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Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells

HATIM HEMEDA[1], BERND GIEBEL[2] & WOLFGANG WAGNER[1]

[1]*Helmholtz-Institute for Biomedical Engineering, RWTH Aachen University Medical School, Aachen, Germany, and* [2]*Institute for Transfusion Medicine, University Hospital Essen, Essen, Germany*

Abstract

Culture media for therapeutic cell preparations—such as mesenchymal stromal cells (MSCs)—usually comprise serum additives. Traditionally, fetal bovine serum is supplemented in basic research and in most clinical trials. Within the past years, many laboratories adapted their culture conditions to human platelet lysate (hPL), which further stimulates proliferation and expansion of MSCs. Particularly with regard to clinical application, human alternatives for fetal bovine serum are clearly to be preferred. hPL is generated from human platelet units by disruption of the platelet membrane, which is commonly performed by repeated freeze and thaw cycles. Such culture supplements are notoriously ill-defined, and many parameters contribute to batch-to-batch variation in hPL such as different amounts of plasma, a broad range of growth factors and donor-specific effects. The plasma components of hPL necessitate addition of anticoagulants such as heparins to prevent gelatinization of hPL medium, and their concentration must be standardized. Labels for description of hPL—such as "xenogen-free," "animal-free" and "serum free"—are not used consistently in the literature and may be misleading if not critically assessed. Further analysis of the precise composition of relevant growth factors, attachment factors, microRNAs and exosomes will pave the way for optimized and defined culture conditions. The use of hPL has several advantages and disadvantages: they must be taken into account because the choice of cell culture additive has major impact on cell preparations.

Key Words: *fetal bovine serum, fetal calf serum, serum, mesenchymal stromal cells, platelet lysate, platelet lysate gel*

Introduction

Overall, the composition of cell culture media still closely resembles formulas developed in the pioneering work of the 1950s: Harry Eagle described a basal medium (Eagle's minimal essential medium), which comprised of a mixture of 29 essential components including 13 amino acids, nine vitamins, D-glucose and six inorganic salts (1). In the early days of cell culture, this basal medium was supplemented with human or horse serum to support the *in vitro* growth of human carcinoma cells or murine fibroblasts. To date, fetal bovine serum (FBS; alternatively termed fetal calf serum [FCS]) is the most commonly used serum additive that is capable of supporting growth of a variety of cell types.

Over the past decades, mesenchymal stromal cells (MSCs) have received much attention for their potential role in regenerative medicine and cellular therapies (2,3). They can be easily culture-expanded, they harbor differentiation capacity toward mesodermal lineages and they reveal a variety of immunomodulatory features. The first clinical trial with the use of culture-expanded MSCs was performed in 1995 (4). In this study, MSCs were obtained from 23 patients who were then reinfused intravenously to demonstrate that these cells can be expanded *in vitro* and were then transplanted without toxicity (4). By 2013, the public clinical trials database (http://clinicaltrials.gov) registered 338 clinical trials that used MSCs for a wide range of therapeutic applications. MSCs can hardly be purified directly from tissue and therefore they are usually culture-expanded *in vitro* to attain sufficient cell numbers for clinical applications. Most of the protocols that have been reported in the literature use FBS-supplemented media to raise and expand human MSCs. However, since concerns have been raised regarding the safety of FBS-based culture media, protocols intended to raise cells for the clinical application should—according to Good Manufacturing Practice—avoid usage of animal sera.

Culture conditions exert major impact on cells cultured *in vitro*. Because of the wide range of

Correspondence: **Wolfgang Wagner,** MD, PhD, Helmholtz-Institute for Biomedical Engineering, Stem Cell Biology and Cellular Engineering, RWTH Aachen University Medical School, Pauwelsstrasse 20, 52074 Aachen, Germany. E-mail: wwagner@ukaachen.de

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therapeutic applications of MSCs and to their worldwide use, we aimed to discuss the relevance of serum substitutes for MSC cultures. Accordingly, this article summarises limitations and improvements of culture media with particular emphasis on human platelet lysate (hPL) (Table I).

