondrogenic lineages are commonly used to demonstrate the multipotency of MSC. We observed a conserved capability of PL-expanded MSC for osteo- and chondrogenic differentiation but a time-delay of fat accumulation and lower amounts of cells converting to adipocytes what is in contrast to published results (Doucet et al., 2005; Müller et al., 2006). The reason for this discrepancy could be the later onset of differentiation (P2 vs. P6) which per se leads to diminished adipogenic differentiation of MSC. Our observation is well in line with our differential gene expression data showing downregulation of genes participating in fatty acid metabolism, e.g. apolipoprotein E (ApoE) and sterol regulatory element binding transcription factor 2 (SREBF2) which promotes adipocyte differentiation.

One of the most interesting properties of hMSC for transplantation is their immunomodulating function shown very imposingly in treatment of acute GvHD (Le Blanc et al., 2004). We found striking evidence for even more immunomodulating activity when cells had no contact to FCS. Recently, we have shown that MSC at low concentrations in MLC (1:0.2) display rather activating than suppressing activity (Fang et al., 2006). In the present study, however, substantial T-cell activation at decreasing MSC numbers was not found when MSC were expanded in PL-supplemented medium. In contrary, Doucet et al. (2005) found comparable suppressive effects of PL- and FCS-generated MSC even at low MSC concentrations. We suggest that the FCS compounds function as strong antigen even at small amounts. Our results are supported by gene expression analysis revealing downregulated genes of both MHC class I and II. Expanded hMSC express MHC I but not II complexes, although MHC II is present intracellularly and can be induced by addition of interferon gamma (IFN- γ) (Le Blanc et al., 2003). The lack of MHC II on hMSC so far has been interpreted as evidence for their nonstimulating properties so that MSC are transplantable between MHC-incompatible individuals (Aggarwal and Pittenger, 2005). However, the MHC I expression bears the risk of CD8-positive killer T cell activation. The downregulation of MHC II as well as MHC I of hMSC expanded in PL-supplemented medium makes the cell preparations even more safe and useful for application in cell-replacement therapies.

One concern in the application of in vitro expanded cell preparation is their genomic stability. For mouse MSC,

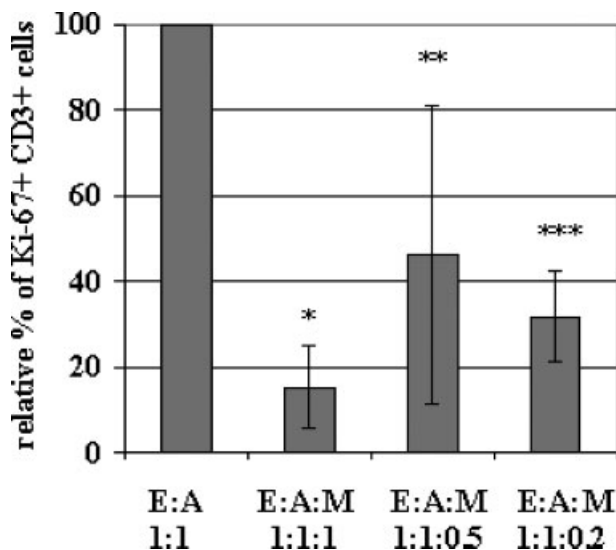


Fig. 7. Immunomodulatory properties of hMSC are preserved after cultivation in PL-supplemented medium. Human PBMC from healthy donors (E) were cocultured with irradiated allogeneic hPBMC (A) and decreasing amounts of hMSC (M) expanded in FCS-free medium M4 for two to four passages as third party. After 96 h, cells were stained with antibodies against surface antigens CD3 and CD56 and intracellular Ki-67. Data represent relative mean values $\pm$ SD of proliferating Ki-67 positive T-cells from six experiments, either cocultivated without (E:A 1:1) or with decreasing amounts of hMSC. P-values: (*) 4×10^{-6} ; (**) 0.013; (***) 1.9×10^{-5} .

