Original Article

Xenogeneic Protein-Free Cultivation of Mesenchymal Stromal Cells – Towards Clinical Applications

(BMP / cell therapy / multipotent mesenchymal stromal cells / growth factors / EGF / PDGF / M-CSF / FGF / differentiation / orthopaedic surgery)

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Abstract. We have studied a rapid cultivation method for human mesenchymal stromal cells based on CellGro™ medium and human serum, supplemented with insulin, ascorbic acid, dexamethasone, epidermal growth factor, platelet-derived growth factor BB, macrophage colony-stimulating factor and fibroblast growth factor 2. This study has shown that rapid expansion of human multipotent mesenchymal stromal cells using human serum could not be achieved without addition of growth factors. Furthermore, we have found that insulin and, quite probably, epidermal growth factor may be omitted from our formula without loss of colony-forming capacity or total cell yield. On the other hand, dexamethasone, ascorbic acid and fibroblast growth factor 2 were necessary for the growth and colony-forming capacity of multipotent mesenchymal stromal cells, while platelet-derived growth factor BB prevented their differentiation into adipogenic lineage. Moreover, multipotent mesenchymal stromal cells cultivated in our system expressed higher levels of bone morphogenetic protein 2, but not bone morphogenetic protein 7, than cells cultivated in α-MEM with foetal bovine serum. This shows that our system promotes differentiation of mesenchymal cells towards osteogenic and chondrogenic lineages, making them more suitable for bone and cartilage engineering than cells grown in conventional media. Furthermore, we have proved that these cells may be conveniently cultivated in a closed system, in vessels certified for clinical use (RoboFlask™), making the transfer of our cultivation technology to good clinical practice easier and more convenient.

Introduction

The revelation of multipotency of human mesenchymal stromal cells (Friedenstein et al., 1968) promised broad applications in numerous areas of medicine (Koc et al., 2000; Le Blanc et al., 2004; Kawate et al., 2006; Lin et al., 2006). Today, however, the reality is far beyond expectations. One of the reasons is the failure to obtain sufficient amounts of human multipotent mesenchymal stromal cells (hMSCs) via the harvesting method (Connolly et al., 1989; Hernigou et al., 2005) or expansion protocols (Abdallah and Kassem, 2009) in a short time period while preserving their multipotency. Several methods of hMSC cultivation have been evaluated, but none of them is absolutely satisfactory (Mannello and Tonti 2007; Tonti and Mannello, 2008; Pal et al., 2009). Another problem is the ability of the cultivation methods to conform with good manufacturing practice (GMP), the criteria and requirements of the Food and Drug Administration (FDA) or similar regulatory authorities (Halme and Kessler, 2006; Unger et al., 2008).

The current laboratory standard expansion protocol for hMSCs is based on various cultivation media supplemented with foetal bovine serum (FBS). Although hMSCs obtained in this way were used for experimental clinical applications (Kawate et al., 2006; Le Blanc et al., 2008), there are certain safety concerns about this

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Abbreviations: α-MEM – minimal essential medium (Eagle) α, BMMCs – bone marrow mononuclear cells, BMP – bone morphogenetic protein, CFU-F – colony-forming unit (fibroblast), EGF – epidermal growth factor, FCS – foetal calf serum, FGF – fibroblast growth factor, FDA – US Food and Drug Administration office, GMP – good manufacturing practice, hMSCs – human multipotent mesenchymal stromal cells, hS – (pooled) human serum, M-CSF – macrophage colony-stimulating factor, PDGF-BB – platelet-derived growth factor BB, PLLA – poly(L-lactic) acid.

technique. The most important is the risk of immune reactions, viral and prion transmission, or the senescence of hMSCs (Lepperdinger et al., 2008; Wagner et al., 2008) caused by FBS. The next critical point is the necessity for multiple passages, which increases the cultivation time and potentially increases the risk of microbial contamination.