Fully defined synthetic culture media: an unmet goal

Efforts have been made to replace serum supplements and to design more standardized and better-defined serum-free formulations. They must comprise all nutrients, amino acids, lipids hormones, vitamins, buffer substances and growth factors that are essential to maintain all physiological functions and to facilitate cellular proliferation. Over the past decade, various serum-free and animal-free media have been described (5–12). In fact, it has been suggested that specific serum-free culture media can be used for expansion of human MSCs (13). However, these media often failed to support the initial isolation and expansion steps: particularly on untreated culture flasks, the cell adhesion and initial outgrowth of fibroblastoid colony-forming units were largely impaired. Therefore, peptides and serum proteins have been used to coat the culture dishes upfront—at the expense of fully standardized and serum-free culture conditions. In general, precisely defined synthetic media provide a better controlled cell culture environment, but optimization of the concentration of individual compounds is a tedious and expensive task that is ongoing (14).

FBS: the gold standard for cell culture

To date, FBS is the most widely used serum supplement for *in vitro* culture of eukaryotic cells. It has been estimated that the annual worldwide production is approximately 500,000 L of raw FBS, which equates harvesting of more than one million bovine fetuses per year (15,16). Generally, FBS is produced from the blood drawn from a bovine fetus that is obtained from pregnant cows sent to slaughter. The fetus—usually at approximately 6 months of fetal development (17)—is separated at the abattoir, and the fetal blood is collected under aseptic conditions. This is usually performed by puncturing of the heart. The blood is chilled, allowed to clot and serum is then separated from the fibrin-clotted mass and red blood cells by centrifugation (Figure 1A). Thereby, FBS can be produced in relatively large quantities, and large batches of pretested serum can be generated and distributed on a commercial basis.

Because of its relatively easy production and rich content of growth factors, FBS became the "most universally applicable cell culture additive for the stimulation of cell proliferation and biological production" (9). Other advantages in the use of FBS in cell culture include the following (Table II): i) it is effective on most types of human and animal cells; ii)

Table I. Different types of culture media.

Type of culture media	Definition
Xeno-free media	All components are either synthetic or derived from the same species corresponding to the species of cellular origin and/or recipient of the transplant. For application with human cells, particularly in clinical therapy, "xeno-free" means that it comprises exclusively human components and chemically defined substances. Addition of recombinant proteins and/or recombinant growth factors is often considered as acceptable for this definition. Human serum or hPL are examples for xenogen-free supplements.
Serum-free media	Does not comprise any serum, either from animals or humans. Per definition, it may include recombinant growth factors and even animal components that are not serum-derived. In this context, hPL comprises plasma but not serum. However, definition of hPL to be "serum-free" might be misleading because plasma has a composition very similar to serum.
Animal-free media	Media are completely devoid of any animal substances. Animal-free media may contain recombinant proteins. Please note that coating of culture dishes is not necessarily animal-free. Often, coating is performed with serum or serum proteins; therefore culture conditions are not "animal-free." For hPL, the label "animal-free" may be misunderstood under the perception that humans are also mammals. However, many groups conventionally use this definition to discern culture condition with supplements derived from animals as opposed to humans.
Animal-free culture conditions	Culture conditions are completely devoid of animal substances, including potential surface coating. They may comprise recombinant proteins, such as growth factors, which are sometimes difficult to standardize and to define.
Fully defined synthetic culture media without growth factors	These media comprise only pure synthetic substances with known activities. They do not rely on recombinant growth factors, which may comprise traces of other proteins or vary in activity. Only such media can be completely standardized.

FBS is implemented in many existing culture protocols for MSCs (18,19); iii) FBS is rich in fetal growth factors and hormones that stimulate cellular proliferation and maintenance (20–23); and iv) it facilitates differentiation toward various lineages. For example, it is traditionally supplemented in differentiation media for osteogenic, adipogenic and chondrogenic lineage. The advantageous effect of FBS in comparison to adult bovine serum might rely on the enormous regenerative needs during fetal development.

