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A whole-cell biosensor as *in vitro* alternative to skin irritation tests

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ABSTRACT

This study presents the time-resolved detection of chemically induced stress upon intracellular signaling cascades by using genetically modified sensor cells based on the human keratinocyte cell line HaCaT. The cells were stably transfected with a HSP72-GFP reporter gene construct to create an optical sensor cell line expressing a stress-inducible reporter protein. The time- and dose-dependent performance of the sensor cells is demonstrated and discussed in comparison to a label-free impedimetric monitoring approach (electric cell-substrate impedance sensing, ECIS). Moreover, a microfluidic platform was established based on μSlides[0.4]Luer to allow for a convenient, sterile and incubator-independent time-lapse microscopic observation of the sensor cells. Cell growth was successfully achieved in this microfluidic setup and the cellular response to a cytotoxic substance could be followed in real-time and in a non-invasive, sensitive manner.

This study paves the way for the development of micro-total analysis systems that combine optical and impedimetric readouts to enable an overall quantitative characterization of changes in cell metabolism and morphology as a response to toxin exposure. By recording multiple parameters, a detailed discrimination between competing stress- or growth-related mechanisms is possible, thereby presenting an entirely new *in vitro* alternative to skin irritation tests.

1. Introduction

Many chemicals can induce irritant contact dermatitis. Several irritation models ranging from single-cell assays, epidermal equivalents, skin equivalents to excised skin have been described (Netzlaff et al., 2005; Schafer-Korting et al., 2008). Keratinocytes, the predominating cell type of the human skin, are the first to encounter chemicals that come into contact with the skin; they are involved in the immune reaction, for example, by producing cytokines. Therefore, keratinocytes represent a valuable model cell type to screen for dermatological effects in response to skin-damaging agents. However, this does not mean that all dermatological effects of the human skin can be studied within this particular cell culture model, because the three-dimensional tissue architecture of the *in vivo* environment and some of its unique transport pathways, *e.g.* penetration of the *stratum corneum*, are missing. Nevertheless, we need to establish standard operating procedures for future *in vitro* assays whose relevance and reliability should be based on internationally recognized protocols.

Equally important to the type of model used to investigate irritancy is the type of biomarker used as readout parameter. Currently those approaches are in focus that aims to investigate the early irritation response, preferably via real-time monitoring before visible cell damage occurs, to provide an alternative to conventional endpoint measurements (Gibbs, 2009). Among these techniques *in vitro* cell culture in microfluidic devices has attracted considerable interest and has led to a substantial impact on cell biology research (Sung and Shuler, 2010; Yeo et al., 2011).

Different microfluidic platforms have been developed for on-chip cell culture (Zhang et al., 2009), allowing quantitative readouts of cell-based assays to study cellular responses to chemical gradients (Hung et al., 2005) or cell/reagent manipulation (Wang et al., 2008) even for incubator-independent conditions (Petronis et al., 2006).

Using genetically modified cells that carry reporter genes under the control of stress-sensitive promoters provide functional information about the impact of cytotoxic reagents on cell physiology (El-Ali et al., 2006). The use of reporter genes, including their individual merits and limitations, has been extensively reviewed (Ghim et al., 2010; Palmer et al., 2011).

It has been reported repeatedly that inducible heat shock protein (HSP) genes are up-regulated in mammalian cells by very different forms of cellular stress via binding of heat shock factor to the heat shock element (Morimoto, 1998). This strategy of cell

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survival is highly conserved in almost every mammalian cell type and has been successfully used in reporter assays (Ait-Aissa et al., 2000; Migita et al., 2010).

With respect to the detection of skin irritation and the use of keratinocytes it has been described that this cell type shows a significant basal HSP72 expression even without any acute cell stress involved. The HSP72 seems to be "superinduced" by underlying continuous environmental stress factors (Jonak et al., 2006). HSP27 has also been reported to be a key player in apoptosis and differentiation (Sur et al., 2007) and it has been suggested to be a useful sensitive marker for skin irritation (Boxman et al., 2002).

Among different irritation models the recently established KeratinoSens seems to be the most promising tool in toxicology to distinguish skin sensitizers from non-sensitizers. The sensor cell holds a luciferase reporter gene under control of a single copy of the antioxidant response element of the human AKR1C2 gene stably inserted into HaCaT keratinocytes (Emter et al., 2010). The major and crucial drawback of this system is the endpoint measurement due to the use of a luciferase reporter.

Besides monitoring cell stress by means of reporter gene expression, several label-free approaches have been described, among which *Electric Cell-Substrate Impedance Sensing* (ECIS) is the most versatile and established sensor device that uses low amplitude AC signals to measure the electrical impedance of small gold-film electrodes that are deposited on the bottom of the culture dish (Giaever and Keese, 1984). When cells attach and spread on the electrode surface, the impedance increases accordingly as the dielectric cell bodies force the current to flow around the cells. Thus, ECIS reports very sensitively on changes in cell shape and electrode coverage. As the three-dimensional cell shape responds to chemical, biological or physical stimuli, ECIS has become an enormously versatile monitoring device for adherent cells that works label-free and non-invasively with a time resolution down to seconds (Arndt et al., 2004).

