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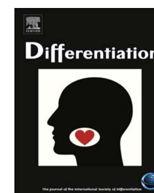

EGF and hydrocortisone as critical factors for the co-culture of adipogenic differentiated ASCs and endothelial cells

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ABSTRACT

In vitro composed vascularized adipose tissue is and will continue to be in great demand e.g. for the treatment of extensive high-graded burns or the replacement of tissue after tumor removal. Up to date, the lack of adequate culture conditions, mainly a culture medium, decelerates further achievements. In our study, we evaluated the influence of epidermal growth factor (EGF) and hydrocortisone (HC), often supplemented in endothelial cell (EC) specific media, on the co-culture of adipogenic differentiated adipose-derived stem cells (ASCs) and microvascular endothelial cells (mvECs). In ASCs, EGF and HC are thought to inhibit adipogenic differentiation and have lipolytic activities. Our results showed that in indirect co-culture for 14 days, adipogenic differentiated ASCs further incorporated lipids and partly gained an univacuolar morphology when kept in media with low levels of EGF and HC. In media with high EGF and HC levels, cells did not incorporate further lipids, on the contrary, cells without lipid droplets appeared. Glycerol release, to measure lipolysis, also increased with elevated amounts of EGF and HC in the culture medium. Adipogenic differentiated ASCs were able to release leptin in all setups. MvECs were functional and expressed the cell specific markers, CD31 and von Willebrand factor (vWF), independent of the EGF and HC content as long as further EC specific factors were present. Taken together, our study demonstrates that adipogenic differentiated ASCs can be successfully co-cultured with mvECs in a culture medium containing low or no amounts of EGF and HC, as long as further endothelial cell and adipocyte specific factors are available.

1. Introduction

White adipose tissue (WAT) comprises about one fourth of the human body weight, interacts with many other organs via paracrine and endocrine signals and absorbs and stores lipophilic substances like the β-adrenergic receptor agonist metoprolol, whereby it impacts drug distribution and available drug levels (Bickel, 1984; Testa et al., 2000). Additionally, WAT gets affected in different diseases, e. g. metabolic syndrome, pancreatitis, Morbus Crohn, steatohepatitis or breast cancer (Nawrocki and Scherer, 2005). Therefore, an *in vitro* adipose tissue model is highly needed as a test system to analyze fundamental biological processes during physiological or diseased state, screen for potential drugs or create affiliated safety profiles. Additionally, adipose tissue substitutes are highly desired to treat lost, deformed or burned subcutaneous fatty tissue in patients (Mizuno et al., 2012).

In vivo adipose tissue is a highly vascularized organ, whereby each adipocyte is in contact with one or more blood vessels (Xue et al., 2009, 2010). Vasculature is composed of endothelial cells (ECs) which supply adipocytes with nutrients and remove waste products. Adipocyte - EC crosstalk itself is thereby of high relevance as a basis for adipose tissue homeostasis and maintenance whereby cell specific activity, fulfillment of functions and viability highly depend on signal molecules produced from each other cell type. Vascularization of adipose tissue constructs is essential as tissue physiology itself relies on the crosstalk of ECs and adipocytes which has previously been reviewed by us (Huber et al., 2015b; Volz et al., 2016). The artificial setup of vasculature within adipose tissue constructs is e.g. needed for several approaches:

- to resemble the native adipose tissue more adequately through physiological tissue maintenance based on EC – adipocyte crosstalk.
- to achieve adipose tissue substitutes of clinically significant dimensions and long term stability by supplying inside located tissue areas with nutrients.
- to facilitate investigations based on vessels and dynamic circulation.

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The major goal in terms of adipose tissue vascularization is the successful maintenance of function and phenotype of both cell types in co-culture which includes the control of excess cell proliferation and dedifferentiation. The applied co-culture medium thereby represents an important adjustment screw.

Currently used adipocyte monoculture media contain factors like biotin, panthothenate, insulin and glucocorticoids (GCs) (Bès-Houtmann et al., 2007; Huber and Kluger, 2015a; Janke et al., 2002; Kern et al., 2006; Korner et al., 2005). In adipose-derived stem cells (ASCs), insulin is activating the CCAAT enhancer binding protein (C/EBP) and peroxisome proliferator activator γ (PPARγ). As a major anabolic hormone it promotes glucose and amino acid uptake, lipogenesis as well as intracellular transport (Rorsman, 2005). GCs like dexamethasone and hydrocortisone are as well addressing PPARγ and thereby initiate adipogenic differentiation (Liu et al., 2012; Moreno-Navarrete and Fernández-Real, 2012; Rosen and Spiegelman, 2000). Their effect on mature adipocytes is discussed controversially. Both, indications for pro- as well as antilipolytic effects have been reported (reviewed in Peckett et al., 2011). The vitamins biotin and panthothenate function as co-enzymes of acetyl-CoA-carboxylase, which is in turn responsible for lipid composition and accumulation (Lee et al., 2004).

While no impact of panthothenate and biotin was reported on ECs, insulin is known to affect vasoconstriction via endothelial nitric oxide synthase (eNOS) release (Barrett and Liu, 2013) and as well cause endothelial dysfunction through oxidative stress (Arcaro et al., 2002). GCs like dexamethasone are next to their general anti-inflammatory effect known to increase the expression of tight junction proteins in mvECs, thereby enhancing endothelial barrier function (Forster et al., 2006), but as well inhibit tube formation (Logie et al., 2011).

