Roles of Spheroid Formation of Hepatocytes in Liver Tissue Engineering

Hu-Lin Jiang¹, You-Kyoung Kim², Ki-Hyun Cho³, Young-Chul Jang³,
Yun-Jaie Choi², Jong-Hoon Chung⁴, Chong-Su Cho²

¹College of Veterinary Medicine, Seoul National University, ²Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, ³Department of Plastic and Reconstructive Surgery, Hangeul Sacred Heart Hospital, Hallym University College of Medicine,

⁴Department of Biosystem Engineering and Biomaterial Sciences, Seoul National University, Seoul, Korea

The liver plays an important role in a broad spectrum of physiological functions and detoxifies endogenous and exogenous substances. The liver failure is associated with a high risk of mortality because it is one of important organs in our body. Various bioartificial liver (BAL) systems have been used for clinical trials as a bridge for liver transplantations in patients with liver failure. Long term and stable liver-specific functions of hepatocytes in the development of BAL support systems should be considered. Spheroid formation of hepatocytes enhances liver-specific functions. In this review, hepatocyte spheroid formation methods such as galactose density, topology of extracellular matrix, micro-molding technique, hanging-drop culture, non-adhesive surface, positive charged surface, spinner culture, rocked technique, medium component, external forces, coculture system and polymeric nanospheres are explained for enhancing liver-specific functions.

Keywords: Spheroid, Hepatocyte, Liver function, Tissue engineering, Extracellular matrix

Introduction

The liver plays an important role in a broad spectrum of physiological functions such as metabolism, storage, synthesis and release of vitamins, carbohydrates, proteins, lipids and cyclic tetrapyrroles (1). The liver also detoxifies and inactivates endogenous and exogenous substances such as toxins and metals, and activates proenzymes and coagulation factors (2). Furthermore, the liver produces bile involved in intestinal lipid absorption and secretes a majority of circulating plasma proteins. Therefore, the liv-

er is one of important organs in our body and liver failure is associated with a high risk of mortality. Currently, the only effective way for liver failure is liver transplantation (3). Because of shortage of organ availability, it is necessary to find alternative ways to temporarily support the failing liver until a compatible donor can be found. Various bioartificial liver (BAL) systems have been used for clinical trials as a bridge for liver transplantations in patients with liver failure (4). Long term and stable liver-specific functions of hepatocytes should be considered in the development of BAL support systems.

Spheroid culture is very effective in the upregulation of liver-specific functions of hepatocytes due to the high similarity to real tissues in many aspects than monolayer cells although the diffusion limitation of oxygen and nutrients by the big size of spheroid of hepatocytes should be considered. In this review, spheroid formation methods of hepatocytes in the extracellular matrix (ECM) are covered for enhancing liver-specific functions.

Accepted for publication September 18, 2010

Correspondence to **Chong-Su Cho**

Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, San 56-1 Sillim-dong, Gwanak-gu, Seoul 151-921, Korea

Tel: +82-2-880-4868, Fax: +82-2-875-2494

E-mail: chocs@snu.ac.kr

Spheroid formation of hepatocytes galactose density

Kobayashi et al. (5) reported that galactose-derivatized polystyrene (PS), poly(*N-p*-vinylbenzyl-4-O- β -D-galactopyranosyl-D-gluconamide (PVLA) is an excellent artificial ECM for hepatocytes because it guides hepatocyte adhesion through the asialoglycoprotein receptors (ASGPR)-galactose interaction between ASGPR of hepatocytes and highly concentrated galactose moieties along the polymer chains. Also, they reported that morphologies of hepatocytes adhered to PVLA at the lower galactose density (0.5 μ g/ml) showed spreaded shapes whereas the morphologies of hepatocytes adhered to PVLA at the higher galactose (100 μ g/ml) showed round ones and the round morphologies of hepatocytes were found to trigger the spheroid formation in the presence of epidermal growth factor (EGF) (6). Yin et al. (7) also reported that hepatocytes formed hepatocyte spheroids 1 day after cell seeding with better liver-functions at a high galactose density of 513 nmol/cm² on the PET surface as an artificial ECM, suggesting that spheroid formation of hepatocytes is dependent on the galactose density in the artificial ECM.

