Morphological changes and molecular expressions of hepatocellular carcinoma cells in three-dimensional culture model

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A B S T R A C T

Metastatic processes of hepatocellular carcinoma (HCC) are highly associated with the breakdown of extracellular matrix (ECM). However, the regular two-dimensional (2D) culture system, in which only little ECM is involved, fails to provide a well-defined microenvironment for HCC functional research. HAb18G/CD147, a HCC-associated antigen, plays important roles in HCC progression, migration and invasion. In this study, we investigated whether HAb18G/CD147 enhanced the HCC migration and invasion in three-dimensional (3D) culture model through affecting the key molecules and enzymes involved in the metastatic processes, such as focal adhesion kinase (FAK), matrix metalloproteinases (MMPs) and cytoskeleton proteins. We found that, compared with those in 2D cell culture model, the expression of HAb18G/CD147 was significantly increased in 3D cell culture model, together with a high production of MMPs (P<0.01), an enhanced expression and activation of FAK (P<0.01) and a changed distribution of F-actin. In addition, the expressions of paxillin and E-cadherin, which enhance the adhesion and migration potentials, were also significantly increased in 3D cell culture model (P<0.01). All the results suggest that the enhanced expressions of HAb18G/CD147, MMPs, paxillin and FAK changed the distributions of cytoskeleton in the 3D reconstituted basement membrane (BM) and increased the adhesion and invasion potentials of HCC cells. © 2009 Elsevier Inc. All rights reserved.

Introduction

Hepatocellular carcinoma (HCC) is a highly malignant tumor characterized by rapid progression, poor prognosis, and frequent tumor recurrence (Xu et al., 2007b). The high potentiality of migration and invasion of HCC is closely correlated with the breakdown of extracellular matrix (ECM). The regular two-dimensional (2D) cell culture system with the involvement of very little ECM fails to provide a well-defined microenvironment for HCC functional research in contrast to the complex host environment provided by an in vivo model (Kim, 2005). Thus, new functionally relevant three-dimensional (3D) cell culture models have been established by using ECM-like biomatrices, such as collagen I (rat tail collagen) and reconstituted basement membrane (BM, Matrigel), etc. (Weaver et al., 1995). The 3D cell culture models provide a well-defined in vitro microenvironment for studying cell–cell and cell–ECM interactions (Petersen et al., 1992).

Abbreviations: HCC, hepatocellular carcinoma; ECM, extracellular matrix; 2D, two-dimensional; 3D, three-dimensional; BM, basement membrane; FAK, focal adhesion kinase; F-actin, filamentous actin; MMP, matrix metalloproteinase; p-FAK, phospho-FAK.

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Some key focal adhesion proteins and adherent junctions have been revealed to regulate the focal adhesion dynamics in response to the microenvironment stimuli (Brown et al., 2002; Romer et al., 2006; Webb et al., 2002). For example, paxillin, a multi-domain adaptor focal adhesion protein, scaffolds a number of signaling molecules such as kinases and other adaptor proteins as well as binding to cytoskeleton proteins (Turner, 2000). Its primary function is to integrate and disseminate signals from integrins to modulate filamentous actin (F-actin) dynamics, assembly/disassembly of focal adhesion, and cell functions (Brown and Turner, 2004). FAK, another key focal adhesion protein, also colocalizes with integrins in focal adhesions and is activated during cell–ECM interaction (Richardson and Parsons, 1996). A line of evidence has shown that the FAK signal pathway in 3D culture model differed from that in 2D culture model as well as being dependent on the 3D substrate (Cukierman et al., 2003). Cadherin, an important adherent junction protein, interacts with the catenin family of intracellular proteins that provide anchorage to the actin cytoskeleton (Aberle et al., 1996). Cadherin expressions are required for the assembly of cells into solid tissues and importantly, they are expressed with a tissue-specific pattern (Shapiro et al., 1995; Takeichi, 1991). 3D cell growth may induce cell adhesion through affecting the expressions of such adhesion molecules and the redistribution of cytoskeleton, which may further enhance the motivation and invasion process of cancer cells.
Matrix metalloproteinases (MMPs), a major family of enzymes degrading ECM, are capable of disrupting the BM and cleaving the ECM components (Bhowmick and Moses, 2005; Egeblad and Werb, 2002; Liotta and Kohn, 2001). In many tumors, MMP expressions are mainly regulated by tumor–stroma interactions via CD147, a highly glycosylated cell surface transmembrane protein belonging to the immunoglobulin superfamily (Toole, 2003). The expression of CD147 is elevated in HCC and is correlated with the histopathologic malignancy grading (Xu et al., 2007c). We previously cloned a hepatoma-associated antigen HAb18G, which has an identical nucleotide acid and amino acid sequence to superfamily (Toole, 2003). The expression of CD147 is elevated in HCC cell surface transmembrane protein belonging to the immunoglobulin family, which has an identical nucleotide acid and amino acid sequence to CD147, by screening human HCC cDNA library using hepatoma monoclonal antibody HAb18 (Chen et al., 2001; 2002; Liu et al., 1991).