On the other hand, FBS bears also a number of disadvantages (Table II): i) many compounds of FBS have not yet been identified, and, for many identified substances, the function of cultured cells is still unclear (24). ii) FBS batches reveal significant lot-to-lot variability; this makes pre-testing of each batch necessary (16). iii) The high endotoxin content of FBS also raises questions regarding the suitability and safety (25). iv) FBS may provoke immunological response against xenogenic serum antigens (19). It has been estimated that approximately 7–30 mg of bovine protein are transferred per 10^8 MSCs (26). Spees *et al.* (26) showed that FBS-expanded MSCs may evoke immune responses in patients even on first administration and

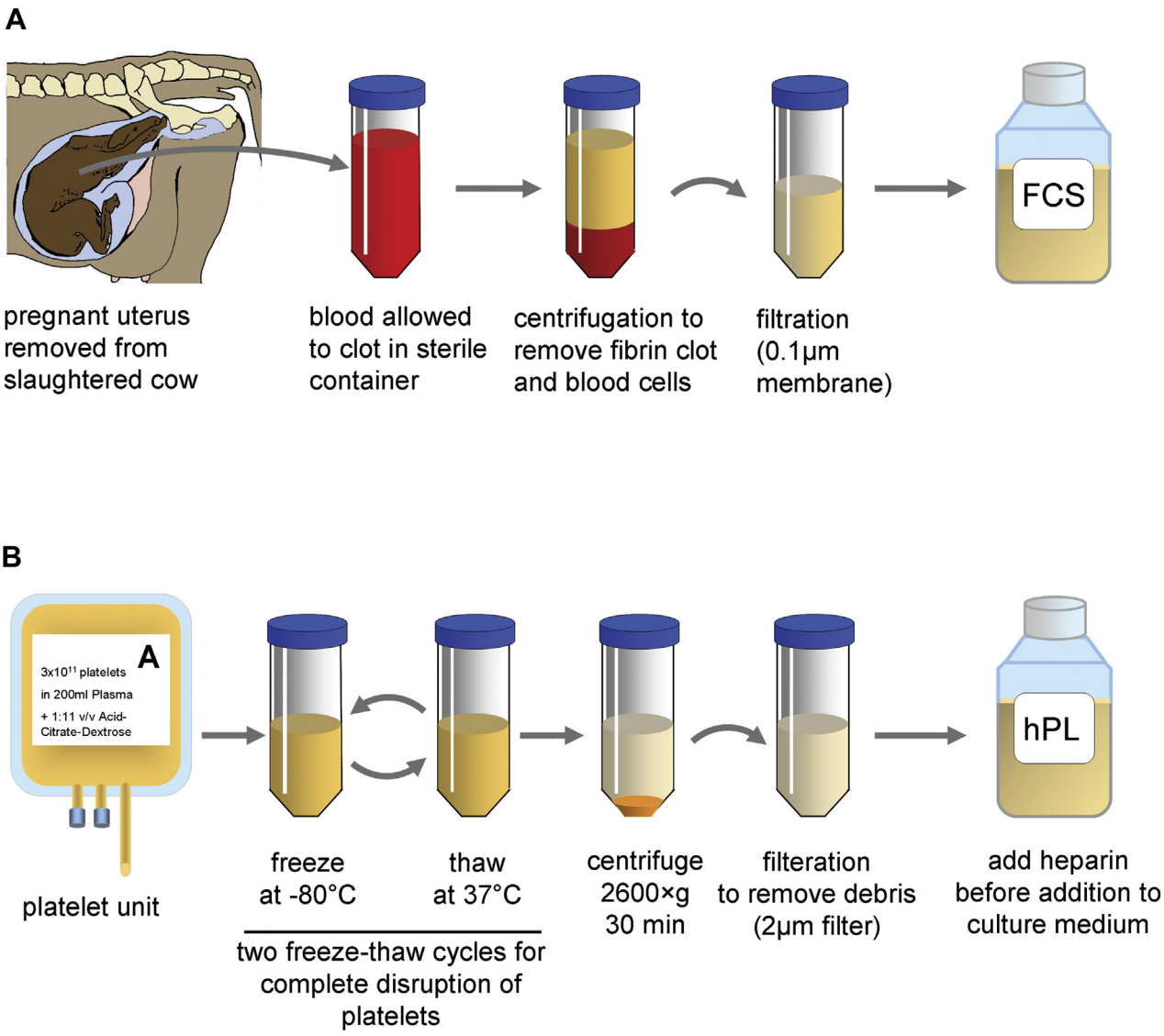

Figure 1. Generation of FBS and human platelet lysate. (A) FBS is produced from the blood of bovine fetuses during slaughter of pregnant cows. The blood is chilled, allowed to clot and serum is then separated from the fibrin-clotted mass and red blood cells by centrifugation and filtration (the calf scheme has been modified with kind permission from Dr William Dee Whittier, Production Management Medicine and Veterinary Extension, VA Tech, Blacksburg, VA, USA). (B) hPL is generated from common platelet units by means of a simple freeze-thaw procedure. Platelet units are aliquoted, twice frozen at −80°C, re-thawed at 37°C and centrifuged to remove cell fragments. The supernatant is then filtered and finally supplemented with heparin to avoid gel formation.

Table II. Advantages and disadvantages of FBS and hPL.

FBS	hPL
Advantages:	
Broadly applicable for many different cell types	Applicable for a wide range of different cell types. Thus far, it is particularly used for human MSCs, endothelial cells and fibroblasts.
Rich in growth factors	Enriched in growth factors of the platelet fraction (such as platelet-derived growth factor).
–	Proliferation of MSCs—particularly of MSCs derived from adipose tissue—is significantly faster than in FBS.
FBS is abundantly available (as a by-product during slaughters of pregnant cows)	hPL is easily generated by freeze-thaw procedures (waste product after expiration date of platelet units).
Commercially available (high lot-to-lot variation necessitates pretesting, facilitating more profit-yielding commercial advertisement).	
Clinical trials have been performed with relatively few side effects. However, human alternatives are preferable.	–
–	Has been used in clinical trials—no critical side effects were reported.
