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Original article

A heparin-modified thermoresponsive surface with heparin-binding epidermal growth factor-like growth factor for maintaining hepatic functions *in vitro* and harvesting hepatocyte sheets

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ABSTRACT

A heparin-modified thermoresponsive surface bound with heparin-binding epidermal growth factor-like growth factor (HB-EGF) was designed to allow creation of transferrable and functional hepatocyte sheets. A heparin-modified thermoresponsive surface was prepared by covalently tethering heparin onto poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide)-grafted tissue culture polystyrene surfaces (Heparin-IC). HB-EGFs were able to stably bind to heparin-IC via affinity interaction. The survival of primary rat hepatocytes was maintained through HB-EGF-bound heparin-IC (HB-EGF/heparin-IC). Moreover, cultured rat primary hepatocytes on HB-EGF/heparin-IC exhibited higher albumin-secretion than hepatocytes cultured on PIPAAm-grafted and collagen-coated surfaces with soluble HB-EGF in the culture medium, regardless of whether soluble EGF was added. These results suggested that HB-EGF/heparin-IC is able to effectively maintain hepatic function via continuous signaling of HB-EGF. After a 4-day cultivation, the cultured hepatocytes on HB-EGF/heparin-IC detached as a cell sheet with fibronectin and HB-EGF only after the temperature was lowered to 20 °C. In addition, higher expression of hepatocyte-specific genes (albumin, hepatocyte nuclear factor 4 alpha, coagulation factor VII, and coagulation factor IX) in hepatocyte sheets was detected on HB-EGF/heparin-IC than on a PIPAAm surface with soluble HB-EGF, indicating that HB-EGF/heparin-IC suppressed the dedifferentiation of cultured hepatocytes. Hence, heparin-modified thermoresponsive surfaces bound with HB-EGF facilitate the fabrication of transferrable hepatocyte sheets with intact hepatic functions and have the potential to provide an *in vitro* culture system using functional hepatocyte sheet tissues, which may serve as an effective hepatocyte-based tissue engineering platform for liver disease treatments.

Abbreviations: IPAAm, *N*-isopropylacrylamide; CIPAAm, 2-carboxyisopropylacrylamide; TCPS, tissue culture polystyrene dishe; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; HB-EGF, heparin-binding EGF-like growth factor; PIPAAm, poly(*N*-isopropylacrylamide) on TCPS; IC, poly(*N*-isopropylacrylamide-*co*-2-carboxyisopropylacrylamide) on TCPS; heparin-IC, heparin-modified IC; HB-EGF$_X$/heparin-IC, HB-EGF-bound heparin-IC; PIPAAm + HB-EGF$_Y$, PIPAAm with soluble HB-EGF; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimetylaminopropyl)-carbodiimide hydrochloride; MES, morpholinoethanesulfonic acid monohydrate; ECM, extracellular matrix; DMEM, Dulbecco's modified Eagle's medium; PBS, Dulbecco's phosphate buffered saline; EDTA, trypsin/ethylenediaminetetraacetic acid; FBS, fetal bovine serum; Alb, albumin; Hnf4α, hepatocyte nuclear factor 4 alpha; F7, coagulation factor VII; F9, coagulation factor IX; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription polymerase chain reaction.

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1. Introduction

Cellular responses including survival, division, differentiation, and apoptosis are regulated by extracellular signaling molecules via receptors on the cellular membrane [1]. Stimulation with the extracellular signaling molecules such as extracellular matrices (ECMs), growth factors, and cytokines triggers intracellular signal transduction and subsequent cellular responses. In conventional cell culture method, soluble signaling molecules including growth factors and cytokines are supplemented in culture medium. However, the frequent dosing of the soluble molecules is required for maintaining the stimulation of cells because of the down-regulation through the endocytosis of the receptors [2]. Covalent immobilization of growth factors and/or ECMs on cell culture surfaces facilitates the long-term stimulation of the receptors without the down-regulation of the receptors [2,3], leading to improved cell growth and maintenance of differentiated state. However, isolation and passage processes using enzymatic treatments such as trypsinization may induce irreversible damage of the receptors, resulting in the reduction of cellular functions [4].

In order to harvest cultured cells nonenzymatically with maintaining cellular functions, our laboratory reported a new type of stimuli-responsive surface that was co-immobilized with ECMs and growth factors [5]. Recently, we designed thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm)-grafted surfaces modified with heparin molecules for switching of sustained stimulation and detachment of cultured cells [6,7]. Heparin has affinity interactions with bioactive molecules such as growth factors, ECMs, and protease [8]. Immobilized heparin on shrunken PIPAAm chains at 37 °C was able to bind basic fibroblast growth factor (bFGF), a heparin-binding growth factor. This heparin-modified surface stabilizes and reinforces the formation of a complex between immobilized heparin and bFGF, thus leading to sustained stimulation of cultured fibroblasts by bFGF and rapid formation of a fibroblast sheet. At 20 °C, by contrast, bound bFGF were released with the cultured fibroblast sheet due to the dynamic motion of heparin accompanied with the swelling of hydrated PIPAAm chains. Thus, the switch from cell proliferation to cell detachment occurred only after the temperature was changed [6].

