Human Platelet Lysates Successfully Replace Fetal Bovine Serum in Adipose-Derived Adult Stem Cell Culture

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Abstract: Fetal bovine serum (FBS) is still the gold standard as a cell culture medium additive due to its high level of growth stimulatory factors. Although supplementation of growth media with FBS is common practice in cell and tissue culture, FBS bears a number of disadvantages and its use has been questioned recently: (1) an ill-defined medium supplement, (2) qualitative and quantitative batch-to-batch variations, and (3) animal welfare concerns regarding the harvest of bovine fetal blood.

Recently, we were able to show the capacity of human platelet α-granule lysates to replace FBS in a variety of human and animal cell culture systems. Thus, lysates of human donor platelets may become a valuable non animal-derived substitute for FBS in cultures of mammalian cells and in human and animal stem cell technology.

Stem cells may become the future for human-based alternative to animal testing, *in vitro* toxicology, and drug safety assessment. New stem cell-based test systems are continuously established, and their performance under animal-derived component free culture conditions has to be defined in prevalidation and validation studies. In order to accomplish these tasks, adipose-derived mesenchymal stem cells (ADSC) were expanded in media supplemented with platelet lysates. Proliferation assays by resazurin and WST-8 compared with direct cell counting confirmed the growth promoting effect of platelet lysate, comparable to high FBS. Furthermore, we established culture conditions that ADSC kept their undifferentiated state as determined by CD73, CD90 and CD105 expression and the lack of negative marker CD45. Preliminary tests whether ADSC can be differentiated towards adipogenic, osteogenic, or chondrogenic phenotypes under platelet lysate supplemented growth conditions were also successful.

Keywords: Fetal bovine serum, platelet lysates, non-animal alternatives, human adult stem cells, stem cell-based approaches.

INTRODUCTION

The supplementation of basal culture media with fetal bovine serum (FBS) is common practice in mammalian cell and tissue culture. FBS provides hormones, growth factors and cytokines, attachment and spreading factors, fatty acids and lipids, vitamins, and trace elements [1-3]. However, the use of FBS has recently been questioned for a number of reasons [4]. Therefore, a major challenge in cell and tissue culture today, aside from the thread of cross-contamination of human cell lines [5, 6] and the contamination of cultures by mycoplasma [7-9], is the search for alternatives to the use of FBS in cell culture media. The arguments for a replacement of FBS are threefold: (1) FBS is an ill-defined supplement with high batch-to-batch variations added to a fully defined basal medium, (2) concerns on animal welfare, when blood is drawn from unborn bovine fetuses, and (3) the dependence from FBS availability at the global market [3, 4, 10, 11].

In addition, there exist additional barriers for the use of FBS in tissue engineering and in future stem cell therapy in regenerative medicine [12-14] as well as in stem cell-based approaches for *in vitro* toxicology and drug testing and safety assessment [15-21]. Aside from a theoretical health risk of using xenogenic serum, FBS is a source for of the non-human sialic acid N-glycolylneuraminic acid (Neu5Gc), which is internalized into the cultured cell membrane, stimulating the immunogenicity of the cells [22]. Thus, xenogenic culture methodology imperils any transplantation success, since an immune response would kill the cells *in vivo*, and any future therapeutic treatment is not feasible [23, 24]. Thus, for any development of stem cell therapies, definition of safe culture conditions is essential. These include animal-derived component-free or chemically defined culture conditions. In this context, considerable efforts are undertaken to grow human embryonic and adult stem cells under strict xeno-free culture conditions to eliminate or reduce the risk of adverse side effects due to FBS constituents [25-32].

It is well recognized, that serum and not plasma supports growth and proliferation of cells in culture [33], which is due to the presence of mitogenic factors in the plasma fraction [34]. During the clotting process *in vitro* - as seen in wound healing *in vivo* [35-37] - a broad spectrum of growth factors and other active molecules are released from the α-granules of activated thrombocytes [38-40].

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A number of recent studies evidenced that human mesenchymal stem cells can be cultivated without FBS in media supplemented with fresh frozen plasma and platelets, platelet lysates, platelet-rich plasma or platelet-derived growth factors (reviewed in [41]). The cells exhibited proliferation and migration capacity, clonogenic efficiency, and the capacity to differentiate towards the adipogenic, osteogenic, or chondrogenic lineage [42-53].

Human adipose tissue, obtained by liposuction, is a promising source for adult mesenchymal stem cells, called adipose-derived stem cells (ADSC) [54-61]. The isolated cells exhibit typical characteristics of mesenchymal stem cells, such as adherence to plastic culture vessels, the ability to form colonies, and the potential to differentiate into specific cell lineages. We recently successfully introduced cell-free human platelet lysates (PL) to grow and maintain anchorage-dependent and -independent human and animal cell lines [38]. In the present work, these studies were extended to human adult mesenchymal stem cells, ADSC.

