J Mater Sci: Mater Med (2011) 22:2071–2078
DOI 10.1007/s10856-011-4394-4

Morphological and functional behaviors of rat hepatocytes cultured on single-walled carbon nanotubes

Haruka Koga · Tsuyohiko Fujigaya · Naotoshi Nakashima · Kohji Nakazawa

Received: 16 March 2011 / Accepted: 9 July 2011 / Published online: 19 July 2011

Abstract This study describes the morphological and functional behaviors of rat hepatocytes on single-walled carbon nanotube (CNT)-coated surfaces. Although the hydrophobic characteristics of CNT-coated surfaces increased with increasing CNT density, hepatocyte adhesion decreased, indicating that the interaction between hepatocytes and CNTs is weak. We found that hepatocytes on a CNT-coated surface gradually gather together and form spheroids (spherical multicellular aggregates). These spheroids exhibit compact spherical morphology with a smooth surface and express connexin-32, an intracellular communication molecule. In contrast, collagen treatment in conjunction with the CNT-coated surface improved hepatocyte adhesion, and the cells maintained a monolayer configuration throughout the culture period. The albumin secretion and ammonia removal activities of hepatocyte spheroids were maintained at elevated levels for at least 15 days of culturing as compared with hepatocyte monolayers. These results indicate that CNTs can be used for the formation and long-term culture of hepatocyte spheroids.

H. Koga · K. Nakazawa (✉)
Department of Life and Environment Engineering, The University of Kitakyushu, 1-1 Hibikino, Wakamatsu-ku, Kitakyushu 808-0135, Japan
e-mail: nakazawa@env.kitakyu-u.ac.jp

T. Fujigaya · N. Nakashima
Department of Applied Chemistry, Graduate School of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

N. Nakashima · K. Nakazawa
JST-CREST, 5 Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan

1 Introduction

Nanostructure scaffolds have the capability to direct and guide cultured cells. Therefore, nanomaterials, such as nanoparticles, nanofibers, and nanotubes, advocated as useful components for cell scaffolding that mimic tissue architecture in the fields of tissue engineering and regenerative medicine. Among the various nanomaterials, carbon nanotubes (CNTs) have attracted attention as one of the most useful scaffolds for biological and medical applications due to their unique structural, electrical, and mechanical properties [1–4]. For example, CNTs are reported to be able to support the attachment, growth, differentiation, and long-term survival of neural cells [5–7]. Other studies show that bone cells can attach and proliferate well on CNT surfaces, confirming that CNTs are useful scaffolds for bone tissue engineering [8–10]. Furthermore, some researchers report that CNTs can be used as a culture substratum for fibroblasts as well as endothelial and smooth muscle cells [11–13]. Although the effects of CNTs on mammalian cell cultures are becoming clearer, research is still limited to a few cell types.

The liver is the main metabolic organ that maintains normal physiology and performs many vital functions. Therefore, primary hepatocytes are used in various applications such as liver tissue engineering as well as pharmacological, toxicological, and fundamental cell biology studies. The design of biomaterials is important for such applications, because primary hepatocytes exhibit different morphological and functional characteristics depending on the surface properties of the biomaterials on which they exist. The efficacy of various nanomaterials is reported among the various approaches for hepatocyte culture. Tanaka et al. [14] report that hepatocytes form a cuboidal shape on a honeycomb-patterned nanofilm while

maintaining their function. Furthermore, recent works show that nanofibers that consist of natural or synthetic polymers promote hepatocyte aggregation and functioning [15–17]. In particular, nanostructural materials can facilitate the formation of in vivo-like hepatocytes morphology and help maintain liver-specific functions. Therefore, CNTs are a very promising material for hepatocyte culture. However, as far as we know, there is no existing report on hepatocyte culture using CNT materials.

In this study, we focus on hepatocyte behavior on single-walled CNT-coated surfaces. The changes in morphology, expression of cell adhesion molecules, and functional activity of primary rat hepatocytes on CNT-coated surfaces were evaluated and their characteristics were compared with those of cells cultured using other methods. The goal of this study is to understand the effects of CNTs on hepatocyte behavior in order to clarify the effects of CNTs in cell culturing.

2 Materials and methods

2.1 CNT-coated dish

The CNT-coated dish was purchased from Meijo Nano Carbon Co., Ltd. (35 mm diameter; Nagoya, Japan). This dish was repetitively coated with CNTs (1.0–2.5 nm in diameter, 5–10 μm in length) that were dissolved in ethanol. Although immobilized on the dish surface by physical adsorption, the CNTs were stably maintained throughout the cell culture period. In this study, we used the CNT-coated dishes at CNT densities of approximately 1, 3, and 6 μg/dish (Fig. 1a).

2.2 Rat hepatocyte culture

This experiment was approved by the Committee of Ethics on Animal Experiments in our institute and was carried out in accordance with the Guidelines for Animal Experiments at our institute.

