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Cell-derived Extracellular Matrix as maintaining Biomaterial for adipogenic differentiation

Abstract: The extracellular matrix (ECM) naturally surrounds cells in humans, and therefore represents the ideal biomaterial for tissue engineering. ECM from different tissues exhibit different composition and physical characteristics. Thus, ECM provides not only physical support but also contains crucial biochemical signals that influence cell adhesion, morphology, proliferation and differentiation. Next to native ECM from mature tissue, ECM can also be obtained from the *in vitro* culture of cells. In this study, we aimed to highlight the supporting effect of cell-derived- ECM (cdECM) on adipogenic differentiation. ASCs were seeded on top of cdECM from ASCs (scdECM) or pre-adipocytes (acdECM). The impact of ECM on cellular activity was determined by LDH assay, WST I assay and BrdU assay. A supporting effect of cdECM substrates on adipogenic differentiation was determined by oil red O staining and subsequent quantification. Results revealed no effect of cdECM substrates on cellular activity. Regarding adipogenic differentiation a supporting effect of cdECM substrates was obtained compared to control. With these results, we confirm cdECM as a promising biomaterial for adipose tissue engineering.

Keywords: extracellular matrix, adipose-derived stem cells, differentiation, biomaterial.

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1 Introduction

Biomaterials should provide an adequate environment for cultures cells and thereby play a crucial role in tissue engineering process. To date there are a variety of available materials for adipose tissue engineering such as gelatine [1], collagen [2], alginate [3] or combinations thereof. However, these materials cannot represent the complexity of the natural

ECM and the typical organization of native tissue. ECM represents the natural environment of cells in an organism, wherein it is synthesized and assembled by tissue-specific cells. The ECM of a tissue provides not only physical support but also delivers crucial biochemical signals that influence cell adhesion, morphology, proliferation and differentiation [4]. The composition and physical characteristics of the ECM vary between different tissues and different stages of cell differentiation. Next to native ECM, derived from mature decellularized tissues, cell-specific matrices can also be obtained from the *in vitro* culture of these cells. Native and cell-derived ECMs (cdECM) are used and studied in numerous applications as potential biomaterials such as cell-influencing coatings [5], hybrid scaffolding materials for tissue engineering [6], and bioinks [7].

In this work, we investigated the potentially supporting effect of cdECM on ASCs regarding cytotoxicity, metabolic activity, proliferation and adipogenic differentiation.

2 Materials and Methods

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

2.1 Cell isolation and expansion

ASCs were isolated from human adipose tissue samples obtained from patients undergoing plastic surgery (Dr. Ziegler, Klinik Charlotenhäuser, Stuttgart, Germany) as described before. ASCs were initially seeded at a density of 5×10^3 cells/cm² in serum-free MSC growth medium (PELOBiotech, #PB-C-MH-675-0511-XF) containing 5 % human platelet lysate.

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2.2 Generation of cdECM and reseeding

ASCs were seeded into 24-well plates at a density of 2.5×10^4 cells/cm² in serum-free MSCGM containing 5 % human platelet lysate. At confluency, medium was changed to either serum-containing growth medium (GM) (Dulbecco's modified Eagle Medium (DMEM) with 10 % fetal calf serum (FCS) = scdECM) or adipogenic differentiation medium (DMEM with 10 % FCS, 1 μ g/mL insulin, 1 μ M dexamethasone, 100 μ M indomethacin, 500 μ M 3-isobutyl-1-methylxanthine = acdECM) both supplemented with 50 μ g/mL Na-L-ascorbate. The medium was changed every other day. At day 7, cells were lysed using hypotonic ammonium hydroxide solution and cdECM was washed with ultrapure water. Direct after cdECM isolation ASCs were seeded on top of the cdECM at a density of 2.5×10^4 cells/cm² in GM. As a control, ASCs were seeded on TCPS.