–	No risk of xenogeneic immune reactions or transmission of bovine pathogens.
–	Can be used in autologous settings to reduce risks of contamination or immune reactions.
Disadvantages:	
The ingredients are not precisely defined.	Not precisely defined; yet, platelet units might be more standardized than bovine fetal blood.
High lot-to-lot variation (even in pooled batches).	Variation exists between individual hPLs, which can be reduced by pooling.
–	Thus far, hPL is rarely distributed commercially.
Can evoke severe immunological reactions against xenogenic serum antigens.	Immunological reactions are possible in allogeneic settings.
High endotoxin content	–
Potential source of microbial contaminants, such as fungi, bacteria, viruses or prions.	Danger of transmission of human diseases by known or unknown viruses such as human immunodeficiency virus and human T-lymphotropic virus (quarantine storage cannot completely exclude this risk). Contamination with mycoplasma should be excluded.
Animal welfare concerns during the bleeding procedure of bovine fetuses.	–

particularly if repeated administrations were required. Several studies reported anaphylaxis and immune reactions in the patients transplanted with cells exposed to animal-derived products (27–29). v) FBS is also a potential source of microbial contaminants, such as fungi, bacteria, viruses or prions (30). It has been estimated that 20–50% of commercially available FBS is virally contaminated, particularly with bovine viral diarrhea virus (25). Even though bovine spongiform encephalopathy is currently not a common threat, the danger of xenogeneic infections—which may cross the species barrier—remains. Thus, expansion of MSCs in a clinical setting should avoid usage of any animal sera. Accordingly, if desired to be translated for a clinical setting, FBS-based protocols that have been successfully applied to raise MSCs for pre-clinical applications must be modified and re-evaluated, which might be very time-consuming.

Apart from safety and scientific concerns, the methods adopted for harvesting blood from the fetal calf have raised animal welfare concerns (16,31). As mentioned above, fetuses probably are exposed to pain and discomfort, which makes the current practice of fetal blood harvest questionable (31). Several measures should be taken to minimize nociception and suffering of the animals, such as ensuring that the calf is unconscious and desensitized during the cardiac puncture and blood collection, but these safeguards are not always warranted (17).