Topology of ECM

Many researches have been focused on the design of ECM topology for the spheroid formation of hepatocytes because the topology of ECM affects cell morphology and this structure resembles the tight cell-cell contact in native liver and tight junctions (8). It is generally known that three-dimensional ECMs more induce spheroid formation of hepatocytes with better differentiated functions than two-dimensional ones due to the provision of better model systems for physiologic situations (9).

Chua et al. prepared highly porous nanofiber scaffolds by electrospinning of poly (epsilon-caprolactone-co-ethyl ethylene phosphate) (PCLEEP) and introduced galactose onto the nanofiber surface (10). The results indicated that hepatocytes cultured on galactosylated nanofiber scaffold formed smaller aggregates of 20~100 microm, resulting in an integrated spheroid-nanofiber construct. Feng et al. prepared nanofibrous galactosylated chitosan (GC) scaffolds by electrospinning and evaluated liver functions (11). The results indicated that hepatocytes cultured on GC nanofibrous scaffold formed stably spheroid formation and exhibited high liver-specific functions.

Recently, Takahashi et al. prepared collagen- or Matrigel-coated nanopillar sheets and evaluated liver functions (12). The results indicated that the hepatocytes cultured on col-

lagen- or Matrigel-coated nanopillar sheets formed spheroids with higher structural polarity and functional bile canaliculi compared with conventional two-dimensional culture method.

Micromolding technique

Microfabrication is an emerging and powerful technique for spheroid formation of hepatocytes with well-controlled size and geometry. Microwells of defined size and aspect ratio can be fabricated by micromolding on cell-non-adhesive inert polymers like agarose (13) and poly (ethylene glycol) (PEG) (14) for the spheroid formation of hepatocytes. Makazawa et al. fabricated polydimethylsiloxane (PDMS) chip having microcavities by a photolithography technique and evaluated liver-specific functions (15). The results indicated that most hepatocytes formed a single spheroid in each cavity until 3 days of culture and the spheroid configuration was maintained for at least 14 days of culture. Also, liver-specific functions such as albumin secretion, ammonia removal and ethoxycorufin O-dealkylase activity of hepatocytes on the PDMS chip were higher than those of a monolayer dish. Miyamoto et al. developed a cell array technique by two combined innovations composed of micropatterning by a hydrophilic polymer and use of bovine carotid artery-derived HH cells as feeder cells for the formation of hepatocyte spheroid (16). The results showed that the rat hepatocytes formed spheroids on the cryopreserved HH cell array and the CYP3A drug metabolism activities of the hepatocytes were well maintained on the cryopreserved and cell arrays. Yoshimoto et al. prepared inverted cell patterned surface using PEG gel by a photolithographic technique and seeded hepatoma cells on the constructed surface (17). The results showed that spheroids of the cells were formed on the unmodified PEG gel domains without feeder cells. Cocultures of HepG2 and Balb/3T3 (18), hepatocytes and mouse embryonic stem (mES) cells (19), and fetal mouse liver cells and non-parenchymal liver cells (20) on the micropatterned surfaces were performed to enhance more liver-specific functions than micropatterned ones with monocultures.

Hanging-drop culture

Lin et al. used hanging-drop culture of hepatocytes to form spheroid formation although the method was initially developed for culture of stem cell embryoid bodies. The technique is useful for generation of spheroid formation of defined sizes, cell numbers and composition al-

though it is labor-intensive and difficult for massive production (21).

Non-adhesive surface

The spheroid formation of hepatocytes was obtained by culturing on a non-adhesive substratum such as poly (N-isopropylacrylamide) (22), alginate (23), and agarose (24) owing to enhancing the cell-cell interaction although it is not easy to control the size of hepatocyte spheroid.

Positive charged surface

Tzanakakis et al. (25) reported that hepatocytes cultured on positively charged surfaces (Falcon Primaria) adhered to form a monolayer with spreading shapes on day 1 and the hepatocytes underwent spheroid formation after 48h through actin filament-mediated tissue reorganization. Verma et al. (26) also reported that HepG2 cells cultured on chitosan surfaces with appropriate seeding density resulted in the spheroid formation and exhibited higher amount of albumin and urea synthesis compared to uncoated dishes. However, Matsushita et al. (27) reported that primary human fetal hepatocytes did not form spheroids on a poly (L-lysine)- or poly (L-arginine)-coated dish that had a positively charged surface whereas the cells formed spheroids on a poly (L-glutamic acid)- or poly (L-aspartic acid)-coated dish that had a negatively charged surface.