Most adequate EC media include vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), hydrocortisone (HC) and ascorbic acid 2-phosphate (A2P). VEGF and bFGF are well characterized mitogens of ECs and thereby responsible for their migration and proliferation (Augustin et al., 2009; Cao, 2010; Fagiani and Christofori, 2013; Gaengel et al., 2009; Marra et al., 2008). IGF fulfills several important functions in ECs. Next to its general contribution to EC metabolism by the increase of amino acid and glucose uptake, IGF stimulates migration and vessel formation through extracellular signal–regulated kinases (ERK) and phosphatidylinositide 3-kinase (PI3K) (reviewed in Bach, 2015). A2P promotes EC growth through an ERK regulated pathway (Ulrich-Merzenich et al., 2007), through nitric oxide (NO) suppression it, however, as well induces an angiostatic effect in high concentrations (Mikirova et al., 2008). EGF is a potent contributor to cell migration, vessel formation and maturation and thereby a key factor for angiogenic processes (Mawatari et al., 1989, 1991).

In ASCs and adipocytes VEGF has shown to support adipogenic differentiation processes (Nishimura et al., 2007). bFGF is known to have a mitogenic effect on stem cells (Schling and Loffler, 2002) but may strongly increase adipogenesis in combination with PPARγ agonists (Neubauer et al., 2004). While IGF is known to support lipogenesis and vice versa block lipolysis in mature adipocytes, it may act either proliferative or induce differentiation in stem cells (Huber et al., 2015b; Schling and Loffler, 2002). Adipogenic differentiation of mesenchymal stem cells was reported to be enhanced by A2P addition (Weiser et al., 2009). In contrast, dose-dependent inhibitory effects of A2P were detected with a preadipocyte cell line (Rahman et al., 2014). However, the used concentrations greatly exceeded the classically set values in EC media. EGFs role in adipocyte development and maintenance is discussed controversially. There is consent on EGFs inhibitory effect on the adipogenic differentiation of precursor cells (Adachi et al., 1994; Hauner et al., 1995; Hebert et al., 2009). Concerning adipocyte maintenance EGF was reported to increase intracellular lipid accumulation in 3T3-cells in low concentrations (Adachi et al., 1994). However, weighty results were also generated on its opposite role. These inconsistent studies demonstrate that EGF does not only reduce the percentage of differentiating cells and block lipid accumulation, but additionally induces lipolysis in terminally differentiated adipocytes (Hauner et al., 1995; Hebert et al., 2009).

Currently performed co-culture attempts were either based on an EC monoculture medium (Bellas et al., 2013; Pellegrinelli et al., 2014) or a simple mixture of different EC and adipocyte monoculture media (Choi et al., 2011). However, according to literature, special attention has to be paid to HC and EGF as these factors and their used concentrations may be the critical parameter since they might allow lipid loss and dedifferentiation of adipogenic differentiated ASCs which might forbid a physiologic arrangement of the co-culture setting. The aim of this study was to evaluate different concentrations of EGF and HC on adipocyte and EC functionality to optimize the co-culture settings.

2. Material & methods

2.1. Human tissue samples

All research was carried out in accordance with the rules for investigation of human subjects as defined in the Declaration of Helsinki. Patients gave a written agreement according to the permission of the Landesärztekammer Baden-Württemberg (F-2012-078; for normal skin from elective surgeries).

2.2. Cell isolation and culture

ASCs were isolated from human subcutaneous adipose tissue plastic surgeries received from Dr. Ziegler (Clinic Charlottenhaus, Stuttgart). The isolation was based on protocols described earlier (Gerlach et al., 2012; Zuk et al., 2001). Briefly, 100g of adipose tissue obtained from surgical operations were cut into small pieces and digested in Dulbecco's modified Eagle medium (DMEM high glucose; Biochrom, Germany) containing 0.1% collagenase type I and II (collagenase NB4, Serva Electrophoresis, Germany) and 1% bovine serum albumin (BSA; Sigma, Germany) overnight at 37 °C under constant motion. The suspension was filtered through a 500 µm sieve and centrifuged for 5 min at 200×*g*. The pellet was suspended in erythrocyte lysis buffer and incubated for 10 min at room temperature (RT). After another centrifugation step the remaining pellet was suspended in phosphate buffered saline (PBS$^-$; Biochrom, Germany) and filtered through a 70 µm meshed sieve. For expansion, cells were suspended in mesenchymal stem cell growth medium (MSCGM; Lonza, Switzerland) containing 2% fetal calf serum (FCS; Life Technologies, Germany). ASCs were used in passages 2–4 in the described experiments.

Dermal microvascular ECs (mvECs) were isolated from adult human skin (Clinic Charlottenhaus, Stuttgart). After careful removal of adipose tissue, the cutis was dissected into small stripes and incubated in dispase solution (2 U mL^{-1}; Serva Electrophoresis, Germany) overnight at 4 °C. Epidermis was removed and remaining dermis was incubated with 0.05% Trypsin in ethylendiaminetetraacetic acid (EDTA), (Life Technologies, Germany) for 40 min at 37 °C. Enzyme reaction was stopped with FCS. Afterwards, each piece was greased with a scalpel from each side into pre-warmed microvascular EC growth medium-2 (EGM-2mv; Lonza, Switzerland) to harvest the cells. The resulting cell medium suspension was filtered through a 70 µm meshed sieve and centrifuged. For expansion, the pellet was suspended in EGM-2mv and cells were plated in cell culture flasks. The next day, cells were incubated with 0.02% EDTA solution (Lonza, Switzerland) to detach possibly entrained fibroblasts. Cells were used up to passage 3 in the following experiments.

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