Biochemical and Biophysical Research Communications 402 (2010) 153–157

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Three-dimensional constructs induce high cellular activity: Structural stability and the specific production of proteins and cytokines

Koji Kadowaki [a], Michiya Matsusaki [a,b], Mitsuru Akashi [a,*]

[a] Department of Applied Chemistry, Graduate School of Engineering, Osaka University, 2-1 Yamada-oka, Suita 565-0871, Japan
[b] PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

ARTICLE INFO

Article history:
Received 22 September 2010
Available online 8 October 2010

Keywords:
Hierarchical cell manipulation
Layer-by-layer assembly
3D-cellular multilayer
Heat shock protein70 expression
Interleukin-6 expression
Tissue engineering

ABSTRACT

The specific properties responsible for the stability and function induced by three-dimensional (3D) cellular constructs were evaluated and compared to a monolayer structure. 3D-cellular multilayers composed of human fibroblast cells (FCs) and human umbilical vascular endothelial cells (ECs) were fabricated by a hierarchical cell manipulation technique. Interestingly, the ECs adhered homogeneously onto four-layered (4L) FCs, and tight-junction formation was widely observed at the centimeter scale, while heterogeneous EC domain structures were observed on the monolayered (1L) FCs. The production of heat shock protein70 (Hsp70) and interleukin-6 (IL-6) from the cellular structures were investigated to elucidate any 3D-structural effect on cellular function. The Hsp70 expression of the ECs decreased after adhesion onto the 4L-FC structure as compared with the EC monolayer. Surprisingly, the Hsp70 production response to heat shock increased drastically by approximately 10-fold as compared with a non-heat shock by 3D structure formation, whereas the monolayer structures showed no change. Moreover, the production of the inflammatory cytokine IL-6 decreased significantly depending on the layer number of FCs. To the best of our knowledge, this is the first report on a basic, 3D-structural effect on cellular stability and function. These findings could be important for not only tissue engineering, but also for basic cell biology.

1. Introduction

Tissues and organs are highly organized hierarchical architectures composed of cells, extracellular matrix (ECM), and signaling molecules. Most cells are surrounded by ECM fibers, which are typically composed of collagen and fibronectin (FN), and the fibrous meshwork of the ECM plays an important role in the control of the cellular interface architecture and function in vivo [1–3]. One of the goals of tissue engineering is the creation of three-dimensional (3D) artificial tissues consisting of various cell types and ECM, which resemble the structure and functions of natural tissues closely. However, an effective methodology to fabricate 3D-engineered tissues composed of cells and ECM, with the appropriate components and thickness, has not been achieved yet. Recently, new technologies such as cell sheet engineering [4], magnetic liposomes [5], chitosan–DNA films [6], and gel layers containing cells [7] have been reported in the fabrication of layered cellular architectures. We also reported a novel hierarchical cell manipulation technique for developing 3D-cellular multilayers by the fabrication of nanometer-sized layer-by-layer (LbL) films composed of FN and gelatin (G) onto cell membranes [8]. Interestingly, a morphological change of the homogeneous FN-G nanofilms to nano-meshworks similar to the native ECM on the cell surface was observed [9]. These nano-meshworks would function analogously to the native ECM for cell adhesion at each layer. This technique enabled the fabrication of various hierarchical structures composed of target cells and ECMs, as well as the precise control of the cell layer number.

Studies on the functions of layered cellular architectures as compared with cell monolayer are valuable not only for understanding how a 3D environment composed of cells, ECM, and signaling molecules regulates functions similar to natural tissues, but also for creating 3D-artificial tissues resembling natural tissues. Recently, some researchers have reported the functions of layered cellular architectures in vitro [5,6,10–12]. For example, Rajagopalan et al. demonstrated an enhancement of liver-specific functions by preparing a bilayer structure composed of hepatocytes and other cells [6]. However, the basic properties induced by 3D-cellular structures, such as the layer number or the cell types, have not been clarified yet.

In this study, we evaluated the structural stability of layered constructs consisting of human fibroblast cells (FCs) and human umbilical vascular endothelial cells (ECs) in relation to their layer number. The production of heat shock protein70 (Hsp70) was also investigated to elucidate the heat stimulus response of the 3D constructs relative to the cell type combination and layer number.