In 2009, we published a one-step, xenogeneic protein-free cultivation protocol for hMSCs, based on clinical-grade CellGro™ Medium for Haematopoietic Stem Cells, human serum and seven supplements (dexamethasone, insulin, ascorbic acid, EGF, PDGF-BB, FGF-2 and M-CSF). Multipotent mesenchymal stromal cells were co-cultivated with haematopoietic cells, with the intention of decreasing the necessity of culture manipulation. In fact, we found that this co-cultivation actually increases the yield of hMSCs, resulting in 40 times higher yields than standard cultivation method based on research-grade α-MEM medium with FCS. Our cells were able to differentiate into three lineages (chondrogenic, adipogenic, osteogenic) and to form bone-like structures on three dimensional scaffolds in immunocompromised mice (Pytlik et al., 2009). In the presented work, we identify essential supplements with relation to further development of our method towards GMP requirements and better characterization of cells obtained by our way of cultivation.

Material and Methods

Mesenchymal stromal cell donors

Patients undergoing diagnostic or staging trephine biopsy for suspected or proven haematological disease were used as hMSC donors. After providing informed consent, 10 ml of bone marrow blood was harvested from the posterior iliac crest. The study was conducted according to the Declaration of Helsinki and under the local ethics committee approval.

Media and supplements

Research-grade cultivation medium (α-MEM) and other reagents (EDTA-trypsin solution, glutamine, antibiotic solutions and FCS) were purchased from Gibco (Invitrogen division, Paisley, Scotland). Clinical grade CellGro™ Medium for Haematopoietic Stem Cells was obtained from CellGenix (Freiburg, Germany).

The following supplements were used: water-soluble dexamethasone (Sigma-Aldrich, Steinheim, Germany), recombinant human insulin for clinical use from Eli Lilli (Prague, Czech Republic), ascorbic acid (Biotica, Prague, Czech Republic), recombinant human epidermal growth factor (EGF, research grade), recombinant human platelet-derived growth factor BB (PDGF-BB, research grade) (both from BD Biosciences, Bedford, MA), and recombinant human macrophage colony-stimulating factor (CSF-1, M-CSF, research-grade) (R&D, Minneapolis, MN). Recombinant human fibroblast growth factor 2 (FGF-2) was purchased either as research grade from Invitrogen (Eugene, Oregon, OR), or as clinical grade from CellGenix. All research grade growth factors were lyophilized and carrier-free (e.g., without bovine albumin), and were reconstituted with tissue grade distilled water supplemented with clinical grade human albumin in normal saline (Baxter AG, Vienna, Austria).

Pooled human serum (hS) was obtained by mixing 5 units of human AB Rh-negative plasma, purchased from the blood bank of the Institute of Haematology and Blood Transfusion (Prague, Czech Republic), with 0.1 M $CaCl_2$ in a 9 : 1 ratio. The mixture was then incubated for 180 min at room temperature. After removal of the fibrin clot by filtration through a metal strainer, the product was incubated for a further 48 h at 4 °C. After residual fibrin removal, serum was sterilized by filtration through 0.22 μm membrane and aliquots were stored in a -80 °C freezer until use.

Human multipotent mesenchymal stromal cell expansion

Human mesenchymal stromal cell expansion was performed as described previously (Pytlik et al., 2009). Briefly, after obtaining the BMMCs (bone marrow mononuclear cell fraction) on Ficoll gradient, standard cultures were established by seeding 10^5 BMMCs/cm^2 culture flask (TPP, Trasadigen, Switzerland) in α-MEM supplemented with 10% FCS, 2 mM glutamine, and 1% penicillin, streptomycin and amphotericin B solution. After 24 hours, non-adherent cells were washed away and the cultures were maintained for two weeks, with change of media twice weekly. Experimental cultures were established by seeding 3.3×10^4 BMMCs/cm^2 culture flask in CellGro™ Medium for Haematopoietic Stem Cells with 10% hS, 2 mM glutamine, and 1% penicillin, streptomycin and amphotericin B solution, insulin (0.25 U/ml), dexamethasone (0.01 μM), ascorbic acid (100 μM), EGF (10 ng/ml) and PDGF-BB (100 ng/ml), M-CSF (25 ng/ml) and FGF-2 (1 ng/ml). Non-adherent cells were not washed away, unless specified otherwise. Medium was not changed for the whole 2-week period of cultivation, but supplements were added twice weekly. In certain experiments, RoboFlasks™ (Corning, Acton, MA) were used. These are rectagonal flasks certified for clinical use, with a 92.6 cm^2 cultivation area, a gas exchange port and a cap with soft rubber septum which can be punctured by a sterile needle for addition of supplements and cell harvests. The amounts of 3.3×10^4 BMMCs/cm^2 were seeded in these flasks in experimental medium without adherent cell removal, and supplements were added as described previously. Cells from these flasks were harvested in a closed system by standard sterile needle and syringe. In all instances, the harvest was performed with 0.25% EDTA – 1% trypsin solution.

