Human-Derived Alternatives to Fetal Bovine Serum in Cell Culture

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Introduction

Within the past years cell therapy and advanced therapy medicinal products (ATMP) became more and more important in terms of clinical use. Several studies are already recruiting, ongoing, or already completed in this field [1]. Especially mesenchymal stem cells (MSC) isolated from bone marrow (BM-MSC) and adipose tissue (ASC) are of great interest. The major problem is the high cell dose needed for clinical applications requiring in vitro expansion. Currently, fetal bovine serum (FBS) is mainly used as medium supplement for the expansion of these cells. FBS is known for batch-to-batch variations and its immunogenicity due to xenogenic proteins as for example N-glycolylneuraminic acid that is known to incorporate in human embryonic stem cells [2]. Another big impact is the possibility of disease transmission due to prions, bacteria, or viruses [3]. The European Commission released a ‘Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products’ (EMA/410/01) that clearly states that first the use of material of non-animal origin should be preferred [4]. Hence, several research groups already tested alternatives to the use of FBS. Bieback et al. [5] used human serum (huS) and human platelet lysate (hPL) for the expansion of BM-MSC and adipose tissue-derived stem cells (ASC). Quality control included cell counts (platelets, red and white blood cells), sterility testing, pH levels, total protein concentrations and growth factor levels. ASC and fibroblasts were expanded for three passages in media supplemented with FBS, huS or hPL and evaluated microscopically. Proliferation in terms of population doubling times (PDT) was determined. In case of ASC, differentiation was performed as well. Results: All three alternatives demonstrated shorter PDT for fibroblasts and ASC compared to FBS. Furthermore, ASC maintained their differentiation potential. Conclusion: We conclude that hPL and huS can be used as alternatives to FBS for the cultivation and expansion of cells intended for human use.

*Karin Witzeneder and Andrea Lindenmair contributed equally to this publication
tion for platelet lysis while Burnouf et al. [11] performed a solvent-detergent treatment to induce the release of growth factors. Mainly, freeze-thaw cycles are performed to induce platelet lysis. Rauch et al. [8] performed three freeze-thaw cycles to obtain higher growth factor levels while Hildner et al. [12] used hPL prepared by a single cycle. Further differences in hPL preparation include the source material (apheresis concentrates, outdated platelet pools or buffy coats (BC)) as well as the medium in which the platelets are suspended (plasma with different anticoagulants, platelet additive solutions or saline). For this study, two types of hPL were prepared and compared to huS and FBS in the expansion of ASC and dermal fibroblasts. Both types of hPL were prepared from BC with the difference that for hPLₘ plasma was substituted by saline, whereas in case of hPLₘₘ the platelets were kept in plasma.

### Material and Methods

**hPL Preparation**

Six batches of hPL from 36 whole blood donations each were prepared after informed consent. The BC were obtained by density gradient centrifugation (3,933 g; 11 min) and separation from red blood cells (RBC) as well as plasma using the Compomat G4 ( Fresenius, Austria). For hPLₘ, 60x BC with identical blood group and Rhesus factor were pooled and centrifuged (442 g; 12 min) to obtain a supernatant comprising of platelets and other cells from the BC in plasma. The supernatant was stored at −80 °C until the donors were tested negative for serology.

Six pools were thawed in a water bath at 37 °C. To remove any cell debris, a final centrifugation step (5,348 g; 7 min) was performed. The supernatants were pooled to make up a pool of 36 individual donors, and aliquots were stored at −80 °C until use. Samples were drawn for the measurement of pH value, the determination of the total protein concentration, and for sterility testing.

For the preparation of hPLₘₘ, saline was added to the pool of 6 BC. Upon two centrifugation steps (442 g for 12 min and 5,348 g for 7 min), a platelet pellet with low RBC and white blood cell (WBC) content was obtained which was re-suspended in saline. The pools were stored at −80 °C until release of serological testing and completed identically to hPLₘ.