The present paper differs considerably from recently published work on this topic [42-45, 47, 48, 52, 62-64], and even goes beyond. (1) The mode of preparation of PL from thrombapheresis donor bags is a cell free extract after ultrafiltration, free of donor serum, each lot thoroughly characterized by growth factor ELISA and determination of protein content [38]. (2) ADSC were cultured on different substrata, comparing negatively and positively charged culture surfaces with collagen-coated culture dishes. (3) The quantification of growth and proliferation of ADSC was performed with WST-8 and resazurin assays, respectively, and was compared with classical growth curves obtained by direct counting of monolayer cell density. (4) The proof of CD marker expression, indicating the mesenchymal undifferentiated state of ADSC was performed in accordance with routine testing protocols [65].

MATERIALS AND METHODS

Platelet Lysate Preparation

The preparation of human platelet lysates (PL) were described elsewhere [38]. The source material were expired human donor thrombocytes, that were immediately used at the end of the shelf life (i.e. 5 days after donation). In brief, human thrombocyte concentrates, obtained by apheresis at the blood bank of the Innsbruck Medical University Hospital, were transferred under sterile conditions into 250 ml centrifugation cups and centrifuged at 6,000 $\times$ g for 20 min in order to remove platelet additive solution (PAS) and donor serum [38]. The supernatant was aspirated and the platelets were washed with 0.9% NaCl. Platelets were resuspended in 15 ml 0.9% NaCl with a final cell count of ~1.5 $\times$ 10^{10} platelets/ml. The suspension was stored at –20° C before lysate preparation by three freeze/thawing cycles. Aliquots of the platelet lysate were again stored at –20° C for use within 4 weeks. Before addition to serum-free culture media, aliquots were thawed and spun at 8,000 $\times$ g for 10 min and supernatants were taken.

Cell Culture

Adipose-derived human stem cells (ADSC), isolated from liposuction material [59], were purchased from Lonza Walkersville, Inc. (Cat. No. PT-5006) (www.lonza.com).

Cultures were incubated at 37°C in a 5% CO_2 and 95% air atmosphere. Routinely, cultures were fed three times a week. Cell culture media were a 1:1 (v/v) mixture of DMEM (Dulbecco`s Modified Eagle`s Medium) base (Cat. No. D5030, Sigma-Aldrich), with 5.5 mM D-glucose, 2 mM L-glutamine, 26.2 mM $NaHCO_3$, and HAM´s F-12 nutrient mixture (Cat. No. N6760, Sigma-Aldrich), supplemented with 50 µg/ml gentamicin (Cat. No. 15750-037, GIBCO), 2.5 µg/ml amphotericin B (Cat. No. A2411, Sigma-Aldrich) and 10% FBS (Biochrom/Berlin, Germany) or 5% PL. Monolayers showing 80% of confluency were subcultured using 0.25% TrypLE™ (GIBCO) and 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free buffered saline. TrypLE™ is a novel recombinant trypsin preparation for serum-free cell culture that does not require inactivation by serum and/or protease inhibitors. Tissue culture plasticware and culture dishes were from Greiner (Greiner Bio-One, Kremsmünster, Austria), Sarstedt (Sarstedt, Inc. Newton, NC) and Becton Dickinson (Becton Dickinson Labware, Two Oak Park, Bedford, MA).

Cell Counting

Light microscopy was used to estimate the number of cells per area (cell density). Cell number was determined by counting the cell nuclei within ten test squares of 0.04 mm^2 on photomicrographs taken randomly over the respective petri dish, considering the “forbidden line rule” [2, 66].

Proliferation Assays

Two test systems were used, a tetrazolium-based assay (WST-8), and the resazurin assay. Both assays measure the activity of dehydrogenases in cultured cells as an indirect parameter of cell viability and proliferation, respectively [67-69]. Assays were performed with cells grown in 24-well plates. The WST-8 assay is a water-soluble tetrazolium-based test system. WST-8 solution (Sigma-Aldrich, Cat. No. 96992) was added at a dilution of 1:10 to cell culture media in 24 wells and incubated for 2 hours at 37°C. WST-8 is reduced by dehydrogenases within living cells to give a yellow-colored product (formazan), which is determined at 450 nm. Formazan formation correlates with the number of metabolically active cells in the culture [2].

The resazurin assay (Alamar Blue, Sigma-Aldrich, Cat. No. R7017) is another method used for the measurement of metabolic activity of living cells. Culture medium of 24-well plate cultures was removed and fresh medium was added containing alamar blue in a final concentration of 44 µM. The bioreduction of the dye reduces the amount of the oxidized form (resazurin, blue) and concomitantly increases the fluorescent intermediate (resorufin, red). Resazurin reduced to resorufin was measured at 540 nm excitation and 590 nm emission. Resazurin reduction is directly proportional to the number of viable cells [70].

