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A three-dimensional *in vitro* HepG2 cells liver spheroid model for genotoxicity studies

Ume-Kulsoom Shah[a], Jefferson de Oliveira Mallia[a], Neenu Singh[b], Katherine E. Chapman[a], Shareen H. Doak[a], Gareth J.S. Jenkins[a,*]

[a] *In vitro Toxicology Group, Institute of Life Science, College of Medicine, Swansea University,Singleton Park, Swansea, Wales, SA2 8PP, UK*
[b] *Faculty of Health Sciences and Life Sciences, School of Allied Health Sciences, De Montfort University, The Gateway, Leicester LE1 9BH, UK*

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ABSTRACT

The liver's role in metabolism of chemicals makes it an appropriate tissue for toxicity testing. Current testing protocols, such as animal testing and two-dimensional liver cell systems, offer limited resemblance to *in vivo* liver cell behaviour, in terms of gene expression profiles and metabolic competence; thus, they do not always accurately predict human toxicology. *In vitro* three-dimensional liver cell models offer an attractive alternative. This study reports on the development of a 3D liver model, using HepG2 cells, by a hanging-drop technique, with a focus on evaluating spheroid growth characteristics and suitability for genotoxicity testing. The cytokinesis-blocked micronucleus assay protocol was adapted to enable micronucleus (MN) detection in the 3D spheroid models. This involved evaluating the difference between hanging vs non-hanging drop positions for dosing of the test agents and comparison of automated Metafer scoring with manual scoring for MN detection in HepG2 spheroids. The initial seeding density, used for all experiments, was 5000 cells/20 µl drop hanging spheroids, harvested on day 4, with > 75% cell viability. Albumin secretion (7.8 g/l) and both CYP1A1 and CYP1A2 gene expression were highest in the 3D environment at day 4. Exposure to metabolically activated genotoxicants for 24 h resulted in a 6-fold increase in CYP1A1 enzyme activity (3 µM B[*a*]P) and a 30-fold increase in CYP1A2 enzyme activity (5 µM PhIP) in 3D hanging spheroids. MN inductions in response to B[*a*]P or PhIP were 2-fold and 3-fold, respectively, and were greater in 3D hanging spheroids than in 2D format, showing that hanging spheroids are more sensitive to genotoxic agents. HepG2 hanging-drop spheroids are an exciting new alternative system for genotoxicity studies, due to their improved structural and physiological properties, relative to 2D cultures.

1. Introduction

Evaluation of the genotoxicity of a chemical substance is best performed in models that closely reflect the characteristics of human tissues. Animal testing and 2D cell cultures have provided important insights into human responses to chemicals. However, such results have only limited relevance to the complexity of human tissues [1]. Whereas *in vivo* tissues have 3D geometries, 2D assays involve cell monolayers, leading to different biochemistries; they have non-physiological microenvironments; they rapidly proliferate and de-differentiate [2]; and they may also have abnormal gene expression profiles [3]. On the other hand, it is argued that, due to differences in animal and human physiological, genetic, and metabolic systems [4,5], animal test results may not serve as accurate predictors of human toxic responses [6]. These limitations motivate the development of 3D human tissue models. Regulations made by the EU Registration, Evaluation, Authorization and restriction of CHemicals (REACH, 1999) also stress the importance of the development of new *in vitro* models that can provide reliable results at lower cost and on a shorter time scale compared to *in vivo* tests [7]. Three-dimensional (3D) cell culture assays involve a cell environment promoting direct cell-cell contact as well as an extracellular matrix (ECM) and are thus considered to be more reflective of *in vivo* cellular responses. The optimization of 3D assays with representative human cell lines can therefore provide very suitable

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; AhR, aryl hydrocarbon receptor; B[*a*]P, benzo[*a*]pyrene; BCG, bromocresol green; CBMN, cytokinesis-blocked micronucleus; CBPI, cytokinesis-block proliferation index; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; MN, micronucleus; OECD, Organization for Economic Co-operation and Development; PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine; PI, propidium iodide; RT-PCR, reverse transcription polymerase chain reaction; SE, standard error

* Corresponding author. correspondence should be addressed. Tel.:+ +44 179 260 2512
E-mail address: G.J.Jenkins@swansea.ac.uk (G.J.S. Jenkins).

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conditions for genotoxicity testing.

The liver is an important site in predicting xenobiotic agent-associated toxicity and the potential impact of drug use on liver failure [8]; this makes it a physiologically and clinically relevant tissue for toxicity testing. Primary hepatocytes have liver-specific functions, particularly cytochrome P450 (CYP450) enzymes, and are therefore currently used for chemical compound testing, but they are expensive and have short life spans in culture. Consequently, immortalised, and more cost-effective, cell lines, such as the human hepatocellular carcinoma (HepG2) cell line, are commonly used [9]. HepG2 cells have low levels of CYP450 enzymes and xenobiotic receptors, compared to normal liver cells; this is noteworthy as these enzymes are often required for metabolism of drugs. The presence of basal enzyme activity and the further induction of CYP450 enzymes are required to assess the toxicity of chemical compounds and, therefore, low expression of these enzymes makes this cell line, at best, a dubious predictor of the toxic effects of pro-carcinogens in humans [10]. The development of 3D assays with higher expression of these enzymes provides an opportunity to develop a cost-effective and reliable test. Several 3D cell culture methods are available for assessment of chemical metabolism, e.g., agitation-based approaches, matrices and scaffolds, hanging-drop, and microfluidic cell culture platforms [11,12]. Most of them are expensive and require specialized equipment. In this study, we report the development and optimization of a hanging-drop 3D spheroid model using the HepG2 cell line, as a relatively simple, inexpensive, and reliable test model for genotoxicity studies [13]. These spheroids provide tightly packed 3D multicellular aggregates with enhanced cell-to-cell contact and extracellular matrix components. The major advantages of this model are that it does not require any specialist materials, equipment, or training, and that cells are not in direct contact with an extracellular matrix. In earlier studies, HepG2 liver cancer cells and MCF-7 breast cancer cells were used to produce 3D spheroids which were described as 'tissue-like' [13], These spheroids can survive up to 28 days and have increased expression of xenobiotic-metabolising enzymes, albumin, and others liver-specific markers, when compared to their monolayer cultures [14]. They also show more physiologically relevant expression of genes involved in xenobiotic metabolism, at levels higher than are typically observed in 2D culture systems, which increases 3D spheroids' sensitivity to hepatotoxic compounds [15].

