Infrared transmission spectra of 30-MWNTs, the 30-MWNTs coated with PVLA, and PVLA were obtained to observe the bonding between 30-MWNTs and PVLA (Figure 2). Compared with the spectrum of 30-MWNTs (Figure 2a), new peaks at around 3430, 2920 and 1640 cm^{-1} on the spectrum of the 30-MWNTs coated with PVLA (Figure 2b) were observed. These peaks were assigned to -OH, $-CH_2$ and C=O, respectively, functional groups derived from PVLA. The presence of these new peaks indicated that there was still a significant amount of PVLA left in the aggregated 30-MWNTs after washing, suggesting that the MWNTs interacted strongly with PVLA.

To observe the interaction between the MWNTs and PVLA more directly, we performed fluorescence CLSM (8, 20) using an FITC-labeled carbohydrate-carrying polymer. In this experiment, small aggregations of the MWNTs, scattered on the slide and faintly visible under the microscope, were targeted to confirm the interaction between the MWNTs and FITC-PVLA in a microscopic field. Transmittance light (left column) and fluorescence (right column) images of aggregates of the MWNTs after centrifugation are shown in Figure 3. As shown in Figures 3a and 3b, we were able to observe aggregations but not each needle shape of the 30-MWNTs; the average diameter of the 30-MWNTs determined by SEM was about 30 nm (Figure 1a). These images showed that the aggregates of the 30-MWNTs were coated with FITC-PVLA. By using 200-MWNTs with diameters of about 100–300 nm, the binding of FITC-PVLA to MWNTs was much more clearly shown (Figures 3c and 3d). These images showed that FITC-PVLA was densely localized around the single 200-MWNTs. Probably due to resolution limit of CLSM, needle shapes of 200-MWNTs coated FITC-PVLA were clearly observed than that of 30-MWNTs. Thus 200-MWNTs were used in the following experiments. Although the strength and the amount of PVLA adsorption to MWNTs were not

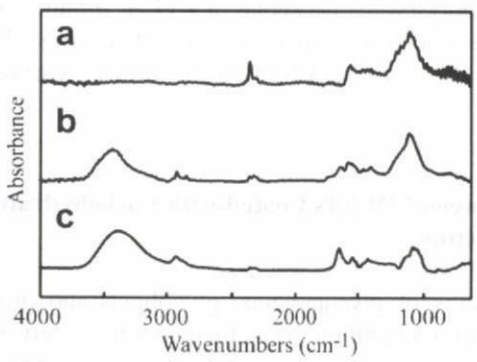


Figure 2. FT-IR spectra of 30-MWNTs (a), the 30-MWNTs coated with PVLA (b), and PVLA (c).

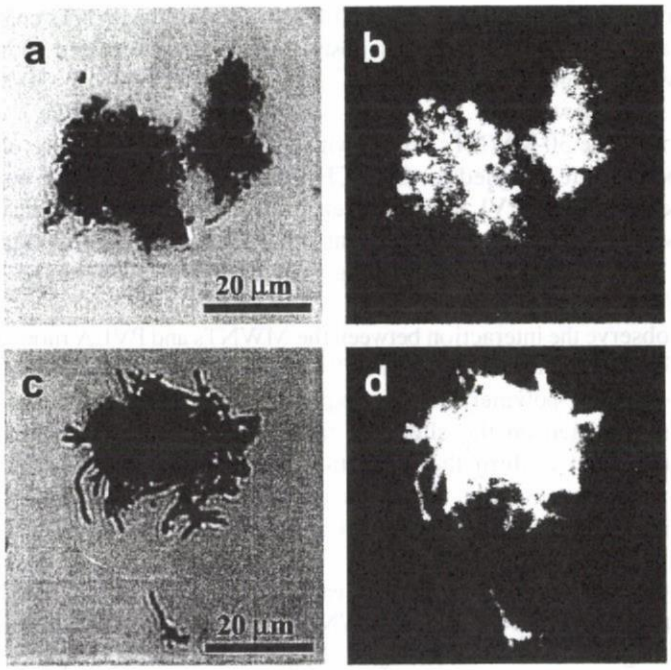


Figure 3. CLSM images of the MWNTs (upper: 30-MWNTs, lower: 200-MWNTs) coated with FITC-PVLA. The left column (a, c) shows images obtained in a transmitted light channel and the right column (b, d) shows images obtained in an FITC channel.

elucidated, PVLA was adsorbed on 30- and 200-MWNTs at sufficient amounts for the modification. Different diameters of MWNTs could effect on PVLA adsorption such as cell adhesion on CNF (35).