* Corresponding author. Fax: +81 6 6879 7359.
E-mail address: akashi@chem.eng.osaka-u.ac.jp (M. Akashi).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.10.007

Moreover, we observed that the layered structure provides a biologically suitable environment for the cells as compared with tissue culture dishes by inflammatory cytokine (IL-6) production experiments. The results of this study gives, for the first time, basic and important information on the effects of 3D structures on cell functions in the biological and biomedical fields.

2. Materials and methods

2.1. Materials

All reagents were used without further purification. Fibronectin (FN) from bovine plasma was purchased from SIGMA–Aldrich (St. Louis, USA). Gelatin (G), Dulbecco's modified Eagle's medium (DMEM), and 10% formalin solution were obtained from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Biowest (Miami, USA). Goat anti-mouse Alexa Fluor 488-conjugated IgG (A11001) was purchased from Invitrogen (CA, USA). The monoclonal mouse anti-human CD31 antibody was purchased from Dako (JC70A, Glostrup, Denmark).

2.2. Cell culture

Human primary dermal fibroblasts (FCs) and umbilical vascular endothelial cells (ECs) (CAMBREX, USA) at passages 4–9 were used in the present study. The FCs were cultured in fibroblast basal medium-2 (FGM-2; CAMBREX, USA) containing hFGF-B, insulin, 2% FBS, and GA-1000, and the ECs were cultured in endothelial basal medium (EGM-2; CAMBREX, USA) containing hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, 5% FBS, hEGF, and GA-1000. The FC and EC cultures were maintained in 5% CO_2 at 37 °C.

2.3. Fabrication of layered structures

For the LbL assembly of FN-G films on a cell surface, a plastic disk (SUMILON Cell Desk, Sumitomo Bakelite Co. Ltd., Japan) for fluorescence microscopy and histological evaluation was used as the substrate. The substrate was immersed into a 50 mM Tris–HCl buffer solution (pH = 7.4) of FN (0.2 mg/ml) for 15 min, and then FCs at 6×10^4 cell/cm^2 were seeded onto the substrate and incubated in FC culture medium for 24 h at 37 °C. The monolayered cells on the substrate were alternately immersed into 0.2 mg/ml FN or G solutions (50 mM Tris–HCl buffer, pH = 7.4) for nine steps, plus a rinse with 50 mM Tris–HCl buffer for 1 min at 37 °C. The thickness of the FN-G films was approximately 6 nm [8]. Thereafter, cells at confluent density were seeded as the second layer, and incubated for 24 h at 37 °C. The FN-G assembly and the cell seeding were repeated for a predetermined time. DMEM containing 10% FBS was used for cell culture after the uppermost layer of cells was seeded.

