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Hydrogels in three-dimensional dendritic cell (MUTZ-3) culture as a scaffold to mimic human immuno competent subcutaneous tissue

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ABSTRACT

The objective of this study was to develop a 3D cell culture model of the human subcutaneous tissue, allowing the prediction of the immunogenicity of subcutaneously injected therapeutic proteins. Several hydrogels were evaluated as scaffolds to mimic the human subcutaneous tissue *in vitro*. Cytocompatibility of the hydrogels with the human myelomonocytic cell line (MUTZ-3) was investigated, as well as their influence on cellular phenotype changes. Elastic Young's moduli in compression of the hydrogels were measured by a texture analyser and compared to *ex vivo* human samples. MUTZ-3 cells were differentiated into dendritic cells before embedding in hydrogels. Agarose at various concentrations (0.5%, 0.35% and 0.25% w/v), Geltrex® matrix and HyStem™ scaffold (1% w/v) displayed a wide range of elastic Young's moduli from 560 kPa to 49 kPa, compared to the reference value of 23 kPa obtained for human tissue. With the exception of HyStem™, good cytocompatibility of hydrogels was shown at the concentrations tested. An optimal combination of MUTZ-3 cells with 0.25% agarose or Geltrex® is suggested.

1. Introduction

Prediction of therapeutic protein immunogenicity is routinely done *in vitro* employing human peripheral blood mononuclear cells (PBMC) stimulation and CD4+ T cells proliferation assays (Jawa et al., 2013). However, use of primary cells necessitates an expensive and time-consuming process to isolate, maintain and differentiate subsets of interest, as well as, to optimize PBMC storage in order to guarantee reproducibility of further analyses. Moreover, those assays are performed in 2D standard cell culture conditions putting new candidate therapeutic proteins in direct contact with the immune cells. This does not necessarily reflect the natural immune response encountered *in vivo* in humans after injection in the subcutaneous tissue. More complex phenomena are involved like potential interactions of the protein with extracellular matrix proteins and ions of the interstitial fluid, or depot effect due to mechanical constraints of the tissue. Of course, *in vivo* studies on animals such as rodents, dogs or pigs are being used (Brinks et al., 2011; Kang et al., 2013), although these models have limitations in their predictive power due to interspecies differences in immune system function. They are now being challenged by the need to replace, reduce and refine the recourse to animal models (3R's principle).

More generally, 3D cell culture is now of increasing interest in attempting to predict the pharmacokinetics, pharmacodynamics and toxicity of drug candidates (Fang and Eglen, 2017; Lelievre et al., 2017; Roth and Singer, 2014). The reason for this being that such complex physiological phenomena may better be mimicked in a 3D cell culture than under standard 2D cell culture conditions (Duval et al., 2017). Several such models exist, comprising co-culture of different cell types in a well insert, organotypic cultures using natural matrices or synthetic scaffolds (Shamir and Ewald, 2014) and cell spheroids formed by using diverse techniques (Verjans et al., 2017). The latter is often carried out using a hydrogel matrix (such as Matrigel®) to provide the mechanical support given *in vivo* by the extracellular matrix.

In this relatively new field, human skin models replicating both epidermis and dermis are in use to test potential skin sensitizers or to find new treatments for burns or wound healing in general (Groeber et al., 2012; Mathes et al., 2014). Most of the skin models include both epidermis and dermis, while full-thickness models including subcutaneous (SQ) tissue are less due to its low significance during the wound healing process. Nevertheless, the increasing number of subcutaneously injected therapeutic proteins, often accompanied by adverse events such as immunogenic reaction and injection site reactions (Murdaca et al., 2013), has triggered a raised interest in better understanding this peculiar tissue (Hendin et al., 2017; Kaiser et al., 2012; Matsui et al., 2017). Recently, an excellent review by Kinnunen and Mrsny (2014) described the composition and physical and physiological

Abbreviations: DC, dendritic cell; MUTZ-3, acute myelomonocytic leukemia cell line; PBMCs, peripheral blood mononuclear cells; SQ, subcutaneous

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properties of the SQ tissue in detail.