We conclude that PVLA is tightly associated with MWNTs because there are interactions between the hydrophobic surface of MWNTs and the hydrophobic polystyrene backbone of PVLA. Binding of a hydrophobic polymer to CNTs is driven by hydrophobic interactions (34) and could be widely utilized for coating CNTs with various carbohydrate-carrying polymers.

Interactions between MWNTs Coated with Carbohydrate-carrying Polymer and Lectins

Carbohydrate chains of glycoproteins, glycolipids and proteoglycans play important roles in recognition phenomena such as fertilization, antigen-antibody reaction, cancer metastasis and infection of viruses and bacteria. These functions are attributable to the biological recognition phenomena induced by lectins and anti-carbohydrate antibodies (28–30).

To estimate the molecular recognition ability of the MWNTs coated with PVLA, interactions with fluorescence-labeled lectins were analyzed using CLSM. Figure 4 shows CLSM images of the 200-MWNTs coated with FITC-PVLA after incubation with Rhod-RCA_{120} [(a) transmitted light channel, (b) FITC channel, and (c) rhodamine channel]. It was found that Rhod-RCA_{120} was densely localized around the 200-MWNTs coated with FITC-PVLA from the rhodamine channel (Figure 4c) and also co-localized with FITC-PVLA adsorbed on the 200-MWNTs from the FITC channel (Figure 4b). In contrast, a control experiment using the combination of Rhod-ConA and the 200-MWNTs coated with FITC-PVLA yielded no rhodamine signal (Figure 5c). These results indicated that the MWNTs coated with PVLA interacted efficiently with the complementary lectin. It is conceivable that the β-galactose moieties of PVLA