Hepatocytes were isolated from the whole livers of adult Wistar rats (males, 7–8 weeks old, weighing approximately 200 g) by liver perfusion using 0.05% collagenase (Wako Pure Chemical Industries Ltd., Osaka, Japan) [18]. Cell viability was determined using the trypan blue exclusion method. Cells with more than 85% viability were used in this study. The culture medium consisted of Dulbecco's modified Eagle's medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10 mg/l insulin (Sigma, St. Louis, MO, USA), 7.5 mg/l hydrocortisone (Wako), 50 μg/l epidermal growth factor (Biomedical Technologies Inc., Stoughton, MA, USA), 60 mg/l proline (Wako), 50 μg/l linoleic acid (Sigma), 0.1 μM $CuSO_4 \cdot 5H_2O$, 3 μg/l H_2SeO_3, 50 pM $ZnSO_4 \cdot 7H_2O$, 58.8 μg/ml penicillin, 100 μg/ml streptomycin, 1.05 g/l $NaHCO_3$, and 1.19 g/l HEPES.

The morphological and functional properties of hepatocytes were compared using the following four culture conditions: a CNT-coated dish, a CNT/collagen-coated dish (in which the CNT-coated dish was coated with 0.3 mg/ml type I collagen solution; Cellmatrix, Nitta Gelatin Co., Osaka, Japan), a tissue culture (TC) dish

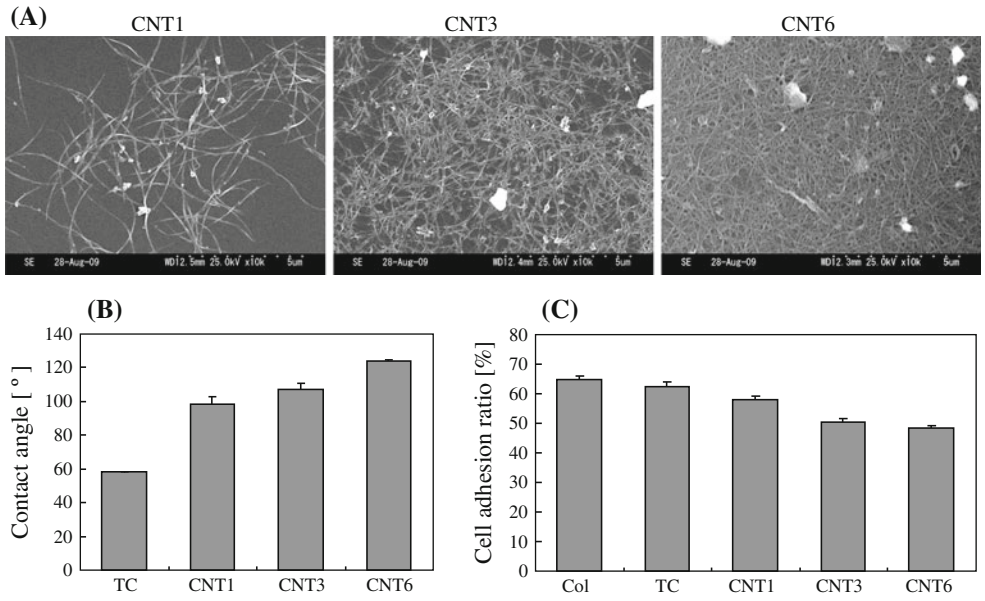

Fig. 1 SEM images (**a**), contact angles (**b**), and cell adhesion ratios (**c**) of each CNT-coated surface. *CNT1* 1 μg CNT-coated dish, *CNT3* 3 μg CNT-coated dish, *CNT6* 6 μg CNT-coated dish, *TC* tissue culture dish, *Col* collagen-coated dish

(35 mm diameter, Corning 430165; Corning Inc., Corning, NY, USA), and a collagen-coated dish (in which the TC dish was coated with 0.3 mg/ml type I collagen solution).

The hepatocyte suspension (cell density, 2.5×10^5 cells/ml) was inoculated into each dish containing 2 ml culture medium for polymerase chain reaction (PCR) analysis and the evaluation of liver function. Cells (5.0×10^4) were inoculated into each dish to measure the cell extension ratio and actin filament staining. The media were changed 24 h after inoculation and at 1-day intervals thereafter. All cells were cultured under a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.3 Cell extension ratio

In order to evaluate changes in the extension area of individual cells over time, images of 30 cells were obtained at 0, 1, 3, 5, 7, 10, and 15 days of culturing by using a phase-contrast microscope. The cell extension area was measured by using a Windows personal computer equipped with a 2-dimensional image analysis program (Win ROOF; Mitani Corp., Fukui, Japan). Using the area of the prepared cells (suspension cells) as a reference, the changes in cell extension ratios under the four culture conditions were calculated.