Our recent study based on 2D cell culture system showed that HAb18G/CD147 has important functions in HCC progression, migration and invasion (Xu et al., 2007a). In order to overcome the disadvantage of 2D cell culture system in studying HCC migration and invasion, the present study aims to investigate the expressions of HAb18G/CD147 and focal adhesion molecules, and the morphological changes of human HCC cells in 3D cell culture model.

Materials and methods

Cell culture

Human hepatoma cell line SMMC-7721 was obtained from the Institute of Cell Biology, Academic Sinica. For 2D cell culture, SMMC-7721 cells were cultured in monolayer in T-flask with RPMI 1640 medium (GIBCO, New York, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, New York, USA), 1% penicillin/streptomycin and 2% L-glutamin, at 37 °C in a humidified atmosphere of 5% CO2. For 3D cell culture, freshly harvested SMMC-7721 cells were resuspended in Matrigel (BD Biosciences, San Jose, CA) with a final density of 1×105 cells/cm3 at a temperature of 0 °C. The cell-containing substrata were gelled (45–60 min, 37 °C) and then covered with RPMI 1640 medium containing 10% FBS, 1% penicillin/streptomycin and 2% L-glutamin. After 6–10 days of culturing, the majority of cells were clustered (Weaver et al., 1997). For the observation of morphogenesis, photos were taken by invert phase-contrast microscope (Olympus CX41, Tokyo, Japan) every other day.

Indirect immunofluorescence

SMMC-7721 cells were cultured in 3D matrix for 6 days. Then, the cell-containing substrata were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at RT, quenched with 0.1 M glycine, and equilibrated first with sucrose and subsequently with Tissue-Tek OCT compound (McCormick, Norcross, USA) before freezing in a dry ice/ethanol bath. 10 μm sections was cut with a cryotome (LEICA CM1900, Wetzlar, Germany), collected on APES-coated (Maixin, Fuzhou, China) glass slides and stored at −70 °C. For relevant antibody immunostaining, the prepared frozen sections were washed 3 times with PBS and air-dried. For 2D monolayer culture, SMMC-7721 cells were grown on acid-washed glass coverslips 1 day before immunostaining.

For F-actin staining, the cells were permeabilized for 5 min in 0.1% Triton X-100, blocked with 5% normal goat serum (30 min, 37 °C) and then incubated with TRITC-phalloidin (1:40, Invitrogen, Carlsbad, USA) for 20 min in the dark at RT. The nuclei were counterstained with DAPI (Biotium, Hayward, USA) for 1 min in the dark at RT.

For FAK, phospho-FAK (p-FAK) and paxillin staining, the permeabilized cells were blocked with 5% normal goat serum (30 min, 37 °C) and respectively incubated (1 h, 37 °C) with monoclonal mouse anti-human pY397-FAK (1:100, BD Biosciences, San Jose, CA), monoclonal mouse anti-human FAK (1:100, BD Biosciences, San Jose, CA) and monoclonal mouse anti-human paxillin (1:500, BD Biosciences, San Jose, CA), and subsequently incubated (30 min, 37 °C) with FITC-conjugated goat anti-mouse secondary antibody (1:500, Pierce Biotechnology, Rockford, USA). The nuclei were counterstained with DAPI (Biotium, Hayward, USA) for 1 min in the dark at RT.

