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Development of complex-shaped liver multicellular spheroids as a human-based model for nanoparticle toxicity assessment *in vitro*

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ABSTRACT

The emergence of human-based models is incontestably required for the study of complex physiological pathways and validation of reliable *in vitro* methods as alternative for *in vivo* studies in experimental animals for toxicity assessment. With this objective, we have developed and tested three dimensional environments for cells using different types of hydrogels including transglutaminase-cross-linked gelatin, collagen type I, and growth-factor depleted Matrigel. Cells grown in Matrigel exhibited the greatest cell proliferation and spheroid diameter. Moreover, analysis of urea and albumin biosynthesis revealed that the created system allowed the immortalized liver cell line HepG2 to re-establish normal hepatocyte-like properties which were not observed under the conditions of conventional cell cultures. This study presents a scalable technology for production of complex-shaped liver multicellular spheroids as a system which improves the predictive value of cell-based assays for safety and risk assessment. The time- and dose-dependent toxicity of nanoparticles demonstrates a higher cytotoxic effect when HepG2 cells grown as monolayer than embedded in hydrogels. The experimental setup provided evidence that the cell environment has significant influence on cell sensitivity and that liver spheroid is a useful and novel tool to examine nanoparticle dosing effect even at the level of *in vitro* studies. Therefore, this system can be applied to a wide variety of potentially hostile compounds in basic screening to provide initial warning of adverse effects and trigger subsequent analysis and remedial actions.

1. Introduction

Cell culture in two dimensions (2D) is the main method of cultivation of cells in thousands of laboratories worldwide for the past decades. However, the culture of cells in two dimensions is primitive and does not reproduce the anatomy and physiology of tissue for informative and useful studies. Moreover two-dimensional cell cultures do not properly represent the functions of the tissues that have specific cell–cell and cell–matrix interactions and completely different transport conditions. Oxygen, nutrient or waste gradients are not present in a monolayer. Coating surfaces with poly-L-lysine, collagen (Niwa et al., 2012), albumin, fibronectin (Dolatshahi-Pirouz et al., 2011; Salmeron-Sanchez et al., 2011) and many other biocompatible materials can mediate more natural basal adhesion; however, cells are still forced into a monolayer morphology. Although establishing co-cultures can increase contact between them (Tape et al., 2014; Tian et al., 2014), the fact that 2D surfaces still inhibit the ability of cells to form multidimensional structures is challenging.

Models for the estimation of cell toxicity must reflect the *in vivo* situation as closely as possible. There is a broad spectrum of three dimensional (3D) cell cultures models that vary widely due to the diverse requirements of different cell lines and applications. Each model comes with its own set of advantages and limitations, and one distinct model is not suitable for all applications (Astashkina and Grainger, 2014; Breslin and O'Driscoll, 2013). Three dimensional cell cultures with spheroid formation is one of the best described models of 3D cell cultures due to its simplicity and similarity to physiological tissues. Spheroids are self-assembled agglomerates of cell colonies that naturally resemble avascular environments with gradients of nutrients, O_2, CO_2 and water soluble wastes (Lin and Chang, 2008; Griffith and Swartz, 2006). Compared to conventional 2D cell cultures, multicellular spheroids better mimic a real tissue. The chemical and physical properties of the cell environment such as wettability, roughness, stiffness, softness (Discher et al., 2005), or microstructure like pore size and pore shape (O'Brien et al., 2005) have been shown to have a crucial effect on the behavior of the cells. Hydrogels with a high water content imitate a natural soft tissue more than any other type of polymeric biomaterials. Toxicological effects are complex and involve a variety of factors. Therefore, it is not surprising that published results on the toxic effects of

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chemicals using *in vitro* 2D and 3D cell culture models showed some differences (Ramaiahgari et al., 2014; Tseng et al., 2015; Sabhachandani et al., 2016; Horning et al., 2008; Sun et al., 2006; Gunness et al., 2013). This fact reveals the importance of cell culture environments for such testing, and indicates that cell dimension is a crucial point for toxicology research.

Nanotechnology is considered as one of the key technologies of the 21st century. The unique physicochemical properties of engineered nanomaterials are attributable to their small size, chemical composition, surface structure, solubility and shape. Indeed, the very same properties that lead to the technical advantages of nanotechnology also lead to distinctive biological effects (Hoyt and Mason, 2008). Therefore, recently studies on the interactions of nanostructures with biological systems have been focused on the relationship between the physical and chemical properties of nanostructures and the induction of toxic biological responses and to determine whether and to what extent these properties may pose a threat to the environment and to human beings (El-Ansary and Al-Daihan, 2009).