2.4 Actin and nuclear staining

In order to evaluate the distribution of actin filaments and nuclei in the hepatocytes, the cells were fixed with a solution containing 2.5% glutaraldehyde and 2.0% paraformaldehyde at room temperature for 10 min. The fixed samples were permeabilized with 0.1% Triton-X for 5 min and blocked in PBS containing 1% BSA for 20 min. The samples were then incubated with 5 units/ml rhodamine–phalloidin (Molecular Probes, Eugene, OR, USA) for 20 min and 1 μg/ml DAPI (Wako) for 5 min to stain the actin filaments and nuclei (DNA) in the cells, respectively. All procedures were performed at room temperature. The samples were observed with a fluorescent microscope (Biorevo BZ-9000™; Keyence, Osaka, Japan).

2.5 Cell number and cell adhesion ratio

The cell number in each culture at a given time was determined using a modified DNA–DAPI fluorescence method [19]. In brief, the DNA from the cells was extracted using a DNA extraction solution consisting of TEN buffer (50 mM Tris–HCl, 5 mM EDTA, and 100 mM NaCl; pH 7.5) with 100 μg/ml proteinase K (Wako). A DAPI solution consisting of TEN buffer with 1 μg/ml DAPI (Wako) was prepared; the extracted DNA solution was mixed with the DAPI solution in a 1:1 ratio. Fluorescence was measured using a fluorescence plate reader with excitation and emission wavelengths at 355 and 460 nm, respectively (Fluoroskan Ascent®; Thermo Electron Corp., Vantaa, Finland). The standard curve of cellular DNA was constructed using a cell suspension with a cell density ranging from 0.5×10^5 to 1.0×10^6 cells/ml. The standard curve was used to convert the DNA–DAPI fluorescence values into cell numbers.

The cell adhesion ratio under each condition was evaluated at 5 days of culture. The ratio was determined as follows: cell adhesion ratio = number of cells remaining on the dish/number of inoculated cells.

2.6 Real-time PCR analysis of cell adhesion molecules

The expression levels of 2 types of cell adhesion molecules—integrin-β1 (a cell–matrix adhesion molecule) and connexin-32 (a cell–cell interaction molecule)—and a housekeeping gene, *GAPDH*, were analyzed by using real-time PCR.

Total RNA was extracted from the hepatocytes at days 1 and 15 of culturing under each condition and from the rat liver tissue by using a spin column (NucleoSpin® RNA II; Nippon Genetics Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 0.2 μg total RNA by using a high-capacity cDNA reverse-transcription kit (Applied Biosystems, Tokyo, Japan). The samples were then stored at −20°C until they were processed for PCR analysis. PCR was performed in an Applied Biosystems StepOne™ real-time PCR system by using TaqMan® Gene Expression Assay Kits (integrin-β1, Rn00566727_m1; connexin-32, Rn01641031_s1; GAPDH, Rn99999916_s1; Applied Biosystems).

The comparative cycle–time method was used to quantify gene expression levels according to the manufacturer's instructions. The gene expression levels of the cell adhesion molecules were normalized to that of *GAPDH*. The change in the gene expression levels under each culture condition was calculated using the corresponding gene expression level in the liver tissue as the reference.

2.7 Liver-specific functions

The liver-specific functions of the hepatocytes were evaluated on the basis of their albumin secretion and ammonia removal activities, which are representative of the synthesis and detoxification functions of the liver, respectively. The concentration of albumin secreted into the culture media over a 24-h period was detected by an enzyme-linked immunosorbent assay (ELISA). To evaluate ammonia removal, the culture media were replaced with fresh media supplemented with 1 mM NH_4Cl. The ammonia

concentration was measured using a commercial kit (Wako). The ammonia removal activity was calculated on the basis of the decrease in ammonia concentration over the 24-h period after the culture media were refreshed.

Albumin secretion and ammonia removal activities were evaluated at days 1, 5, 10, and 15 of culturing, and the values were normalized with the cell numbers at each time point.

2.8 Statistical analysis

The data obtained from the functional activities of the rat hepatocytes are presented as the mean (SD) of five experiments. Statistical analysis of numerical variables was performed using repeated-measures ANOVA. A value of $P < 0.05$ was considered significant.

3 Results

3.1 Cell adhesion on the CNT-coated surface

The CNT-coated surface exhibited hydrophobic characteristics as compared with the TC surface, and the contact angles increased with increasing CNT density (Fig. 1b). However, the cell adhesion ratios decreased with increasing CNT density (Fig. 1c), indicating that the interaction between hepatocytes and CNTs is weak, and the hydrophobic characteristics of CNTs lead to weak hepatocyte adhesion. Because the cell adhesion ratios at CNT densities greater than 3 μg/dish were almost the same, the following experiments used CNT-coated surfaces at a density of 3 μg/dish.