Immunofluorescence Staining of State of Differentiation

Cells were seeded on glass cell culture slides from Becton Dickinson (Becton Dickinson Labware, Two Oak Park, Bedford, MA). After seven days of incubation with 10% FBS, 5% PL or serum-free medium, cells were fixed in 100% ice-cold methanol for 30 min at –20° C. Fixed cells were incubated at room temperature in blocking buffer (1% (w/v) BSA in PBS). Subsequently cells were washed with phosphate-buffered saline (PBS) and incubated with primary antibodies for one hour at room temperature. The primary antibodies (mouse anti-human CD45, No. 640265, BD Transduction Laboratories™, mouse anti-human CD105, No. 611314, BD Transduction Laboratories™, mouse anti-human CD73, No. bs-0372R, Bioss Inc., and mouse anti-human CD90, No. bs-0778R, Bioss Inc.) were diluted to a final concentration of 10 µg/ml in PBS with 0.1% BSA (w/v). Thereafter, the cells were washed with PBS and incubated for 30 min with 10 µg/ml of an Alexa488-conjugated anti-mouse secondary antibody (Invitrogen, A11059). Culture slides were mounted in 3 mg/ml p-phenylene-diamine glycerol on a microscope slide.

RESULTS

Influence of Different Surfaces on the Growth of Adipose-Derived Stem Cells

In order to determine the most supportive culture substratum in terms of surface charge and coating with extracellular matrix material, respectively, adipose-derived stem cells (ADSC) were grown on negatively charged polystyrol (Becton-Dickinson), on positively charged dishes (Cell$^+$, Sarstedt), on poly-D-lysine-treated dishes, which resulted in a positive surface charge, and on culture dishes coated with collagen type I (BD BioCoat™, Becton-Dickinson). Poly-D-lysine-treated dishes are equivalent to Cell$^+$ dishes in terms of a positive surface charge. ADSC were seeded into DMEM/Ham F-12 medium, supplemented with 10% FBS, 24 hours before switching to the respective culture media (10% FBS, 5% platelet lysates, PL or serum-free, SF). In this series of experiments, PL treated cells show the highest proliferation rates and no significant differences between the culture surfaces tested, including Cell$^+$ dishes (data not shown), could be detected (Figure **1**).

Comparison of FBS Batches with Platelet Lysates on Growth of Adipose-Derived Stem Cells

In the growth and proliferation experiments summarized in Figure **1** it was noted, that growth curves obtained with cultures in FBS-supplemented DMEM/Ham F-12 media differed only slightly ($p<0.1$) from growth curves of serum-free cultures, although growth and proliferation under FBS was apparent at daily microscopical inspection. However, the results obtained with PL could never be achieved. Therefore, FBS batches of different vendors were tested. The results of a representative series of experiments are shown in Figure **2**. As can be seen, batches of FBS differed in their growth promoting capacity, determined by WST-8 assays, however, PL were still superior in all experiments tested.

Growth Promoting Effect of Platelet Lysates on Mesenchymal Stem Cells

ADSC were grown in DMEM/Ham F-12 media, containing either 10% FBS or 5% PL. In addition, a 1% FBS/5% PL combination was tested for additive effects. Serum-free culture conditions (SF, no additions) served