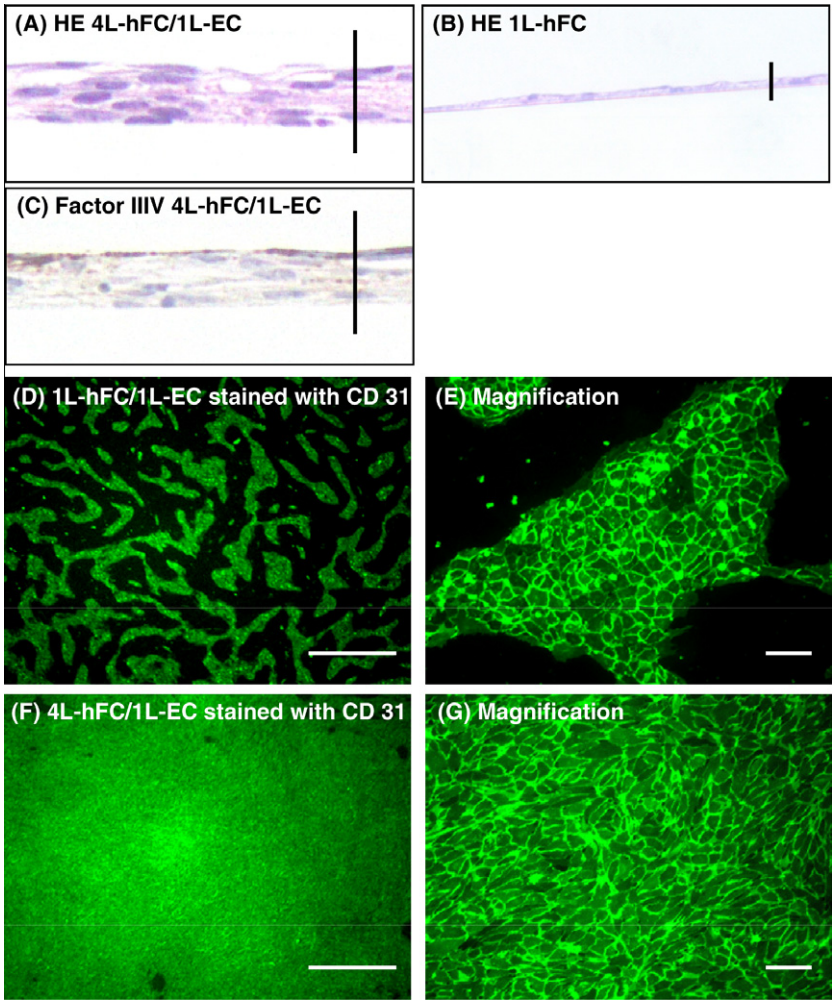

Fig. 1. Histological images: (A) stained with HE and (C) immunostained for factor VIII of the 4L-FC/1L-EC construct as compared to (B) HE images of the 1L-FC construct. Fluorescent immunostaining images with an anti-CD31 antibody of (D and E) 1L-FC/1L-EC and (F and G) 4L-FC/1L-EC constructs. The scale bars are (A and C) 40 μm, (B) 20 μm, (D and F) 3000 μm, and (E and G) 100 μm, respectively.

2.4. Fluorescence microscopic observation of layered structures

For the fluorescence observation of the EC layers on mono- or four-layered (1L or 4L) FCs, the layered structures on a plastic disk were immunostained. Briefly, the samples were fixed with 10% formalin and then rinsed with phosphate buffered saline (PBS) 3 times. After immersion in PBS containing 0.2% Triton X-100 for 10 min at room temperature, the samples were rinsed with PBS 5 times, and then blocked with PBS containing 1% BSA for 1 h at room temperature. The samples were next immersed into PBS containing 1% BSA plus a monoclonal mouse anti-human CD31 antibody (1:40) for 1 h at room temperature, rinsed with PBS 3 times, and then immersed into PBS containing 1% BSA plus goat anti-mouse Alexa 488-conjugated IgG (1:200) for 1 h at room temperature. After rinsing with PBS 5 times, and the nuclei were labeled with 4′-6-diamidino-2-phenylin-dole (DAPI), the obtained samples were observed by a DSU-IX81-SET fluorescence microscope (Olympus, Japan).

2.5. Measurement of total DNA content of layered structures

The amount of DNA in the various layered structures was determined with a DNA assay kit (DNeasy Tissue Kit, QIAGEN, Germany). The fabrication of various layered structures was performed in the above-mentioned manner, and the DNA in the layered structures was extracted at 48 h of incubation after the uppermost layer of cells was seeded. The total DNA content was subsequently measured with the DNA assay kit.

2.6. Measurement of Hsp70 and IL-6 production from layered structures

The expressions of Hsp70 and IL-6 from various layered structures were detected with a Hsp70 enzyme-linked immunosorbent assay (ELISA) kit (Assay Designs, Inc., USA) and a Quantikine Human IL-6 ELISA kit (R&D Systems, USA), respectively, according to the manufacturer's recommended procedures. Briefly, various layered structures composed of FCs and ECs were fabricated as above. At 48 h of incubation after the uppermost layer of cells was seeded, the samples were rinsed with PBS, and then cell lysis was performed. For the heat shock experiments [13], the layered structures were heated by immersing the culture dishes, which were sealed with Parafilm, into a water bath. After heating at 45 °C for 20 min, the layered structures were incubated at 37 °C for 2 h, and then cell lysis was performed. The cell lysates were kept at −80 °C until assayed, and the amount of Hsp70 in the cell lysates was quantified by the ELISA kit. For the IL-6 assay, the supernatant from the culture medium of the layered structures was collected after 48 h of incubation, and was kept at −80 °C until assayed. The amount of IL-6 was determined by the ELISA kit. The total amount of protein from the layered structures in the cell lysates was analyzed using a Protein Quantification Kit-Rapid (Doj-

