Effect of serum replacement with Plysate on cell growth and metabolism in primary cultures of human skeletal muscle

David K Krämer^{1,2}, Karim Bouzakri¹, Olle Holmqvist³, Lubna Al-Khalili^{1,2} and Anna Krook^{2,*}

¹*Department of Surgical Science, Karolinska Institutet S-171 77 Stockholm, Sweden;* ²*Department of Physiology and Pharmacology, Karolinska Institutet S-171 77 Stockholm, Sweden;* ³*ProLiff AB, SE-392 35 Kalmar, Sweden;* **Author for correspondence (e-mail: Anna.Krook@fyfa.ki.se; phone: +46-8-728-7824; fax: +46-8-33-54-36)*

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Abstract

Cell- and tissue culture methodology form an important base for biological, biochemical and biomedical research. Most cell culture techniques require the use of animal sera for the successful propagation of cells. However, the varying composition between batches has fuelled the need for alternatives. In the case of serum derived from animal foetuses, ethical concerns have also been raised. Here we compare the use of a platelet derived lysate (Plysate), which is currently under development as a serum substitute, in the culturing of primary human muscle cells to foetal bovine serum (FBS). In cells cultured with Plysate, differentiation into myotubes, glucose-uptake, phosphatidylinositol 3-kinase (PI3K) activity and expression and phosphorylation of ERK1/2 MAPK and PKB/Akt was impaired. Thus for primary human skeletal muscle Plysate is a sub-optimal substitute for FBS.

Introduction

Tissue and cell culture form an important base for many areas of research, spanning from basic science to tissue engineering applications. In comparison to animal models or human investigations, cell cultures are more amenable to manipulations (such as the introduction of novel genes) and offer advantages such as control, containment and cost. The advantage of containment, allows for the use of radioisotopes for metabolic tracer studies, potentially hazardous or toxic chemicals, such as carcinogens in toxicology studies, and pathogenic or infectious micro-organisms.

Cells require a range of supplements in media in order to proliferate. One common supplement is serum collected from blood as it provides a broad spectrum of macromolecules, carrier proteins for lipid substances, trace elements, attachment and spreading factors, low molecular weight nutrients, hormones and growth factors (Gstraunthaler 2003). Foetal bovine serum (FBS) is the most widely used supplement for *in vitro* culture media (Jayme and Blackman 1985; Liselott et al. 2003). The collection of serum from bovine foetuses has been the issue of moral concerns (Jochems et al. 2002; van der Valk et al. 2004). Aside from moral concerns, there are several scientific and technical

challenges with animal sera in cell culture. Serum is a supplement of unknown and varying composition and as a consequence of this variability experimental results may vary with the batch of serum used. This also includes the possibility of contamination with unwanted factors, which may pose a risk to the person handling the cultures (van der Valk et al. 2004). Additionally, availability and therefore high costs pose an important issue for research institutions (Liselott et al. 2003). Considerable efforts have been made to substitute FBS and similar sera with supplements obtained by alternative production processes with more controllable qualitative properties (Gstraunthaler 2003; Kwon et al. 2004; Lebkowshi et al. 1995). In this regard, developments towards reduced- and serum-free media, chemically defined media, maintenance media, and suspension culture media for anchorage-dependent cell lines have been realized. However, the substitutes have not managed to gain wide-spread acceptance, often due to high-cost, problems with availability, and major differences in the nutritional requirements between different cell-lines (Liselott et al. 2003).

Plysate has been patented (Olov and Westermarck 1989, 2003) as a serum-substitute which is produced from the platelet fraction from animal sources and is currently under further development. While Plysate shares the disadvantage of batch to batch variations and a possible health risk for the user with FBS, it is ethically less controversial and has a cost advantage (Liselott et al. 2003). Platelets are present in blood at high concentrations (0.1–0.25% (w/w)), and primarily aggregate at a site of injury and assist in the clotting mechanism and releasing growth factors, as well as other mediators to initiate the wound healing (Liselott et al. 2003). Platelets are a primary source of a number of growth factors (Ross et al. 1990; Sporn and Roberts 1989). Of special importance is the concentrated supply of platelet-derived growth factor (PDGF) (Ross et al. 1990) and transforming growth factor- β (TGF- β) (Fukamizu and Grinnell 1990). These two growth factors play an important role in cell culture as they are participating in many diverse cellular functions.