In present paper, we focused on the cultivation of hepatocytes on the heparin-modified thermoresponsive surfaces. We have previously succeeded in the fabrication and subcutaneous transplantation of hepatocyte sheets [9,10]. However, it is difficult to culture functional primary hepatocytes because the hepatocytes rapidly lose their specific function as the cultivation time is increased. In previous studies, the long-term maintenance of hepatic morphologies and functions have been achieved by co-culturing with nonparenchymal cells [11] and using ECMs such as collagen [12] and Matrigel [13]. However, the conventional culture systems for hepatocytes are not suitable for manipulation and transplantation. In contrast, co-culturing of hepatocyte sheets with nonparenchymal cells, including endothelial cells, on thermoresponsive cell culture surfaces with two-dimensional micropatterning [14] or three-dimensional layering techniques [15] facilitates both the transfer and the functional maintenance of hepatocytes. One of the reasons for this maintenance of hepatic functions is considered to be the continuous stimulation of cellular signaling by bioactive molecules containing growth factors, which are continuously secreted from nonparenchymal cells. If the signaling for the activation of hepatocytes were to continuously be supplied from immobilized growth factors on thermoresponsive cell culture surfaces, the cultured hepatocyte sheet would be able to effectively maintain its functions. In addition, the creation of transplantable hepatocyte sheets with high hepatic functionality would lead to an improvement in the quality of cell-sheet based therapies for liver diseases.

The purpose of this study was the development of a functional thermoresponsive cell culture system that maintains hepatocyte specific functions without non-parenchymal cells and allows for the recovery of a cell sheet. Under typical culture conditions of hepatocytes and their sheets, the addition of soluble EGF, a potent hepatocyte mitogen [16,17], to the culture medium is essential for hepatocyte survival. Here, we utilize affinity-immobilization of heparin-binding epidermal growth factor-like growth factor (HB-EGF), which has the ability to bind to heparin with a specific affinity interaction [18–20] on a heparin-modified thermoresponsive surface for the maintenance of hepatic function. HB-EGF activates EGF receptors (ErbB1 and ErbB4) and, similarly to EGF, acts as a potent hepatocyte mitogen [21–24]. In this study, rat primary hepatocytes were cultured on a heparin-modified thermoresponsive surface with HB-EGF, and the hepatic function was investigated. To demonstrate the effectiveness of affinity-bound HB-EGF in a hepatocyte culture, two culture conditions were examined as follows: 1) hepatocytes on an HB-EGF-bound heparin-modified thermoresponsive surface (Fig. 1a) and 2) hepatocytes on a thermoresponsive surface in a culture medium containing heparin and soluble HB-EGF (Fig. 1b). The albumin secretion of hepatocytes under each condition was determined by enzyme-linked immunosorbent assay (ELISA). After a 4-day cultivation, the hepatocytes were detached as a cell sheet from each of the surfaces. To evaluate the cell function of each hepatocyte sheet, several hepatic mRNA expression levels were determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) analyses.

2. Experimental section

2.1. Chemicals and materials

N-Isopropylacrylamide (IPAAm) was obtained from Kohjin (Tokyo, Japan) and purified by recrystallization from *n*-hexane. 2-Carboxyisopropylacrylamide (CIPAAm) was synthesized using the method described previously [25]. Tissue culture polystyrene dishes (TCPS) (Nunc™, culture area: 8.8 cm^2) were purchased from Thermo Fisher Scientific (Roskilde, Denmark). *N*-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimetylaminopropyl)-carbodiimide hydrochloride (EDC), Triton-X 100, 2-propanol, dexamethasone, and insulin were purchased from Wako Pure Chemicals (Osaka, Japan) and used as received. 2-Morpholinoethanesulfonic acid monohydrate (MES) was purchased from Dojindo (Kumamoto, Japan). Heparin sodium salt from porcine intestinal mucosa (grade I-A, 180 USP units/mg), Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (PBS), and trypsin/ethylenediaminetetraacetic acid (EDTA) solution were obtained from Sigma–Aldrich (St. Louis, MO, USA). Heparin-binding epidermal growth factor-like growth factor (HB-EGF) was purchased from R&D Systems (Minneapolis, Minnesota, USA). L-Proline was obtained from ICN Biomedicals (Aurora, OH, USA). Nicotinamide was obtained from Kanto Chemicals (Tokyo, Japan). Epidermal growth factor (EGF) and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Japan Bioserum (Nagoya, Japan). [^{125}I]-labeled HB-EGF (191 Ci/μg) was obtained from Anawa (Wangen, Switzerland). The ultrapure water used for all experiments was prepared by an ultrapure water purification system (synthesis A10; Millipore, Billerica, MA, USA). Male Wistar rats from 5 to 6 weeks of age were purchased from Japan Laboratory Animals (Tokyo, Japan) and used for isolating primary hepatocytes. The rats were housed under temperature-controlled conditions with a 12-h light/dark cycle and also had *ad libitum* access to rat chow and water. All animal experiments were approved by the Animal Care and Use Committee