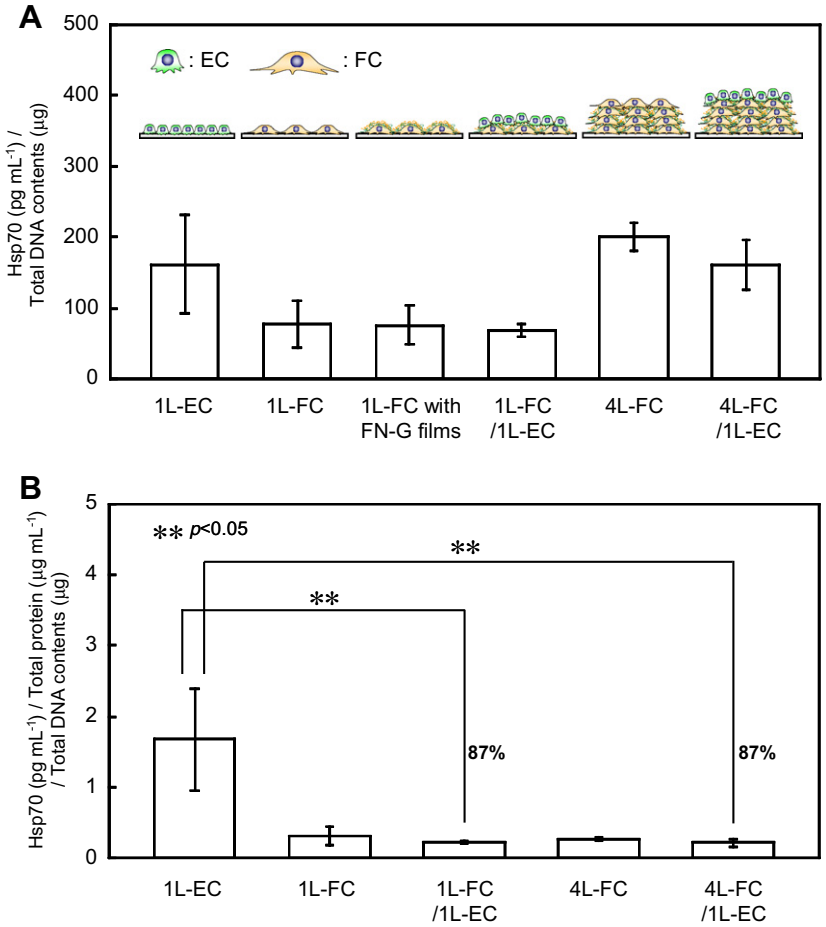

Fig. 2. (A) Production of Hsp70 versus the amount of DNA and (B) Hsp70 production versus the total amount of protein of non-heat shocked layered structures composed of FCs and ECs at 48 h of incubation after the top layer of cells was seeded ($n = 4$). The asterisks (**) denote a statistically significant difference between the samples calculated by a two sample t-test.

indo, Japan). The amount of Hsp70 or IL-6 versus the total protein from the layered structures was estimated from these results.