Determination of the yield of human multipotent mesenchymal stromal cells

The overall count of the cell product was calculated in a standard haematological analyser (Beckman-Coulter AcTdiff2, Fullerton, CA). Further analysis of the adherent cells was performed by FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA). Cells were labelled with anti-CD45 and anti-CD235a antibodies (DakoCytomation, Brno, Czech Republic) and 7-AAD (Sigma-Aldrich), and the fraction of $CD45^{-}$ $CD235a^{-}$ cells were considered hMSCs while 7-AAD negative cells were considered living cells. The total yield of hMSCs was determined by the formula:

$$hMSCs\ count = \frac{(overall\ cell\ count) \times (\%CD45^{neg}CD235a^{neg}cells)}{100}$$

BMP-2, BMP-7 and osteocalcin studies

hMSCs were stained with a 3-colour antibody panel involving biotinylated Anti-Human BMP-2, biotinylated Anti-Human BMP-7 (both PeproTech, Rocky Hill, NJ), anti-osteocalcin PE (clone 190125, R&D Systems) and biotinylated anti-ALP (Clone B4-78, R&D Systems). Lineage-specific (Lin) dump channel consisted of anti-CD45 PE-Cy5 (Clone T29/33, DakoCytomation, Denmark). Biotinylated antibodies were detected by streptavidin FITC (R&D Systems). Trypsinized cells were resuspended in phosphate-buffered saline containing 2% foetal bovine serum and permeabilized by an Intrastain kit (DakoCytomation). Isotypic immunoglobulins IgG1 FITC, IgG1 PE, IgG2b FITC, IgG2b PE and IgG1 PE-Cy5 (all DakoCytomation) were used as controls. Positivities were adjusted at the 0.5% confidence level.

Statistical analysis

For statistical analysis, normal data distribution was tested by Kolmogorov-Smirnov and Lillefors tests. As most of the data followed normal distribution, the results are presented as mean and standard deviations. Paired *t*-tests were used for group comparisons, as in all experiments multiple samples from the same patient were used. Therefore, this test corrected for inter-patient variations. In supplemental omission studies, a standard group (no omission) was compared to each of the experimental groups in these paired tests. P values lower than 0.05 (two-side) were considered as statistically significant.

Results

Effect of supplement deprivation on haematopoietic cells cultivated in CellGro™ with human serum

In our previous experiments, we found that when hMSCs were cultivated in α-MEM with human serum with dexamethasone, ascorbic acid, insulin, PDGF-BB and EGF, their yields did not differ from the yields of cells cultivated in α-MEM with human serum without supplements. After addition of M-CSF, the yields grew insignificantly, while significantly more hMSCs were obtained only with FGF-2 addition (unpublished data). CellGro™ with human serum and all seven supplements gave better yields of hMSCs than similarly supplemented α-MEM (Pytlik et al., 2009). However, until now we have not studied the effects of individual supplements on CellGro™-based cultivation medium. We have chosen a different approach than in the previous set of experiments – this time we omitted supplements from the whole formula rather than adding them to the basal medium.

The results of these manipulations are shown in Tables 1 and 2. In a series of seven experiments, we omitted one supplement from the CellGro™ and human-serum based medium. Compared to fully supplemented medium, both the number of colonies and total number of adherent cells were lower when dexamethasone, ascorbic acid, FGF-2, or PDGF were omitted. After omission of M-CSF, the number of colonies was significantly lower ($P = 0.03$), but the total number of adherent cells was not ($P = 0.90$).