Primary human skeletal muscle cultures are usually cultured with a supplement of FBS (Al-Khalili et al. 2004; Cha et al. 2005). The purpose of this study was to investigate the applicability of platelet lysate (Plysate) for culture

of primary human muscle cells. Thus, cell growth, differentiation and insulin-stimulated glucose-metabolism were examined in cells grown in either media supplemented with FBS or Plysate.

Materials

Dulbeccos minimum essential medium (DMEM), Ham's F-10 medium, fetal bovine serum (FBS), penicillin, streptomycin, and fungizone were obtained from GibcoBRL.¹ Plysate was provided from ProLiff AB, and the production of Plysate has been described (Olov and Westermarck 1989, 2003). Radiochemical, 2-[G-³H]deoxy-D-glucose (6.0 Ci/mmol/l), D-[U-¹⁴C] glucose (310 mCi/mmol/l), were from Amersham.² All other chemicals were analytical grade and from Sigma-Aldrich.³ Phosphospecific antibodies against PKB (Ser473) and ERK1/2 MAP Kinase (Thr202 and Tyr204) were from New England Biolabs.⁴ Monoclonal antibodies against pan ERK1/2 (Clone 16, Catalog number 612641) were from Transduction Laboratories⁵ and polyclonal PKB/Akt was from New England Biolabs⁶ was raised in rabbits. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G was from Bio-Rad Laboratories.⁷ Reagents for enhanced chemiluminescence (ECL) were from Amersham².

Procedures

Human primary skeletal muscle cell cultures

Skeletal muscle biopsies were obtained from healthy individuals who underwent general surgery. None of the subjects had known metabolic disease. Satellite cells were isolated and primary muscle cultures were established (Al-Khalili et al. 2003; Al-Khalili et al. 2004). The ethical committee at Karolinska Institutet approved

¹ GibcoBRL, Life-Technologies, Stockholm, Sweden

² Amersham Life Science, Sweden

³ Sigma-Aldrich Sweden AB, Stockholm, Sweden

⁴ New England Biolabs Beverly, MA

⁵ Transduction Laboratories Lexington, KY

⁶ New England Biolabs, Beverly, MA

⁷ Bio-Rad Laboratories, Richmond, CA

protocols. Cells were grown in DMEM (1000 mg/l glucose) with 20% FBS or Plysate and 1% Penicillin/Streptomycin (PeSt) in non-coated dishes. Proliferation of cells was assessed by microscopy, estimating the density of cells grown in 100 mm dishes, seeded at an initial density of 30% confluency. In order to differentiate human myoblasts into myotubes, dishes with a cell density of 80–90% were grown in DMEM with 4% FBS or Plysate for 2 days to induce myotube formation, and then grown in DMEM with 2% FBS or Plysate for 2 days. Before utilization, the cells were controlled optically for formation of elongated myotubes and serum-starved over night.

Production of platelet lysate (Plysate)

The development of Holmqvist and Westermarck has been described in the in EC- and US-patents (Olov and Westermarck 1989, 2003). Briefly, the platelets are collected from whole blood in a two-step centrifugation procedure. The precipitate is then lysed with water and coagulated with CaCl_2 . After another centrifugation step, the platelet lysate is sterile filtered through $0.2 \mu\text{m}$ and is then ready for use. Platelet lysate was stored frozen at -18°C .

Glucose uptake and PI 3-kinase activity

Glucose uptake was performed as described for primary human muscle cells (Al-Khalili et al. 2003). Phosphatidylinositol 3-kinase (PI3K)-activity assay was assessed as described previously (Al-Khalili et al. 2004).

Western blot analysis

Cells for Western blot analysis were harvested at day 4 after initiation of the differentiation. Cell monolayers were washed once in ice-cold PBS and harvested directly by scraping into ice-cold lysis buffer (135 mM NaCl, 1 mM MgCl_2 , 2.7 mM KCl, 20 mM Tris pH 8.0, 0.5 mM Na_3VO_4 , 10 mM NaF, 1% Triton X-100, 10% v/v glycerol, 0.2 mM phenylmethanesulfonyl (PMSF), 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ antipain, and 10 $\mu\text{g}/\text{ml}$ aprotinin). Lysates (20 μg) were separated by

SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and probed with primary phospho-specific or protein specific antibodies and secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

Statistics

Statistical differences were determined Student's *t*-test. Significant differences were accepted at $p < 0.05$.

