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Test system for evaluating the influence of polymer properties on primary human keratinocytes and fibroblasts in mono- and coculture

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ABSTRACT

Interactions of cells with polymer-based biomaterials are influenced by properties of the substrate. Polymers, which are able to induce cell specific effects, gain increasing importance for biotechnology and regenerative therapies. A test system was developed, which allows studying primary human keratinocytes and fibroblasts in mono- and cocultures to analyze and operate the effect of polymer properties. This system offers to identify polymers for keratinocyte cultivation or wound dressings, since adherence, viability and functionality can be analyzed. Especially the coculture system enables the characterization of potential cell specific effects of polymer-based biomaterials. To establish a coculture test system, it is challenging to find a suitable culture medium, to identify initial seeding densities for comparable cell growth and to develop methods to distinguish and characterize both cell types.

Poly(*n*-butyl acrylate) networks (cP*n*BAs) as model biomaterials were used to demonstrate the applicability of our newly developed co-culture screening system for differential cell growth. The apparent Young's modulus of the cP*n*BAs differentially regulated fibroblasts and keratinocytes. Particularly, cP*n*BA73 with an apparent Young's modulus of 930 ± 140 kPa measured in phosphate buffered saline (PBS) solution at ambient temperature (Yoshikawa et al., 2012) seemed to have favoring properties for keratinocyte adhesion, while fibroblast adhesion was not affected. For keratinocytes the concentration of some pro-inflammatory cytokines was lower on cP*n*BA73 and a decreased deposition of collagen, elastin and fibronectin was observed in the coculture.

1. Introduction

Polymer-based biomaterials are increasingly explored for biotechnological applications and regenerative therapies. Biofunctional polymeric materials, designed to induce cell specific effects, are proposed as next generation of biomaterials. To obtain such cell specificity, chemical and physical properties of polymers have to be adjusted (Scharnagl et al., 2010; Shastri and Lendlein, 2009). The specificity of materials for different cell types can only be tested in coculture systems. For cutaneous cells these are e.g. keratinocytes and fibroblasts. In vivo, keratinocytes of the epidermis and fibroblasts of the dermis are separated by the basement membrane, but communicate by soluble factors (Kanitakis, 2002; Stark et al., 1999). Furthermore, during wound healing their proper interaction plays a critical role (Werner et al., 2007). To isolate keratinocytes from skin biopsies, the epidermal and dermal layer needs to be separated (Aasen and Izpisua Belmonte, 2010). Despite enormous improvements, the cultivation of keratinocytes is still challenging and a substrate favoring keratinocyte adhesion and proliferation over fibroblasts could overcome the need to separate the cell layers before seeding. Because of promoting paracrine interactions fibroblasts can be used as feeder layer for keratinocyte cultures, but their proliferation has to be limited to prevent overgrowth (Jubin et al., 2011; Rheinwald and Green, 1975, 1977). To evaluate potential cell specific effects of polymers aiming applications for the improved cultivation of keratinocytes and for skin regeneration, an appropriate test system would be useful. Systematic coculture studies for the effect of polymers on primary human keratinocytes and human fibroblasts are missing, but are required to clearly identify cell specific effects of the tested polymers. Therefore, a test system for the evaluation of effects on both cell types in mono- and cocultures was developed in the current study.

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Besides the chemical composition of the substrate and the topography (roughness and geometry) (Jiang et al., 2011; Stoppato et al., 2013), the apparent Young's modulus can influence cell adherence, viability and function (Georges and Janmey, 2005). Hydrophobic, transparent poly(*n*-butyl acrylate) networks (cP*n*BAs) in medical grade quality have been developed as potential candidate polymers for active regulation of several cell types. Their apparent Young's modulus can be adjusted in a wide range by variation of its crosslinking density (Cui et al., 2011). In contrast to hydrogel-based biomaterials, the benefit of such hydrophobic polymers is that the apparent Young's modulus is not influenced by exposure to physiological conditions (Braune et al., 2011). cP*n*BA networks with apparent Young's moduli of $E = 120 \pm 10$ kPa (cP*n*BA04) and $E = 930 \pm 140$ kPa (cP*n*BA73) (Yoshikawa et al., 2012) were selected as first model polymers to be analyzed with the new test system (Trescher et al., 2012a). The viability and the density of adherent cells, as well as their ratio in coculture and their functionality in terms of deposition of components of the extracellular matrix (ECM) were analyzed 48 h after seeding the cells on the polymer samples. Since cP*n*BAs are flexible enough to be aligned to body curvatures and their transparency would enable the monitoring of the healing process, they could be potentially used as wound dressings. Therefore also the secretion of different cytokines and growth factors by the cells cultured on the polymers was studied.

2. Materials and methods

2.1. Cultivation of primary human keratinocytes and primary human dermal fibroblasts

Primary human keratinocytes and primary human dermal fibroblasts from adult donors (cryopreserved cells; Provitro, Berlin, Germany) were used for the experiments. Before seeding on the cP*n*BA samples, keratinocytes were cultured in serum free keratinocyte growth medium (KGM adv., Provitro) and fibroblasts in Dulbecco's modified Eagle Medium (DMEM; LifeTechnologies, Darmstadt, Germany) supplemented with 10 vol% fetal bovine serum (FBS) superior (Biochrom, Berlin, Germany). To subcultivate keratinocytes and fibroblasts, the cells were rinsed with phosphate buffered saline without calcium and magnesium ($PBS^{-/-}$; Biochrom) and incubated with a 0.5 wt% EDTA-solution (ethylenediaminetetraacetic acid in $PBS^{-/-}$; Sigma–Aldrich, Munich, Germany) for 10 min. Afterwards, the cells were loosen with 0.025 vol% trypsin/0.053 mM EDTA (ATCC/LGC Standards GmbH, Wesel, Germany). The enzymatic activity of trypsin was stopped for fibroblasts and keratinocytes with DMEM + FBS10% and defined trypsin inhibitor (Invitrogen, Darmstadt, Germany), respectively. After centrifugation fibroblasts were resuspended in DMEM + FBS10% and keratinocytes were resuspended in KGM adv.

