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Three Dimensional Mixed-Cell Spheroids Mimic Stroma-Mediated Chemoresistance and Invasive Migration in hepatocellular carcinoma

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Abstract

Interactions between cancer cells and cancer-associated fibroblasts (CAFs) within the tumor microenvironment (TME) play an important role in promoting the profibrotic microenvironment and epithelial-mesenchymal transition (EMT), resulting in tumor progression and drug resistance in hepatocellular carcinoma (HCC). In the present study, we developed a mixed-cell spheroid model using Huh-7 HCC cells and LX-2 stellate cells to simulate the *in vivo* tumor environment with respect to tumor-CAF interactions. Spheroids were cultured from cancer cells alone (monospheroids) or as a mixture (mixed-cell spheroids) in ultra-low-attachment plates. Compact, well-mixed, and stroma-rich mixed-cell spheroids were successfully established with heterotypic cell-cell contacts shown by the presence of gap junctions and desmosomes. Mixed-cell spheroids showed enhanced expression of collagen type-I (Col-I) and pro-fibrotic factors such as, transforming growth factor beta1 (TGF-β1), and connective tissue growth factor (CTGF) compared to the levels expressed in mono-spheroids. The EMT phenotype was evident in mixed-cell spheroids as shown by the altered expression of E-cadherin and vimentin. Differential drug sensitivity was observed in mixed-cell spheroids, and only sorafenib and oxaliplatin showed dose-dependent antiproliferative effects. Simultaneous treatment with TGF-β inhibitors further improved sorafenib efficacy in the mixed-cell spheroids, indicating the involvement of TGF-β in the mechanism of sorafenib resistance. In 3D matrix invasion assay, mixed-cell spheroids exhibited fibroblast-led collective cell movement. Overall, our results provide evidence that mixed-cell spheroids formed with Huh-7 and LX-2 cells well represent HCC tumors and their TME *in vivo* and hence are useful in studying tumor-stroma interactions as mechanisms associated with drug resistance and increased cell motility.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and third most common cause of cancer-related mortality worldwide, and the incidence rate is increasing continuously [1]. The treatment modalities include surgical resection, liver transplantation, transarterial

Abbreviations: CTGF, connective tissue growth factor; Col-I, collagen type-I; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; TGF-β1, transforming growth factor beta1; TME, tumor microenvironment.

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chemoembolization, and systemic chemotherapy in patients with advanced HCC. However, conventional chemotherapy cannot be considered a standard-of-care option for advanced-stage HCC due to low survival advantage as demonstrated in a large, randomized trial [2, 3]. HCC is well known for its intrinsic resistance to systemic cytotoxic chemotherapy and local regional therapies [4]. Sorafenib (multikinase inhibitor), the only molecular targeted drug approved for advanced-stage HCC, provides a survival advantage of only 3 months [5]. Such a poor response has been attributed to a number of key factors including abnormal tumor microenvironment (TME), induction of epithelial-mesenchymal transition (EMT), HCC heterogeneity, activation of multiple signaling pathways, and epigenetic dysregulation [6]. Among these factors, TME is being widely studied with respect to its major role in cancer progression and therapeutic resistance [7, 8]. Hence, the TME has been widely utilized as a marker for the identification of novel therapeutic targets and agents, and several TME-targeting drugs are in clinical trials to determine their potential to improve therapeutic efficacy for HCC [9].

The HCC TME comprises cancer and stromal cells, including cancer-associated fibroblasts (CAFs), hepatic stellate cells (HSCs), immune and inflammatory cells, and endothelial cells [10]. CAFs are the crucial components of TME that promote HCC growth and progression by secreting several cytokines and growth factors. CAFs can be derived from different sources such as resident and bone marrow-derived fibroblasts, but HSCs constitute the major source of activated CAFs in the HCC TME [11, 12]. Following activation, HSCs secrete significant amounts of growth factors including transforming growth factor β1 (TGF-β1), connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF) into the TME. These factors promote activation of both HSCs and HCC cells *via* autocrine and paracrine mechanisms [13, 14]. Bidirectional cancer-stroma activation leads to enhanced cancer cell proliferation, excessive ECM synthesis, EMT and invasion, as well as drug resistance [15]. Targeting HCC-HSC cell interactions has already shown promise for HCC growth suppression in various models; therefore, stellate cells are implicated as a key component of future preclinical drug screening models designed to develop new and effective anti-HCC therapies [14, 16].