**Serum Preparation**

For the preparation of a serum pool 6 volunteer blood donors were asked for a whole blood donation after informed consent. The blood was collected without any addition of anticoagulants and was allowed to clot for at least 2 h. Upon two centrifugation steps (both at 3,000 g for 15 min), the formed clot and residual RBC were removed. The sera were stored at −80 °C until release. For the final preparation, the six sera were thawed in a water bath at 37 °C followed by a heat inactivation step at 56 °C for at least 2 h. Centrifugation (5,348 g; 7 min) was performed in order to remove any formed aggregates, and the supernatant of 6 donations was pooled. Samples were drawn for pH measurement, total protein concentration, and sterility testing. Six pools of huS were stored in aliquots at −80 °C until use.

**Quality Control of the Production of hPL and huS**

Samples of hPL and huS were taken before freezing at −80 °C for analysis of residual WBC and RBC. In case of hPL, the platelet concentration before freezing was determined at the Sysmex XE-2100 (Sysmex, Kobe, Japan) as well. Total protein content (ARCHITECT®, Abbott, Ludwigshafen, Germany) and pH level (IL Synthesis 20; Instrumentation Laboratory, Vienna, Austria) were determined after product finalization. Samples for growth factor analysis (ELISA detecting EGF, PDGF-BB, TGF-β1, IGF-1 and basic FGF; R&D Systems, Abingdon, UK) were retained. Additionally, microbiological testing using BactAlert 3D Select Link (bioMérieux France, Craponne, France) was done for each batch of hPL and huS. All pools of hPL and huS had to be negative for sterility and serological testing to be used in cell culture.

**Cell Culture**

The collection of adipose tissue material was approved by the local ethical board. ASC were isolated as previously described [13] and stored in LN₂ tanks until use. Evaluating batch-to-batch variabilities of huS and hPL, only one ASC donor (female, at time of donation 46 years old) was used for all cell culture experiments. For expansion ASC were seeded at a density of 4 × 10⁵ cells/cm² and cultured in DMEM/Ham’s-F12 medium supplemented with 1% penicillin/streptomycin and 1 ng/ml rhFGF basic (R&D Systems). Either 10% FBS or 10% huS or 5% hPL was added to the medium. Human foreskin fibroblasts (ATCC® CRL-2522™; LGC Standards, Teddington, UK) were cultivated in alpha-MEM supplemented with 1% penicillin/streptomycin, 1% L-glutamine and either 10% FBS or 10% huS or 5% hPL at a seeding density of 3.2 × 10⁴ cells/cm². In case of hPLₘ additional heparin (4 U/ml; Biochrom, Berlin, Germany) was added to the media in order to prevent clot formation. Cell cultures were maintained at 37 °C in a humidified 5% CO₂ incubator. ASC were subcultured at a confluence of 80% to prevent spontaneous differentiation while fibroblasts were passaged at 100% confluence. Proliferation doubling time (PDT) was determined by trypan blue staining. All media, reagents, and supplements except huS and hPL were provided by PAA Laboratories (Pasching, Austria) if not otherwise stated.

**Differentiation**

Differentiation capacity of human ASC expanded in different media was evaluated in passage 4 (P4) seeding the cells in 24-well-plates (Iwaki, Tokyo, Japan). For adipogenic differentiation, human ASC were seeded at a density of 7.0 × 10⁵ cells/cm² and induced by a protocol based on Pitot of the expansion of ASC and dermal fibroblasts. Both types of hPL were prepared from BC with the difference that for hPLₘ plasma was substituted by saline, whereas in case of hPLₘₘ the platelets were kept in plasma.