Lately, a broad range of studies has been performed to establish *in vitro* models based on cultured human cells as an alternative for traditional animal-based models. Although *in vivo* animal models can mirror many aspects of human responses, they progressively show their limits as well. Interspecies differences in metabolism and responses to the regulatory signals are increasingly recognized (Sivaraman et al., 2005; Rangarajan et al., 2004; Hartung, 2009). Additionally, the European Union insists to reduce, refine and replace experiments on animals (3R principle; EU-Directive 2010/63). This demands the development of human-cell based models for toxicity testing, which may reflect with the best possible efficiency living tissue and give highly predictive results to *in vivo*. Here we present a promising liver multicellular spheroids model as a tool to assess the cytotoxicity of nanoparticles (NPs) in three different environments of hydrogels: gelatin (cross-linked with transglutaminase), collagen type I and Matrigel- to show the significant influence of the microenvironment on toxic effects. Even though a variety of toxicological research on 3D cultures has recently been published, the results were mostly compared with conventional 2D cultures and not with different conditions of 3D cultures (Chia et al., 2014; Wenzel et al., 2014; Tung et al., 2011). Hence there is a lack of information concerning the comparison of various 3D systems regarding the crucial question as to what impact the proper selection of the type of 3D environment has for cells and, thus, for the outcome of cytotoxicity measurements. The choice of environment should be preceded by experiments that allow for the identification of suitable conditions to grow the selected cell line. Because the liver is a major organ for NP accumulation, we examined this aspect by developing a 3D-liver-tissue-spheroids model for the evaluation of NP cytotoxicity. Moreover, the entire system including time point of NPs application was considered to be closer natural conditions. Additionally, to visualize three-dimensional cultures and to show the real 3D effect, were conducted observations of cells on hydrogels under the scaning electron microscope with stereo reconstruction of their shape.

2. Materials and methods

2.1. Cell line

The human hepatoblastoma cell line HepG2 was obtained from Cell Line Service GmbH (CLS Eppelheim, Germany). Cells were maintained in Dulbecco's modified Eagle's medium with low glucose (DMEM) supplemented with 10% (*v*/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin. All mentioned reagents were purchased from Biowest. The cells were kept at 37 °C in a cell incubator under a humidified atmosphere with 5% CO_2. With the exception of cytotoxicity assay (Almar Blue), cells were deprived of serum (1% FBS) for at least 24 h prior to the treatment with chemicals.

2.2. 3D cell culture

Three different hydrogels were developed to generate liver spheroids: Matrigel without growth factors and phenol red (Corning, Netherlands), gelatin type A from porcine skin (Sigma) and collagen type I (Sigma). A stock of Matrigel was prepared in a concentration of 1 mg/ml in DMEM and diluted in ratio 1:1 with cell culture medium before cell seeding. Gelatin solution (10% v/v in DMEM) was sterilized using a syringe filter with 0.22 μm pore size (Carl Roth) incubated 30 min. at 37 °C prior to use to obtain liquid consistency. The gelatin solution was then cross-linked with 1% v/v transglutaminase (100 u/g, Ajinomoto). Collagen gel was obtained by mixing DMEM and collagen type I solution (10 mg/ml in 0.1% acetic acid). pH was adjusted to 7.4 with 1 M NaOH (Sigma). HepG2 cells were mixed with the above mentioned hydrogels and cultured at 37 °C in a cell incubator.

2.3. Metabolic activity of HepG2 cells

Albumin and urea in the cells lysates were measured using the BCG Albumin Assay Kit and the Urea Assay Kit (both from Sigma). Samples were collected after 3, 5, 7 and 10 days of initial cell seeding from 2D and 3D cultures. Changing the culture media every 2–3 days was essential for optimal growth of spheroids in 3D culture and for cells growing in monolayer. Cells were extracted from the hydrogels and dissociated directly before the experiment by using collagenase (400 u/ml) and dispase (4 u/ml) (Gibco). The number of living cells was estimated at each time point using Trypan Blue (Carl Roth) and the data was normalized to 1×10^6 cells. Cultures were briefly washed with PBS and homogenized with urea assay buffer (urea measurements) or Cellytic M/Protease Inhibitor Cocktail (Sigma) (albumin measurements) and analyzed according to protocols provided by the manufacturer. Known quantities of human albumin and urea were used as standards.

2.4. Proliferation assay

The colorimetric assay CellTiter 96 Aqueous One Solution (Promega) was used to determine the number of viable cells. The assay was based on a modified Mosmann's method (Mosmann, 1983). 2×10^4 cells were seeded on the surface of each well of 96-well plates to obtain 2D cell cultures. For 3D cell cultures, cells were first mixed with previously prepared hydrogels and subsequently seeded on well plates. Assay was performed after 1, 3, 7, and 10 days of cultivation of the cells in the 2D and 3D cultures by adding 20 μl of the CellTiter reagent directly to the culture wells. After 3 h of incubation in a cell culture incubator, absorbance was recorded at 490 nm with a plate reader (Tecan). The growth of spheroids was estimated by measuring their diameters (over 16 days) on the images obtained with an LSM 710 microscope (ZEISS) in orthogonal view after staining the nuclei with DAPI (1 μg/ml, AppliChem). An EC Plan-Neofluar 20×/0.50 M27 objective (ZEISS) was used. Error bars represent the standard deviation of three independent experiments with 100 images taken at each time point.

2.5. Assessment of nanoparticle induced cytotoxicity

The cells viability was determined using the Almar Blue Assay (Alfa Aesar). 15,000 cells were seeded in each well of 96-well plates in the form of monolayer or spheroids. After 8 days the cells were treated with varying concentrations of silver nanoparticles (AgNPs, 10 nm, PlasmaChem), zinc oxide nanoparticles (ZnONPs, 25 nm, PlasmaChem) or silicon dioxide nanoparticles (SiO_2NPs, 10 nm, PlasmaChem); sterilized using a syringe filter with a 0.22 μm pore size (Carl Roth). All NPs were sonicated (Bandelin, Sonorex super RK 102H) for 15 min at room temperature directly before use to resuspend formed agglomerates. After 24 h or 72 h of incubation all wells were washed 3 times with PBS and Almar Blue (50 μM) was added. Absorbance was recorded at 540 nm with reference at 630 nm by using a plate reader. Cytotoxicity