At confluency medium was changed to differentiation medium. For this study, two different adipogenic differentiation media were used. A full supplemented adipogenic differentiation medium (DMEM with 10 % FCS, 1 μ g/mL insulin, 1 μ M dexamethasone, 100 μ M indomethacin, 500 μ M 3-isobutyl-1-methylxanthine = Diff. medium) and an adipogenic differentiation medium supplemented with 1 μ g/mL insulin (GM + insulin). As control, ASCs were cultured in GM.

2.3 Assays for cellular activity

At day three after reseeding, assays were performed to determine the influence of cdECM substrates on ASCs activity. For evaluation of cytotoxicity of the substrates a lactate dehydrogenase (LDH) assay was performed. LDH is released into the cell culture medium after cell death. Metabolic activity of ASCs cultured on the different substrates was evaluated by WST I assay. WST I is a tetrazolium salt which is cleaved to formazan by cellular mitochondrial dehydrogenase and therefore can be used to measure metabolic activity. ASCs proliferation was determined by colorimetric BrdU assay (Sigma Aldrich, Germany). Within this assay, bromolated nucleotides are incorporated into the DNA during replication and can therefore be used to determine the proliferation rate of ASCs. Incorporated BrdU was detected by horse radish peroxidase conjugated antibody and TMB turnover was measured using a plate reader at 650nm.

2.4 Oil red O staining and quantification

To determine the amount of accumulated lipids and thereby the degree of adipogenic differentiation, ASCs were stained with Oil red O. Cells were fixed with Roti-Histofix for 15 min. Subsequently, cells were incubated with 60 % isopropanol for 5 min followed by 10 min incubation with Oil red O solution (60% Oil red O stock solution (5 mg/mL) in Millipore water). For quantification, the staining solution was extracted with 100 % isopropanol for 15 min under shaking conditions. Absorbance was measured at 520 nm with a plate reader (Tecan safire²). Light microscopic pictures were taken before extracting Oil red O off the cells with an Axio Observer (Carl Zeiss).

2.5 Statistics

All experiments were performed with cells from three different biological donors. Data was compared by a one-way analysis of variance and a Tukey post-hoc test using OriginPro 2018b.

3 Results

3.1 ASC activity

To determine potential cytotoxic effects of cdECM, LDH release was measured at day three after reseeding (Figure 1). Results revealed no cytotoxic effect of cdECM on ASCs (TCPS: 100.0 (\pm 8.8) %; acdECM: 123.8 (\pm 48.4) %; scdECM: 89 (\pm 23.3) %). Influence of cdECM on metabolic activity and proliferation was determined by WST I assay (TCPS: 100.0 (\pm 1.0) %; acdECM: 99.2 (\pm 14.2) %; scdECM: 130.2 (\pm 6.9) %) and BrdU assay (TCPS: 100.0 (\pm 4.6) %; acdECM: 148.0 (\pm 61.8) %; scdECM: 98.2 (\pm 22.2) %), respectively.

3.2 Adipogenic differentiation

For determining a potential effect of cdECM on adipogenic differentiation, ASCs were seeded on top of acdECM and scdECM and were cultured in GM, GM with insulin (insulin) or fully supplemented adipogenic differentiation medium (full suppl.). As a control, ASCs were cultured on TCPS. To analyse lipid accumulation as an indicator for adipogenic differentiation Oil red O staining was performed at day 14 (Figure 3). Light microscopic analysis revealed no adipogenic differentiation in GM on all substrates. For insulin, some lipid