Despite these disadvantages, FBS usage is still tolerated—at least in phase I clinical trials (32)—but its use must be highly regulated in regenerative medicine (24). For therapeutic applications, FBS derived from countries without any incidence of bovine spongiform encephalopathy is preferred. Veterinary control of animal-derived products largely follows the regulations set by the European Union (DG SANCO; EU 142/2011) and the United States Department of Agriculture (USDA). Thus, it is possible to use FBS for culture expansion of therapeutic cell preparations; however, human alternatives are preferable (9,33,34).

Human alternatives for FBS

Over the past 15 years, various human alternatives have been tested for their ability to sustain proliferation and differentiation of cells in culture. The use of human serum (HS) may be the most straightforward "humanized" approach. Several studies demonstrated that the use of autologous HS for culture expansion of MSCs is feasible without compromising differentiation capacity or the MSC cell surface immunophenotype (35–37). However, it has also been reported that this method is not always reliable, particularly in studies with allogeneic HS: proliferation of MSCs was often rather low, and the cells hardly reached confluence (26,33). Platelet-rich plasma (PRP) has been shown to enhance proliferation of MSCs in culture (38). However, the debris in PRP may disturb cell culture, and not all growth factors are released without thrombocyte activation. Bieback *et al.* (34,39) demonstrated the use of thrombin-activated platelet-rich plasma (tPRP) for isolating and expanding human MSCs. They demonstrated that pooled human HS and tPRP provide a significantly higher proliferative effect on adipose tissue–derived MSCs than FBS: in early passages, HS and tPRP MSCs showed a 66-fold and 68-fold expansion, respectively, whereas expansion in FCS was only 24-fold. For generation of tPRP, platelets were activated by human thrombin (34). The fibrin clot can be removed subsequently by means of centrifugation (17). tPRP contains several growth factors derived from platelets that have been shown to enhance proliferation of MSCs (34,40–42). Furthermore, thrombin-cleaved osteopontin promotes cell attachment and spreading (43). It should be noted that several groups freeze their PRP before addition to culture medium, which then closely resembles human platelet lysate (hPL), and thus the nomenclature should be used accordingly.

Generation of hPL

The use of hPL for MSC expansion was first described by Doucet *et al.* (44) in 2005. Since then, hPL has been proven as an extremely effective cell culture additive that is concurrently used in many laboratories and clinical trials (45). hPL is prepared from PRP, either derived from pooled buffy coat–derived platelet concentrates of whole blood or from apheresis (46–48). In contrast to tPRP, which requires a more complicated manufacturing process, hPL can be generated from common platelet units by a simple freeze-thawing procedure (Figure 1B). This procedure is very simple, fast and effective. Variation between individual platelets can be reduced by pooling of platelet units. Within one single bag, usually a pool of five harvests from fresh blood are combined unless apheresis from single donors has been performed. hPL is often used at a concentration of 10%. A single platelet unit of approximately 250 mL would consequently facilitate generation of 2.5 L of culture medium. Protocols have been developed to generate large pools of hPL to balance the lot-to-lot variation (47,49,50). For example, pooling of more than 50 units counteracts variation between individual hPLs. Furthermore, it provides larger volumes for generation of homogeneous culture medium. Alternatively, it is possible to use autologous hPL to minimize the risk of immunological reactions or infections (48). hPL can be produced according to Good Manufacturing Practice procedures and permits scale-up production of MSCs for clinical applications (41,44,47,51–53). Furthermore, hPL can be used for isolation of a broad range of different cell types such as human endothelial cells (54,55), human fibroblasts (56) and MSCs from various tissues (44,47,53,57).