The main objective of this study was the development of a 3D hanging-drop procedure using HepG2 cells to evaluate the genotoxic effects of carcinogenic chemicals. The compounds benzo[*a*]pyrene (B[*a*]P) and 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) were selected on the basis of their ability to form DNA adducts as a result of metabolic activation to a DNA-reactive form [16]. A currently recommended Organization for Economic Co-operation and Development (OECD) 487 [17] guideline for *in vitro* MN tests was selected as the basis for evaluating the reliability of 3D models for genotoxicity investigations and to study the dose-responses of B[*a*]P and PhIP in both 2D and 3D models. CYP450 expression and activity, and albumin production, were used as indicators of the enhanced metabolic capability of the 3D models.

2. Material and Methods

2.1. Chemicals

Benzo[*a*]pyrene (B[*a*]P) (Sigma, UK) and 2-amino-1-methyl-6-phenylimidazo(4,5-b) pyridine (PhIP) (Calbiochem, EMD Chemicals Inc. Germany) were used in this study and stored according to the manufacturer's instructions. B[*a*]P and PhIP were both diluted in DMSO (Fisher Scientific). Fresh dilutions from the master stocks were made for each replicate.

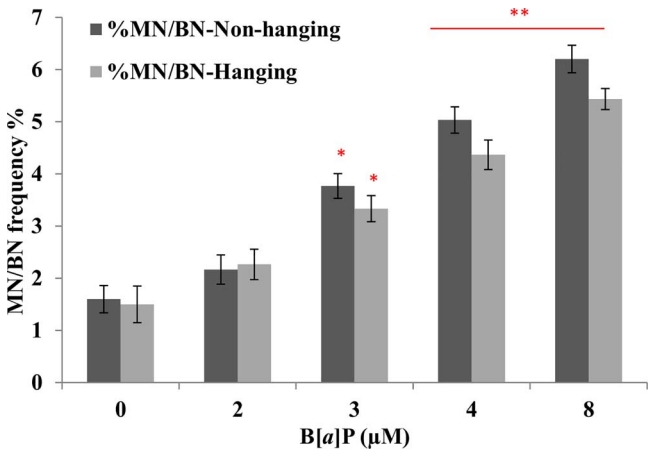

Fig. 1. Comparison between hanging vs. non-hanging position of drops, exposed to B[*a*]P for 24 h. No statistically significant difference was observed between the orientations of drop. The first significant increase in micronucleus frequency P relative to control was observed at 3 μM B[*a*]P. 1,000 binucleated cells were scored manually. Statistically significant results are denoted by * = $p < 0.05$, ** = $p < 0.01$. Error bars are SE; $n = 3$.

2.2. Cell culture

The human hepatocellular carcinoma cell line HepG2, obtained from ECACC, was cultured in DMEM (GIBCO®, Paisley, UK) supplemented with 10% foetal bovine serum (FBS, GIBCO®, Paisley, UK) and penicillin/streptomycin 100X (GIBCO®, Paisley, UK). Sub-culturing or processing of HepG2 cells was performed by trypsinisation with trypsin/EDTA (0.05%) solution (GIBCO®, Paisley, UK). The cells were maintained in culture at a density between 1- 3 × 10^5 cells/ml or cm^2 and sub-cultured every 5 d, until they reached confluency. Cultures were examined using x40 objective on a Zeiss Axiovert 25 light microscope.

2.3. Hanging spheroid preparation from different cell density of HepG2 monolayers

HepG2 monolayers (Fig. 1A) were used to form the hanging spheroids using a hanging drop method. Initially, cell densities of 5000 and 10000 per 20 μl drop of growth medium were made. The drops were placed on the inner side a 9.4 cm Petri dish (Greiner bio-one, UK) lid as shown in (Fig. 1B). Lids contained either 50 or 100 drops, depending on the analysis. The cellular suspension was gently mixed via pipetting to ensure the cells were thoroughly suspended with the media. To prevent the drops from drying, the 9 cm Petri dish was filled with 20 ml PBS (inner compartment). The whole setup was placed very gently in the incubator at 37 °C and 5% CO_2 atmosphere.

The cell viability in the hanging drop was maintained by adding growth medium, 6 μl, to each drop on day 3 and day 6. The light colour of the hanging spheroid (indicator of the live and dead cells colonization) and its size (indicator of cell growth) were observed and measured on day 4 and day 7 using a Nikon Eclipse 50i (Nikon) attached to a computer (Windows 7) using Cellsense software (OLYMPUS). The experiments were done three times, and area/diameter are shown as the mean of three replicates.

2.4. Cell viability of hanging spheroids

To determine the viability of hanging spheroids within the hanging drops, trypan blue was used. Five hanging spheroids were harvested on day 4 and day 7, transferred into a centrifuge tube and centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the cell pellet was re-suspended in 1 ml PBS (GIBCO®, Paisley, UK), centrifuged and again re-suspended in trypsin/EDTA (200 μl). This suspension was incubated for 8 min at 37C, followed by mixing with a pipette to ensure