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**Differentiation**

Differentiation capacity of human ASC expanded in different media was evaluated in passage 4 (P4) seeding the cells in 24-well-plates (Iwaki, Tokyo, Japan). For adipogenic differentiation, human ASC were seeded at a density of 7.0 × 10⁵ cells/cm² and induced by a protocol based on Pitot et al. [14]. Cells were cultured in 3 repetitive cycles of 48–72 h in adipogenic differentiation medium (DMEM-high glucose, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 10 µg/ml insulin (Sigma, Vienna, Austria), 1 µmol/l dexamethasone (Sigma), 0.5 µmol/l 3-isobutyl-1-methylxanthine (Sigma), 100 µmol/l indomethacin (Sigma)) followed by 24 h in adipogenic medium (DMEM-high glucose, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 10 µg/ml insulin). Osteogenic differentiation was induced in osteogenic medium according to De Girolamo et al. [15] (DMEM-low glucose, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 150 µmol/l ascorbate-2-phosphate (Sigma), 10 nmol/l dexamethasone (Sigma), 10 nmol/l 1,25-dihydroxy-vitamin D₃ (Sigma), 10 nmol/l β-glycerophosphate (STEMCELL Technologies, Cologne, Germany)) at a seeding density of 10 × 10³ cells/cm². Additionally, adipogenic as well as osteogenic differentiations were induced using the same supplement as for expansion, substituting FBS by huS, hPLₘ or hPLₘₘ. Parallel cultures in expansion media or control medium (DMEM-low glucose, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin) served as negative controls. For media supplemented with hPLₘₘ, 4 U/ml heparin were added to avoid clotting. Differentiation media were applied for up to 3 weeks with two media changes per week. Adipogenic differentiation was detected by staining of lipid droplets with Oil Red O. Osteogenic differentiation was demonstrated by Alizarin Red S and von Kossa staining of mineral depositions [16]. After photo-documentation, Alizarin Red S was dissolved using an aqueous solution containing 20% methanol and 10% acetic acid [17]. Samples were measured at 450 nm and compared to controls in corresponding expansion medium.
Results

Production and Quality Control of huS and hPL

Each batch of hPLP, hPLN, and huS was tested negative for microbiological contamination using the BactAlert System. For hPLP and hPLN mean platelet counts were determined. Furthermore, residual WBC and RBC were determined by flow cytometry. In both cases low amounts of RBC and WBC per unit were obtained (table 1). In case of huS, platelets, RBC, and WBC were determined as well, resulting in cell numbers smaller than for hPLP and hPLN (table 1). For all three cell types significantly lower values were obtained compared to hPLP and hPLN (p < 0.001).

Concerning pH values, significant differences were determined between all three supplements (hPLP 7.29 ± 0.09, hPLN 6.68 ± 0.05, huS 7.53 ± 0.01). For all three supplements, total protein concentrations were significantly different (p < 0.001; table 1).

Table 1. Production of hPL and huS was controlled by cell counts of platelets as well as residual WBC and RBC; furthermore, total protein concentrations and pH values were determined.

<table>
<thead>
<tr>
<th>Tested parameter</th>
<th>hPLP</th>
<th>hPLN</th>
<th>huS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets, cells per unit</td>
<td>3.58 ± 0.13 × 10^10</td>
<td>3.34 ± 0.13 × 10^10</td>
<td>9.49 ± 1.90 × 10^8</td>
</tr>
<tr>
<td>RBC, cells per unit</td>
<td>8.79 ± 0.91 × 10^6</td>
<td>4.73 ± 0.38 × 10^6</td>
<td>7.98 ± 1.88 × 10^7</td>
</tr>
<tr>
<td>WBC, cells per unit</td>
<td>1.15 ± 0.30 × 10^7</td>
<td>8.85 ± 2.07 × 10^6</td>
<td>1.63 ± 1.00 × 10^7</td>
</tr>
<tr>
<td>pH values</td>
<td>7.29 ± 0.09</td>
<td>6.68 ± 0.05</td>
<td>7.53 ± 0.01</td>
</tr>
<tr>
<td>Total protein, g/dl</td>
<td>6.12 ± 0.06</td>
<td>0.77 ± 0.05</td>
<td>6.66 ± 0.04</td>
</tr>
</tbody>
</table>

*Differences in total protein and pH levels were significant between all three supplements (total protein: p < 0.001 for each case, pH: hPLN vs. huS p < 0.05, hPLP vs. huS and hPLP vs. hPLN p < 0.001).