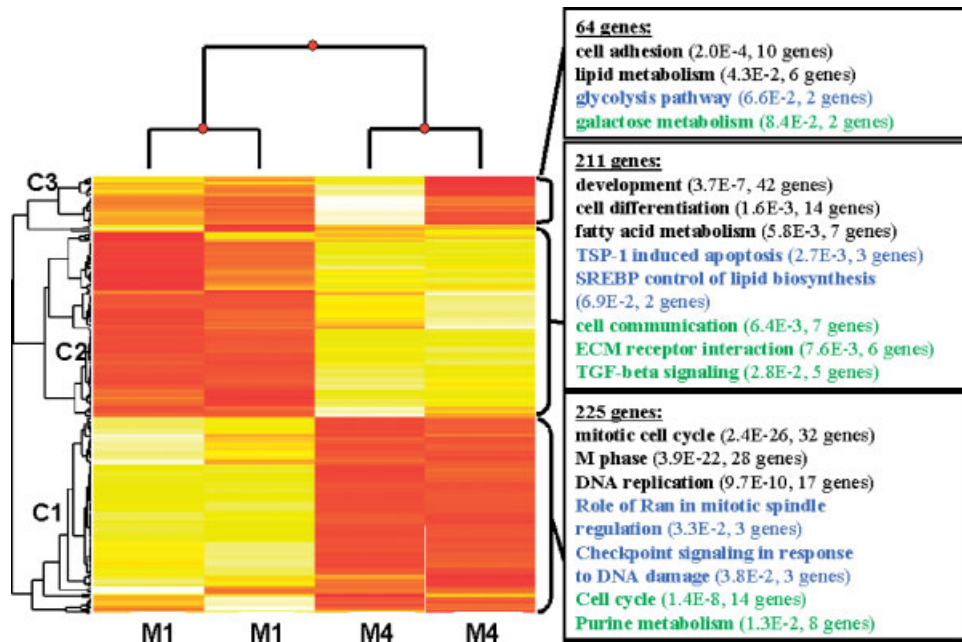


Fig. 8. Hierarchical clustering using the top 500 genes with high variability throughout all four samples (two hMSC preparations cultured in medium M1 and two hMSC preparations cultured in M4) using the deviation from the first principle component as a figure-of-merit. Gene expression values are shown as heat colors (yellow, low expression; red, high expression). Cluster stability >95% is depicted as red dots on the dendrogram nodes. Samples and genes were clustered using *manhattan* metric and average distance. Genes clustered in three distinct groups (C1–C3), which were analyzed in respect to GO terms, Biocarta, and KEGG pathways (Right part; letters in black, blue, and green, respectively. In parentheses: number of genes and *P*-value from the overrepresentation analysis).

accumulation of chromosomal abnormalities has been described repeatedly (Miura et al., 2006). For hMSC, spontaneous transformation is an uncommon process. However, immortalization of hMSC with telomerase reverse transcriptase has been shown to initiate transformation of the cells in vitro and in vivo (Serakinci et al., 2004). Additionally, long-term in vitro expansion of hMSC over 4–5 months eventually leads to spontaneous transformation (Rubio et al., 2005). With our expansion protocol, after six passages a high cell number will be generated expectedly adequate for larger clinical trials. Therefore, the expansion could be limited to maximum six passages not exceeding 12 weeks. In all investigated samples, these cells were normal without karyotype abnormalities and should provide a safe cell preparation.

In summary, we show here that expansion of hMSC without FCS is feasible and has several advantages. Replacement of FCS by PL regularly increases MSC growth and cell numbers. The basic medium seems not to be the decisive factor, however a GMP-produced medium should be the primary choice. We propose a standardized protocol using 5% PL derived from 1.5×10^9 platelets/ml. Gene expression data support that MSC expanded under these conditions display a less immunogenic potential in vitro even at low cell numbers. Expansion with PL is safe and produces cells with a normal karyotype at least for six passages. Our microarray data strengthen the observation of rapid growth since expressions of genes for apoptosis, differentiation, ECM interaction, and TGF- β signaling are decreased and, conversely, expression of genes for cell cycle components is increased. Altogether, we suggest to omit FCS from expansion media for clinical grade hMSC to generate safe cell therapeutics available from small BM donations.

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