3. Results and discussion

3.1. Histological and morphological evaluations of layered structures

To elucidate the structural stability of the layered constructs, five-layered (5L) constructs consisting of 4L-FCs plus an uppermost layer of ECs (4L-FC/1L-EC) was fabricated, and the histological and morphological evaluations were performed. Approximately 6 nm of the FN-G films was prepared on cell a surface based on our previous studies to provide a cell adhesive scaffold for the second layer of cells [8]. Hematoxylin and eosin (HE) staining images clearly showed precise 5L-structures (Fig. 1A), and immunostaining for factor VIII of the ECs revealed the presence of ECs in the uppermost layer (Fig. 1C). To clarify the detailed morphology of the EC layer in relation to the layer number of the constructs, fluorescence microscopic observations of ECs fluorescently labeled with the anti-CD31 antibody were performed on the 1L-FC/1L-EC and 4L-FC/1L-EC constructs after 48 h of culture (Fig. 1D–G). Interestingly, the ECs on the 1L-FC showed domain structures, even though homogeneous primary adhesion was observed after 24 h of incubation, whereas the homogeneous adhesion of ECs on the 4L-FC structures was observed at the centimeter scale. The magnified image in Fig 1G clearly shows tight-junction formation of the ECs in the 5L-structures. These effects of the layer number on the morphology of the adhered ECs are probably due to the basal layers. The monolayer structure of the FCs seems to be insufficient to attenuate the effect of a plastic substrate on the upper layer of ECs. Furthermore, the difference in cytokine production from the basal FC layers may affect the EC layers. The 3D-abundant cells and the ECM protein structures of the 4L-FC construct would provide satisfactory environment for the uppermost ECs. In order to clarify in detail the cellular functions related to the 3D-cellular structure, the production of Hsp70 and IL-6 from variously-layered structures was investigated.

3.2. Hsp70 production from layered structures

The Hsp70 families are expressed in response to different types of cellular stress, such as heat elevation, mechanical trauma, chemical reagents, and heavy metals [14,15]. Thus, when cells suffer physical and physicochemical stress from the environment, the production of Hsp70 should increase to serve as molecular chaperones in protein folding and transport [16]. In our previous paper, we found that Hsp70B mRNA expression is useful for studying cell-polymer interactions and the biocompatibility of materials [13]. In addition, it is widely accepted that the functions of the Hsp70 families are related to cellular activation. Therefore, the production of Hsp70 from the

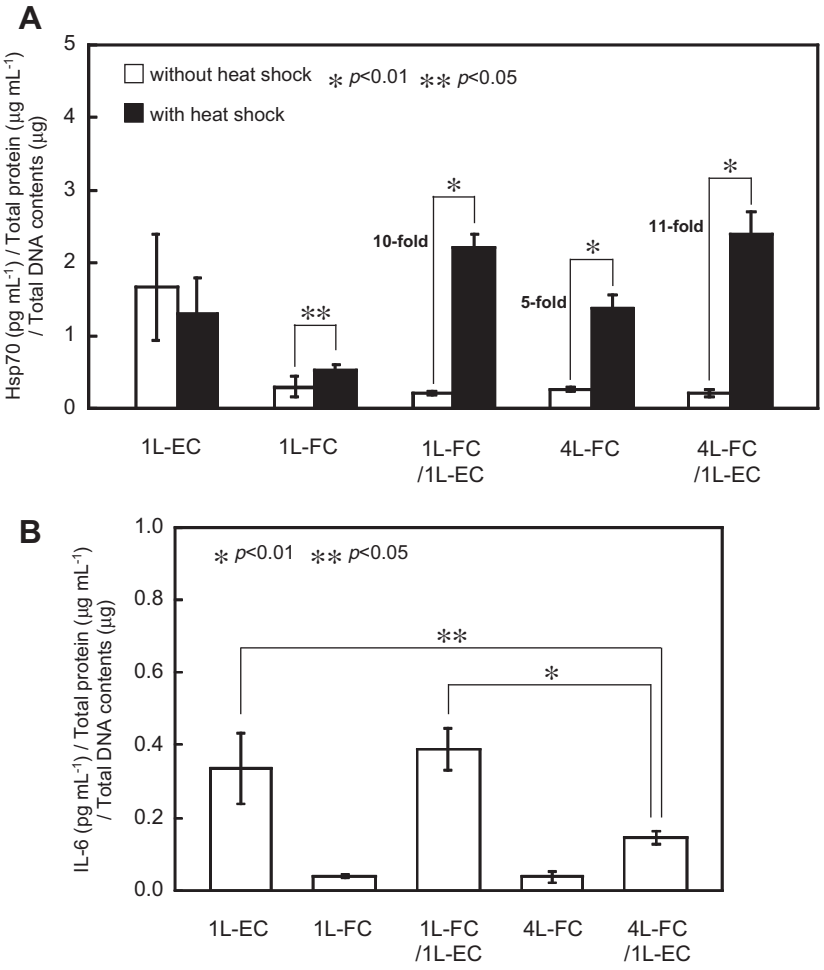

Fig. 3. (A) Hsp70 production versus the total protein from non-heat shocked and heat shocked layered structures composed of FCs and ECs ($n = 4$). The heat shock condition is defined as 20 min of incubation at 45 °C, followed by a 2 h recovery period. (B) IL-6 production versus the total protein from layered structures composed of FCs and ECs ($n = 3$). The asterisks (∗, ∗∗) denote a statistically significant difference between the samples calculated by a two sample t-test.