Spinner culture

Spinner culture systems were used to form large numbers of hepatocyte.

Spheroids (28) although they can experience a strong shear force. To overcome the problem, a rotational cell culture was recently used (29).

Rocked technique

Brophy *et al.* (30) used rocked technique to induce spheroid formation of hepatocytes. The spheroid formation by rocked technique was more rapid and more efficient than rotational technique. The hepatocyte spheroids in rocked culture showed stable expression of over 80% of 242 liver-related genes.

Medium component

Medium components have been used to enhance spheroid

formation of hepatocytes. The round shapes of hepatocytes were found to trigger the spheroid formation in the presence of EGF (6), HGF and sodium butylate (31), collagen (32), dexamethasone (33) and Eudragit (34).

External forces

External forces such as dielectrophoresis (35), magnetic fields (36) and ultrasound trap (37, 38) were used to enhance cell aggregation and spheroid formation from single cells although specialized equipment is necessary in spheroid formation of the cells. Spheroid formation of HepG2 adhered on a PHEMA-coated surface and encapsulated in alginate capsules was formed within 30 and 5 min, respectively, by the ultrasound trap with good liver-specific functions (37).

Coculture system

Heterotypic cell interactions between parenchymal cells and nonparenchymal neighbors are of fundamental importance in physiology (39) and tissue engineering (40) because coculture of hepatocytes with other cells enhances spheroid formation of hepatocytes and liver-specific functions. Coculture of rat hepatocytes with pancreatic islets (41) and rat hepatocytes with NIH/3T3 fibroblasts (42, 43) enhanced spheroid formation and functions of hepatocytes.

Polymeric nanosphere

Gwak et al. (44) developed a new method for the effective spheroid formation of hepatocytes by addition of poly (lactic-co-glycolic acid) nanospheres to hepatocyte cultures in spinner flasks although the mechanism is not clear.

Conclusion and perspectives

Accumulated evidence showed that spheroid formation of hepatocytes is very effective in the enhancement of liver-specific functions of hepatocytes. The spheroid formation of hepatocytes will contribute to liver tissue engineering because they induce long term and stable liver-specific functions of hepatocytes although several obstacles should be overcome. First, diffusion limitation of nutrients and oxygen to the big size of spheroid should be considered. Second, spheroid studies are needed to correlate how well the system represents real tissues because spheroid formation is an artificially reconstructed system. Spheroid formation-based 3-D culture systems continue to

advance in all aspects, including new applications in combination with micro-electro-mechanical systems and new drug evaluation.

Acknowledgement

This work was supported by the National Research Laboratory Project (ROA-2010-00-8428) from the Ministry of Education, and Science and Technology.

Potential conflict of interest

The authors have no conflicting financial interest.

