REVERSIBLY-ASSEBMLED PERFUSION CULTURE CHIP WITH MICROWELL ARRAY FOR CONTROLLABLE SPHEROID CULTURE AND POST-CULTURE ANALYSIS

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ABSTRACT

This paper reports a microfluidic device composed of a reversibly-assembled microfluidic chamber chip and multi-microwell array chip. This device enabled perfusion culture of spheroid in a microarray format, and detailed post-culture analysis by collection of spheroids from the disassembled device. The device provides four advantages simultaneously; the uniform spheroid formation, growth analysis in a microarray format, controlled proliferation by flow rate of perfusion, and post-culture analysis by collection of spheroids from the disassembled device. Thus, this method is a promising tool for fundamental and application research in drug screening, tissue engineering, and regenerative medicine.

KEYWORDS

Microwell array, Spheroid, Perfusion culture, HepG2 cells, Post-culture analysis

INTRODUCTION

Three-dimensional cell aggregates emulate *in vivo* microenvironments more accurately than do conventional two-dimensional monolayer cultures.[1] In particular, spherical multicellular aggregates (spheroids) display tissue-like architectures and can maintain the high liver-specific functions of hepatocytes,[2] provide hypoxic conditions for cancer cells in therapeutic studies,[3] and induce controlled differentiation of stem cells.[4] Recently, we and several research groups developed spheroid arrays using a microwell structure, which enables mass production of spheroids with controlled diameters.[5–11]

Generally, nutrients and oxygen must be supplied to maintain spheroid cultures.[12, 13] Toward this end, some research groups have created perfusion cultures of spheroids formed in the microfluidic devices to realize controlled mass transfer of nutrients and oxygen.[14–17] Even though array-formatted microchambers are essentially required for high-throughput analysis on a single chip, none of those groups reported the perfusion culture of spheroids in such microchambers, because of the difficulty associated with loading spheroids uniformly. Furthermore, most of the reported perfusion culture microfluidic devices have difficulty in collecting the cultured spheroids for further analysis, such as DNA and protein expression analysis, because the microchambers are covalently bonded to the culture surface.[14–16,18] These disadvantages have limited the applicability of microfluidic devices in practical drug screening. In this study, we circumvented the disadvantages associated with perfusion culture of spheroid array by creating a microfluidic device composed of reversibly-assembled microfluidic chambers and microwells.

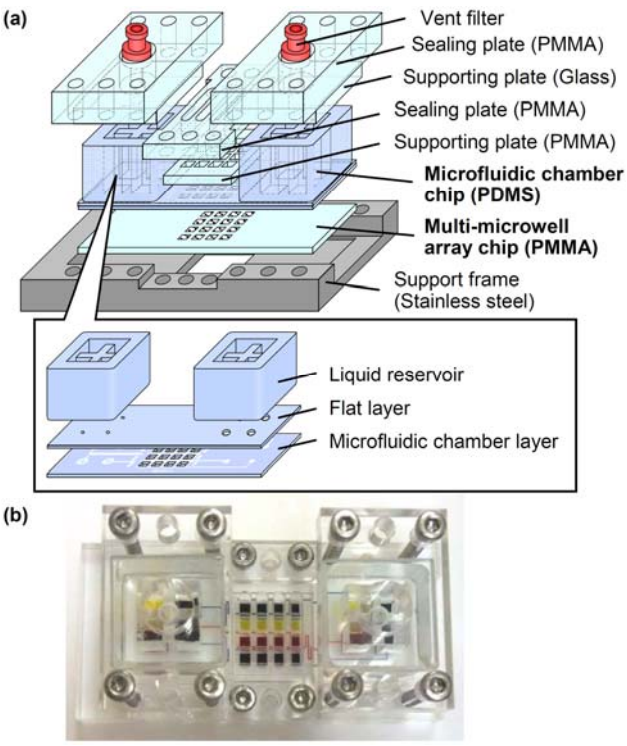

Figure 1. (a) Schematic diagrams of the microfluidic device for perfusion culture of spheroid arrays. (b) Four differently colored dye solutions were loaded into the microfluidic network on the device.

EXPERIMENT

The microfluidic device for perfusion culture of spheroid array is composed of a poly(dimethylsiloxane) (PDMS) microfluidic chamber chip and a poly(methyl methacrylate) (PMMA) multi-microwell array chip (Fig. 1a). The microfluidic chamber chip consists of a microfluidic chamber layer, which has a microfluidic channel network and a 4×4 array of quadrilateral through-holes that serve as culture chambers. The multi-microwell array chip consists of a 4×4 array of microwell regions, and each microwell region consists of an 8×8 array of microwells. A suspension of HepG2 cells was manually inoculated onto the microwell regions on the multi-microwell array chip. After the cells

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fell into each microwell, the two chips were assembled with the fixtures, and the assembled device was used for perfusion culture (Fig. 1b).

HepG2 spheroids were formed and cultured in the assembled microfluidic device by following procedures of cell inoculation, chip assemblage and perfusion culture. A suspension of HepG2 cells was manually inoculated onto the microwell regions on the multi-microwell array chip at a density of 6.4×10^3 cells/chamber/3.8μL (100 cells/microwell) using a PDMS stencil. After the cells fell into each microwell, the culture medium was removed and the PDMS stencil was peeled off. The microfluidic chamber chip was placed in contact with the multi-microwell array chip with alignment of the through-holes and the microwell region. The two chips were placed on a stainless steel support frame and held in place with the fixtures.

Perfusion culture of the spheroid array was carried out by applying a pressure. The flow rate was easily controlled by regulating the pressure applied through the vent filter.