Results and discussion

Growth and morphology

Differentiation of primary human myoblasts into elongated and multinucleated myotubes is essential for the validity and functionality of the human muscle cell culture as a model (Al-Khalili et al. 2003). The morphology of the muscle cells was followed during differentiation. A marked difference in cell growth was noted between cells differentiated with FBS and the platelet lysate (Plysate). Parallel cultures of cells (originating from the same donor) were grown using either FBS or Plysate as supplement. Proliferation rate was reduced in cells grown and differentiated with platelet lysate. Cells grown in FBS supplemented media grew approximately 1.3 times faster (Figure 1a). Moreover, cells grown and differentiated with platelet lysate lacked clear signs of differentiation into myotubes (Figure 1b). Thus, Plysate is insufficient to support adequate proliferation and differentiation of primary human myoblasts into myotubes.

The sub-optimal effects we observed using Plysate supplemented media on myotube growth and metabolism could be due to either the presence of inhibitory factors that prevent optimal proliferation and differentiation or due to the lack of important differentiation factors. Although satellite cells from muscle of various organisms have been utilized extensively for cell culture, little is known about the actual factors needed for the process of differentiation (reviewed by (Peng and Huard 2004)). Thus, key factors promoting differentiation may be absent from the Plysate-conditioned media.

Figure 1. (a) Growth rate of myoblasts in medium supplemented with FBS or Plysate (content 20%). (b) Microscopy capture of primary human muscle cell morphology after 5 days of differentiation (2 days at 4% serum/Plysate, 3 days at 2% serum/Plysate). FBS-treated cells on the left picture, Plysate treated cells on the right picture. Arrows indicate the ends of one myotube for size-comparison.

To determine which factor or factors are lacking in Plysate versus FBS-conditioned media would likely require extensive investigations. However, platelet derived growth factors (PDGF) are the major components of Plysate (Liselott et al. 2003) and this may contribute to the compromised phenotype. Although the effects of PDGF on cell-proliferation varies depending on species, cell type, method of stimulation, and level of receptor expression (reviewed in (Tallquist and Kazlauskas 2004)), PDGF is known to have differential effects on proliferation in primary human cells in culture, with measurable increases in growth in some, but not all cell-types (Mumford et al. 2001). PDGF act on a variety of proteins involved in cell-repair and differentiation (reviewed in (Tallquist and Kazlauskas 2004)).

The orchestration of cell growth and differentiation processes initiated by PDGF may be counter-active for the differentiation of myoblasts into myotubes. Interestingly, platelet-derived growth factor receptor (PDGFR) signalling is down-regulated during muscle differentiation (Fiaschi et al. 2003). Moreover, growth factor signalling is impaired in differentiated cells, due to the withdrawal of growth factors or due to transcriptional down-regulation of their receptors (Fiaschi et al.

2003). Therefore the lack of differentiation in the Plysate-treated cells may occur in response to an abundance of PDGF.

Phosphatidylinositol 3-kinase (PI3K)-activity

Skeletal muscle is a primary site of insulin action, and plays a key role in whole body glucose homeostasis. Insulin-mediated activation of Phosphatidylinositol 3-kinase is a critical step regulating insulin-stimulated glucose uptake. Thus we assessed insulin-stimulated PI3-kinase activity in primary human muscle cells grown in media supplemented with Plysate or FBS. Basal and insulin induced PI3K-activity increases during differentiation of primary muscle cultured cells from myoblasts to myotubes (Al-Khalili et al. 2004). As expected, insulin increased PI3K-activity in cells cultured in FBS (Figure 2, $p < 0.05$). Conversely, in cells cultured in Plysate, basal PI3K-activity was increased, with no further stimulation noted after insulin exposure. Basal and insulin-stimulated PI3K activity was significantly different between the cells cultured with Plysate and cells cultured with FBS ($p < 0.05$).

Expression and phosphorylation of key-proteins involved in insulin action

Insulin activates both mitogenic and metabolic signalling pathways (Krook et al. 2004). The extent of insulin stimulated ERK phosphorylation was assessed as a marker of mitogenic pathways while insulin stimulated PKB (also called AKT) activation was assessed as a marker of metabolic

Figure 2. PI3-Kinase activity in myotubes (at 5 days differentiation) in the basal state and after 30 min of stimulation with 120 nM insulin. Filled bars represent Plysate-treated cells; empty bars represent FBS-treated cells. * $p < 0.05$ vs. basal, # $p < 0.05$ Plysate vs. FBS.