Several animal models (ectopic, orthotropic, and genetically engineered) have been developed to study HCC pathogenesis and investigate the outcomes of potential therapies; however, the high cost as well as the prolonged time period required for their implementation and, most importantly, the lack of availability of human fibroblasts limit their usefulness as efficient preclinical models [17]. *In vitro* two-dimensional (2D) co-culture models show the tumor-CAF interactions [18] but lack the potential to accurately mimic the *in vivo* TME; thus, three-dimensional (3D) *in vitro* models have emerged as promising tools for this purpose. Tumor spheroids are now commonly used 3D models, which retain the *in vivo* tumor conditions in terms of morphology, functional phenotype, and specialized microenvironment [19]. These structures exhibit numerous *in vivo* features that make them suitable for use in HCC development studies [20, 21]. 3D co-culture models of liver, breast, and pancreatic cancer established by incorporating cancer and stromal cells have been used to verify the role of stromal cell-mediated phenotypic alterations such as EMT and enhanced mobility that ultimately cause drug resistance [22–25].

In this study, we successfully established a stoma-rich 3D mixed-cell spheroid model by culturing Huh-7 (HCC cell line) and LX-2 (HSCs) cells. We then used this model to demonstrate the role of HSCs in establishing *in vivo*-like HCC TME characteristics including a profibrotic microenvironment and EMT phenotype. The mixed-cell spheroid model was validated by low drug activity and enhanced cell migration. Our findings show that this stroma-rich 3D mixed-cell spheroid model can be considered a clinically relevant *in vitro* HCC tumor model for the investigation of novel stroma-related mechanisms involved in drug resistance and enhanced cell migration and to develop effective anti-HCC therapies.

Materials and Methods

Reagents

Huh-7 cells (HCC cell line) were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB), Tokyo, Japan. LX-2 cells (human HSC cell line) were provided by Dr. S. L. Friedman (Mount Sinai School of Medicine, NY, USA). LX-2 cells were developed by spontaneous immortalization of primary HSCs and can be maintained for minimum 50 passages. LX-2 cells showed to express α-SMA, vimentin, and several other profibrotic factors when cultured under low serum conditions [26]. LX-2 cells and Huh-7 cells were maintained in DMEM (Welgene, Daegu, Korea) supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, 250 ng/ml amphotericin B, and 5% and 10% heat-inactivated fetal bovine serum (Welgene, Daegu, Korea), respectively, in a humidified atmosphere (5% CO_2/95% air) at 37°C. Drugs used in present study include sorafenib (Biovision, CA, USA), oxaliplatin (Hanmi Pharmaceutical, Seoul, Korea), gemcitabine (Korea United Pharm Inc., Seoul, Korea), 5-fluorouracil (5-FU) (Sigma-Aldrich, St. Louis, USA), doxorubicin (Korea United Pharm Inc., Seoul, Korea), TEW-7197 (a TGF-β1 inhibitor, provided by Dr. D.K. Kim, Ewha Womans University, Korea), and pentoxifylline (Sigma-Aldrich). The acid phosphatase (APH) substrate p-nitrophenyl phosphate (PNPP) was obtained from Thermo Fisher Scientific (Rockford, USA). All other chemicals, including the cell tracker PKH26 red fluorescent cell linker kit, were obtained from Sigma-Aldrich unless noted otherwise.

Culture and Analysis of Tumor Spheroids

A liquid overlay technique was used to generate tumor spheroids in 96-well ultra-low-attachment (ULA) plates (Corning, MA, USA). Mixed-cell spheroids were generated by seeding Huh-7 and LX-2 cells at a 1:3 ratio (750: 2250) in ULA plates and incubating for 5 days with daily media changes. Monospheroids were generated by seeding 750 cells of Huh-7 or 2250 cells of LX-2. For mixed-cell spheroids, the mixing ratio of 1:3 (Huh-7: LX-2) was selected based on our preliminary data as well as literature data [27]. For cell tracking experiment, LX-2 cells were stained with cell tracker PKH26 (cell membrane binding dye), prior to mixing with Huh-7 cells, using a standard protocol provided by the manufacturer. The stability of PKH26 loading was confirmed in our preliminary test for cell tracking experiments (data not shown). For preparation of histological sections, spheroids were embedded in OCT compound for cryosections, or fixed in 3.7% formaldehyde and subjected to routine tissue processing for paraffin sections.

Human Tumor Tissue and Xenograft

Clinical study protocols were approved by the Institutional Review Board (project number MC16SISI0027) at the College of Medicine, The Catholic University of Korea. Five tissue samples of human HCC (three HCC and two mixed hepatocellular and