3.2 Changes in hepatocyte morphology

A weak interaction between hepatocytes and CNTs led to the formation of hepatocyte spheroids (spherical multicellular aggregates), which are formed by the compaction of cell aggregates (Fig. 2). Although the hepatocytes in the CNT-coated dish attached to and spread over the surface until 2–3 days of culturing, they then gradually gathered together and formed spheroids. Spheroid formation increased with time, and most hepatocytes formed spheroids. An almost identical phenomenon occurred on the TC dish. In contrast, in the collagen- and CNT/collagen-coated dishes, the hepatocytes adhered to the surface and assumed a monolayer configuration after 1 day of culturing that was maintained throughout the culturing period (Fig. 2).

To understand the morphological properties, hepatocytes were cultured at low cell density (5.0×10^4 cells/dish). Although hepatocytes on the CNT-coated and TC dishes spread slightly over each surface, they maintained their cuboidal shape (Fig. 3a). In contrast, cells on the collagen- and CNT/collagen-coated dishes spread over the entire surface, and actin stress fibers were strongly expressed in each cell.

The cell extension ratios on the CNT-coated and TC dishes increased slightly until 1 day of culturing; they were almost constant throughout the remainder of the culture

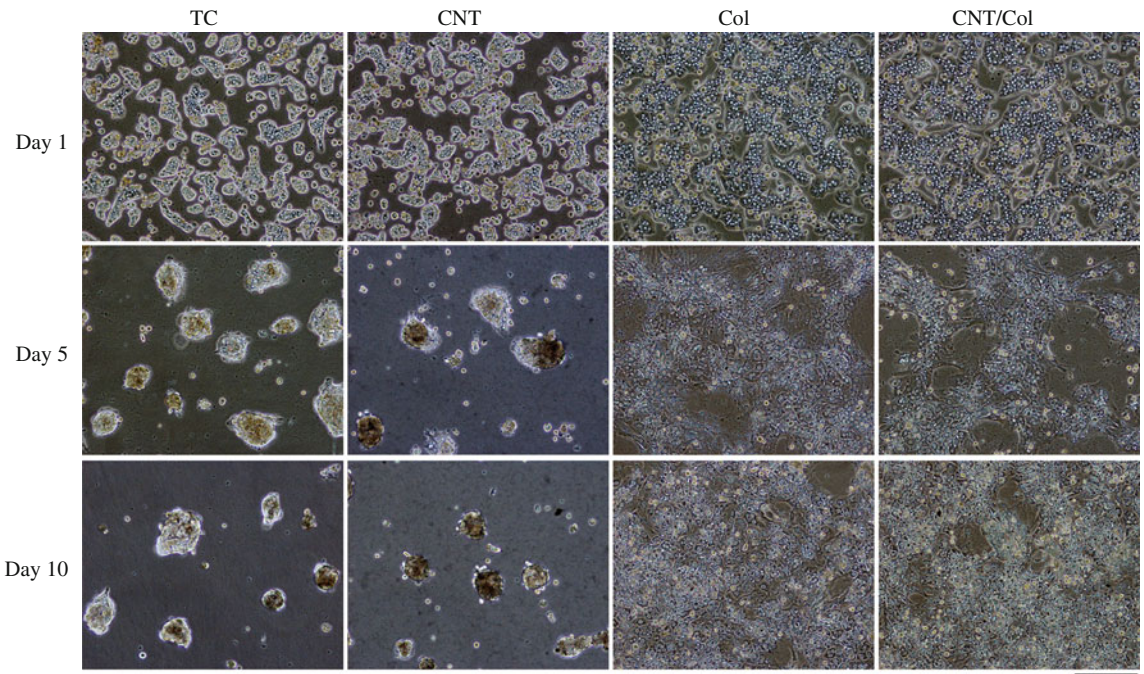

Fig. 2 Phase-contrast micrographs of hepatocyte configurations in each culture condition. *Bar* = 300 μm

