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Research paper

Tuning liver stiffness against tumours: An in vitro study using entrapped cells in tumour-like microcapsules

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ABSTRACT

Liver fibrosis is a reversible pathology characterized by the up-regulated secretion and deposition of ECM proteins and inhibitors of metalloproteinases, which increase the stiffness and viscosity of this organ. Since recent studies have shown that fibrosis preceded the generation of hepatocellular carcinomas, we hypothesize that liver fibrosis could play a role as a mechanism for restricting uncontrolled cell proliferation, inducing the mortality of cancer cells and subsequent development of primary tumours.

With this purpose, in this work we analysed *in vitro* how the modulation of stiffness can influence proliferation, viability and aggregation of hepatocarcinoma cells ($HepG_2$) embedded in 3D micromilieus mimicking values of elasticity of fibrotic liver tissues.

Experiments were performed by immobilizing up to 10 $HepG_2$ cells within microcapsules made of 0.8%, 1.0% and 1.4% w/v alginate which, besides having values of elasticity from the lower-healthy to the upper-fibrotic range liver tissues, lacked domains for proteases, mimicking the micromilieu existing in hepatic primary tumours.

Our results show that entrapped cells exhibited a short duplication phase followed by an irreversible decay stage, in which cell mortality could be mediated by two mechanisms: mechanical stress, in the case of cells entrapped in a stiffer micromilieu; and mass transfer limitations produced by pore coarsening at the interface cell–matrix, in softer micromilieus.

According to the authors' knowledge, this work represents the first attempt to elucidate the role of liver fibrosis during Hepatocarcinoma pathologies, suggesting that the generation of a non-biodegradable and mechanically unfavourable environment surrounding cancer cells could control the proliferation, migration of metastatic cells and the subsequent development of primary tumours.

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Nomenclature

dX/dt:	Variation of the cell number with the culture time (cell number day^{-1}).
μ:	Proliferation rate constant (day^{-1}).
μ_{app}:	Apparent proliferation rate constant (day^{-1}).
K_d:	Decay rate constant (day^{-1}).
X:	Cell number (n).

1. Introduction

In several liver diseases (e.g. Hepatitis B, C, or Hepatocarcinomas), a very remarkable observation is the variation of the organ's stiffness in early versus late stages of fibrosis and tumour development (Wells, 2008; Georges et al., 2007; Masuzaki et al., 2007; Yeh et al., 2002; Seow et al., 2001).

Liver fibrosis is mostly produced by hepatic stellate cells, which differentiate into fibroblast-like cells, up-regulating the expression of some proteins of the extracellular matrix (mostly collagen Types I, III and IV), and the secretion of tissue inhibitor of metalloproteinases (e.g. TIMP-1). This accumulation results in the liver increasing its stiffness, inducing apoptosis and necrosis in cell-conforming affected tissues (Hemmann et al., 2007; Georges et al., 2007; Malhi et al., 2006).

Hepatocellular carcinoma (HCC) is one of the most recurrent types of cancer, largely attributed to the hepatitis B and C virus infection (Willatt et al., 2008). Recently Gordon-Walker et al. (2010) showed that advanced levels of fibrosis preceded the generation of hepatocellular carcinomas (HCC), indicating that modifications of mechanical properties of the liver could be related to the development of this type of cancer. Moreover, it has been reported that the increased stiffness in the case of relevant liver pathologies (e.g. carcinoma), the modification on the elasticity is not attributed to the tumoural tissue itself, rather than the fibrotic stroma surrounding this tumour (Mazza et al., 2007), suggesting that the fibrotic response could be a potential mechanisms to control the development of primary tumours.

Taking into account that *in vivo* the biophysical characteristics of 3D milieus surrounding malignant cells can influence their viability, proliferation and metabolic activity (Bearer et al., 2009; Butcher et al., 2009; Yamada and Cukierman, 2007), in this work we hypothesize that, in case of HCC, liver fibrosis could play a main role, not as a pathology, but as a biological mechanism for restricting the proliferation and migration of Hepatocarcinoma cells, generating a non-biodegradable and a mechanically unfavourable micromilieu surrounding cancer cells, to induce their apoptosis/necrosis.

Since *in vivo* the tumour microarchitecture is constantly subjected to modifications, it is extremely challenging to generate an experimental system which recreates *in vitro* all these conditions. Nevertheless, several attempts to mimic *in vitro* this 3D microenvironment have already been made by using polymers with similar properties to the extracellular matrix (Liang et al., 2011; Gurski et al., 2010; Ulrich et al., 2010; Hutmacher, 2010; Horning et al., 2008).

In this work we immobilized the hepatocarcinoma cell line $HepG_2$ in microcapsules made of alginate which, besides mimicking values of elasticity from the lower-healthy to the upper-fibrotic range liver tissues, lack domains for proteases (tumour-like microcapsules), allowing us the analysis of cell viability, aggregation, and cell proliferation/decay rates in a similar microenvironment to primary tumours.

According to the authors' knowledge, this is first attempt to elucidate the influence of the mechanical tuning and tissue biodegradability on the surviving of HCC cells, suggesting that liver fibrosis could act as a mechanisms for controlling the proliferation and migration of cancer cells, as well as the subsequent development of primary tumours.

2. Materials and methods

Culture of $HepG_2$: The hepatocarcinoma cell line $HepG_2$ was sourced from the departmental cell bank of the Stem Cell Biology Laboratory, University of Leipzig. $HepG_2$ cells were cultivated in DMEM (Biochrom AG, Germany) supplemented with 15% v/v foetal bovine serum (GIBCO, Scotland, UK), 100 ng/ml sodium pyruvate (Sigma, Germany) and 50 μg/ml Gentamycin (PAA laboratories GmbH, Austria).

Cell encapsulation: $HepG_2$ cells were immobilized in 0.8%, 1.0% and 1.4% w/v Alginate–$CaCl_2$ microcapsules according to methods described previously (Leal-Egaña et al., 2006, 2010). Briefly, after trypsinization cells were seeded in a filter of 40 μm diameter (BD Falcon, USA) with the purpose of avoiding the immobilization of aggregates. A commercially available encapsulation system (Innotech, IE-50R) with a 250 μm nozzle was used. This system produces capsules with a diameter of up to 500 μm. In all cases, the initial number of immobilized $HepG_2$ per mL was $1.5 \cdot 10^5$, which corresponds to approximately 10 cells per capsule. The viability observed for the immobilized cells before the process of encapsulation was determined by Tripan Blue exclusion method. In all cases, the measured viability exceeded 95%.

Experimental system: Experiments were carried out over 8 days in a 6 well plate placed inside an incubator with controlled temperature (37 °C), CO_2 (5%), humidity (95%) and constant agitation (150 rpm). Each well contained approximately 3000 capsules immersed in 3 mL of culture media, which was changed once a day. Every experiment was repeated at least three times. Statistical analyses were performed according to the publication of Cumming et al. (2007). During the whole experiment, no damaged capsules or cells leaving a capsule were observed.

Analysis of cell proliferation: For cell proliferation the rate of resazurin reduction was analysed according to methods previously reported (Leal-Egaña et al., 2010). Briefly, the method is based on the transformation of resazurin into the fluorescent product resorufin (λ_{ex} 579 nm and λ_{em} 584 nm) by living cells, which can be measured using a UV–Vis spectrophotometer (Tecan Safire; Germany).

Determination of proliferation rate constant: Cell proliferation and decay rate constants were determined using the cell viability assay "Cell Titer-Blue" (Promega, USA) according to the protocol described by Leal-Egaña et al. (2010). Values of μ_{app} and K_d, were obtained after fitting curves to the