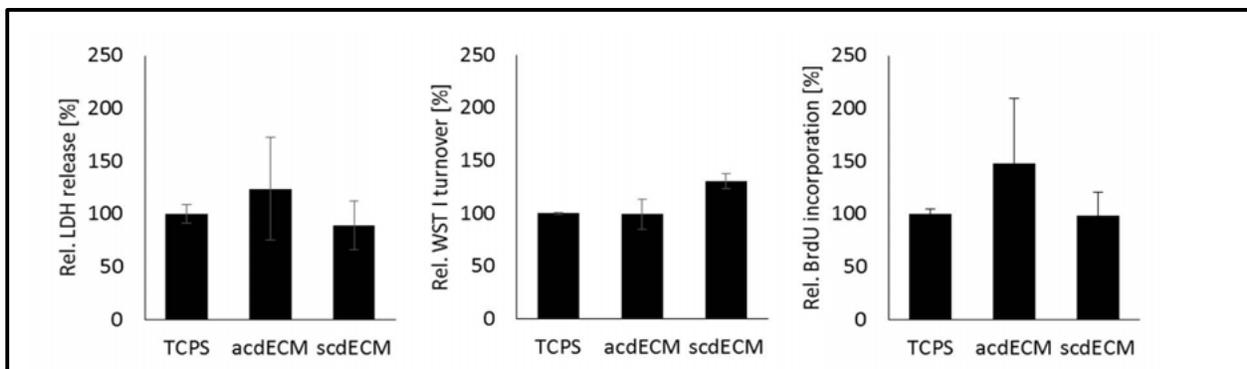


Figure 1: Cellular activity of ASCs cultured on cdECM substrates. ASCs were seeded on top of TCPS, acdECM and scdECM at a density of 2.5×10^4 cells/cm² in GM. At day three LDH, WST I and BrdU assays were performed. None of the assays revealed any influence of cdECM substrates on cell death, metabolic activity or proliferation rate of ASCs.

accumulation can be observed on acdECM and scdECM but not on TCPS. Full supplemented medium led to extensive lipid accumulation on all substrates. Quantification of Oil red O staining was performed at day 14 (Figure 3). No adipogenic differentiation can be observed in GM on all substrates (TCPS: 100.0(±23.0) %; acdECM: 107.6 (± 23.7) %; scdECM: 103.2 (±22.2) %). For insulin supplemented medium a significantly higher lipid accumulation can be found in ASCs cultured on cdECM substrates compared to TCPS (TCPS: 100.0 (± 20.3) %; acdECM: 163.0 (± 18.1) %; scdECM: 150.4 (± 19.5) %). In fully supplemented adipogenic differentiation medium a higher degree of lipid accumulation can be observed on

acdECM compared to scdECM and TCPS (TCPS: 100.0 (± 9.2) %; acdECM: 146.7 (± 9.1) %; scdECM: 91.9 (± 37.3) %).

4 Discussion

The present study confirms cdECM as promising biomaterial for adipose tissue engineering. ASCs cultured in insulin supplemented medium led to a significantly higher lipid accumulation on cdECM substrates compared to TCPS. These results indicate a supporting effect of cdECM on adipogenic differentiation in combination with soluble differentiation factors. The incorporation of lipids, analysed in this study, is a process occurring at a late stage of adipogenic differentiation. Insulin is well known to induce adipogenic differentiation and thus accelerate the supporting effect of cdECM, but does not induce differentiation as strong as fully supplemented adipogenic differentiation medium [8, 9]. These results are in line with Guneta et al. who found the expression of genes associated with adipogenic differentiation in ASCs cultured on cdECM [5]. Interestingly in fully supplemented adipogenic differentiation medium a supporting effect of acdECM on adipogenic differentiation can be found indicating the promising effect of acdECM. The complex composition of the ECM is not fully characterized. Individual components of the ECM like collagen and laminin are widely used in tissue engineering approaches [10]. Next to structural proteins native ECM is also decorated with growth factors and degradation products of the ECM are known to induce cellular response [4]. A major advantage of cdECM over ECM from mature tissue is the independence of yielded ECM amount from the size of donated tissue samples. In conclusion, these results highlight the promising effect of cdECM as biomaterial for adipose tissue engineering by supporting adipogenic differentiation.