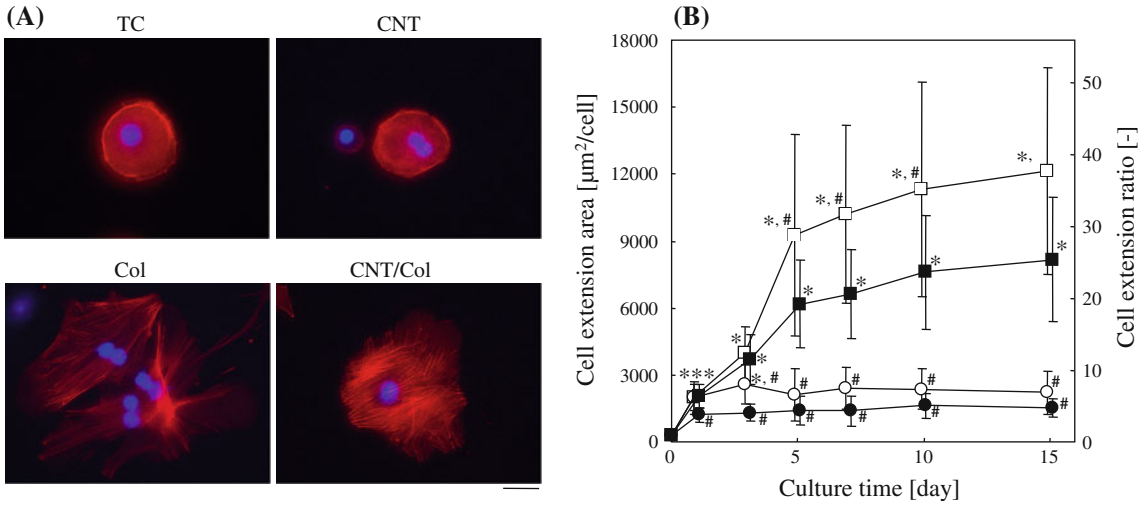

Fig. 3 **a** Distribution of actin filaments and nuclei in hepatocytes in each culture condition at 5 days of culturing. Actin cytoskeleton (*red*) with nuclear stain (*blue*). *Bar* = 20 μm. **b** Changes in cell extension ratios in each culture condition. Tissue culture dish (TC), *open circles*; CNT-coated dish (CNT), *closed circles*; collagen-coated dish (Col), *open squares*; and CNT/collagen-coated dish (CNT/Col), *closed squares*. *Error bars* represent SD. $^{*}P < 0.05$ as compared with the value of the CNT-coated dish. $^{\#}P < 0.05$ as compared with the value of the CNT/collagen-coated dish (Color figure online)

period (Fig. 3b). However, the CNT-coated dish tended to suppress cell extension more than the TC dish. In the collagen- and CNT/collagen-coated dishes, the cell extension ratio drastically increased until 5 days of culturing and gradually increased thereafter. Although hepatocyte adhesion to the CNT-coated surface improved with collagen treatment, significant differences were observed in the cell extension ratios between the collagen- and CNT/collagen-coated dishes after 5 days of culturing. These results indicate that cell extension on the dish surfaces is suppressed by the presence of CNTs.

3.3 Expression of cell adhesion molecules

Cell adhesion properties were compared at the gene expression level. Integrin is a major cell–matrix adhesion molecule that mediates cell–matrix anchoring junctions such as focal adhesions and hemidesmosomes. Although the expression of integrin-β1 in the collagen- and CNT/collagen-coated dishes increased with culture time, these increases were only slight in the CNT-coated and TC dishes (Fig. 4a). These results correspond with the changes in cell morphology (Figs. 2, 3). The hepatocytes that formed monolayers strongly expressed the integrin molecule. Connexin-32 is a molecule that forms gap junctions for cell–cell communication. Connexin-32 expression was higher in the CNT-coated and TC dishes than in the collagen- and CNT/collagen-coated dishes (Fig. 4b). These findings demonstrate that hepatocytes on the CNT-coated and TC dishes developed cell–cell communication. Furthermore, no significant differences were observed regarding connexin-32 expression between the CNT-coated

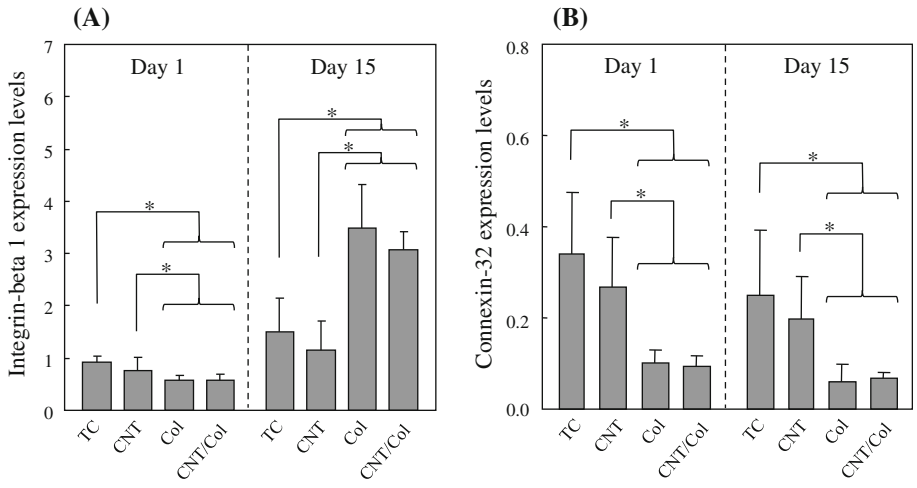

Fig. 4 mRNA expression levels of the cell adhesion molecule, integrin-β1 (**a**) and the cell interaction molecule, connexin-32 (**b**). *Error bars* represent SD. $^{*}P < 0.05$