Pros and cons of hPL

Human platelet lysate has several advantages and disadvantages in comparison to FBS (Table II): i) many studies indicated that hPL supports culture expansion of MSCs even better than FBS, HS, PRP or tPRP (33,41,48,53). Particularly, proliferation of adipose tissue–derived MSCs is largely increased with hPL (58–60). This growth advantage was less pronounced with the use of bone marrow–derived MSCs, which indicates that these cell preparations may differ in their nutritional requirements. ii) Platelet units can be used after the maximal shelf life time of 5 days. Thereafter, because of the increased risk of platelet aggregation and particularly because of the increased risk of bacterial contamination, they should not be considered for platelet transfusion; however, the waste product is still suitable for generation of hPL (61). iii) hPL has been used for therapeutic applications, and no severe side effects were observed (62,63). iv) MSCs expanded in the presence of platelet-released growth factors retain their immunosuppressive properties (44). v) Because hPL is derived from humans, neither bovine viruses nor immune reactions against bovine proteins are a concern (44,64). vi) hPL can be derived from the patient's blood without immunological problems or risk of infection with human diseases.

Despite the above-mentioned advantages, hPL also has some limitations (Table II): i) hPL—just as FBS—is not precisely defined. Several factors contribute to variation in hPLs derived from individual platelet units (Figure 2) (48,58). ii) To date, hPL is rarely distributed commercially. The ease of

hPL generation and the high profit production of FBS make it less attractive for companies to commercialize platelet lysate. Furthermore, the use of human-derived products raises ethical constrains that must be considered. iii) Immunological reactions are less likely with the use of hPL than with FBS, but they may still occur, particularly in an allogeneic setting. iv) hPL may transmit human diseases such human immunodeficiency virus, hepatitis B and C, syphilis, human T-lymphotropic virus and cytomegalovirus. All blood products are routinely tested for these diseases, but this does not exclude the risk of infections. To further minimize the risk, it has been recommended to quarantine stored hPL and reanalyze the donor after 3 months for potential serum conversions (46,65). However, such testing and storage is hardly feasible in daily research routine. Furthermore, hPL might harbor mycoplasma contamination, which is not routinely tested but must be excluded for cell culture (authors' observation).

What are the relevant ingredients of hPL?

Activated platelets are known to deliver a broad spectrum of cytokines involved in tissue repair (66,67) such as epidermal growth factor, basic fibroblast growth factor, insulin-like growth factor 1, platelet-derived growth factor, platelet factor 4, transforming growth factor-β and vascular endothelial growth factor (44,48,58,68,69). Such growth factors have been shown to enhance the MSC proliferation rate and to maintain their multilineage differentiation potential under *in vitro* conditions in the absence of FBS and exogenous growth factors (46,70–73). In addition, the impact of hPL on MSC osteoblastic differentiation is supported by numerous growth factors, which include bone morphogenic proteins 2, 4 and 6, interleukin 1, osteonectin, platelet-derived endothelial growth factor, platelet factor 4, transforming growth factor-β, insulin-like growth factor 1, basic fibroblast growth factor and platelet-derived growth factor, some of which are known to mediate osteoinductive effects (74–76). Without question, growth factors are essential, but they may not be the only relevant compounds in hPL which attribute to growth stimulation.

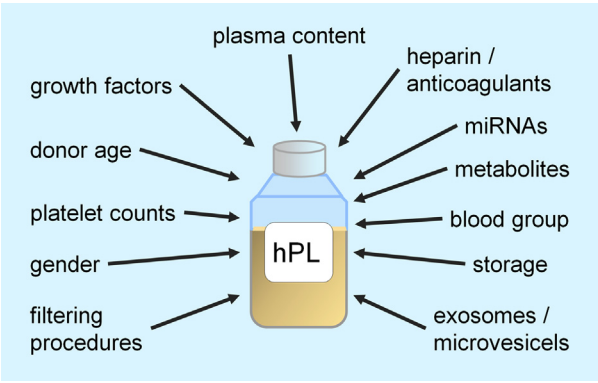

Figure 2. Relevant parameters for the activity of human platelet lysates in MSC cultures.