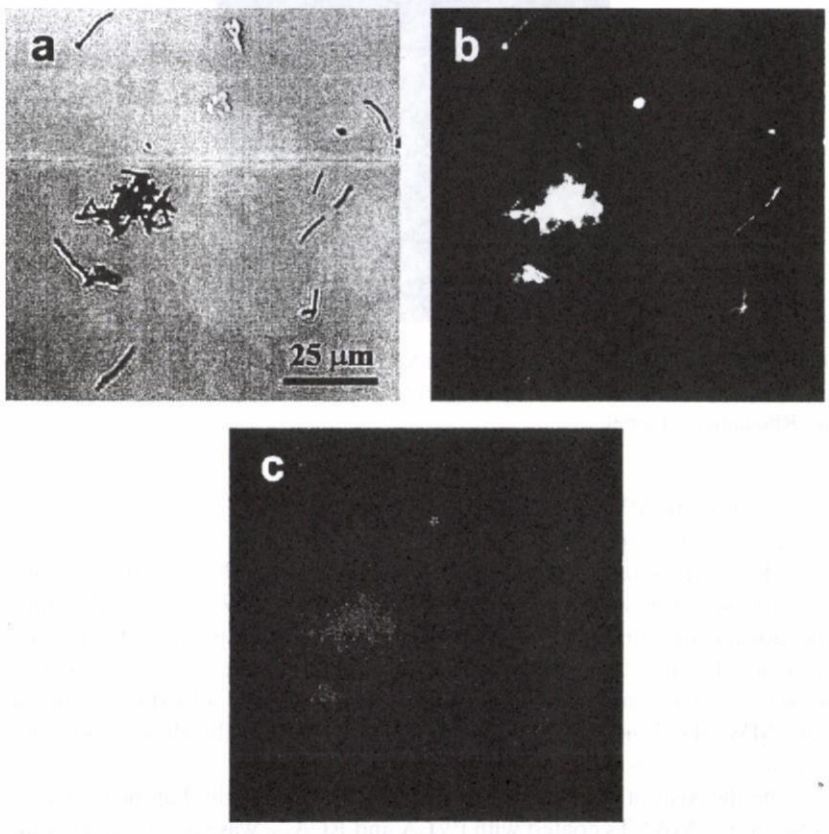


Figure 4. CLSM images of the 200-MWNTs coated with FITC-PVLA after incubation with Rhod-RCA_{120}. (a) Transmitted light channel, (b) FITC channel, and (c) Rhodamine channel.

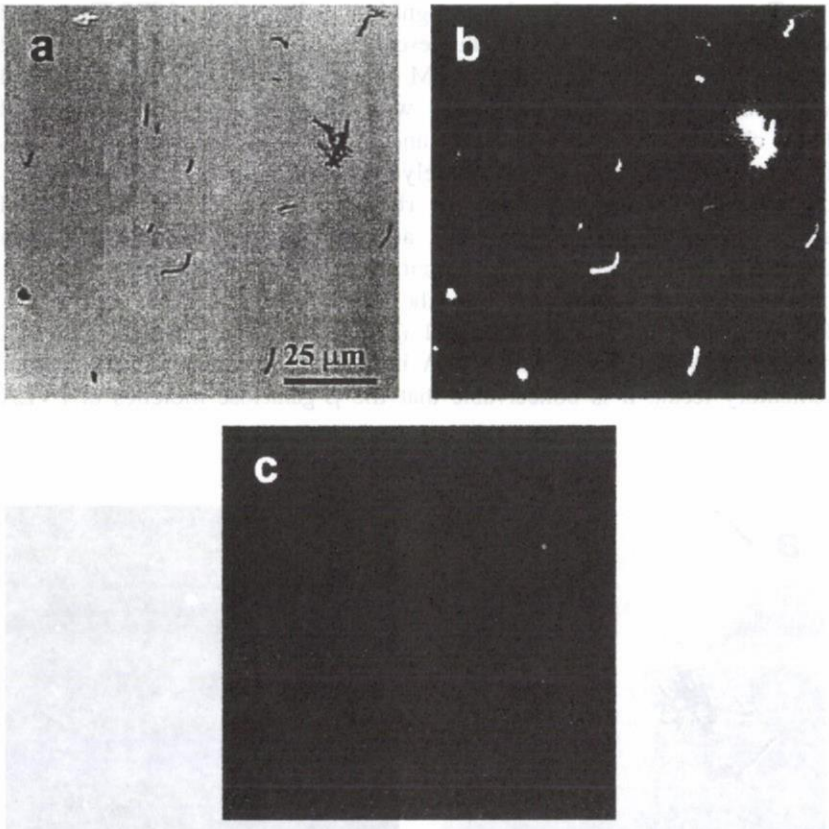


Figure 5. CLSM images of the 200-MWNTs coated with FITC-PVLA after incubation with Rhod-ConA. (a) Transmitted light channel, (b) FITC channel, and (c) Rhodamine channel.

adsorbed on the MWNTs are recognized specifically by β-galactose-specific RCA_{120} lectin (36).

However, certain proteins were found to bind to CNTs via hydrophobic interactions even without sidewall functionalization (9, 11). To determine the nonspecific binding of lectins as a control experiment, bare MWNTs were incubated with lectins. Nonspecific bindings of lectins were observed as shown in Figure 6, in which CLSM images show adsorbed lectins on bare MWNTs. Binding of lectins to CNTs could also be driven by hydrophobic interactions.

On the basis of our experimental results, it was thought that the interaction between the MWNTs coated with PVLA and RCA_{120} was specific and not due to a variation in physical adsorption, since ConA did not interact with the coated MWNTs. This also indicated that the entire hydrophobic area on the surface of the MWNTs was coated and blocked with PVLA.

