Biochemical Engineering Journal 48 (2010) 332–336

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Review

Practical and functional culture technologies for primary hepatocytes

Hiroyuki Ijima *

Department of Chemical Engineering, Faculty of Engineering, Graduate School, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka, 819-0395, Japan

ARTICLE INFO

Article history:
Received 28 August 2009
Received in revised form 1 October 2009
Accepted 3 October 2009

Keywords:
Animal cell culture
Biomedical
Bioreactor
Hepatocyte
Immobilization
Liver tissue engineering

ABSTRACT

The liver is a central organ for metabolism in the body, and it is therefore important to develop effective culture methods for the expression of liver-specific functions. The development of effective primary hepatocyte culture methods for the expression of these functions has been attempted by focusing on cell–cell and cell–matrix interactions. Two-dimensional organoid culture methods using substrata with the immobilized arginine–glycine–aspartic acid sequence, a well-known cell-adhesive sequence, have been developed. Furthermore, a drug metabolism simulator and a hybrid-type blood purification system as applications of this type of two-dimensional organoid culture method have been developed. On the other hand, growth factor/heparin-immobilized culture substrata and cell-embedded hydrogels in extracellular matrix-filled biocompatible macroporous scaffold culture systems for liver tissue engineering have been developed. Promotion of organoid formation and high expression of the liver-specific functions of primary rat hepatocytes will be realized by using these substrata and culture systems.

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1. Introduction

The development of functional animal cell culture methods is very important for cell function analyses, the pharmaceutical industry, artificial organs and regenerative medicine. In particular, the development of functional and practical culture methods for hepatocytes is strongly desired because the liver is a central metabolic organ in the body. However, it is difficult to maintain the survival of hepatocytes and the expression of their liver-specific functions in vitro. Hepatocytes lose their liver-specific functions even on collagen-coated surface [1]. Therefore, it was thought that the development of an adequate culture medium [2] and the construction of microenvironment around hepatocytes as in our body were necessary. It was thought that effective microenvironments for functional animal cell cultures involve homotypic or heterotypic cell–cell interactions, cell–matrix interactions, soluble growth factors and mass transfer around cells. Based on this hypothesis, organoid culture [3], collagen gel sandwich culture [4], matrigel culture [5] and coculture [6] were developed, and they achieved the high expression of liver-specific functions in vitro. Furthermore, practical hybrid-type artificial livers [3,7] and drug metabolism simulators [8] were developed by focusing on functional organoid formation methods for hepatocytes [9,10]. These organoid culture methods are suitable for the development of hybrid artificial liver

* Tel.: +81 92 802 2758; fax: +81 92 802 2758.
E-mail address: ijima@chem-eng.kyushu-u.ac.jp.

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doi:10.1016/j.bej.2009.10.003

systems, but have many associated problems, such as mass transfer for the development of cell function simulators and tissue engineering. Furthermore, the requirements differ depending on the purpose. In this review, I summarize the functional hepatocyte culture methods, with the exception of hybrid-type artificial liver development which has already been reported [3,7].

2. Functional culture methods for primary hepatocytes

2.1. Functional monolayer cultures

Extensive spreading of functional animal cells such as hepatocytes occurs on natural extracellular matrix (laminin, fibronectin, type I collagen, type IV collagen)-coated culture substrata, and the cells quickly lose their organ-specific functions [11]. However, observation of individual cells and the creation of uniform culture conditions around the cells can be more easily realized in monolayer cultures than in organoid cultures. Therefore, monolayer cultures are effective as estimation tools for cell functions and responses to various stimulations. On the other hand, extensive spreading is inhibited and albumin production activity is enhanced in hepatocyte cultures on synthetic culture substrates consisting of an adequate spacer sequence and the arginine-glycine-aspartic acid (RGD) sequence, a well-known cell-adhesive sequence of fibronectin [12,13].

Although Pronectin F (PnF) is a synthetic substratum containing the RGD sequence, it was reported that PnF did not exert the above-mentioned effects [13]. However, the cell morphology was dependent on the immobilized density of PnF [14]. Furthermore, primary rat hepatocytes and a renal proximal tubular cell line (PCTL-MDR) formed functional monolayers with uniform and cuboidal cell morphologies (two-dimensional organoids) on a high density PnF-immobilized (0.445 nmol RGD cm^{-2}) culture substratum [14–16]. In other words, the organ-specific functions of hepatocytes (ammonia metabolism [9], albumin production [16] and drug metabolism [16]) and renal proximal tubular cells (active transport of drugs (Fig. 1) [15]) can be well expressed and maintained. These improvements were brought about by the formation of cuboidal cell structures on the high density RGD-immobilized substratum [14,16]. Cell shape is controlled by cell–cell interactions, cell–ligand affinities and cell/ligand–substrate interactions [13,14,17]. However, it is currently unclear which of these components is the main reason for the above-mentioned effects of the high density RGD-immobilized substratum. I expected that the high expressions of organ-specific functions of parenchymal cells would be strongly affected by the cell morphology, which is controlled by