References

1. Zakim D, Boyer TD, eds. *Hepatology. A Textbook of Liver Disease* (Vol. 1). Philadelphia: WB Saunders Co; 1990
2. Davis MW, Vacanti JP. Toward development of an implantable tissue engineered liver. *Biomaterials* 1996;17:365-372
3. Millis JM, Losanoff JE. Technology insight: liver support systems. *Nat Clin Pract Gastroenterol Hepatol* 2005;2:398-405
4. Yu CB, Pan XP, Li LJ. Progress in bioreactors of bio-artificial livers. *Hepatobiliary Pancreat Dis Int* 2009;8:134-140
5. Kobayashi A, Kobayashi K, Akaike T. Control of adhesion and detachment of parenchymal liver cells using lactose-carrying polystyrene as substratum. *J Biomater Sci Polym Ed* 1992;3:499-508
6. Tobe S, Takei Y, Kobayashi K, Akaike T. Receptor-mediated formation of multilayer aggregates of primary cultured adult rat hepatocytes on lactose-substituted polystyrene. *Biochem Biophys Res Commun* 1992;184:225-230
7. Yin C, Liao K, Mao HQ, Leong KW, Zhuo RX, Chan V. Adhesion contact dynamics of HepG2 cells on galactose-immobilized substrates. *Biomaterials* 2003;24:837-850
8. Berthiaume F, Moghe PV, Toner M, Yarmush ML. Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration. *FASEB J* 1996;10:1471-1484
9. Wu FJ, Friend JR, Remmel RP, Cerra FB, Hu WS. Enhanced cytochrome P450 IA1 activity of self-assembled rat hepatocyte spheroids. *Cell Transplant* 1999;8:233-246
10. Chua KN, Lim WS, Zhang P, Lu H, Wen J, Ramakrishna S, Leong KW, Mao HQ. Stable immobilization of rat hepatocyte spheroids on galactosylated nanofiber scaffold. *Biomaterials* 2005;26:2537-2547
11. Feng ZQ, Chu X, Huang NP, Wang T, Wang Y, Shi X, Ding Y, Gu ZZ. The effect of nanofibrous galactosylated chitosan scaffolds on the formation of rat primary hepatocyte aggregates and the maintenance of liver function. *Biomaterials* 2009;30:2753-2763
12. Takahashi R, Sonoda H, Tabata Y, Hisada A. Formation of hepatocyte spheroids with structural polarity and functional bile canaliculi using nanopillar sheets. *Tissue Eng Part A* 2010;16:1983-1995
13. Dean DM, Napolitano AP, Youssef J, Morgan JR. Rods, tori, and honeycombs: the directed self-assembly of microtissues with prescribed microscale geometries. *FASEB J* 2007;21:4005-4012
14. Fukuda J, Sakai Y, Nakazawa K. Novel hepatocyte culture system developed using microfabrication and collagen/polyethylene glycol microcontact printing. *Biomaterials* 2006;27:1061-1070
15. Nakazawa K, Izumi Y, Fukuda J, Yasuda T. Hepatocyte spheroid culture on a polydimethylsiloxane chip having microcavities. *J Biomater Sci Polym Ed* 2006;17:859-873
16. Miyamoto Y, Ikeya T, Enosawa S. Preconditioned cell array optimized for a three-dimensional culture of hepatocytes. *Cell Transplant* 2009;18:677-681
17. Yoshimoto K, Ichino M, Nagasaki Y. Inverted pattern formation of cell microarrays on poly(ethylene glycol) (PEG) gel patterned surface and construction of hepatocyte spheroids on unmodified PEG gel microdomains. *Lab Chip* 2009;9:1286-1289
18. Kikuchi K, Sumaru K, Edaheiro J, Ooshima Y, Sugiura S, Takagi T, Kanamori T. Stepwise assembly of micro-patterned co-cultures using photoresponsive culture surfaces and its application to hepatic tissue arrays. *Biotechnol Bioeng* 2009;103:552-561
19. Torisawa YS, Mosadegh B, Luker GD, Morell M, O'Shea KS, Takayama S. Microfluidic hydrodynamic cellular patterning for systematic formation of co-culture spheroids. *Integr Biol (Camb)* 2009;1:649-654
20. Kojima R, Yoshimoto K, Takahashi E, Ichino M, Miyoshi H, Nagasaki Y. Spheroid array of fetal mouse liver cells constructed on a PEG-gel micropatterned surface: upregulation of hepatic functions by co-culture with nonparenchymal liver cells. *Lab Chip* 2009;9:1991-1993
21. Lin RZ, Chou LF, Chien CC, Chang HY. Dynamic analysis of hepatoma spheroid formation: roles of E-cadherin and beta1-integrin. *Cell Tissue Res* 2006;324:411-422
22. Takezawa T, Yamazaki M, Mori Y, Yonaha T, Yoshizato K. Morphological and immuno-cytochemical characterization of a hetero-spheroid composed of fibroblasts and hepatocytes. *J Cell Sci* 1992;101:495-501
23. Yang J, Goto M, Ise H, Cho CS, Akaike T. Galactosylated alginate as a scaffold for hepatocytes entrapment. *Biomaterials* 2002;23:471-479
24. Hasebe Y, Okumura N, Koh T, Kazama H, Watanabe G, Seki T, Ariga T. Formation of rat hepatocyte spheroids on agarose. *Hepatol Res* 2005;32:89-95
25. Tzanakakis ES, Hansen LK, Hu WS. The role of actin filaments and microtubules in hepatocyte spheroid self-assembly. *Cell Motil Cytoskeleton* 2001;48:175-189
26. Verma P, Verma V, Ray P, Ray AR. Formation and characterization of three dimensional human hepatocyte cell line spheroids on chitosan matrix for in vitro tissue engineering applications. *In Vitro Cell Dev Biol Anim* 2007;43:328-337
27. Matsushita T, Nakano K, Nishikura Y, Higuchi K, Kiyota A, Ueoka R. Spheroid formation and functional restoration