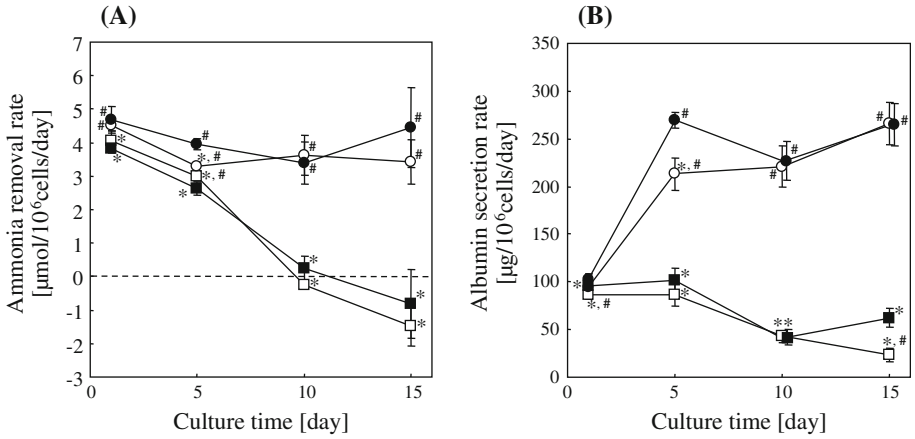

Fig. 5 Time courses of the ammonia removal activity (**a**) and albumin secretion activity (**b**) of hepatocytes on the TC (*open circles*), CNT-coated (*closed circles*), collagen-coated (*open squares*), and CNT/collagen-coated dishes (*closed squares*). *Error bars* represent SD. $^{*}P < 0.05$ as compared with the value of the CNT-coated dish. $^{\#}P < 0.05$ as compared with the value of the CNT/collagen-coated dish

and TC dishes, indicating that degree of development of cell–cell communication is almost the same under both conditions.

3.4 Liver-specific functions

Although the cell extension ratios on the collagen- and CNT/collagen-coated dishes were significantly different (Fig. 3), the difference did not appear to affect the expression of liver-specific functions (i.e., ammonia removal and albumin secretion activities) (Fig. 5). The expression of liver-specific functions in the CNT-coated and TC dishes was almost the same; both dishes maintained higher expression levels than both the collagen- and CNT/collagen-coated dishes (Fig. 5). This may be responsible for the difference in hepatocyte morphology, because hepatocytes formed spheroids in the CNT-coated and TC dishes but assumed a monolayer configuration in the collagen- and CNT/collagen-coated dishes.

4 Discussion

Many researchers report that mammalian cells, such as neural cells, bone cells, and fibroblasts, form monolayer configurations on CNT surfaces [5–13]. However, in this study, we found that primary rat hepatocytes exhibit different behaviors, spheroid configurations, as compared to other cell species. This difference is due to the presence or absence of serum in culture medium. We used the serum-free medium in order to clear the interaction between hepatocytes and CNTs in this study, because it is known that serum components contain cell attachment proteins and they promote cell adhesion. Actually, we confirmed that hepatocytes in the serum-containing medium adhered well on the CNT surface and formed monolayer configuration (data not shown). Furthermore, some studies report that serum-free medium induces the spheroid formation of rat hepatocytes irrespective of material wettability [20, 21] and hydrophobic surfaces repress hepatocyte adhesion [22–24]. Our results were identical to these reports. Although the detailed mechanism involved in the relationship between hepatocyte adhesion and material wettability is not clear, the combination of hydrophobic characteristics of CNTs and serum-free medium may lead to weak hepatocyte adhesion, and spheroid formation may be promoted.

Because the process of spheroid formation on the CNT-coated dish was almost the same to that on the TC dish, it may have been promoted by the same mechanism. The rat hepatocytes adhered to the CNT-coated and TC dishes at the initial stage of culturing, and then they formed spheroids by the acceleration of cell–cell adhesion (Fig. 2). In the initial stage of culture, the hepatocytes adhesion may be supported by the adsorption of proteins and/or extracellular matrices secreted from cells. As next steps, different cell secretions may promote the cell–cell adhesion. Recent studies reveal that the plasminogen activator/plasmin system plays important roles in the modulation of cells from adherent monolayers to multicellular configurations; its expression promotes the spheroid formation of hepatocytes on TC dishes in serum-free condition [25, 26]. Furthermore, Koide et al. [20] report that spheroid-forming factors like proteoglycan fraction are secreted from cells and settle onto the dish surface during the spheroid formation. Therefore, the spheroid formation may be controlled by the reconstruction of such cell secretions.