In a series of eight experiments with omission of two supplements, we did not omit dexamethasone, as it seemed to be essential for the growth of hMSCs in CellGro™ and human serum-based medium. In these experiments, all combinations where ascorbic acid was omitted had a significantly lower number of colonies than complete medium, and also lower total numbers of adherent cells. On the other hand, when insulin was omitted, only the combination with ascorbic acid omission led to lower numbers of colonies and adherent cells than complete medium. All other omissions essentially maintained the numbers of colonies, but when either FGF-2 or PDGF-BB was missing, colonies were small, mostly with one or two layers of cells only (in comparison to multilayered colonies in the full formulation), and total numbers of cells were significantly lower.

In the next series of experiments, where more supplements were omitted, we maintained the presence of both dexamethasone and ascorbic acid, as these seemed to be most important for hMSC growth. On the other hand, we omitted insulin, which seemed to be dispensable. Because of this we could minimize the number of our experiments while maximizing the amount of obtained data. Therefore, in the next series of nine experiments, either four or five supplements were omitted (in the last case, only dexamethasone and ascorbic acid were left in the medium). In all these cases the numbers of colonies and total yields of nucleated cells were lower compared to fully supplemented medium. Most notably, when only dexamethasone and ascorbic acid were left in the CellGro™ with human serum, the yields of nucleated cells were the lowest achieved in any series of these experiments, showing that these two supplements, while indispensable, cannot have full effect without the help of cytokines.

Table 1. Omission of supplements: number of colonies per 10^6 seeded BMMCs

Supplement combination	Mean (± standard deviation)	P (compared to complete medium)
One supplement omitted		
Complete medium	33.4 ± 12.2	-
CM – ascorbic acid	15.9 ± 9.9	**0.0008**
CM – dexamethasone	9.1* ± 5.2	**0.0006**
CM – insulin	29.5 ± 10.3	0.19
CM – EGF	29.5 ± 12.1	0.13
CM – FGF-2	21.8 ± 7.8	**0.003**
CM – M-CSF	25.9 ± 11.7	**0.03**
CM – PDGF-BB	25.2 ± 8.8	**0.01**
Two supplements omitted		
Complete medium (CM)	31.5 ± 11.4	-
CM – ascorbic acid – insulin	20.5 ± 13.2	**0.0015**
CM – ascorbic acid – EGF	17.1 ± 13.3	**0.001**
CM – ascorbic acid – FGF-2	10.8 ± 13.6	**0.0005**
CM – ascorbic acid – M-CSF	15.5 ± 11.2	**0.0002**
CM – ascorbic acid – PDGF-BB	10.6 ± 6.8	**0.0002**
CM – insulin – EGF	34.3 ± 12.5	0.29
CM – insulin – FGF-2	31.9 ± 14.8	0.89
CM – insulin – M-CSF	35.1 ± 14.6	0.10
CM – insulin – PDGF-BB	29.0 ± 11.8	0.36
CM – EGF – FGF-2	34.1 ± 23.1	0.58
CM – EGF – M-CSF	35.3 ± 12.6	0.11
CM – EGF – PDGF-BB	33.1 ± 13.3	0.68
CM – FGF-2 – M-CSF	28.5 ± 17.4	0.33
CM – FGF-2 – PDGF-BB	27.4 ± 20.6	0.37
CM – M-CSF – PDGF-BB	31.8 ± 18.5	0.94
Four supplements omitted		
Complete medium (CM)	30.1 ± 23.6	-
CM – ins – EGF – FGF-2 – M-CSF	25.8 ± 23.5	**0.0005**
CM – ins – EGF – FGF-2 – PDGF-BB	13.8 ± 16.2	**0.0035**
CM – ins – EGF – M-CSF – PDGF-BB	22.7 ± 16.2	0.08
CM – ins – FGF-2 – M-CSF – PDGF-BB	23.0 ± 20.9	**0.002**
Five supplements omitted		
Only ascorbic acid and insulin left	15.0 ± 16.4	**0.001**

Apart from the quantitative results, qualitatively, the omission of PDGF-BB (either alone or in combination with other supplement) frequently led to spontaneous adipogenic differentiation of hMSCs, which was not observed if other combinations of cytokines were missing.