In this study, we aim to develop a 3D cell culture model encapsulating the major immune cells found in the SQ tissue, namely sentinel dermal dendritic cells (DCs) (Fathallah et al., 2013), in a simple but well-defined hydrogel scaffold mimicking the SQ tissue mechanical properties. Our objective was to reproduce the (immune) microenvironment into which therapeutic proteins are usually injected in order to better understand their potential *in situ* aggregation, uptake by immune cells and pro-inflammatory cytokines release. We investigated and evaluated the combination of several hydrogels and a human acute myelomonocytic leukemia cell line (MUTZ-3), in terms of cytocompatibility (cell viability) and potential cellular phenotype changes induced by the hydrogels used. Furthermore, elastic Young's moduli of these hydrogels were measured and compared to those of human SQ tissue samples with the intention to achieve a good representation of the mechanical properties of the SQ tissue.

2. Materials and methods

2.1. MUTZ-3 cell line differentiation in dendritic cells

The human acute myelomonocytic leukemia cell line MUTZ-3 (ACC 295) and the human urinary bladder carcinoma cell line 5637 (ACC 35) were purchased from DSMZ (Braunschweig, Germany).

MUTZ-3 cells were maintained in a "routine" cell culture medium without phenol red composed of 60% minimum essential medium α (MEMα) containing ribonucleosides and desoxyribonucleosides, supplemented with 20% heat inactivated fetal bovine serum (FBS), of 5637 cell line-conditioned medium and penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively). All products were obtained from Gibco® (Life Technologies, Grand Island, NY, USA). The 5637 conditioned medium (RPMI 1640 supplemented with 10% FBS and penicillin-streptomycin) was collected after at least 48 h conditioning, centrifuged to remove cell debris and pooled. Aliquots of adequate volume were frozen and stored at −20 °C after sterile filtration through 0.22 µm cellulose acetate low protein binding filters (Corning Inc., Corning, NY, USA). Cell incubation was performed at 37 °C, in an atmosphere of 95% humidity and containing 5% CO_2.

Differentiation of MUTZ-3 into dendritic cells (MUTZ3-DCs) was induced by adding recombinant human granulocyte macrophage–colony stimulating factor (GM-CSF, 100 ng/mL), interleukin-4 (IL-4, 10 ng/mL) and tumor necrosis factor alpha (TNFα, 2.5 ng/mL, all from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to the 80% MEMα supplemented with 20% heat inactivated FBS and penicillin-streptomycin, for 7 days. Cells were seeded at an initial concentration of 2×10^5 cells/mL in a 12-well plate (1 mL/well). The same volume of fresh differentiation medium was added on day 3 (cell dilution 1:2). Finally, in order to check the maturation capacity of the MUTZ3-DCs, high concentration of TNFα (75 ng/mL) was added to the medium and the cell incubated for 48 h. This protocol was adapted from Masterson et al. (2002).

2.2. Flow cytometry analyses

The immunophenotype of MUTZ-3 cells before and after differentiation was analyzed using an Accuri™ C6 cytometer and CFlow® Plus software (Becton-Dickinson Biosciences, San Jose, CA, USA). The cytometer was used at the following settings: one blue laser with an excitation wavelength (λ_{ex}) of 488 nm using three different filters at 533/30, 575/40 and 670LP (λ_{em}/BP); and one red laser with a λ_{ex} of 635 nm using one filter at 675/25. Cells were stained with the following mouse monoclonal antibodies (mAbs): FITC-labeled antihuman-CD14; APC-labeled antihuman-CD83; PE-labeled antihuman-langerin (CD207); and the recombinant PE-Vio770-labeled antihuman-CD209 (DC-SIGN). These antibodies and their corresponding isotype controls were used according to supplier's instructions (Miltenyi Biotec GmbH).