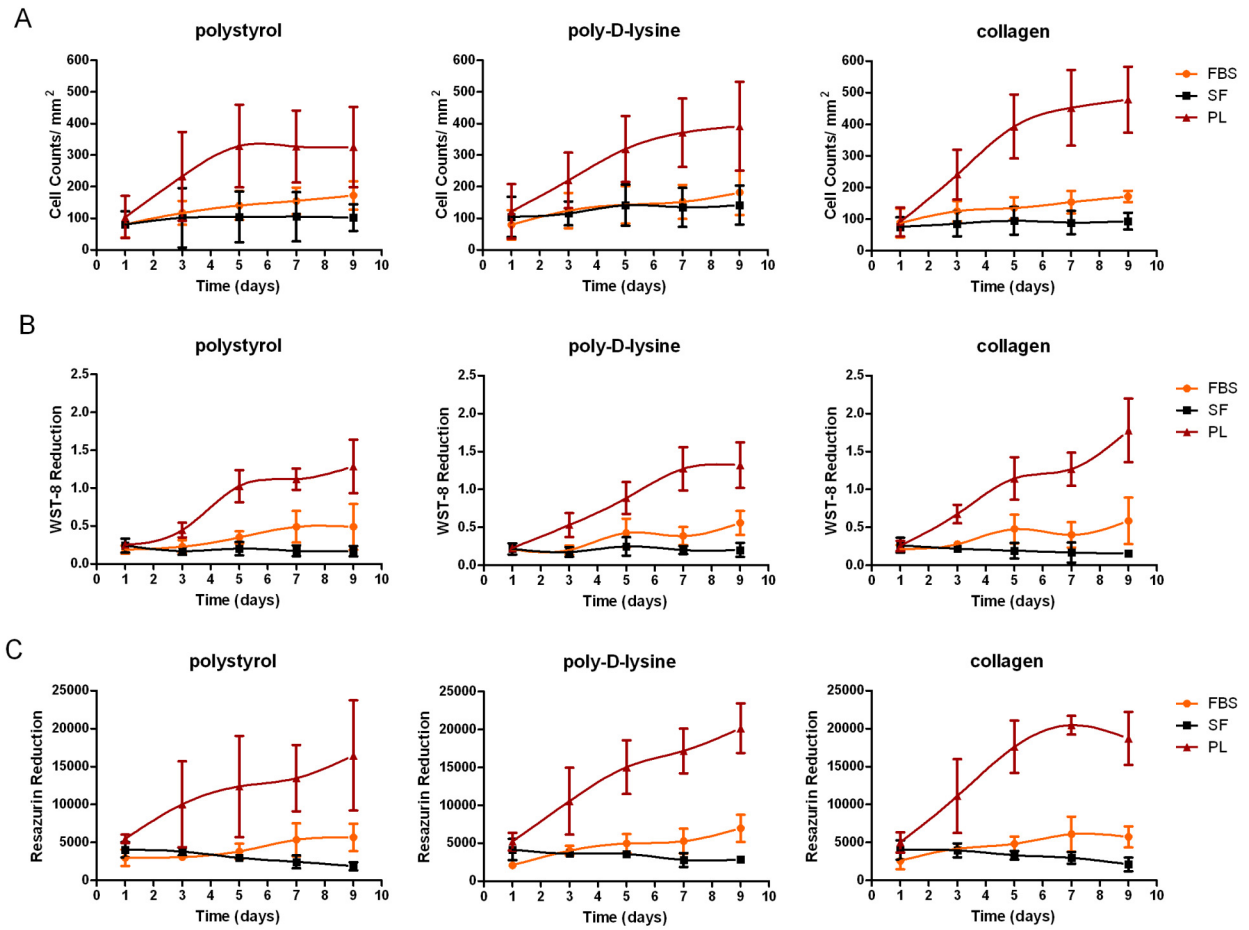

Figure 1: Cells were grown on polystyrol, on poly-D-lysine coated and on collagen type I-coated dishes. Quantification of cell growth experiments was performed by *in situ*-cell counting (**A**), and by WST-8 (**B**) and resazurin assays (**C**) [2]. Cell counts were determined from 10 randomly taken photomicrographs (means ± SD). WST-8 and resazurin values are expressed as means ± SD of 3 - 6 independent series of experiments. The growth curves with FBS are statistically significant from serum-free curves with p-values <0.1 in unpaired Student's *t*-test. Growth curves with PL differ from FBS with p<0.05.

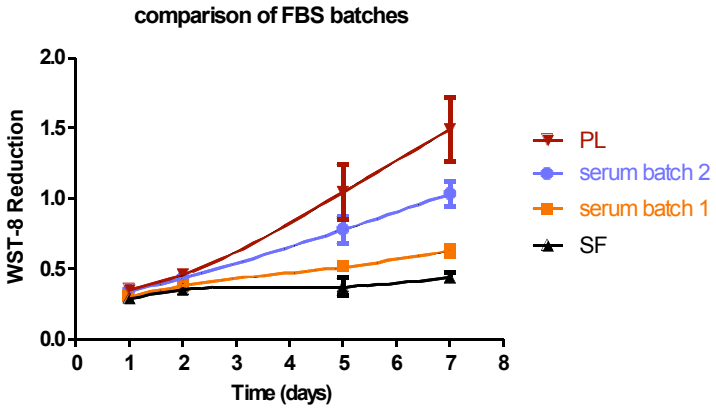

Figure 2: Comparison of FBS batches on growth and proliferation of ADSC. Cells were grown on polystyrol dishes in DMEM/Ham F-12 supplemented with either 10% FBS from different vendors (batch 1, Biochrom; batch 2, GIBCO), or 5% PL. SF, serum-free media without any supplementation. WST-8 read-outs are given as means ± SD of four independent series of experiments.

as controls. Cells were seeded onto polystyrol either with FBS containing, or serum-free media 24 h before starting the experiment. As depicted in Figure **3**, cells seeded in FBS-containing medium before switching to experimental media exhibit a shorter lag-phase, which can be explained by an improved cell attachment due