Osteogenic markers on human multipotent mesenchymal stromal cells

In six experiments, we compared the expression of BMP-2, BMP-7 and osteocalcin on hMSCs cultivated in α-MEM with FCS (haematopoietic cells washed away) with hMSCs cultivated in CellGro™ with human serum and supplements (haematopoietic cells washed or unwashed). The results of these experiments are shown in Table 3. Expression of BMP-2 in osteocalcin-negative fraction was higher in hMSCs grown in CellGro™ with human serum and supplements (either washed or unwashed) compared to α-MEM with FCS. In the osteocalcin-positive fraction, when compared with α-MEM and FCS, the differences were statistically significant in washed CellGro™ cultures, while for unwashed CellGro™ cultures they reached only marginal significance (P = 0.055). Washing away haematopoietic cells did not have any influence on BMP-2 expression if cells were cultivated in CellGro™ with human serum and supplements.

For BMP-7, on the other hand, no significant differences were found among hMSCs cultivated in α-MEM-based or CellGro™-based media.

RoboFlasks™ vs. TPP plastics

We performed 12 paired cultivations of hMSCs in CellGro™ medium with human serum and supplements in 75 cm^2 TPP cultivation flasks and closed system RoboFlask™. The results of cultivations in RoboFlasks™ vs. TPP cultivation vessels are shown in Table 4. The yields of both adherent and $CD45^-CD235a^-$ cells were slightly but significantly lower in RoboFlasks™ than in TPP vessels (adherent cells in RoboFlasks™, mean 1.23×10^6 per 10^6 seeded BMMCs vs. mean 1.55×10^6 per 10^6 seeded BMMCs in TPP vessels, P = 0.002; $CD45^-CD235a^-$ cells in RoboFlasks™, mean 1.14×10^6 per 10^6 seeded BMMCs vs. mean 1.44×10^6 per 10^6 BMMCs in TPP vessels, P = 0.003). There was no difference between the percentages of $CD45^-CD235a^-$

Table 2. Omission of supplements: number of adherent cells per 10^6 seeded BMMCs

Supplement combination	Mean (± standard deviation) $\times 10^6$	P (compared to complete medium)
One omitted supplement		
Complete medium (CM)	$2.3 \pm 1.7 \times 10^6$	-
CM – ascorbic acid	$1.0 \pm 1.0 \times 10^6$	**0.02**
CM – dexamethasone	$0.4 \pm 0.1 \times 10^6$	**0.02**
CM – insulin	$2.3 \pm 1.4 \times 10^6$	0.75
CM – EGF	$2.1 \pm 1.3 \times 10^6$	0.41
CM – FGF-2	$1.1 \pm 0.7 \times 10^6$	**0.03**
CM – M-CSF	$2.3 \pm 1.5 \times 10^6$	0.9
CM – PDGF-BB	$0.9 \pm 0.6 \times 10^6$	**0.048**
Two omitted supplements		
Complete medium (CM)	$3.3 \pm 2.2 \times 10^6$	-
CM – ascorbic acid – insulin	$1.2 \pm 1.4 \times 10^6$	**0.002**
CM – ascorbic acid – EGF	$1.0 \pm 1.1 \times 10^6$	**0.002**
CM – ascorbic acid – FGF-2	$0.6 \pm 0.7 \times 10^6$	**0.002**
CM – ascorbic acid – M-CSF	$0.9 \pm 1.1 \times 10^6$	**0.001**
CM – ascorbic acid – PDGF-BB	$0.5 \pm 0.5 \times 10^6$	**0.004**
CM – insulin – EGF	$3.7 \pm 1.5 \times 10^6$	0.29
CM – insulin – FGF-2	$2.5 \pm 1.6 \times 10^6$	0.07
CM – insulin – M-CSF	$3.2 \pm 2.0 \times 10^6$	0.98
CM – insulin – PDGF-BB	$1.6 \pm 1.1 \times 10^6$	**0.007**
CM – EGF – FGF-2	$1.7 \pm 1.1 \times 10^6$	**0.007**
CM – EGF – M-CSF	$3.0 \pm 1.8 \times 10^6$	0.46
CM – EGF – PDGF-BB	$0.8 \pm 0.5 \times 10^6$	**0.006**
CM – FGF-2 – M-CSF	$1.9 \pm 1.7 \times 10^6$	**0.008**
CM – FGF-2 – PDGF-BB	$0.6 \pm 0.4 \times 10^6$	**0.005**
CM – M-CSF – PDGF-BB	$1.4 \pm 1.2 \times 10^6$	**0.001**
Four supplements omitted		
Complete medium (CM)	2.9 ± 2.4	-
CM – ins – EGF – FGF-2 – M-CSF	1.7 ± 1.8	**0.044**
CM – ins – EGF – FGF-2 – PDGF-BB	0.4 ± 0.4	**0.011**
CM – ins – EGF – M-CSF – PDGF-BB	1.2 ± 1.2	**0.011**
CM – ins – FGF-2 – M-CSF – PDGF-BB	0.6 ± 0.4	**0.011**
Five supplements omitted		
Only ascorbic acid and insulin left	0.3 ± 0.2	**0.01**