layered structures was investigated to elucidate the effect of the 3D structures on cellular function. FN-G films prepared on the cell surface did not show any effect on the production of Hsp70 (Fig. 2A) probably due to the high cytocompatibility of the FN-G films [9]. The production of Hsp70 from 4L-FC and 4L-FC/1L-EC constructs was slightly higher than that of 1L-FC and 1L-FC/1L-EC, but there were no significant differences between 1L-EC, 1L-FC, and 1L-FC/1L-EC. However, the production of total protein from 4L-FC and 4L-FC/1L-EC was significantly different from the monolayers (ca. 1.5 ~2.0-fold higher), and thus the amount of Hsp70 should be analyzed in relation to the total protein expression. Fig. 2B shows the results of Hsp70 expression per total protein per cell in each sample. The EC monolayer induced the highest Hsp70 production, and both 1L- and 4L-FC basal layers effectively suppressed the Hsp70 production (about 90% reduction) from the upper EC layers, suggesting a comfortable and stress-free 3D-environment for the ECs.

To elucidate the Hsp70 production of the layered structures in response to heat shock, the samples were incubated at 45 °C for 20 min, which is the general condition defined as heat shock [13]. In the case of monolayers of ECs and FCs, the Hsp70 production was almost the same as under the non-heat shock conditions. Surprisingly, the layered constructs showed significantly higher Hsp70 production, 5- to 11-fold higher than those under non-heat shock conditions. These results suggested that the 3D-layered structures dramatically enhanced cellular thermosensitivity to produce the proteins, although the monolayered cells directly adhered onto the plastic disk revealed quite low sensitivity, probably due to the effect of the substrate. The 3D-environment consisting of cells and nano-meshworks like the ECM should provide comfortable conditions similar to the native tissues. On the other hand, plastic disks or dishes such as tissue culture polystyrene (TCPS) are widely used for cell culture due to their high cell adhesive property, but induction of inflammation has been reported [17]. Thus, we evaluated the production of inflammation cytokine from the layered structures.

3.3. Production of IL-6 from layered structures

The production of the inflammatory cytokine IL-6 from the layered structures and monolayers was detected by an ELISA method, because it is known that IL-6 plays an essential role in intercellular communication [18]. The production of IL-6 from the 1L-EC was 8-fold higher than that from the 1L-FC (Fig. 3B), suggesting that the ECs suffered from an inflammatory response due to the substrate as compared to the FCs. Accordingly, the Hsp70 production of the 1L-EC was also higher than that of the 1L-FC, because Hsp70 is known to be related to the inflammatory response to protect the structure and functions of unfolded proteins as a molecular chaperone protein [16]. The IL-6 production of 1L-FC/1L-EC bilayered structures was slightly higher than that of 1L-EC, indicating that the bilayered structure was not sufficient to suppress the inflammatory response from the substrate. On the other hand, about 60% lower IL-6 production as compared to the 1L-EC and 1L-FC/1L-EC constructs was observed in the 4L-FC/1L-EC construct, probably due to the biocompatible 3D-constructs consisting of abundant cells and proteins. These results demonstrated that 3D-layered constructs of fibroblast cells would be a good basal layer for primary cell culture to maintain their native properties and functions.

In conclusion, we investigated for the first time the effects of 3D-cellular structures on cell stability and function in relation to their layer number. These results suggested that 4L-FC provided a more favorable environment for ECs than a cell culture plastic disk to induce high thermosensitivity, and to suppress the inflammatory response from the substrate. The layered construct would be an analogous environment to natural tissues. Although it is difficult to identify which kinds of cells expressed these Hsp70 and IL-6, the results from this study provide basic and valuable information in the biological and biomedical fields. We are now performing further experiments using 3D-cellular constructs, and layered architectures consisting of various types of cells may be useful as a tissue model for cell biology and pharmaceutical assays.