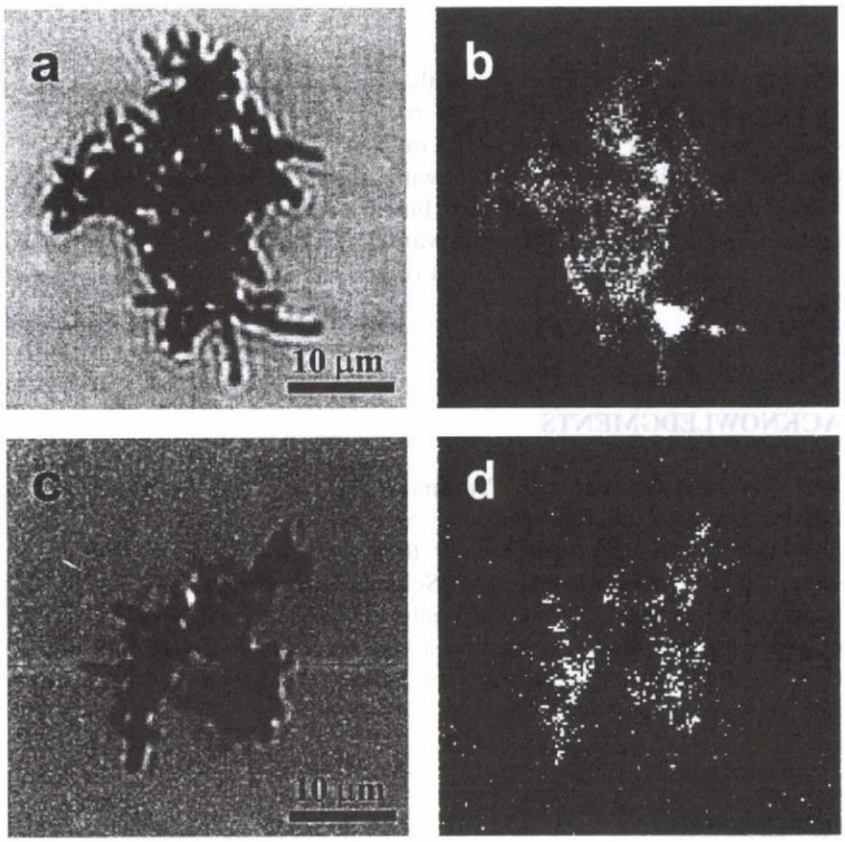
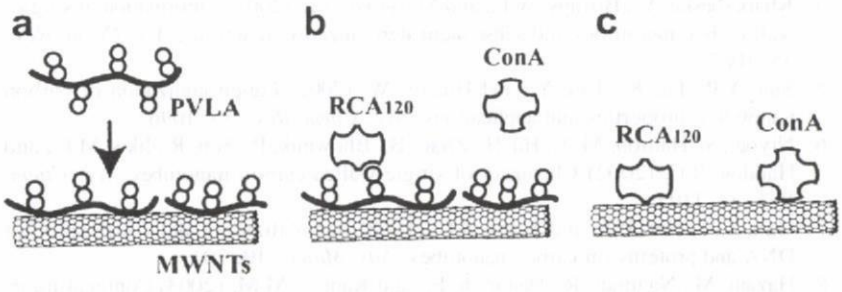


Figure 6. CLSM images of bare 200-MWNTs after incubation with Rhod-RCA_{120} (upper) and Rhod-ConA (lower). (a, c) Transmitted light channel, (b, d) Rhodamine channel.



Scheme 1. Illustrations of (a) adsorption of PVLA onto the surface of MWNTs, (b) specific interaction between the MWNTs coated with PVLA and lectins, and (c) non-specific interaction between MWNTs and lectins.

CONCLUSION

In summary, we have demonstrated that the MWNTs coated with a carbohydrate-carrying polymer for use as biological recognition signals can be easily prepared by a non-covalent method via hydrophobic interactions (Scheme 1). The MWNTs coated with a carbohydrate-carrying polymer were found to acquire a selective binding affinity to the corresponding lectin. Modification of CNTs with various carbohydrate chains will be a useful protocol for molecular designs of biomaterials, nanoarchitecture, and biosensors.