pathways. Expression of both PKB Akt (Figure 3b) and ERK1/2 MAPK (Figure 3d) was higher in primary human muscle cells grown in FBS supplemented media as compared to cells grown in Plysate supplemented media ($p < 0.05$). Independent of the serum culture conditions, insulin increased PKB phosphorylation (Figure 3a) and ERK1/2 phosphorylation (Figure 3c). However, when comparing the intensity of the phosphorylation signal of both culture conditions, basal phosphorylation of ERK1/2 MAPK was greater in cells cultured in FBS ($p < 0.05$). Moreover, there was a tendency for greater phosphorylation of ERK1/2 MAPK after insulin stimulation ($p = 0.07$). A similar result was observed for PKB phosphorylation; with a tendency for increased basal ($p = 0.08$) and a more profound insulin-stimulated phosphorylation ($p < 0.05$).

When normalizing ERK1/2 MAPK phosphorylation for the observed protein expression changes (data not shown), the magnitude of

insulin-stimulated protein phosphorylation was similar for Plysate and FBS. In contrast, normalizing PKB phosphorylation for the observed protein expression changes, suggested that insulin action on PKB phosphorylation in Plysate treated cells was impaired as compared to cells cultured in FBS. Thus, the magnitude of PKB phosphorylation in response to insulin was significantly higher in cells cultured with FBS compared to Plysate ($p < 0.05$). Thus muscle cultures grown in Plysate supplemented media had preserved insulin action on MAP kinase activation, while insulin stimulation of metabolic pathways (exemplified by PI 3-kinase and PKB) was impaired.

Glucose-uptake

An important metabolic endpoint of insulin action in vivo is to mediate insulin-stimulated glucose disposal in skeletal muscle, thus insulin action on glucose uptake was determined in

Figure 3. (b) Protein expression (quantified by western blot analysis) and (a) phosphorylation of PKB/Akt and (d) expression and (c) phosphorylation of ERK1/2 MAPK in myotubes (at 5 days differentiation). Filled bars represent Plysate treated cells; empty bars represent FBS treated cells. * $p < 0.05$ vs. basal. ** $p < 0.01$ vs. basal. # $p < 0.05$ comparing equal conditions of Plysate vs. FBS.

Figure 4. Glucose-uptake in Plysate and FBS treated myotubes (at 5 days differentiation) under basal (filled bars) and insulin-stimulated (empty bars) conditions. * $p < 0.05$ vs. basal, # $p < 0.05$ comparing equal conditions of Plysate vs. FBS.

primary human muscle cells grown in FBS or Plysate as supplement (Figure 4). Insulin increased glucose uptake 1.9 fold ($p < 0.05$ versus basal conditions) in FBS treated cells and 1.2 fold in Plysate treated cells ($p < 0.05$ versus basal conditions) (Figure 4). Insulin stimulated glucose uptake magnitude was significantly ($p < 0.05$) higher in cells cultured with FBS compared to Plysate. These results strongly suggest insulin action on glucose metabolism is compromised in primary human muscle cells cultured in media supplemented with Plysate.

In conclusion, culturing primary human skeletal muscle in Plysate supplemented media results in changes in cell morphology, growth rate and response to insulin. Thus, for studies on skeletal muscle insulin action, Plysate with its current composition is a sub-optimal substitute for FBS and would require composition adjustments. This effect appears to be cell-specific and is in contrast to reports of positive effects for other cell lines, including Sp2/0 (myeloma cell), 39.5 (hybridoma cell), Hel-299 (fibroblast cell), HepG2 hepatocytic cell) and Vero (African green monkey transformed kidney epithelial cells) (Olov and Westermarck 1989, 2003; Liselott et al. 2003). One possibility is that this reflects a difference between established immortalized cell lines and primary muscle cells (Perez-Reyes et al. 1992) with their ability to undergo cell-differentiation and eventually senescence (Di Donna et al. 2003). Thus, FBS is the preferred media complement to promote appropriate growth, differentiation and insulin action on glucose metabolism in primary human skeletal muscle myotubes.