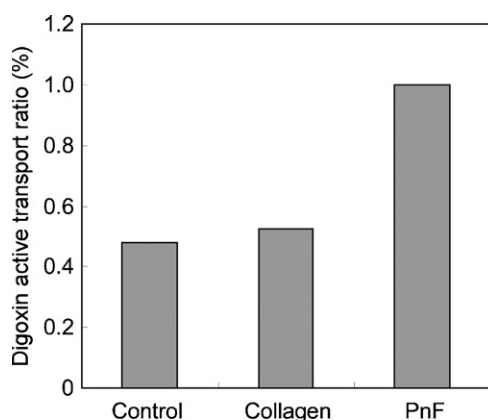

Fig. 1. Digoxin active transport ratios via renal proximal tubule cells (PCTL-MDR)-covered porous membranes after 26 days of culture. The membranes were coated with 0.445 nmol RGD cm^{-2} or type I collagen (adsorption).

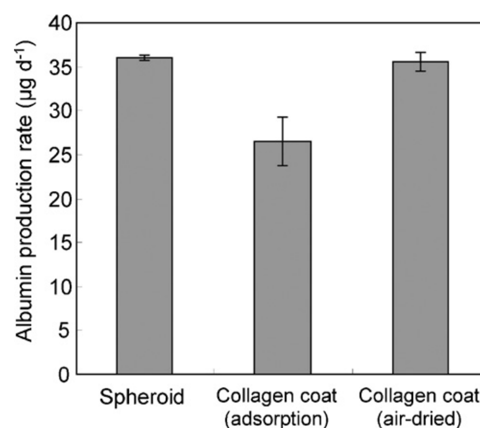

Fig. 2. Albumin production rates of primary rat hepatocytes after 5 days of culture. The hepatocytes were inoculated into 35-mm dishes at 5×10^5 cells $dish^{-1}$.

differences in cell–cell and cell–matrix interactions, rather than by specific signals from cell-adhesive molecules.

Therefore, an air-dried collagen film (10 μg collagen cm^{-2} substrate) was used for hepatocyte cultures, instead of collagen-coated (adsorption) dishes on which extensive spreading and rapid decreases in liver-specific functions of hepatocytes occurred. The morphology and liver-specific functions of hepatocytes on the air-dried collagen film were similar to those on the RGD-immobilized culture substratum (Fig. 2). In other words, the previous description that a synthetic substratum was superior for the expression of liver-specific functions compared with the natural extracellular matrix [11] was inadequate. A culture substratum that inhibits the extensive spreading of hepatocytes, such as the high density RGD-immobilized culture substratum or the air-dried collagen film, is important. This information will facilitate the development of various substrata for functional monolayer cultures for various purposes. There are two possibilities for the inhibition of extensive spreading of hepatocytes on the air-dried collagen film, namely that the number of cell-adhesive points was increased on the substratum and/or that the adhesive force of the hepatocytes to the air-dried collagen film was decreased. Although the answer remains unclear at present, I expect that extensive spreading of hepatocytes is inhibited by an increased number of cell-adhesive points on the substratum, since similar inhibition was observed to depend on the PnF density in the culture substratum.

2.2. Spheroid cultures on functional molecule-immobilized substrata

Media containing high concentrations of hepatocyte growth factor (HGF) and epidermal growth factor (EGF) are effective for quick formation of spheroids and high expressions of liver-specific functions [18] in polyurethane foam/spheroid culture systems [19]. Therefore, functional molecules as growth factors are important and indispensable for functional animal cell cultures. However, these requirements are obstacles to the widespread use of functional animal cell culture methods because the functional molecules are very expensive.

An HGF/heparin-immobilized collagen film was developed by using the affinity between HGF and heparin [20]. Heparin will be a suitable biomaterial for hepatocytes because it is found in the liver as a functional glycosaminoglycan and has a high affinity for HGF. Primary rat hepatocytes formed monolayers on a collagen film, but formed spheroids on the HGF/heparin-immobilized collagen film [20]. Furthermore, primary hepatocytes strongly expressed and maintained their albumin production activity on the HGF/heparin-immobilized film even in growth factor-free medium,

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