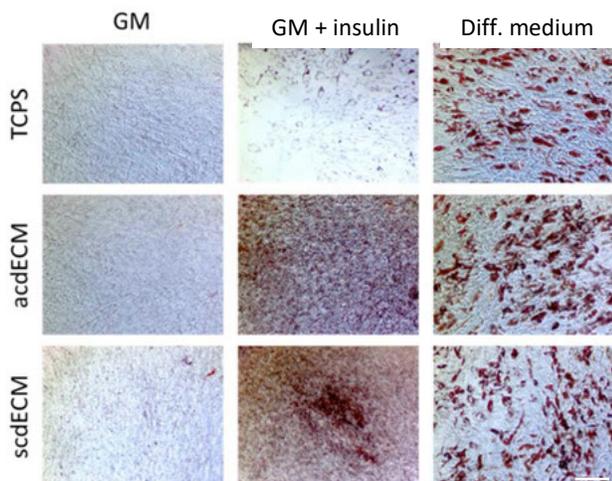


Figure 2: Oil red O staining of ASCs cultured on different substrates. ASCs were seeded on top of cdECM substrates and TCPS at a density of 2.5×10^4 cells/cm² and were cultured in GM, insulin supplemented medium and fully supplemented adipogenic differentiation medium for 14 days. ASCs cultured in GM exhibit no lipid accumulation. ASCs cultured in insulin supplemented medium exhibit higher degree of lipid accumulation on cdECM substrates compared to TCPS. In fully supplemented medium lipid accumulation can be found on all substrates. Scale bar: 200µm

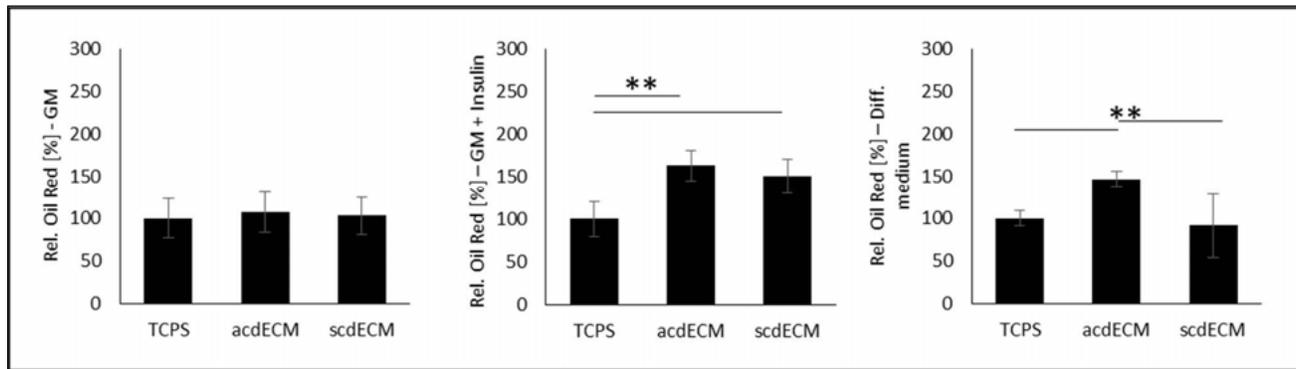


Figure 3: Adipogenic differentiation on different substrates. ASCs were seeded on top of cdECM substrates and TCPS at a density of 2.5×10^4 cells/cm² and were cultured in GM, GM supplemented with insulin and fully supplemented adipogenic differentiation medium for 14 days. In GM no lipid accumulation can be observed on all substrates. In insulin supplemented medium significantly increased lipid accumulation can be found on cdECM substrates compared to controls for both time points. In fully supplemented adipogenic differentiation medium, enhanced lipid accumulation can be observed at day 14 on acdECM. (** $p \leq 0.01$)

Author Statement

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Informed consent: Informed consent has been obtained from all individuals included in this study. Ethical approval: The research related to human use complies with all the relevant national regulations, institutional policies and was performed in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee.

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