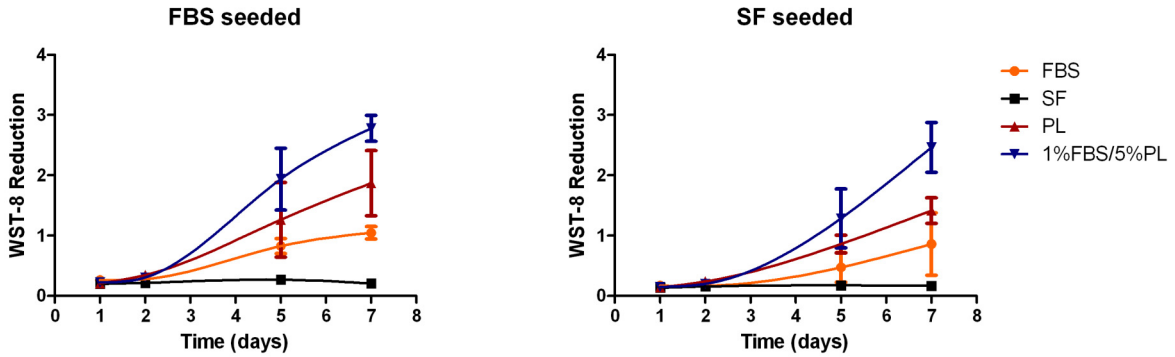

Figure 3: ADSC were grown on polystyrol and seeded either with FBS (left) or serum-free (SF) media (right) 24 hours before switching to experimental media (10% FBS, 5% PL, SF, or 1% FBS/5% PL). Cells seeded with FBS show a shorter lag-phase due to increased attachment and higher proliferation rates than cells seeded with SF media, which is retained in all experimental conditions. WST-8 data are given as means ± SD of four independent experiments.

to serum factors. Cells cultivated with 1% FBS/5% PL exhibited the highest proliferation rates, whereas serum-free cultures did not show any proliferation.

These results were further confirmed by resazurin assays at fixed time points (24 - 96 h) after addition of PL-supplemented medium (Figure **4**). Again, proliferation of ADSC – in terms of resazurin reduction – significantly increased in PL-medium compared to FBS controls. ADSC in serum-free, unsupplemented medium showed almost no dehydrogenase activity and stasis, respectively.

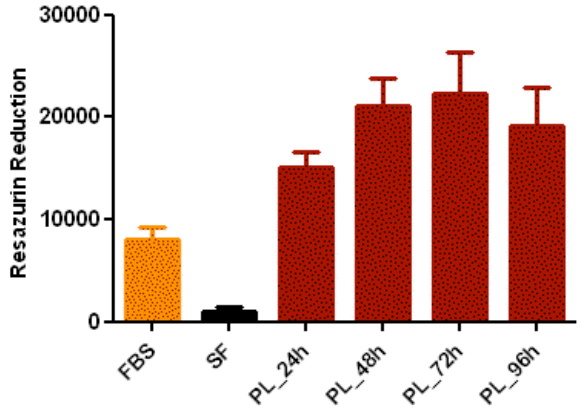

Figure 4: Resazurin assays of ADSC cultures after addition of PL-supplemented medium. ADSC were seeded in FBS-containing medium (FBS) and after 24 h, cultures were either continued in FBS or were switched to PL-supplemented medium (PL) or to serum-free unsupplemented medium (SF). Cultures were maintained for 96 h with a medium change at 48 h. At the time points indicated, parallel cultures were assayed for resazurin reduction. FBS and SF cultures were measured after 96 h. Data are means ± SD (n = 3).

Effect of FBS-, and PL-Coated Surfaces on Cell Growth

To examine if cell adherence and hence cell growth can be improved by using pre-coated tissue culture material, dishes were coated with FBS or PL before seeding. Best results could be achieved with the combination of FBS coating and FBS containing media and PL coating with either FBS or PL containing media (Figure **5**). Furthermore, cells grown on uncoated surfaces and in PL-supplemented media also showed high proliferation rates.

Cells Kept their Undifferentiated State During Treatment with Platelet Lysates

ADSC were grown in FBS containing, non-supplemented and PL-supplemented DMEM/Ham-F12 media. After seven days of treatment the cells were examined upon their expression of the mesenchymal positive stem cell surface markers CD73 (ecto-5'-nucleotidase), CD90 (Thy-1) and CD105 (endoglin, SH2) and the absence of the hematopoietic marker CD45. As can be seen in Figure **6**, both, FBS- and PL-grown cells show a strong expression of CD73, CD90 and CD105. Serum-free grown cells also express the three positive markers, but there are only very few cells left. ADSC do not show any CD45 expression at the three culture conditions. Only the strong autofluorescence of nuclear DNA is visible.