Table 3. Comparison of the expression of BMP-2 and BMP-7 in hMSCs cultivated in different media

	% of positive cells	P (vs. CellGro™ + hS + S, washed)	P (vs. CellGro™ + hS + S, unwashed)
Osteocalcin-negative fraction			
BMP-2 positive			
α-MEM + FCS	7.5 ± 8.0%	**P = 0.0037**	**P = 0.0039**
CellGro™ + hS + S, washed	26.6 ± 10.9%	P = 1.00	P = 0.71
CellGro™ + hS + S, unwashed	28.7 ± 6.3%	P = 0.71	P = 1.00
BMP-7 positive			
α-MEM + FCS	9.0 ± 8.7%	P = 0.29	P = 0.37
CellGro™ + hS + S, washed	5.2 ± 3.9%	P = 1.00	P = 0.78
CellGro™ + hS + S, unwashed	4.3 ± 5.4%	P = 0.78	P = 1.00
Osteocalcin-positive fraction			
BMP-2 positive			
α-MEM + FCS	3.6 ± 1.5%	**P = 0.033**	P = 0.055
CellGro™ + hS + S, washed	19.6 ± 14.1%	P = 1.00	P = 0.58
CellGro™ + hS + S, unwashed	22.6 ± 18.9%	P = 0.58	P = 1.00
BMP-7 positive			
α-MEM + FCS	6.3 ± 4.1%	P = 0.77	P = 0.97
CellGro™+ hS + S, washed	7.2 ± 6.7%	P = 1.00	P = 0.75
CellGro™ + hS + S, unwashed	6.2 ± 7.7%	P = 0.75	P = 1.00

S = supplements, washed = non-adherent cells removed, unwashed = non-adherent cells not removed. Results are given as means ± standard deviation. Paired *t*-tests were used for the comparisons.

Table 4. Comparison of the yields of hMSCs in TPP culture vessels and in RoboFlasks™

	TPP flasks	RoboFlasks™	P
Adherent cells (× 10^6/10^6 BMMCs)	1.55 ± 0.72	1.23 ± 0.64	**0.0027**
% of CD45$^-$CD235a$^-$ cells in adherent cells	92.7 ± 4.7	93.2 ± 5.7	0.37
CD45$^-$CD235a$^-$ cells (× 10^6/10^6 BMMCs)	1.44 ± 0.69	1.14 ± 0.61	**0.004**

Yields are expressed as millions of harvested cells per million of seeded BMMCs. Results are given as means and standard deviations. P = two sided.

cells in RoboFlasks™ and TPP vessels. Of 12 paired samples, the yields of CD45$^-$235a$^-$ cells were higher than 10^6/10^6 BMMCs in six RoboFlasks™ vs. eight TPP flasks (P = ns). The yields of adherent cells, CD45$^-$CD235a$^-$ cells, and percentages of CD45$^-$CD235a$^-$ cells were all highly correlated in the paired samples (r = 0.91–0.95, P < 0.0001 in all cases).