New research perspectives: microRNAs and microvesicles in hPL

MicroRNAs (miRNAs) are small non-coding RNA molecules that modulate protein expression by degrading messenger RNA or repressing messenger RNA translation (77). They have been shown to modulate MSC differentiation (78) and may contribute to regulation of the bone marrow niche (79). Thus far, miRNA content has rarely been addressed in hPL, but it may be relevant for MSC cultures. Apparently, miRNAs are trafficed at least by two types of extracellular vesicles (EVs), exosomes and microvesicles (MVs), which are formed during platelet activation or secreted into the plasma by other cell types (80). Exosomes are rather small vesicles (70–120 nm) of endosomal origin that correspond to the intraluminal vesicles of multivesicular bodies (MVBs), which, on fusion of MVBs with the plasma membrane, are released as exosomes into the environment (81). Carrying a variety of different molecules, including miRNAs, exosomes appear as extracellular organelles involved in intercellular communication processes (82,83). In contrast, MVs are larger EVs—ranging from 100–1000 nm—which are shed from the plasma membrane. Even though exosomes and MVs can hardly be discriminated experimentally, MVs are believed to contain a different molecular composition than that of exosomes. However, without consideration of whether they are predominantly exosomes or MVs, platelet-derived EVs have been shown to participate in agglutination. EV fractions are enriched in coagulation factors and have been reported to contain a 50–100-fold higher procoagulant activity than do activated platelets (84). Depending on the platelet unit and processing, different amounts of EVs appear to be released in stored platelet concentrates (85). Further research will be required to better understand the role of miRNAs, exosomes and MVs for MSC growth, which may explain the above-mentioned limitations of synthetic culture conditions.

Cellular aging of MSCs and hPL

MSCs undergo replicative senescence during culture expansion (86,87), and their long-term growth

curves are affected by serum supplements: in particular, adipose tissue–derived MSCs reach a higher number of cumulative population doublings with hPL in comparison to FBS. However, because of the faster proliferation rate, they enter the senescent state after fewer days in the presence of hPL than in the presence of FBS (59). It has been speculated that the enormously enhanced growth rates in hPL might favor chromosomal aberrations, but this effect has not been proven thus far (88). Long-term culture of MSCs in media supplemented either with FBS or pooled hPL induce similar gene expression (89) and DNA methylation changes (90) in expanded cells. Interestingly, the stimulatory effect of hPL was found to be dependent on the donor age of the platelet unit: we have demonstrated that hPLs generated from platelet units derived from younger donors stimulate proliferation and enhance osteogenic differentiation of MSCs more than do hPLs derived from elderly donors (58). The nature of these age-associated factors is unclear. Yet, the same effect may also contribute to the above-mentioned predominance of FBS in comparison to serum from adult cows (91).

Heparin concentration is critical

In contrast to FBS, hPL comprises plasma with fibrinogen and all other clotting factors. The anticoagulant effect of citrate is reduced on addition to calcium-containing culture medium. Therefore, further additives, such as heparin, must be added to prevent gelatinization. Heparins are highly sulfated glycosaminoglycans that activate anti-thrombin III, an inhibitor of several enzymes of the coagulation cascade. They are subdivided into unfractionated heparins (3000–30,000 Da) and low-molecular-weight heparins (2000–12,000 Da) featuring different clinical effects, pharmacokinetics and pharmacodynamics (92). We have recently demonstrated that unfractionated heparins and low-molecular-weight heparins can be used as additive in hPL media. However, their concentration was found to be critical (93): expansion and differentiation of MSCs was significantly impaired with the use of heparins at high concentrations (93). This is of particular relevance for cellular therapies that require optimized and standardized cell culture procedures.