DISCUSSION

Adult stem cells reside in so-called stem cell niches in almost all tissues [71-73]. Their role is believed to compensate for tissue loss by generating new cells. In order to fulfill this regenerative capacity throughout life, adult stem cells on the one hand must retain the tissue stem cell pool, and on the other hand, must be able to differentiate into tissue-specific somatic cells. A specific feature of stem cells is their asymmetric cell division. First, when stem cells divide, daughter cells can remain in undifferentiated state to replenish the stem cell pool,

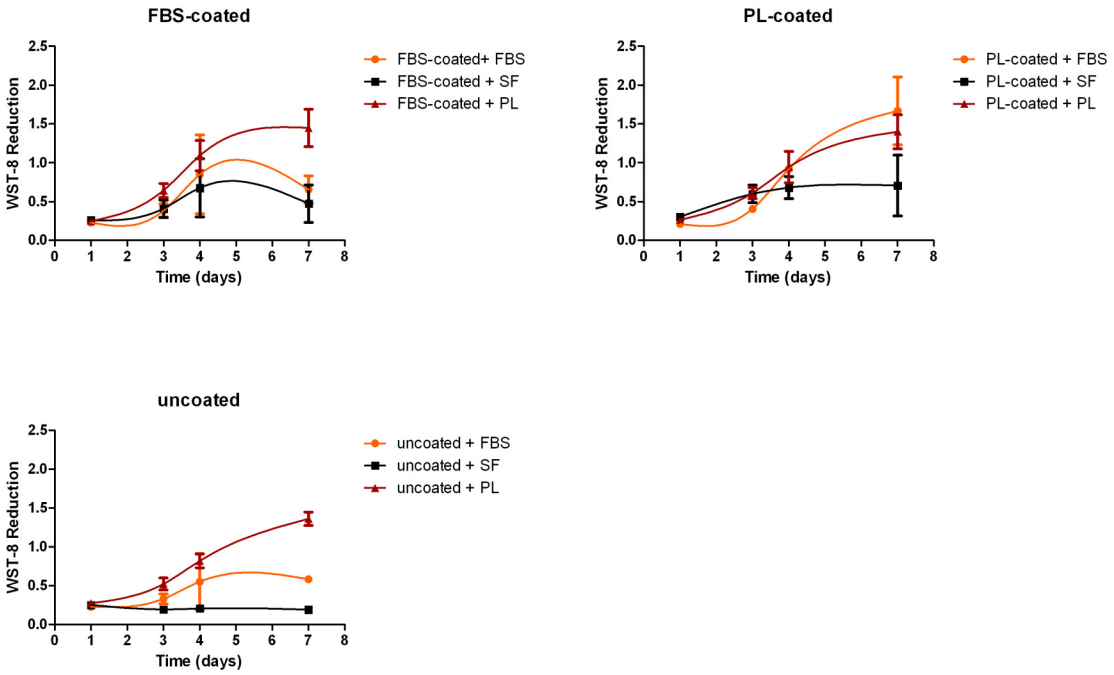

Figure 5: Cells were seeded on uncoated, FBS- and PL-coated cell culture dishes. The combinations of FBS coating and FBS containing media, PL coating with either FBS or PL containing media and uncoated surfaces and PL supplemented media show the best growth results. WST-8 values are expressed as means ± SD of three independent experiments.

a property called *self-renewal*. Second, daughter cells can differentiate into specific cell types to supply all the somatic cells of the tissue, called *multipotency*. Self-renewal and multipotency are the defining characteristics of stem cells [74].

When innovative culture systems for human stem cells are applied i.e. the replacement of high contents of FBS in culture media by non-animal derived components, like human platelet lysates (PL) [41], the stem cell attributes of self-renewal and multipotency have to be retained under those culture conditions. Before applying PL as an alternative to FBS, the following questions have to be answered as prerequisites for a successful stem cell culture system: (1) Can adult human stem cells e.g. ADSC, be cultured in the presence of PL? (2) Can the cells be maintained in undifferentiated state? (3) Can the cells be triggered to differentiate into specific lineages?

The first two questions are positively answered in the present study, also a preliminary answer for the third question can delivered. However, a full answer about the differentiation potential of ADSC in PL-supplemented media would go beyond the present paper, and will be given in a subsequent publication.

Bone marrow aspirates and adipose tissue, derived from liposuction, are rich sources for adult human mesenchymal stem cells. The generation of this type of cells is – in contrast to human embryonic stem cells – ethically acceptable, that paved the way for a vast amount of research concerning their potential use in regenerative medicine. However, any future clinical application of adult stem cells is impeded by the use of FBS as an animal-derived growth supplement in expansion culture media, due to the possibility of introducing xenogenic molecules into human stem cells [22]. This fact called for the search and the development of alternatives to FBS that are animal-derived component-free and safe for any therapeutic application.

In addition, human stem cell cultures gained importance as innovative human-based alternative to animal testing, *in vitro* toxicology, and drug testing and safety assessment [15-21, 75-78]. Thus, also for a successful application of human stem cell-based testing systems under fully humanized test conditions, animal-derived component-free culture protocols are mandatory.