Table 2. For product characterization, growth factor analysis was performed for PDGF-BB, basic FGF, TGF-β1, EGF and IGF-1.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>hPLP</th>
<th>hPLN</th>
<th>huS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-BB, ng/ml</td>
<td>18.11 ± 5.33</td>
<td>22.14 ± 3.52</td>
<td>2.38 ± 0.57</td>
</tr>
<tr>
<td>basic FGF, pg/ml</td>
<td>363.47 ± 23.55</td>
<td>173.67 ± 13.15</td>
<td>0</td>
</tr>
<tr>
<td>TGF-β1, ng/ml</td>
<td>32.74 ± 7.07</td>
<td>54.86 ± 5.39</td>
<td>4.89 ± 0.36</td>
</tr>
<tr>
<td>EGF, pg/ml</td>
<td>13.96 ± 0.05</td>
<td>14.07 ± 0.02</td>
<td>13.71 ± 0.01</td>
</tr>
<tr>
<td>IGF-1, ng/ml</td>
<td>33.14 ± 2.26</td>
<td>4.42 ± 0.60</td>
<td>85.98 ± 9.75</td>
</tr>
</tbody>
</table>

*aAll parameters were significant except PDGF-BB and EGF in case of hPLP vs. hPLN.

Activity of Alkaline Phosphatase and Determination of Calcium Content

The activity of intracellular alkaline phosphatase (AP) was determined at the end of differentiation by adding PBS and freezing the plate for at least half an hour. After permeabilizing the cells using 0.25% Triton-X100 (Sigma), p-nitrophenyl phosphate (p-NP; Sigma) was added. Samples and controls were measured at 405 nm and calculated using p-NP standard curve. For determination of the calcium content, cells were lysed after 3 weeks adding 250 μl of 0.5 mol/l hydrochloric acid (Roth, Karlsruhe, Germany) per well. This solution together with cell fragments were transferred into tubes and shaken at 488 rpm at room temperature for at least 3 h. After centrifugation, samples were evaluated with the Calcium CPC FS Kit (DiaSys Diagnostic Systems, Holzheim, Germany) according to manufacturer’s instructions.

Statistics

Results are presented as mean ± SEM and analyzed using ANOVA one-way analysis of variance followed by Tukey’s multiple comparison test or Dunn’s multiple comparison test (GraphPad PRISM, version 3.02). Differences were considered significant for p < 0.05. Six batches of each supplement were evaluated.
tion could be demonstrated for most cases, except for ASC being expanded in hPLP and afterwards differentiated in FBS and for those being cultured in FBS throughout culture. The most intensive staining was shown by using huS for expansion as well as for differentiation. Quantification of Alizarin Red S confirmed these data revealing a 44.4 ± 8.5-fold increase of induction in osteogenic differentiated ASC (fig. 3B).

ASC being expanded and differentiated in hPLP showed the highest activity of AP being able to produce 393.3 ± 128.7 μmol p-NP (fig. 3A). Quantifying the calcium content, again huS was the most potent supplement used for osteogenic differentiation producing 15.9 ± 0.8 mg/dl calcium (fig. 3C).

Using huS for expansion as well as differentiation was also demonstrated to be the best for adipogenic differentiation. ASC expanded with both types of hPL could be induced to produce lipid vacuoles, when using FBS for differentiation. However, when hPL was used for differentiation as well as for expansion, ASC could not be differentiated towards the adipogenic lineage (fig. 4).

Cell Culture
ASC and fibroblasts were expanded for three passages. Cell morphology showed the expected spindle-shaped structures. Although cells expanded in FBS attached more rapidly to the plastic surface of the flasks, the mean PDT was higher compared to the alternative supplements. In case of fibroblasts, hPLP followed by huS led to the shortest PDT (fig. 1).

ASC proliferated best in presence of huS while no significant difference was detectable between hPLP and hPLN (fig. 2). Expanding both cell types, hPLN resulted in the development of aggregates thus complicating the microscopic evaluations.

Osteogenic and Adipogenic Differentiation Capacity
Mineralization was shown by von Kossa (data not shown) as well as Alizarin Red S staining (fig. 3D). Mineral deposition could be demonstrated for most cases, except for ASC being expanded in hPLP and afterwards differentiated in FBS and for those being cultured in FBS throughout culture. The most intensive staining was shown by using huS for expansion as well as for differentiation. Quantification of Alizarin Red S confirmed these data revealing a 44.4 ± 8.5-fold increase of induction in osteogenic differentiated ASC (fig. 3B).