Hepatocyte adhesion to the CNT-coated surface was improved with the collagen treatment (Figs. 2, 3). However, the cell extension ratio on the CNT/collagen-coated surface was inhibited compared to that on the collagen-coated surface (Fig. 3b). Many researchers report that nanostructure of surface has the capability to direct and guide cultured cells, and the orientation of cultured cells is arranged by surface nanotopography [27, 28]. Therefore, while the collagen treatment of surface enhances the adhesion of hepatocytes, the surface roughness by presence

of CNTs may regulate the cell extension on the CNT/collagen-coated surface.

The expression of the liver-specific functions of hepatocytes is closely related to their cell morphology, and it is known that the formation of adequate intracellular communication between cells is important for their functions [29–32]. The fact that the expression levels of connexin-32 were higher on the CNT-coated and TC surfaces than on the collagen- and CNT/collagen-coated surfaces indicates the development of intracellular communication between cells under those conditions (Fig. 4b). Because spheroids possess adequate intercellular communication, they may be able to perform highly liver-specific functions compared to hepatocyte monolayers.

Recently, there are many reports addressing the potential toxicity of nanomaterials like CNTs, reporting that CNTs induce inhibition of cell proliferation and cell death [33, 34]. However, in this study, the variety of cell morphology and functional expressions of hepatocytes were almost same irrespective of the presence of the CNTs. This difference may be due to the working condition of CNTs. Although CNTs show the cytotoxicity when they are suspended in the culture medium, its risk seems to be avoided by the immobilization to culture substratum like this study. Therefore, the material design which closely immobilized CNTs may be important for TC applications.

5 Conclusions

In this study, we established the effects of CNTs as a culture scaffold on hepatocytes. We showed that the interaction between primary hepatocytes and CNTs is weak depending upon CNT density, and that CNTs promote spheroid configuration. The liver-specific functions of hepatocytes that formed spheroids on the CNT-coated surface were more elevated than those that formed conventional monolayers; these elevated functions were maintained for at least 15 days of culture. These results indicate that CNTs can be used the formation and long-term culture of hepatocyte spheroids.

Acknowledgment This work was partly supported by the Nanotechnology Network Project implemented by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT).

References

1. Dresselhaus MS, Dresselhaus G, Jorio A. Unusual properties and structure of carbon nanotubes. Annu Rev Mater Res. 2004;34:247–78.
2. Ouyang M, Huang JL, Lieber CM. Fundamental electronic properties and applications of single-walled carbon nanotubes. Acc Chem Res. 2002;35:1018–25.
3. Dai H. Carbon nanotubes: synthesis, integration, and properties. Acc Chem Res. 2002;35:1035–44.
4. Niyogi S, Hamon MA, Hu H, Zhao B, Bhowmik P, Sen R, Itkis ME, Haddon RC. Chemistry of single-walled carbon nanotubes. Acc Chem Res. 2002;35:1105–13.
5. Hu H, Ni Y, Montana V, Haddon RC, Parpura V. Chemically functionalized carbon nanotubes as substrates for neuronal growth. Nano Lett. 2004;4:507–11.
6. Jan E, Kotov NA. Successful differentiation of mouse neural stem cells on layer-by-layer assembled single-walled carbon nanotube composite. Nano Lett. 2007;7:1123–8.
7. Galvan-Garcia P, Keefer EW, Yang F, Zhang M, Fang S, Zakhidov AA, Baughman RH, Romero MI. Robust cell migration and neuronal growth on pristine carbon nanotube sheets and yarns. J Biomater Sci Polym Ed. 2007;18:1245–61.
8. Shi X, Sitharamana B, Pham QP, Liang F, Wu K, Billups WE, Wilson LJ, Mikos AG. Fabrication of porous ultra-short single-walled carbon nanotube nanocomposite scaffolds for bone tissue engineering. Biomaterials. 2007;28:4078–90.
9. Zanello LP, Zhao B, Hu H, Haddon RC. Bone cell proliferation on carbon nanotubes. Nano Lett. 2006;6:562–7.
10. Chlopek J, Czajkowska B, Szaraniec B, Frackowiak E, Szostak K, Beguin F. In vitro studies of carbon nanotubes biocompatibility. Carbon. 2006;44:1106–11.
11. Edwards SL, Church JS, Werkmeister JA, Ramshaw JAM. Tubular micro-scale multiwalled carbon nanotube-based scaffolds for tissue engineering. Biomaterials. 2009;30:1725–31.
12. Takahashi K, Shizume R, Uchida K, Yajima H. Improved blood biocompatibility of composite film of chitosan/carbon nanotubes complex. J Biorheol. 2009;23:64–71.
13. MacDonald RA, Laurenzi BF, Viswanathan G, Ajayan PM, Stegemann JP. Collagen–carbon nanotube composite materials as scaffolds in tissue engineering. J Biomed Mater Res A. 2005;74:486–96.
14. Tanaka M, Nishikawa K, Okubo H, Kamachi H, Kawai T, Matsushita T, Shimomura M. Control of hepatocyte adhesion and function on self-organized honeycomb-patterned polymer film. Colloids Surf Physicochem Eng Asp. 2006;284–285:464–9.
15. Chu XH, Shi XL, Feng ZO, Gu ZZ, Ding YT. Chitosan nanofiber scaffold enhances hepatocyte adhesion and function. Biotechnol Lett. 2009;31:347–52.
16. Feng ZO, Chu XH, Huang NP, Leach MK, Wang G, Wang YC, Ding YT, Gu ZZ. Rat hepatocyte aggregate formation on discrete aligned nanofibers of type-I collagen-coated poly(L-lactic acid). Biomaterials. 2010;31:3604–12.
17. Yamaguchi T, Sakai S, Watanabe R, Tarao T, Kawakami K. Heat treatment of electrospun silicate fiber substrates enhances cellular adhesion and proliferation. J Biosci Bioeng. 2010;109:304–6.
18. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol. 1976;13:29–83.
19. Nakazawa K, Izumi Y, Fukuda J, Yasuda T. Hepatocyte spheroid culture on a poly-dimethylsiloxane chip having microcavities. J Biomater Sci Polym Ed. 2006;17:859–73.
20. Koide N, Sakaguchi K, Koide Y, Asano K, Kawaguchi M, Matsushita H, Takenami T, Shinji T, Mori M, Tsuji T. Formation of multicellular spheroids composed of adult rat hepatocytes in dishes with positively charged surfaces and under other nonadherent environments. Exp Cell Res. 1990;186:227–35.
21. Nakazawa K, Izumi Y, Mori R. Morphological and functional studies of rat hepatocytes on a hydrophobic or hydrophilic polydimethylsiloxane surface. Acta Biomater. 2009;5:613–20.
22. Catapano G, Lorenzo MCD, Volpe CD, Bartoro L, Migliaresi C. Polymeric membranes for hybrid liver support devices: the effect of membrane surface wettability on hepatocyte viability and functions. J Biomater Sci Polym Ed. 1996;11:1017–27.