For HAb18/CD147 and E-cadherin staining, fixed cells were blocked with 5% normal goat serum (30 min, 37 °C) and respectively incubated (1 h, 37 °C) with monoclonal mouse anti-human pY397-FAK (1:100, BD Biosciences, San Jose, CA), monoclonal mouse anti-human FAK (1:100, BD Biosciences, San Jose, CA) and monoclonal mouse anti-human paxillin (1:500, BD Biosciences, San Jose, CA), and subsequently incubated (30 min, 37 °C) with FITC-conjugated goat anti-mouse secondary antibody (1:500, Pierce Biotechnology, Rockford, USA). The nuclei were counterstained with DAPI (Biotium, Hayward, USA) for 1 min in the dark at RT.

Finally, the cells were mounted using glycerol and analyzed using a laser scanning confocal microscope (Olympus FV1000, Tokyo, Japan).

Western blotting

SMMC-7721 cells in 3D cell culture model were firstly recovered by cell recovery solution (BD Biosciences, San Jose, CA) at 0 °C, and then harvested in a RIPA lysis buffer (Beyotime, Haimen, China). Finally, equal
Amount of cellular proteins were loaded onto 12% SDS-PAGE for electrophoresis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Pierce Biotechnology, Rockford, USA) and blots were probed with HAb18 mAb (0.5 μg/ml, developed in our laboratory), mouse anti-human FAK mAb (1:1000, BD Biosciences, San Jose, CA) and mouse anti-human paxillin mAb (1:10,000, BD Biosciences, San Jose, CA), respectively. β-actin was chosen as internal control and the blots were probed with mouse anti-actin mAb (1:1000, Chemicon International Inc., Temecula, CA). After washing with Tris-buffered saline/0.1% Tween 20 and incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, Pierce Biotechnology, Rockford, USA) for 1 h, immunodetection was performed by using the Western-Light chemiluminescent detection system (Amersham Bioscience, Piscataway, USA).

Fig. 2. Distribution of F-actin in SMMC-7721 cells in 2D (left) and 3D (right) culture models analyzed by laser scanning confocal microscopy. Magnification: ×600.

Fig. 3. Confocal images of HAb18G/CD147 (A), the expression of HAb18G/CD147 protein (B) and the gray scale analysis of HAb18G/CD147 (C) in SMMC-7721 cells in 2D and 3D cell culture models. The expressions of HAb18G/CD147 protein were tested by using Western blot analysis and normalized by human β-actin expression respectively. Results are the means ± SD from three independent experiments, each with triplicates. *, P < 0.01, t test. Magnification: ×400.
After 8 h culturing in 2D and 3D cell culture systems with serum-free medium, conditioned medium was collected and separated on 12% acrylamide gels containing 0.1% gelatin (Sigma, St. Louis, USA). The gels were incubated in 2.5% Triton X-100 solution at RT with gentle agitation and then soaked in reaction buffer (0.05 mol/l Tris–HCl [pH 7.5], 0.2 mol/l NaCl, and 0.01 mol/l CaCl₂) overnight at 37°C. The gels were stained with Coomassie blue and destained in a solution of 7.5% acetic acid and 5% methanol. The zones of gelatinolytic activity were shown by negative staining.

**Statistical analysis**

Results are expressed as mean values ± SD and representative for at least three independently performed experiments. Statistical significance between the different values was analyzed by Student’s t test for unpaired data and was set at \( P < 0.05 \) (SPSS 13.0 statistical software).

**Results**

**Morphological changes of SMMC-7721 cells in 3D cell culture model**

Profound differences in morphology became evident after only 1 day following their culturing within a 3D reconstituted BM (Fig. 1A). Within 8 days, SMMC-7721 cells formed monolayer, anchorage-dependent and polygon colonies in 2D cell culture, while in 3D cell culture model, SMMC-7721 cells gradually formed large, spheroidal and unpolarized invasive colonies along the culture process (Fig. 1). In addition, SMMC-7721 cells formed filopodia similar to tentacles on the surface of the colonies in 3D cell culture model after 5 days (Figs. 1C and D).