Quickly, 10^5 cells were centrifuged in Eppendorf tubes at $300 \times g$ for 10 min. The supernatant was aspirated and cells were washed with rinsing buffer (DPBS pH 7.2, 0.5% BSA, 2 mM EDTA, degassed and filtered through 0.22 µm cellulose acetate filter) and centrifuged again. Supernatant was discard and cells were resuspended and incubated 30 min at 4 °C with 1 mL per tube of rinsing buffer supplemented with 10% human plasma to avoid non-specific Fc receptor binding of the mAbs. Cells were then centrifuged, resuspended with 100 µL rinsing buffer, and incubated with 10 µL of the appropriate surface antibody or the corresponding isotype control for 10 min in the dark at 4 °C. After a last washing step, cells were centrifuged and resuspended in 1 mL rinsing buffer. As CD207 (Langerin) is expressed intracellularly, the staining was done after cell fixation with 4.2% formaldehyde (w/w) and permeabilization with saponin using Cytofix/Cytoperm™ Kit (Becton-Dickinson Biosciences). Dead cells were excluded by forward and side scatter characteristics, and doublets (cell clumps) were removed using area forward scatter *vs.* height forward scatter. At least 10^4 cells were counted in the "live" gate for each sample.

2.3. MUTZ3-DCs encapsulation in hydrogels

Once differentiated, MUTZ3-DCs were embedded in three different hydrogels: a basement membrane extract Geltrex® Matrix (Gibco®), cross-linked hyaluronic acid HyStem™, and low-gelling temperature agarose (A9045 – Sigma-Aldrich, St. Louis, MO, USA). All the hydrogels were first mixed with 4×10^5 cells/mL as detailed below, poured in flat bottom 96-well plates and once set the same volume of full differentiation medium was added on top. The same procedures were followed for the preparation of the gel blank, and the same proportions of medium or DPBS were used for the standard 2D cell culture conditions used as reference.

The Geltrex® Matrix was slowly thawed overnight in the fridge (5–8 °C) and then kept on ice before mixing at a 1:3 ratio with cells diluted in differentiation medium pre-warmed at 37 °C. The final protein concentration was ≤ 9 mg/mL allowing the gelation within 30 min at 37 °C.

The HyStem™ scaffold kit was prepared according to the manufacturer's instructions. Briefly, hyaluronic acid and crosslinking agent stored at −20 °C were allowed to warm to room temperature for 1 h before being reconstituted and fully dissolved. Cells washed and resuspended in DPBS were then mixed with 1% (w/v) HyStem™ (thiol-modified hyaluronan) by pipetting up and down several time. Finally, thiol reactive crosslinker (PEGDA) was added and the mixture was directly poured into 96 well-plates. Gelation was observed after one hour.

For the preparation of low gelling temperature agarose, a 1% (w/v) stock solution was prepared by dissolving powdered agarose in sterile DPBS pH 7.2 in a boiling water bath, allowed to cool to 37 °C before dilution and addition of the cells. Agarose was used at three different final concentrations of 0.5, 0.35 and 0.25% w/v. Plates were placed in a 25 °C incubator for 1 h for gelation before being transferred to a 37 °C incubator, in a humidified (95%) atmosphere containing 5% CO_2.

2.4. Optical microscopy

In order to visualize eventual changes in the phenotype and morphology of MUTZ3-DCs once embedded in hydrogels, pictures were taken daily over 3 days with an Axiovert 200 inverted microscope (Carl Zeiss, Feldbach, Switzerland) in bright-field, equipped with a QIClick CCD camera (QImaging, Surrey, BC, Canada) using phase contrast objectives to better discriminate differences between cells and hydrogels.

2.5. WST-1 colorimetric viability assay

Viability of cells embedded in hydrogels was assessed by a colorimetric assay using the enzymatic conversion of tetrazolium salts (WST-1) to formazan. Thirty microliter of WST-1 (Roche, Mannheim,