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Effect of carbon nanotubes on cellular functions *in vitro*

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Abstract: Carbon nanotubes (CNTs) have been shown to affect cell behavior. But how and why the CNTs affect potential differentiation of the attached cells has not been largely known. In this study, multiwalled carbon nanotubes (MWNTs) and graphite (GP) were pressed as compacts. Higher ability of CNTs to adsorb proteins, compared with GP, was shown. Myoblastic mouse cells (C2C12) were cultured and the cell responses to the two kinds of compacts were compared *in vitro*. Meanwhile, we used cell culture on the culture plate as a control. During the conventional culture, significantly better cell attachment, proliferation, and differentiation of cells on the MWNTs were found. To confirm the hypothesis that the larger amount of protein adsorbed on the CNTs was crucial for this, we made the compacts adsorb more proteins in culture medium with 50% fetal bovine serum (FBS) before cell culture. With the adsorption of the proteins in advance, the increments of the total-protein/DNA and alkaline phosphatase (ALP)/DNA for the MWNTs was respectively as about 11 times and 18 times as the increments of those for GP and the control at both day 4 and day 7. Therefore, the CNTs might induce cellular functions by adsorbing more proteins, which indicated that the CNTs might be a candidate for scaffold material for tissue engineering. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 00A: 000–000, 2008

Key words: carbon nanotubes; cell differentiation; alkaline phosphatase (ALP); protein; tissue engineering

INTRODUCTION

The practice of using tissue engineering to repair tissue damages is attracting more and more attention.[1–7] Scaffolds, lying at the heart of all the new tissue engineering approaches, act as a substrate for cellular attachment, proliferation, and differentiation.[5–8] In the imminent ageing society of the 21st century, nanomaterials have been widely investigated for potential application in the medical field.[9,10] The nano-dimensionality of nature has logically given rise to the interest in using nanomaterials to prepare scaffolds for tissue engineering. These materials have the potential to have a significant impact on tissue engineering.[11–14] Carbon nanotubes (CNTs), one of the most representative nanomaterials, with unique electrical, mechanical, and surface properties, were first reported in the year of 1991 and up to now appear well suited as a biomaterial.[14–27]

Several studies have been carried out on the interaction between CNTs and a variety of cells including osteoblasts, showing CNTs to be excellent substrates for cellular attachment and growth.[28–36] But few (if any) studies have elucidated the effect of CNTs on the protein content and potential differentiation of the attached cells, although cell differentiation is also very important for tissue repair.[37–39]

To start the differentiation of cells, it has been suggested that nanostructures of the biomaterials are critical.[40–46] In other words, the microenvironment around the cells may be crucial.[45,46] Fujibayashi et al. suggested that even a nonsoluble metal that contains no calcium or phosphorus can be an osteoinductive material when treated to form an appropriate nanostructure.[41] Popat et al. presented osteogenic differ-

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entiation of C57 BJ mice marrow stromal cells on nanoporous alumina surfaces, suggesting the ability of nanostructured biomaterials to enhance cell differentiation.[47] Yim et al. reported the nanopatterns, reproduced on poly (dimethylsiloxan) (PDMS) using soft lithography on the nanoimprinted poly (methyl methacrylate) (PMMA)-coated Si master mold, played an important role in directing differentiation of adult stem cells into neuronal lineage.[48]

In this study, myoblastic mouse cells (C2C12) were cultured on multiwalled CNTs (MWNTs) compacts, comparing on graphite (GP) compacts, with and without the adsorption of fetal bovine serum (FBS) in advance. Cell differentiation was examined and compared, as well as cell attachment and proliferation. Meanwhile, we used cell culture on the culture plate as a control.