RESULTS AND DISCUSSION

Perfusion culture of the spheroid array was carried out at low and high flow rates of 64.0 ± 2.7 and 154.3 ± 16.2 μL/chamber/day, respectively. Fig. 2(a)–2(i) show phase-contrast micrographs obtained under static, low-flow-rate perfusion, and high-flow-rate perfusion culture conditions at 3, 7, and 10 days of culture. Under all culture conditions including static culture, HepG2 cells spontaneously formed spheroids with uniform diameter in each microwell after 3 days of culture and the diameter of the spheroids increased for at least 10 days of culture.

Proliferation of the HepG2 spheroids in 8×8 arrays of microwells in the 4×4 array of the culture chambers was quantitatively analyzed by conducting image analysis of both the static and perfusion cultures. Throughout the 10 days of cultivation, the high-flow–rate perfusion culture condition led the largest diameter probably because this condition provided the largest supply of nutrients to the spheroids (Fig. 3). The high-flow–rate perfusion culture condition led the largest diameter probably because this condition provided the largest supply of nutrients to the spheroids.

After 15 days of culture, the microfluidic device was disassembled and spheroids were collected from each culture chamber. The number of cells in the collected spheroids was counted by means of DNA-4',6-diamino-2-phenylindole (DAPI) fluoresce, and mRNA expressions in the cells was measured by real-time polymerase chain reaction (PCR) analysis. The cell numbers obtained for the static and low-flow-rate perfusion cultures were almost the same, whereas a significantly higher cell number was observed for the high-flow-rate perfusion culture (Fig. 4a). The mRNA expression levels for E-cadherin and connexin-32 were the same for both the static culture and the low-flow-rate perfusion culture; however, high-flow-rate perfusion culture exhibited low expression of E-cadherin (Fig. 4b and 4c). These results indicate the potential of the perfusion culture to control

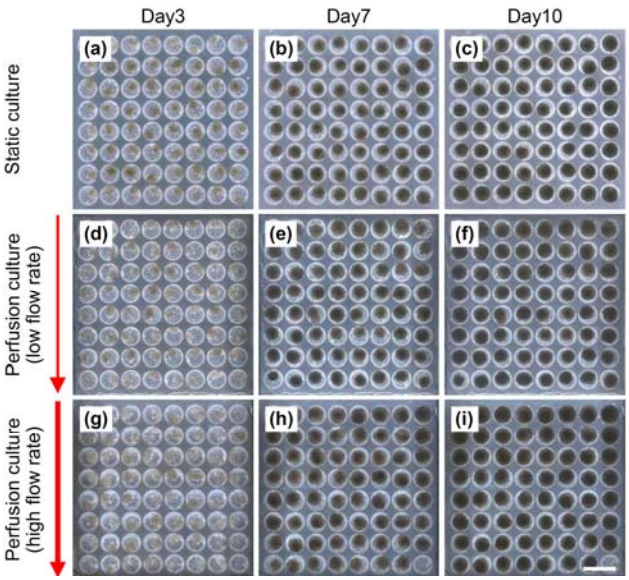

Figure 2. Phase-contrast micrographs of spheroids under static culture (a–c), low-flow-rate perfusion culture (d–f), and high-flow-rate perfusion culture (g–i). Micrographs were obtained at 3 days (a, d, g), 7 days (b, e, h), and 10 days of culture (c, f, i). Scale bar: 500 μm.

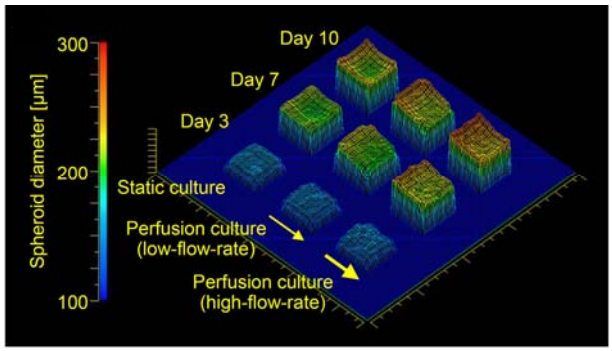

Figure 3. Distributions of spheroid diameters in the 8×8 microwells in the culture achambers (j); diameters are expressed as the means of at least 8 chambers. Red and yellow arrows indicate flow direction through the culture chamber.

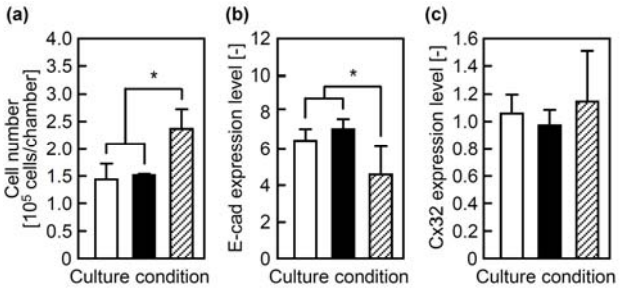

*Figure 4. Cell numbers (a) and mRNA expression levels for E-cadherin (b), and connexin-32 (c) at day 15 of culture for static (white columns), low-flow-rate perfusion (black columns), and high-flow-rate perfusion (striped columns) cultures. The expression level of the HepG2 cell suspension was set as 1.0. Error bars represent SD. *P < 0.01.*

the culture environment in each culture chamber.

In summary, we developed a novel microfluidic device for controlled perfusion culture of uniformly formed spheroids. The reversibly-assembled device enabled the collection of spheroids from its array-formatted culture chambers, thus overcoming one of the major limitations of microfluidic cell-culture chips. The collected spheroids can be used for further traditional cell assays such as PCR, cell sorting, and immunohistochemistry assays.

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