For the growth curves and the test system, KGM adv. was used as consistent medium for both cell types in mono- and coculture for resuspension and cultivation.

2.2. Determination of initial seeding densities (pilot study)

Growth curves were performed to determine the baseline seeding densities for keratinocytes and fibroblasts to reach comparable cell numbers per ml or cm^2 in mono- and coculture after 48 h of cultivation.

To determine the optimal growth for monoculture conditions, cells were initially seeded at 1×10^4, 2×10^4 and 3×10^4 cells/cm^2 on glass cover slips (hydrolytic class 1 glass; Ø 14 mm, thickness # 1 = 0.13–0.16 mm; Gerhard Menzel GmbH, Brunswick, Germany) in 24 well plates. After 12, 24, 36, 48, and 60 h, cells were detached from the culture plates and viable cell numbers per ml were determined by the CASY system (Innovatis Systems/Roche Diagnostics, Mannheim, Germany).

To determine the optimal growth for coculture conditions, cells were initially seeded with 5×10^3 cells/cm^2 and 1×10^4 cells/cm^2 of each cell type. After 24, 48, 72, and 96 h cells were stained for keratin 14 and vimentin (see Section 2.4) to clearly identify and count the cell types by ImageJ (National Institutes of Health).

2.3. Polymer networks

Poly(*n*-butyl acrylate) networks (cP*n*BAs) were synthesized by polymerization of *n*-butyl acrylate and a low molecular weight poly(propylene glycol) dimethacrylate (PPGDMA, $M_n = 560$ g mol^{-1}) as crosslinker (Cui et al., 2011). For the synthesis of cP*n*BA04 a PPGDMA content of 0.4 wt% in the starting reaction mixture was applied, and 7.3 wt% PPGDMA for cP*n*BA73. The obtained cP*n*BAs exhibited similar surface properties with advancing contact angles ranging from $\theta_{advancing} = 111–123°$, a surface roughness ($R_q$) below 40 nm and an isoelectrical point at pH 3.7 observed in streaming potential investigations (Hiebl et al., 2012).

The local mechanical properties were determined by an atomic force microscopy (AFM) based indentation method performed in a 10 mM phosphate buffered saline (PBS) solution with pH 7.4 at ambient temperature. Here, apparent Young's moduli of $E = 120 \pm 10$ kPa for cP*n*BA04 and $E = 930 \pm 140$ kPa for cP*n*BA73 were obtained (Yoshikawa et al., 2012). Before biological tests started, all samples were sterilized by gas sterilization using ethylene oxide.

2.4. Cell adherence and viability

Keratinocytes and fibroblasts were seeded on 13 mm diameter disks of both polymers in 24 well plates, either in monoculture (3×10^4 cells/cm^2) or coculture (1×10^4 cells/cm^2 each type, both cell types were seeded as one suspension). The cells were cultured for 48 h in KGM adv. (5% CO_2, 37 °C, humidified atmosphere). Glass coverslips (Menzel) were used as control material.

Cell viability was analyzed by live/dead staining as previously described (Trescher et al., 2012b) using fluorescein diacetate (FDA, 25 μg/ml; Sigma, Munich, Germany) and propidium iodide (PI; 2 μg/ml; LifeTechnologies). FDA visualized viable cells in green and PI the DNA of dead cells in red. Thereafter, one image was scanned from each sample by a confocal laser scanning microscope (cLSM Axiovert 200 M, Zeiss, Jena, Germany) with 5-fold magnification ($n = 3$; see typical image at Fig. 1a). From the densities of viable and dead cells, determined using the ImageJ software, the relative cell density (reflects the density of adherent cells on the respective sample related to the density of adherent cells on the glass control) and viability (density of viable cells × 100/(density of viable cells + density of dead cells)) were calculated.

Additional samples ($n = 3$) were stained for keratin 14, vimentin, and DNA as described previously (Trescher et al., 2012b), as well as keratin 10 (monoclonal rabbit antibody to human keratin 10, ab76318, 1:250 (v/v), Abcam, Cambridge, UK and IgG anti-rabbit Cy5, Jackson Immuno Research, Newmarket, UK, 1:200 (v/v)) in addition. From each sample 5 fields of view were randomly chosen and scanned with the cLSM in 10fold magnification. Keratin 14 was used as a specific marker of undifferentiated (basal) keratinocytes and vimentin was used to detect fibroblasts (Lammers et al., 2009). Thus both cell types could be clearly distinguished on coculture samples (see typical image at Fig. 1b; both cell types stained on glass) and counted with the ImageJ-Software in order to calculate the ratio of keratinocytes to fibroblasts types. In the monocultures the purity of cell population was confirmed by the keratin14/vimentin stain.

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