Acknowledgments

This work was supported mainly by the Industrial Technology Research Grant Program in 2006 (06B44017a) from NEDO of Japan, partly by the Mitsubishi Chemical Corporation Fund, PRESTO-JST, and by The Noguchi Institute.

References

[1] N.J. Boudreau, P.L. Jones, Extracellular matrix and integrin signalling: the shape of thing to come, Biochem. J. 339 (1999) 481–488.
[2] R.O. Hynes, Fibronectins, Springer Verlag Inc., New York, 1990.
[3] E.W. Raines, The extracellular matrix can regulate vascular cell migration, proliferation, and survival: relationship to vascular disease, Int. J. Exp. Pathol. 81 (2000) 173–182.
[4] T. Sasagawa, T. Shimizu, S. Sekiya, Y. Haraguchi, M. Yamato, Y. Sawa, T. Okano, Design of prevascularized three-dimensional cell-dense tissue using a cell sheet stacking manipulation technology, Biomaterials 31 (2010) 1646–1654.
[5] A. Ito, H. Jitsunobu, Y. Kawabe, M. Kamihira, Construction of heterotypic cell sheets by magnetic force-based 3-D coculture of HepG2 and NIH3T3 cells, J. Biosci. Bioeng. 104 (2007) 371–378.
[6] P. Rajagopalan, C.J. Shen, F. Berthiaume, A.W. Tilles, M. Toner, M.L. Yarmush, Polyelectrolyte nano-scaffolds for the design of layered cellular architectures, Tissue Eng. 12 (2006) 1553–1563.
[7] L. Grossin, D. Cortial, B. Saulnier, O. Felix, A. Chassepot, G. Decher, P. Netter, P. Schaaf, P. Gillet, D. Mainard, J. -C. Voegel, N. Benkirane-Jessel, Step-by-step build-up of biologically active cell-containing stratified films aimed at tissue engineering, Adv. Mater. 21 (2009) 650–655.
[8] M. Matsusaki, K. Kadowaki, Y. Nakahara, M. Akashi, Fabrication of cellular multilayers with nanometer-sized extracellular matrix films, Angew. Chem. Int. Ed. 46 (2007) 4689–4692.
[9] K. Kadowaki, M. Matsusaki, M. Akashi, Control of cell surface and functions by layer-by-layer nanofilms, Langmuir 26 (2010) 5670–5678.
[10] M. Harimoto, M. Yamato, M. Hirose, C. Takahashi, Y. Isoi, A. Kikuchi, T. Okano, Novel approach for achieving double-layered cell sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes, J. Biomed. Mater. Res. 62 (2002) 464–470.
[11] M. Ohno, K. Motojima, T. Okano, A. Taniguchi, Maturation of the extracellular matrix and cell adhesion molecules in layered co-culture of HepG2 and endothelial cells, J. Biochem. 145 (2009) 591–597.
[12] N. L'Heureux, J. -C. Stoclet, F.A. Auger, G.J. Lagaud, L. Germain, R. Andriantsitohaina, A human tissue-engineered vascular media : a new model for pharmacological studies of contractile responses, FASEB J. 15 (2001) 515–524.
[13] S. Kato, T. Akagi, K. Sugimura, A. Kishida, M. Akashi, Evaluation of biological responses to polymeric biomaterials by RT-PCR analysis III: study of HSP 70, 90 and 47 mRNA expression, Biomaterials 19 (1998) 821–827.
[14] M. Ashburner, J.J. Bonner, The induction of gene activity in drosophila by heat shock, Cell 17 (1979) 241–254.
[15] M.J. Schlesinger, Heat shock proteins: the search for functions, J. Cell Biol. 103 (1986) 321–325.
[16] J.G. Kiang, G.C. Tsokos, Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology, Pharmacol. Ther. 80 (1998) 183–201.
[17] S. Sawada, S. Sakaki, Y. Iwasaki, N. Nakabayashi, K. Ishihara, Suppression of the inflammatory response from adherent cells on phospholipid polymers, J. Biomed. Mater. Res. 64A (2003) 411–416.
[18] S.J. Van, Interleukin-6: an overview, Annu. Rev. Immunol. 8 (1990) 253–278.