Cell-free extracts of human donor thrombocytes (PL) have been established as a human-based, xeno-free surrogate for FBS (reviewed in [41, 79, 80]). The use of PL in culturing human cells may overcome some of the critical aspects in the FBS dilemma [3, 4, 28], like the presence of bovine sialic acids, that may act as xenoantigens [22] which imperils any future therapeutic use of FBS-grown (stem) cells [24], or the limited global

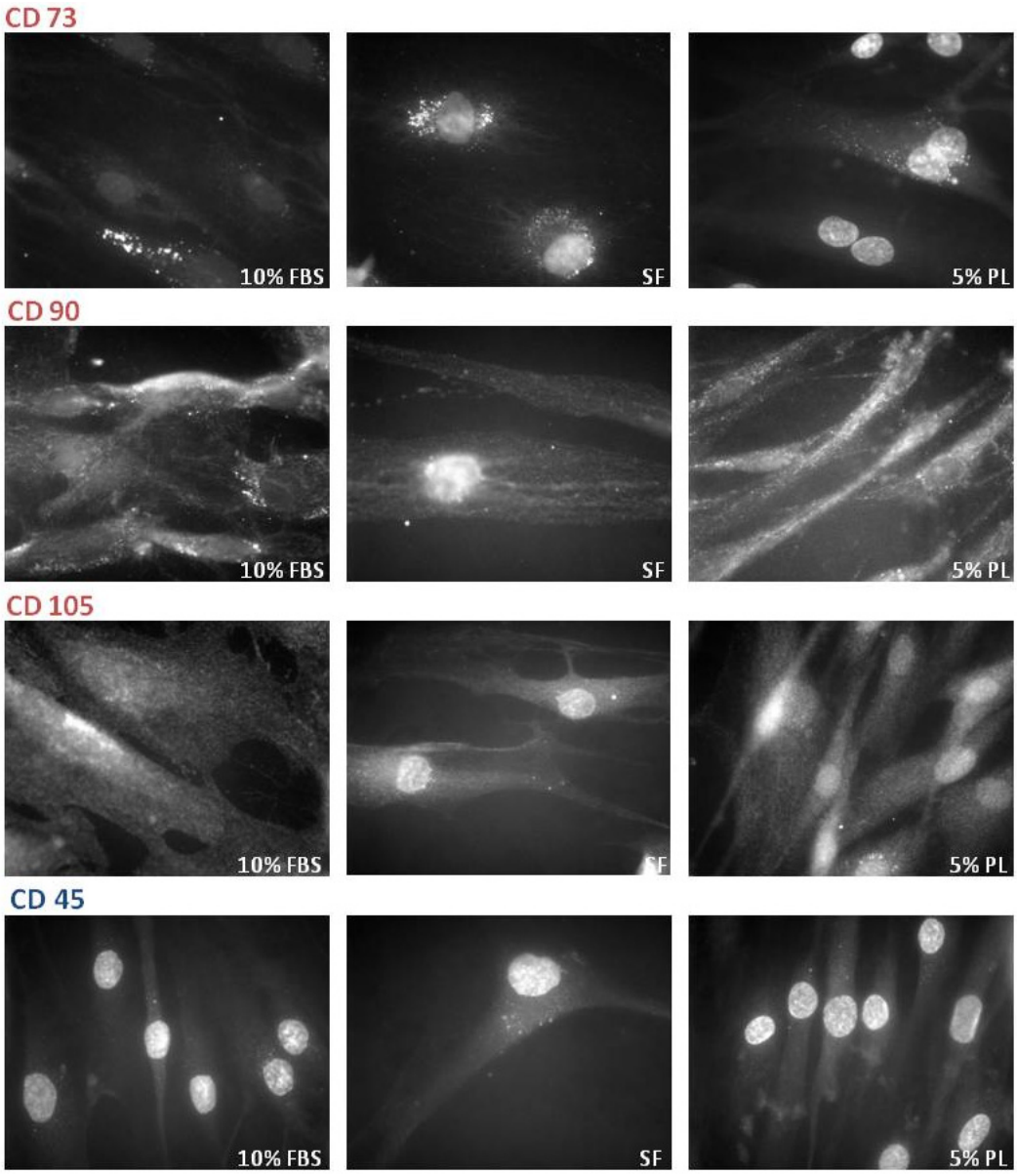

Figure 6: ADSC were grown with FBS-, PL- and non supplemented, serum-free media for one week. Three positive (CD73, CD90 and CD105) and one negative mesenchymal stem cell marker (CD45) were examined and confirmed the expression of all positive markers on ADSC at the three culture conditions. Positive stains of CD73, CD90 and CD105, respectively, are well developed at the cell surface.

supply and the questionable quality of FBS [11, 81]. However, PL are still ill-defined. Better definition and decreased batch-to-batch variation of PL preparations can be obtained by standardization of the manufacturing process, the determination of α-granule growth factors by ELISA, and the pooling of PL preparations, respectively [38]. Concerns about carrying infectious agents are neglectible, since PL are isolated at the date of expiration from donor thrombocyte bags, that were originally designed for therapeutic applications and were clinically tested for a broad spectrum of human viruses. The ultimate goal, however, still is the serum-free cell culture in chemically defined media [1, 3], although PL can be advantageous in autologous cell expansion for personalized cell therapy.