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References

- Al-Khalili L., Chibalin A.V., Kannisto K., Zhang B.B., Permert J., Holman G.D., Ehrenborg E., Ding V.D., Zierath J.R. and Krook A. 2003. Insulin action in cultured human skeletal muscle cells during differentiation: assessment of cell surface GLUT4 and GLUT1 content. *Cell Mol. Life Sci.* 60: 991–998.
- Al-Khalili L., Krämer D., Wretenberg P. and Krook A. 2004. Human skeletal muscle cell differentiation is associated with changes in myogenic markers and enhanced insulin-mediated MAPK and PKB phosphorylation. *Acta Physiol. Scand.* 180: 395–403.
- Cha B.S., Ciaraldi T.P., Park K.S., Carter L., Mudaliar S.R. and Henry R.R. 2005. Impaired fatty acid metabolism in type 2 diabetic skeletal muscle is reversed by PPAR(γ) agonists. *Am. J. Physiol. Endocrinol. Metab.*
- Di Donna S., Mamchaoui K., Cooper R.N., Seigneurin-Venin S., Tremblay J., Butler-Browne G.S. and Mouly V. 2003. Telomerase can extend the proliferative capacity of human myoblasts, but does not lead to their immortalization. *Mol. Cancer Res.* 1: 643–653.
- Fiaschi T., Chiarugi P., Buricchi F., Giannoni E., Taddei M.L., Magnelli L., Cozzi G., Raugeri G. and Ramponi G. 2003. Down-regulation of platelet-derived growth factor receptor signaling during myogenesis. *Cell Mol. Life Sci.* 60: 2721–2735.
- Fukamizu H. and Grinnell F. 1990. Spatial organization of extracellular matrix and fibroblast activity: effects of serum, transforming growth factor beta, and fibronectin. *Exp. Cell Res.* 190: 276–282.
- Gstraunthaler G. 2003. Alternatives to the use of fetal bovine serum: serum-free cell culture. *Altex* 20: 275–281.
- Jayme D.W. and Blackman K.E. 1985. Culture media for propagation of mammalian cells, viruses, and other biologicals. *Adv Biotechnol Processes* 5: 1–30.
- Jochems C.E., der Valk J.B., Stafleu F.R. and Baumans V. 2002. The use of fetal bovine serum: ethical or scientific problem? *Altern. Lab Anim.* 30: 219–227.
- Krook A., Wallberg-Henriksson H. and Zierath J.R. 2004. Sending the Signal: Molecular Mechanisms Regulating Glucose Uptake. *Med. Sci. Sports Exerc.* 36: 1212–1217.
- Kwon M.S., Dojima T. and Park E.Y. 2004. Use of plant-derived protein hydrolysates for enhancing growth of

- Bombyx mori* insect cells in suspension culture. *Biotechnol. Appl. Biochem.*
- Lebkowski J.S., Schain L.R. and Okarma T.B. 1995. Serum-free culture of hematopoietic stem cells: a review. *Stem Cells* 13: 607–612.
- Liselott Johansson J.K., Olov H. and Ohlson S. 2003. Platelet lysate: a replacement for fetal bovine serum in animal cell culture? *Cytotechnology* 67–74.
- Mumford J.H., Carnes D.L., Cochran D.L. and Oates T.W. 2001. The effects of platelet-derived growth factor-BB on periodontal cells in an in vitro wound model. *J. Periodontol.* 72: 331–340.
- Olov H. and Westermarck B. 1989. Blood Platelet Lysate, method of its preparation and a cell culture medium containing blood platelet lysate. European Patent Cooperation 413727.
- Olov H. and Westermarck B. 2003. Preparation of a blood platelet lysate for use in a cell culture medium for hybridoma cells. Patent USA.
- Peng H. and Huard J. 2004. Muscle-derived stem cells for musculoskeletal tissue regeneration and repair. *Transpl. Immunol.* 12: 311–319.
- Perez-Reyes N., Halbert C.L., Smith P.P., Benditt E.P. and McDougall J.K. 1992. Immortalization of primary human smooth muscle cells. *PNAS* 89: 1224–1228.
- Ross R., Bowen-Pope D.F. and Raines E.W. 1990. Platelet-derived growth factor and its role in health and disease. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 327: 155–169.
- Sporn M.B. and Roberts A.B. 1989. Transforming growth factor-beta. Multiple actions and potential clinical applications. *JAMA* 262: 938–941.
- Tallquist M. and Kazlauskas A. 2004. PDGF signaling in cells and mice. *Cytokine Growth Factor Rev.* 15: 205–213.
- van der Valk J., Mellor D., Brands R., Fischer R., Gruber F., Gstraunthaler G., Hellebrekers L., Hyllner J., Jonker F.H., Prieto P., Thalen M. and Baumann V. 2004. The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. *Toxicol. In Vitro* 18: 1–12.