- of human fetal hepatocytes on poly-amino acid-coated dishes after serial proliferation. *Cytotechnology* 2003;42:57-66
28. Nyberg SL, Hardin J, Amiot B, Argikar UA, Rimmel RP, Rinaldo P. Rapid, large-scale formation of porcine hepatocyte spheroids in a novel spheroid reservoir bioartificial liver. *Liver Transpl* 2005;11:901-910
 29. Nelson LJ, Walker SW, Hayes PC, Plevris JN. Low-shear modelled microgravity environment maintains morphology and differentiated functionality of primary porcine hepatocyte cultures. *Cells Tissues Organs* 2010;192:125-140
 30. Brophy CM, Luebke-Wheeler JL, Amiot BP, Khan H, Rimmel RP, Rinaldo P, Nyberg SL. Rat hepatocyte spheroids formed by rocked technique maintain differentiated hepatocyte gene expression and function. *Hepatology* 2009;49:578-586
 31. Huang H, Hanada S, Kojima N, Sakai Y. Enhanced functional maturation of fetal porcine hepatocytes in three-dimensional poly-L-lactic acid scaffolds: a culture condition suitable for engineered liver tissues in large-scale animal studies. *Cell Transplant* 2006;15:799-809
 32. Meng Q, Wu D, Zhang G, Qiu H. Direct self-assembly of hepatocytes spheroids within hollow fibers in presence of collagen. *Biotechnol Lett* 2006;28:279-284
 33. Abu-Absi SF, Hu WS, Hansen LK. Dexamethasone effects on rat hepatocyte spheroid formation and function. *Tissue Eng* 2005;11:415-426
 34. Kamihira M, Yamada K, Hamamoto R, Iijima S. Spheroid formation of hepatocytes using synthetic polymer. *Ann N Y Acad Sci* 1997;831:398-407
 35. Sebastian A, Buckle AM, Markx GH. Tissue engineering with electric fields: immobilization of mammalian cells in multilayer aggregates using dielectrophoresis. *Biotechnol Bioeng* 2007;98:694-700
 36. Ino K, Ito A, Honda H. Cell patterning using magnetite nanoparticles and magnetic force. *Biotechnol Bioeng* 2007;97:1309-1317
 37. Liu J, Kuznetsova LA, Edwards GO, Xu J, Ma M, Purcell WM, Jackson SK, Coakley WT. Functional three-dimensional HepG2 aggregate cultures generated from an ultrasound trap: comparison with HepG2 spheroids. *J Cell Biochem* 2007;102:1180-1189
 38. Bazou D. Biochemical properties of encapsulated high-density 3-D HepG2 aggregates formed in an ultrasound trap for application in hepatotoxicity studies: Biochemical responses of encapsulated 3-D HepG2 aggregates. *Cell Biol Toxicol* 2010;26:127-141
 39. van Breemen C, Skarsgard P, Laher I, McManus B, Wang X. Endothelium-smooth muscle interactions in blood vessels. *Clin Exp Pharmacol Physiol* 1997;24:989-992
 40. L'Heureux N, Pâquet S, Labbé R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel. *FASEB J* 1998;12:47-56
 41. Lee KW, Lee SK, Joh JW, Kim SJ, Lee BB, Kim KW, Lee KU. Influence of pancreatic islets on spheroid formation and functions of hepatocytes in hepatocyte-pancreatic islet spheroid culture. *Tissue Eng* 2004;10:965-977
 42. Ryu KH. Liver stem cells derived from the bone marrow and umbilical cord blood. *Int J Stem Cell* 2009;2:97-101
 43. Seo SJ, Kim IY, Choi YJ, Akaiki T, Cho CS. Enhanced liver functions of hepatocytes cocultured with NIH 3T3 in the alginate/galactosylated chitosan scaffold. *Biomaterials* 2006;27:1487-1495
 44. Gwak SJ, Choi D, Paik SS, Cho SW, Kim SS, Choi CY, Kim BS. A method for the effective formation of hepatocyte spheroids using a biodegradable polymer nanosphere. *J Biomed Mater Res A* 2006;78:268-275