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hPLN, and huS were sterile and could be used in cell culture, compared to hPLP. Burnouf et al. [11] induced the release of higher yields in PDGF-BB, TGF-β1, and EGF. These growth factors are platelet-secreted factors, whereas IGF-1 and basic FGF are plasmatic factors. In hPLN, higher yields in PDGF-BB, TGF-β1, and EGF were obtained compared to hPLp. Burnouf et al. [11] induced the release of growth factors by solvent-detergent treatment, resulting in an increase in concentrations of PDGF-AB, TGF-β1, and EGF. We therefore conclude that the addition of saline improves the release of these three growth factors as well.

Discussion

In order to enable the evaluation of batch variations, six pools of each alternative cell culture supplement were prepared. Quality control was performed throughout processing according to criteria for hPL as previously defined [18]. Microbiological testing confirmed that all batches of hPLp, hPLN, and huS were sterile and could be used in cell culture, thus making it suitable for the expansion of cells intended for cell therapy or ATMP. Processing was performed in a closed system, and aliquots were prepared in a laminar air flow (GMP grade A). All supplements were produced according to GMP requirements, and hPL production is already certified by the competent authorities. Besides sterility, further criteria for hPL were high platelet as well as low RBC and WBC counts before induction of platelet lysis. Comparing hPLN to hPLp, less platelets were found in hPLN (table 1) but the difference was not significant (p > 0.05). RBC and WBC were significantly less in hPLN (p < 0.001 and p < 0.01 respectively). Addition of saline to the BC pool before the first centrifugation step resulted in an increased volume that might have improved the separation of platelets from RBC and WBC.

As expected, hPLN obtained far lower total protein concentrations due to the exchange of plasma by saline (table 1). Hence, it can be assumed that about a tenth of the total protein concentration is made up by factors released by platelets and causes the positive effects observed in cell culture. As expected, hPLN obtained far lower total protein concentrations due to the exchange of plasma by saline (table 1). Hence, it can be assumed that about a tenth of the total protein concentration is made up by factors released by platelets and causes the positive effects observed in cell culture.

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As a quality control of our expanded cells, we evaluated the differentiation capacity under standard conditions, i.e., at present applying protocols with FBS. Furthermore, we wanted to verify the differentiation potential of our supplements reflecting conditions more likely to the in vivo environment or if cells need to be applied pre-differentiated towards a specific lineage. Comparing huS- and FBS-supplemented media, Josh et al. [24] demonstrated that human ASC expanded with huS proliferated more rapidly while retaining their differentiation capacity. In our hands, adipogenic as well as osteogenic differentiation capacity could even be improved when using huS compared to standard FBS cultures. Investigating an extended panel of human alternatives to FBS, also platelet rich plasma was reported to preserve differentiation capacity throughout long-term culture of human ASC [25]. Concordantly, we could demonstrate adipogenic as well as osteogenic differentiation capacity of ASC independent of the medium additive. Furthermore, osteogenic differentiation was even improved by application of the same supplement for differentiation and for the expansion compared to the use of standard FBS induction medium. To our knowledge, this is the first announcement reporting expansion and differentiation of human ASC with hPL. Interestingly, when differentiating hPL-expanded human ASC towards the adipogenic lineage, we could only detect lipid vacuoles when FBS was present during induction, suggesting that some factors in hPL may inhibit adipogenic differentiation. This is not depending on the presence of serum as it was shown for hPL as well as for hPL-RC. FGF may contribute to this inhibition as it is not present in huS but in both hPL and has been reported to inhibit osteogenic as well as chondrogenic differentiation in mouse BM-MSCs [26]. Likewise, our results suggest that, depending on the cell type and the intended clinical application, the ideal substitute needs to be determined individually.

Conclusion

Cell therapy and ATMP production require the safe expansion of cells. Thus FBS should be replaced by human alternatives or defined supplements. We could show that production of huS and hPL according to GMP requirements is feasible resulting in highly potent alternatives to FBS.

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Disclosure Statement

The authors did not provide a conflict of interest statement.

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