**Distributions of F-actin in two different cell culture models**

F-actin distributions have been used to characterize the relative intensity of the cell–cell and cell–substratum interactions experienced by hepatocytes (Tzanakakis et al., 2001). In 2D monolayer cell culture model, intense F-actin stress fibers distributed throughout the SMMC-7721 cells (Fig. 2, left) indicating that there were strong cell–substratum interactions for SMMC-7721 cells forming anchorage-dependent colonies under this condition. However, F-actin stress fibers surrounded the SMMC-7721 cells surfaces in 3D cell culture model (Fig. 2, right). The results suggested that there were not only cell–substratum interactions but also cell–cell interactions in 3D cell culture model.

**Expression of HAb18G/CD147 in 3D cell culture model**

As shown in Figs. 3B and C, the expression of HAb18G/CD147 was significantly increased in 3D cell culture model by more than 4-fold as compared with that in 2D culture model (\( P < 0.01 \)). Similar results were obtained by laser scanning confocal microscopy analysis (Fig. 3A).

**Expression of paxillin in 3D cell culture model**

To determine whether the 3D culture model affected focal adhesion, the expression of paxillin was detected. As shown in
Figs. 4B and C, the protein expression of paxillin was significantly elevated in 3D cell culture model with more than 5-fold in SMMC-7721 cells as compared with that in 2D culture model ($P<0.01$). Similar results were obtained by using laser scanning confocal microscopy analysis (Fig. 4A).

**Expressions and activation of FAK in two different cell culture models**

FAK, another key focal adhesion molecule, was also detected to determine whether the 3D culture model affected focal adhesion. Both the laser scanning confocal microscopy and Western blot analysis showed that the protein expression of FAK was obviously increased in 3D cell culture model (Fig. 5). The expression of FAK was increased more than 2-fold in SMMC-7721 cells in 3D model compared with that in 2D model ($P<0.01$). Activities of FAK are regulated by a complex set of phosphorylation sites (Schaller et al., 1994). The expression of p-FAK was tested by laser scanning confocal microscopy, too. As shown in Fig. 5, the punctuate p-FAK cluster signals were also stronger in 3D cell culture model than that in 2D model.

**Expression of E-cadherin in two different cell culture models**

The E-cadherin expression is a specific indicator of cell–cell interaction during the formation of 3D colonies. As shown in Fig. 6, E-cadherin was highly expressed at the cell–cell boundary in 3D cell culture model (red arrow), leading to the formation of new spheroidal colonies. However, in 2D cell culture model, SMMC-7721 cells expressed E-cadherin sparingly and did not form 3D colonies (Fig. 6).

**Secretion of MMPs in 3D cell culture model**

To determine whether the 3D culture model affected MMP secretion, SMMC-7721 cells were respectively cultured in 2D and 3D models, and the secretion levels of MMPs were detected by gelatin
Fig. 6. Expression of E-cadherin in SMMC-7721 cells in 2D and 3D culture models analyzed by laser scanning confocal microscopy. The expression of E-cadherin was obviously elevated at the cell–cell boundary in 3D cell culture model (red arrow). Magnification: ×400.

Fig. 7. MMP productions of SMMC-7721 cells at the 2nd day (A), 3rd day (B), 4th day (C) and 5th day (D) were tested by gelatin zymography analysis in 2D and 3D cell culture models successively. The gray scale analysis of pro-MMP2 (E), MMP2 (F), pro-MMP9 (G), MMP9 (H) and total MMPs (I) in two different cell culture models. Values are the means ± SD from four independent experiments, each with triplicates. *, P<0.01, t test.
zymography at different time points. As shown in Fig. 7, The MMPs secretion was significantly and abidingly increased in SMMC-7721 cells in 3D cell culture model. Total MMPs production of SMMC-7721 cells was elevated more than 80-fold in 3D model as compared with that in 2D model (Fig. 71) (P<0.01).

Discussion

The previous study suggested that certain cytoarchitecture features of normal tissue cells and malignant tumor cells were lost in 2D culture (Weaver et al., 1995). To simulate the 3D condition of the tissue microenvironment for culture experiments, several ECM-like biomatrices have been used. In the present study, a 3D reconstituted BM was used to establish a 3D HCC cell culture model and the reconstituted BM finally determined the cellular phenotypes following culture progress. There were remarkable differences in morphology between the 2D and 3D cell culture models in this study. SMMC-7721 cells grew inside the reconstituted BM, attached BM and formed three-dimensional structures, thus simulating the colonies of an in vivo model. None of phenotypes described above appeared in 2D monolayer cell culture model. We also found that the organization and distribution of F-actin cytoskeleton had obvious changes in 3D cell culture model. The reorganization and redistribution of F-actin was consistent with the change of morphological characteristics.