Discussion

In this work, we have tried to develop our cultivation method further, to better conform to the GMP requirements and to better characterize the cells that are obtained by our cultivation method.

We have confirmed that it is possible to cultivate hMSCs in the CellGro™-based medium in a fully closed system, as RoboFlasks™ allow addition of cells, cultivation medium and supplements, as well as harvest of the cells without opening the cultivation flask. The yields of hMSCs were slighthy lower than in TPP flasks, but still on average more than 10^6 adherent and CD45$^-$ CD235$^-$ cells were obtained per million of seeded BMMCs, thus confirming our previous findings. Why the yields were lower in the flasks approved for clinical use remains unclear. Both types of vessels are tissue-culture treated; however, as they represent patented technology, we do not have access to the information that could explain this fact.

BMPs play a crucial role in osteogenic differentiation. BMP-2 seems to play an essential role in hMSC recruitment during fracture repair (Tsuji et al., 2006; Yu et al., 2010). BMP-2 increases during differentiation towards chondrogenic lineage and osteoblasts, except for the terminal stage. BMP-7 does not seem to be expressed by hMSCs in large amounts. Osteocalcin is one of the extracellular matrix proteins expressed in post-proliferative osteoblasts. Therefore, we have compared the expression of BMP-2, BMP-7 and osteocalcin in our cells. In cells grown in CellGro™-based media, both BMP-2$^+$ osteocalcin$^+$ and BMP-2$^+$ osteocalcin$^-$ fractions were more abundant than in the cells grown in standard, α-MEM-based medium. This supports our hypothesis that during the expansion of hMSCs in full GellGro™-based medium, a significant number of cells are differentiated towards osteogenic or chondrogenic lineage but still retain their mitotic capacity (BMP-2$^+$ osteocalcin$^-$ cells), while some of them are already post-mitotic pre-osteoblasts or osteoblasts (BMP-2$^+$ osteocalcin$^+$ cells). Differentiation experiments have shown previously that our cells are able to differentiate to both osteogenic and chondrogenic lineages (Pytlik et al., 2009). Further studies are needed to determine whether the potential of chondrogenic differentiation may be evaluated in this early stage of hMSC expansion. These experiments show that the expression of BMP-2 and osteocalcin may be an important marker for the quality control of hMSC-based cellular product.

On the other hand, we did not find any difference in BMP-7 expression on hMSCs grown in either media and the number of BMP-7-positive cells was small.

We were quite surprised by the results of our supplement-deprivation experiments. In the CellGro™-based medium, dexamethasone and ascorbic acid seemed to be by far the most important supplements for both clonogenicity and expansion of hMSCs. On the other hand, these two supplements alone were unable to maintain the clonogenic capacity and cell yields without the help of additional cytokines. Dexamethasone and ascorbic acid are commonly added to osteoblast-differentiating media, which may explain preferential differentiation of our cells towards osteoblastic lineage, but until now, the special importance of the former for the growth and expansion did not seem to have been fully appreciated.

While glucocorticoids are used in many protocols for hMSC differentiation, their role in hMSC proliferation is unclear. It is known that glucocorticoids suppress bone formation and stimulate osteoblast apoptosis. We have found that in our conditions, dexamethasone is essential for both colony formation and hMSC expansion. This is supported by the report of Purpura, who observed similar beneficial effects of dexamethasone in rat calvaria cell culture (Purpura et al., 2004). However, the mechanisms of glucocorticoid action in hMSCs are unclear and seem to modulate the action of cytokines (Liberman et al., 2007) via autocrine and paracrine feedback loops.

On the other hand, ascorbic acid is known for its pro-proliferative as well as differentiation properties. Except for osteoblast differentiation, ascorbic acid was used for smooth muscle and even for adipogenic differentiation (Sato et al., 2006) of hMSCs. Moreover, it was found that hMSC proliferation is increased proportionally to the amount of ascorbic acid added to the medium (Yoo et al., 2008), and in this work, hMSCs seemed to lose their contact inhibition and grew in multilayers, similar to our cells. From this point of view, it is interesting that in our system, the effect of ascorbic acid seemed to be less important than the effect of dexamethasone.