A number of studies have proven PL as a suitable substitute in cell expansion media, including adult human stem cells [42, 43, 45, 47, 48, 52, 62, 63]. Recently, we succeeded in using PL as an alternative for FBS in the cell culture of a number of continuous human and animal cell lines. The lysates were prepared as cell-free extracts from activated thrombocytes obtained by thrombapheresis. For each lot, growth factor content was determined by ELISA [38].

In the present study, ADSC, derived from adipose tissue after liposuction, were used as adult stem cell model [59]. The minimal criteria defining multipotent mesenchymal stromal cells, proposed by Dominici *et al*. [65], include – among others – the cells' potency to adhere to plastic culture surfaces. However, neither more precise information is given about the surface treatment and/or charge of the culture plastic, nor a systematic study on the nature of the plastic surface has been performed yet [82]. To this end, ADSC were cultured on different substrata, comparing negatively and positively charged culture surfaces with collagen-coated culture dishes in culture media containing FBS or PL. In order to quantify growth and proliferation of ADSC, WST-8 and resazurin assays were performed and the data compared with classical growth curves after direct counting of monolayer cell density. As can be seen from Figure **1**, no significant differences in rates of cell proliferation could be observed with respect to cell surface treatment. Clear differences, however, can be seen in terms of culture media supplements (for comparison of different FBS batches see also Figure **2**). Highest proliferation rates of ADSC were found in media supplemented with 5% PL. In addition, the diagrams within each assay method to determine the rate of proliferation (cell counting, WST-8, resazurin) are well comparable, as are the growth curves among each experimental group. This is, for the first time, a systematic comparison of classical growth curves, obtained by direct counting of cell density, with two indirect proliferation assays, measuring the activity of cellular dehydrogenases as indicators for cell viability [2, 68, 69].

However, considerable differences in initial cell adhesion were observed depending on the composition of the seeding medium (Figures **3** and **4**). It is a well known fact, that FBS, aside from hormones, growth factors and cytokines, vitamins, and trace elements, also contains attachment and spreading factors, facilitating the attachment of cells after seeding into fresh culture vessels [2, 3]. Therefore, in some serum-free culture protocols for sensitive cells, pre-coating of culture dishes is required [1]. These facts are well reflected by the different lag-phases of ADSC, when either seeded in FBS-containing or in serum-free medium (Figure **3**).

In order to further support these findings and to test whether PL may contain attachment and spreading factors, culture dishes were coated with either FBS or PL before seeding (Figure **5**). The results are in good accordance with the data of the previous series of experiments. Again, cell attachment, and growth and proliferation, respectively, of ADSC was improved after pre-coating with FBS or PL, shown by a shorter lag-phase and steeper log-phases, in contrast to seeding of cells into uncoated plastic culture dishes.

A prerequisite for successful application of adult stem cells in cell replacement therapy and *in vitro*-toxicology, respectively, is the expansion and maintenance of the cells in their undifferentiated state, thus to retain the multipotential nature and the proliferative capacity of the cells. In order to test whether ADSC maintain their undifferentiated state under PL culture conditions, cells were grown in the respective experimental media and the expression of specific surface markers, characteristic for defining multipotent mesenchymal stem cells, was determined [65]. ADSC cultured in FBS- or in PL-supplemented culture media stained positive for the mesenchymal markers CD73, CD90, and CD105, while cells were negative for the hematopoietic marker CD45 (Figure **6**).

To summarize, human thrombocyte extracts (platelet lysates, PL) can successfully replace the high contents of FBS in human adult mesenchymal stem cell cultures, as shown previously for continuous human and animal cell lines [38]. In the present study, in culture media supplemented with 5% PL, ADSC attached to polystyrene culture dishes, grew out and proliferated at rates equal or higher than control cultures with 10% FBS. In addition, under PL culture conditions, ADSC retained their undifferentiated phenotype. Thus, stem cell culture media supplemented with PL provide a fully "humanized" culture system for human adult mesenchymal stem cells (hMSC). In the future, stem cells under appropriate culture conditions may transform the way in which therapeutics are discovered and validated [83]. In addition, this culture system can also be applied for human induced pluripotent stem cells (iPS) [84]. The unique attributes of iPS are the generation of patient- and disease-specific cells for early drug discovery and safety assessment [85-89]. A promising approach is the setup of an European iPS Cell Bank by the recently initiated StemBANCC Project, funded by IMI, the Innovative Medicines Initative [90]. Also in this multinational project xeno-free culture conditions are mandatory prerequisites for successful applications of iPS for drug screening and safety assessment studies. A consistent source of cells cultured at the highest quality available to guarantee powerful test results will be the future challenge in *in vitro* toxicology [91, 92]. Or, as pointed out recently, "Are our cell cultures good enough for regulatory decision taking?" [93, 94].

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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