HAb18G/CD147 is particularly over-expressed on the surface of malignant tumor cells and enhances the production of various MMPs by stimulating fibroblasts and endothelial cells, but it also acts in an autocrine fashion to increase MMP synthesis and promote the invasion of tumor cells themselves (Jiang et al., 2001; Xu et al., 2007c). Our previous studies have indicated that the overexpression of HAb18G/CD147 stimulated MMPs (including MMP-1, MMP-2 and MMP-9) production and enhanced metastatic potentials in human HCC cells (Jiang et al., 2001). In the present study, the expressions of HAb18G/CD147 in SMMC-7721 cells were obviously increased in 3D cell culture model together with the elevated production of MMPs, especially the production of MMP2 during the 3D culture process. These findings suggested that the reconstituted BM provides a more suitable microenvironment to support the interactions between HCC cell and ECM and induces expression of HAb18G/CD147 and secretion of MMPs, and these interactions make HCC cells being more susceptible to migration and invasion.

E-cadherin is an important component of adherent junction involved in carcinoma cells metastasis and its expression is spatially and temporally dynamic in metastasis process. E-cadherin is down-regulated when carcinoma cells separate from primitive neoplastic focus, and then is up-regulated to form new carcinoma colonies (Christofori, 2006; Thiery and Sleeman, 2006). In this study, the expressions of E-cadherin on the plasma membrane of SMMC-7721 cells were increased in newly-formed colonies in the 3D reconstituted BM. The recent study showed that CD147 is an additional subunit of gamma-secretase complexes increased the activity of Abeta-cadherin, within their transmembrane regions. Removal of CD147 peptides to cleave E-cadherin (Zhou et al., 2006). This suggested that CD147 may indirectly regulate the expression of E-cadherin. The results of present study have shown that the increased expressions of HAb18G/CD147 accompanied the enhanced expressions of E-cadherin in HCC cells in the 3D reconstituted BM. This finding is partially consistent with the previous report (Zhou et al., 2006). But the correlation between HAb18G/CD147 and E-cadherin in HCC cells needs to be further investigated.

In the present study, the expression of paxillin and FAK were both obviously increased in SMMC-7721 cells in 3D cell culture model. The elevated expressions of these two focal adhesion proteins stimulated the formation of focal adhesions which directly linked to F-actin cytoskeleton. The high expressions of the two focal adhesion proteins and the redistributions of F-actin may enhance the adhesion and migration potentials of SMMC-7721 cells. We have reported that silencing HAb18G/CD147 inhibits cells mobility, FAK and F-actin expression in HCC cells via an ERK1/2-dependent signaling pathway (Xu et al., 2007a). The present study showed that the increased expression of HAb18G/CD147 was obviously accompanied by an enhanced expression of FAK and redistribution of F-actin in the 3D reconstituted BM. Our recent study and several reports showed that HAb18G/CD147 is associated with integrins which mediated cell migration and invasion processes (Berditchevski et al., 1997; Curtis et al., 2005; Tang et al., 2008). Paxillin and FAK also bind to integrins and interact with cytoskeletal and other signaling proteins to regulate the signaling of focal adhesion. HAb18G/CD147 may affect the expression of paxillin and FAK through interacting with integrins. The exact mechanisms of HAb18G/CD147, paxillin and FAK in the adhesion and invasion processes of HCC cells are not clear, and the investigation is currently underway in our laboratory.

In conclusion, we established 3D HCC cell culture model by using a 3D reconstituted BM and investigated the phenotypic characteristics and the expressions of key molecules involved in HCC cells adhesion and invasion. In the 3D reconstituted BM, enhanced expressions of HAb18G/CD147, MMPs, paxillin and FAK change the distributions of cytoskeleton and increase the adhesion and invasion potentials of HCC cells. The 3D cell culture model provides a more well-defined microenvironment for cancer research.

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