Of the rest of the supplements, there seemed to be an effect of FGF-2 and PDGF-BB on both the colony num-

ber and the total cell number. Omission of M-CSF seemed to diminish the number of colonies but not total numbers of hMSCs. On the other hand, insulin and EGF did not seem to have any role for hMSCs growth in our system. Insulin seems to be present in the CellGro™ Haematopoietic Stem Cell Medium in adequate dose. The basal secretion of PDGF in the bone is low (Rydziel et al., 1994). Despite the activity of PDGF pathways during osteogenic, adipogenic and chondrogenic differentiation (Goff et al., 2008), the primary effect is mitogenic and inhibited differentiation in bone cells. This was confirmed in our studies, where cultures without PDGF-BB were spontaneously differentiating towards adipogenic lineage. PDGF-BB therefore seems to be important in our culture system, not only for cell expansion, but also for prevention of the differentiation of hMSCs in an unwanted direction. PDGF may act in concert with FGF-2 and EGF, as these factors increase PDGFRα expression in osteoblastic cells (Seko et al., 1991).

The action of EGF on hMSCs may overlap with that of PDGF. EGF together with PDGF-BB acted synergistically on hMSC expansion in one report (Gronthos and Simmons, 1995), while in another, EGF and PDGF-BB acted antagonistically (Geng et al., 2008). It was shown that EGF and PDGF signalling led to phosphorylation of a similar set of proteins (Kratchmarova et al., 2005), with one important exception, the proteins of the PI3K pathway, which are phosphorylated by PDGF only. Kratchmarova also found that EGF induces osteoblastic differentiation of hMSCs, while PDGF does not. In our experiments, the omission of EGF did not lead either to lower colony-forming capacity or to lower yield of hMSCs, in contrast to PDGF-BB. This is in contrast to another report (Tamama et al., 2010), where addition of EGF increased the colony-forming capacity of hMSCs by 25 %. However, PDGF was not used in this work. Whether EGF in our system supported osteoblastic differentiation of hMSCs is to be proved by other experiments. In this system EGF seems to be dispensable.

M-CSF was found to increase the number of hMSC colonies by 25 % (Jin-Xiang et al., 2004). This was confirmed in our experiments, as omission of M-CSF did lead to a significant drop in hMSC colony-forming ability; however, the total numbers of adherent cells were not impaired. M-CSF is produced by osteoblasts and, together with RANKL, stimulates the osteoclast development (Yoshida et al., 1990; Anderson, et al., 1997). Whether M-CSF acts on hMSCs themselves in an autocrine way as well or its supplementation helps hMSC expansion has yet to be determined. In any case, the omission of M-CSF seemed to have a less detrimental effect on the number of cells in our CellGro™-based cultures than the omission of other supplements, except for insulin and EGF.

According to some authors FGF-2 maintains stem cell characteristics and growth in hMSCs (Ng et al., 2008) and self-renewal capacity in osteo- and chondroprogenitors (Tsutsumi et al., 2001; Benavente et al., 2003; Solchaga et al., 2005). In one report, it was found to increase the colony size but not the colony number (Martin et al., 1997). In our previous experiments, addition of FGF-2 to other six supplements to α-MEM and human serum-based media increased the yield of hMSCs significantly (Pytlik et al., 2005). In this set of experiments, we have found not only a decrease in total numbers of cells with FGF-2 omission, but also a decrease in colony numbers. It seems that intermittent administration of FGF-2 plays an important role in our cultivation system.

In summary, we proved that hMSCs can be expanded in medium using human serum, but only with the addition of growth factors. Furthermore, we have made several steps towards further clinical use of hMSCs in human medicine. We have confirmed that these cells may be expanded in a closed system in vessels certified for clinical use. We have found that in CellGro™-based medium, insulin, EGF and possibly M-CSF may be omitted from the formula without impairment of yields of hMSCs. This may be important, as currently we do not know of any clinical-grade recombinant hEGF preparation. Finally, BMP-2 and osteocalcin were found to be useful for evaluating the quality of hMSCs, especially when they are to be used in bone or cartilage engineering.

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