23. Krasteva N, Groth TH, Fey-Lamprecht F, Altankov G. The role of surface wettability on hepatocyte adhesive interactions and function. J Biomater Sci Polym Ed. 2001;12:613–27.
24. Grant MH, Morgan C, Henderson C, Malsch G, Seifert B, Albrecht W, Groth T. The viability and function of primary rat hepatocytes cultured on polymeric membranes developed for hybrid artificial liver devices. J Biomed Mater Res A. 2005; 73:367–75.
25. Hasebe Y, Akao M, Okumura N, Izumi T, Koh T, Seki T, Ariga T. Plasminogen activator/plasmin system regulates formation of the hepatocyte spheroids. Biochem Biophys Res Commun. 2003;308:852–7.
26. 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.
27. Rebollar E, Frischauf I, Olbrich M, Peterbauer T, Hering S, Preiner J, Hinterdorfer P, Romanin C, Heitz J. Proliferation of aligned mammalian cells on laser-nanostructured polystyrene. Biomaterials. 2008;29:1796–806.
28. Liliensiek SJ, Wood JA, Yong J, Auerbach R, Nealey PF, Murphy CJ. Modulation of human vascular endothelial cell behaviors by nanotopographic cues. Biomaterials. 2010;31: 5418–26.
29. Hou DX, Arimura M, Fukuda M, Oka T, Fujii M. Expression of cell adhesion molecule and albumin genes in primary culture of rat hepatocytes. Cell Biol Int. 2001;25:239–44.
30. Shimoyama Y, Hirohashi S. Cadherin intercellular adhesion molecule in hepatocellular carcinomas: loss of E-cadherin expression in an undifferentiated carcinoma. Cancer Lett. 1991; 57:131–5.
31. Fukuda J, Sakai Y, Nakazawa K. Novel hepatocyte culture system developed using microfabrication and collagen/polyethylene glycol microcontact printing. Biomaterials. 2006;27:1061–70.
32. Vinken M, Henkens T, Rop ED, Fraczek J, Vanhaecke T, Rogiers V. Biology and pathobiology of gap junctional channels in hepatocytes. Hepatology. 2008;47:1077–88.
33. Tian F, Cui D, Schwarz H, Estrada GG, Kobayashi H. Cytotoxicity of single-wall carbon nanotubes on human fibroblasts. Toxicol In Vitro. 2006;20:1202–12.
34. Rotoli BM, Bussolati O, Bianchi MG, Barilli A, Balasubramanian C, Bellucci S, Bergamaschi E. Non-functionalized multi-walled carbon nanotubes alter the paracellular permeability of human airway epithelial cells. Toxicol Lett. 2008;178:95–102.