In the present study we describe the time-resolved detection of chemically induced stress upon intracellular signaling cascades by using genetically modified sensor cells based on the human keratinocyte cell line HaCaT. The cells were stably transfected with a HSP72-GFP reporter plasmid to create an optical sensor cell line for prescreening applications in cosmetic and pharmaceutical industry. The time- and dose-dependent performance of the sensor cells is demonstrated and will be compared to real-time monitoring using electric cell-substrate impedance sensing (ECIS) measurements as well as endpoint measurements via MTT. Our studies will pave the way to establish combined optical and impedimetric readouts for skin irritation tests.

2. Material and methods

2.1. Cell culture

HaCaT cells (Boukamp et al., 1988) were grown in DMEM Low Glucose medium (Biowest) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (100 μg/ml), and penicillin (100 μg/ml) in a humidified atmosphere/cell culture incubator with 5% CO_2 at 37 °C. With the exception of cytotoxicity assay (MTT) cells were deprived of serum (1% FBS) for at least 24 h prior to treatment with $CdCl_2$ or heat shock (43 °C). For time-lapse microscopy of cells on μSlidesI^{0,4}Luer (Ibidi) DMEM Low Glucose was replaced by CO_2-independent medium.

2.2. Cytotoxicity testing

Cell viability was essentially determined using the MTT assay as described (Mosmann, 1983). 15,000 cells were seeded in each well of a 96-well plate. After 24 h the cells were treated with varying concentrations of $CdCl_2$ (0–1 mM) for 24 h in medium containing either 1% or 10% FBS. After the incubation time the assay was performed as described previously, but dissolving the blue crystals was achieved using DMSO and Soerensen's buffer. Cytotoxicity (in percent) was expressed by dividing the absorbance detected for treated cells by that for untreated controls.

2.3. RNA extraction and two step quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from HaCaT cells using peqGOLD TriFast (Peqlap) according to the manufacturer's protocol. One microgram of total RNA was reversely transcribed using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) applying oligo(dT) primers following the standard protocol. Resulting cDNAs were diluted to a total volume of 100 μl with water (PCR grade). The reaction mixture for quantitative RT-PCR consisted of qPCR GreenMaster (Jena Bioscience), cDNA and 0.2 μM of the specified primers (MWG operon). The protocol included an initial denaturation step at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Data obtained on a Corbett Rotor-Gene 6000 were analyzed with the delta-Cq quantification model (Hellemans et al., 2007) using GAPDH as reference gene. Primer sequences for quantitative RT-PCR were: HSPA1A fwd 5′-ACCACTTCGTGGAGGAGTTCAAGA-3′ and rev 5′-ACGTGTAGAAGTCGATGCCCTCAA-3′; HSPB1 fwd 5′-AACGAGATCACCATCCCAGTCACCTT-3′ and rev 5′-TAAGGCTTTACTTGGCGGCAGTCT-3′; GAPDH fwd 5′-TTCGACAGTCAGCCGCATCTTCTT-3′ and rev 5′-GCCCAATACGACCAAATCCGTTGA-3′. All RT-PCR expression values were determined at least three times from three independent experiments. Data was analyzed by the Student's *t*-test.

2.4. Engineering the sensor cell line

The DNA segment containing the region from nt −165 to +91 of the *HSPA1A* gene was amplified from the human genome by PCR using 5′-AAAAACTCGAGCACTCTGGCCTCTGATTGGT-3′ and 5′-AAAAACCCGGGACAG GTTCGCTCTGGGAAG-3′ as primers. The PCR amplicon was ligated into pJET1.2/blunt. After amplification the plasmid was digested with the restriction enzymes *XhoI* and *XmaI*. The fragment containing the functional promoter region of *HSPA1A* (Morgan et al., 1987) was then subcloned into the *XhoI* and *XmaI* sites of pAcGFP1-1 (Clontech) to create pHSP72$_p$-AcGFP. Stable transfection of the cells was achieved with TurboFect (Thermo Fisher Scientific) according to the manufacturer's protocol and subsequent selection in medium containing 1 mg/ml G418. Resistant cells were harvested and initially screened by fluorescence-activated cell sorting (FACSAria IIU, Becton Dickinson) to eliminate cells that had a constitutively high GFP expression without induction of the functional promoter (negative screening). Cells demonstrating low GFP expression were again maintained under normal culture conditions. Cells were cultured for 2 h at 43 °C in an incubator followed by 12–16 h recovery at 37 °C. Only cells responding to heat shock with a high level of GFP expression were collected for single clone selection (positive screening). Cells were diluted to 30 cells/ml and seeded into 6-well tissue culture plates using culture medium supplemented with 0.5 mg/ml G418. Well-separated, G418-resistant single colonies from each dish were harvested using trypsin-impregnated filter paper (5 mm diameter) and placed in 24-well plates with selective medium including 0.5 mg/ml G418. Inducibility of single cell lines was again checked by microscopy and FACS to select a cell line showing the strongest response to heat shock (2 h, 43 °C) and $CdCl_2$ (6 h, 25 μM).