hPL can provide culture medium and biomaterial

Traditionally, cell culture of adherent cells is performed on tissue culture plastic. On these culture dishes—which usually resemble biofunctionalized polystyrene surfaces—growth of MSCs is restricted to two dimensions and occurs preferentially at the rim of colonies as the result of contact inhibition (59). There is a growing perception that cell cultures can be improved by suitable three-dimensional scaffolds. Hydrogels and fibrous scaffolds facilitate cell growth without direct contract with rigid biomaterials (94,95). In particular, fibrin gels raise high hopes for therapeutic application because they are naturally occurring, biocompatible and biodegradable. Furthermore, they have been shown to support proliferation of various cell types (96,97). Without adding anticoagulants, hPL-supplemented media form soft and translucent gels within 1 hour. These hPL gels provide a suitable matrix to largely increase culture expansion of MSCs (60). In addition, the very soft and viscous mechanical consistency of hPL gel allows a simple passaging procedure without the need of separating cells from their matrix or additional washing steps (Figure 3). Expansion rates on passaging with the use of this method were similar as compared with the conventional method on tissue culture plastic with trypsin. In our previous work, cell recovery rates after passaging with the use of the pipetting method were very high, and we did not observe obvious impact on cellular activity and viability; however, this aspect should be further analyzed and optimized in future studies. Cell culture with hPL gel provides various new perspectives: it supports formation of MSC colonies and increases their proliferation rate. Furthermore, higher cell densities can be obtained and passaging procedures for culture expansion of MSCs can be performed non-enzymatically (60). It is even possible to directly apply cells embedded in hPL gel to further optimize cellular integration in a three-dimensional context; yet, proof of concept must be demonstrated in a clinical setting.

Conclusions

The right choice of serum or serum replacement is crucial for cell culture. Each type of culture supplement has advantages and disadvantages, and they convey severe effects on function and composition of cell preparations. hPL-cultured cells have been used in the clinic; for example, they have been used for treatment of graft-versus-host disease (NCT01764100) and for lumbar intervertebral degenerative disc disease (NCT01513694). Thus far, no critical side effects have been reported on treatment with such cell preparations. However, the relevant molecular factors are not fully unraveled. Batch-to-batch variation can be minimized by generation of large pools of approximately 40–50 donations per batch (33,47,98); yet, this also increases the risk of infection with human viruses. Ongoing

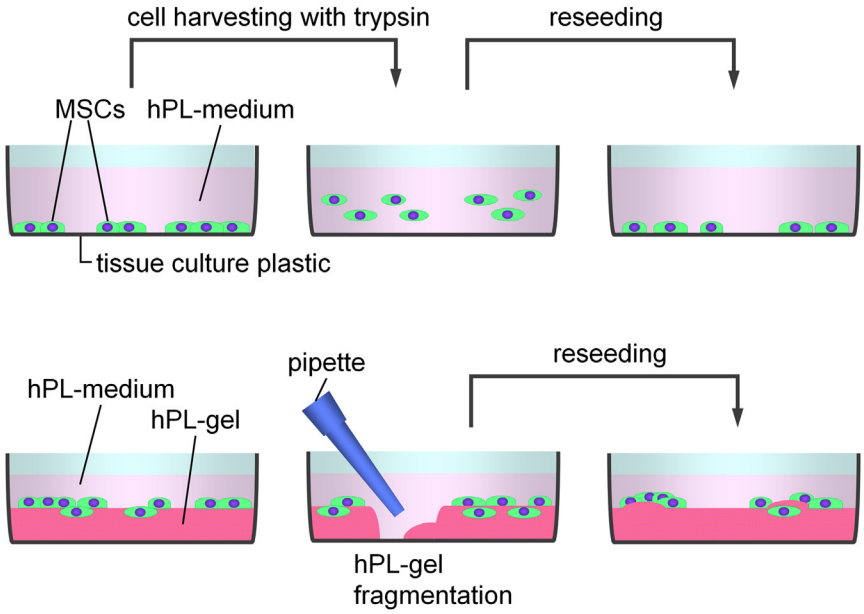

Figure 3. Culture expansion and passaging of MSCs in hPL gel. Scheme of culture expansion on tissue culture plastic (TCP) or gelatinized medium-hPL mixture (hPL-gel): MSCs on TCP are conventionally passaged after reaching semi-confluence with trypsin-ethylenediaminetetraacetic acid. On hPL gel, the cells reveal less contact inhibition and can be harvested together with the gel by pipetting without separation of MSCs from their matrix (60).

research is required to understand the interplay of growth factors, metabolites and possibly other constituents such as miRNAs or EVs. This will open new perspectives that seem to be required for further development of fully defined synthetic culture media.

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