MATERIALS AND METHODS

Materials

MWNTs used in this study were obtained from Nano-Lab (Brighton, MA). The MWNTs of curled shape with about 90 nm in diameter were produced by the chemical vapor deposition (CVD) method. The purification procedure for MWNTs was as follows: First, MWNTs were heated to ~500 C for 90 min under atmospheric conditions. Next, the cooled MWNTs were transferred into a flask containing 6*M* HCl and treated at 60 C for 2 h, and then washed thoroughly with deionized water and completely dried. The GP particles used in this study were about 4.5 μm in diameter.

Fabrication of compacts

MWCTs and GP with the same weight were separately compacted serially in a steel-tool die via a uniaxial pressing cycle (0.09 GPa for 2 min, then 0.22 GPa for 3 min, finally 0.36 GPa for 3 min) at room temperature. The compacts were washed ultrasonically with acetone, 70% ethanol, and RX-water for 15 min and then dried at 60 C. All the compacts were sterilized by ultraviolet radiation for 48 h prior to experiments with cells. The morphology of the compacts was examined by scanning electron microscopy (SEM; S-4000, Hitachi, Japan).

Evaluation of protein adsorption on the compacts

Before cells culture, ability to adsorb proteins of the compacts was evaluated. At first, 0.25% FBS (250 μL FBS in 100 mL 25 ppm NaN3 solution) were sterilized with 0.22 μm filter. After immersing the compacts respectively for 1, 4, and 7 days, the residual protein content (P_1) of the FBS solution (3 mL per sample, $n = 5$) was determined with the QuantiPro™ BCA Assay Kit (TaKaRa BIO INC, Japan) according to the guideline of the company. The fluorescence was measured with a BIO-TEK automate microplate reader at 620 nm. The adsorbed protein (P_a) was determined by the formula of $(0.25\% - P_1)/0.25\%$.

Conventional cell culture on the samples

The compacts were placed in the cell culture plates. Then C2C12 were respectively seeded on the compacts with a cell density of 2.0×10^4 per sample. Then the samples were put into an incubator at 37 C in a humidified atmosphere with 5% CO_2 and 95% air for 4 h. Finally, 2.5-mL culture medium, Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% fetal bovine serum (FBS; Biowest) and 1% penicillin/streptomycin (100 U/mL penicillin, 10 μg/mL streptomycin), was added into the walls of the plates and then, the plates were put back to the incubator. The culture mediums were refreshed twice a week.

SEM observation

At the prescribed time, the samples were rinsed with PBS to remove nonadherent cells, fixed in a solution of 2% glutaraldehyde, and postfixed in a 1% osmium tetroxide solution. Then, the samples were dehydrated in a series of solutions with increasing ethanol concentrations, followed by critical-point drying at 40 C. Finally, the morphology of the cells on the compacts was examined by SEM (S-4000, Hitachi, Japan).

DNA, ALP, and total protein analyses

After cell culture, the samples with cells were washed by PBS for three times after the cultured medium was totally removed. Then the samples were stored in the freezer at −80 C for at least 12 h for the biochemical analyses. As soon as the plates were taken out from the freezer, they were kept on the ice, prepared in advance. And then 0.5 mL 0.2% triton was put into each wall with samples in the plates. The plates were shook gently for 45 min. Finally, the solutions were analyzed for DNA, ALP, and protein content.

DNA content was determined with the CyQuant Cell Proliferation Assay Kit (Invitrogen) according to the guideline of the company. About 0.1 mL of each sample ($n = 4$) were diluted in TE to a final volume of 1.0-mL test tubes. Then 1.0-mL of aqueous working solution (dye) was added to each sample. After the tubes were incubated for about 3 min, the fluorescence using instrument parameters was measured at an emission wavelength of 520 nm and excitation of 480 nm. The DNA content of cells attached on the porous samples was counted through a premade standard DNA curve. DNA content was expressed as mean ± SD.

For the determination of ALP content, 20 μL of each sample ($n = 4$) was added to the walls of a 96-wall plate and then 100 μL Paranitrophenylphosphate (PNP) solution was added. After shaken gently, the plate was incubated at 37 C for 15 min. After 80 μL stop solution (0.2 mol/L sodium hydroxide) was added, the plate was read with a

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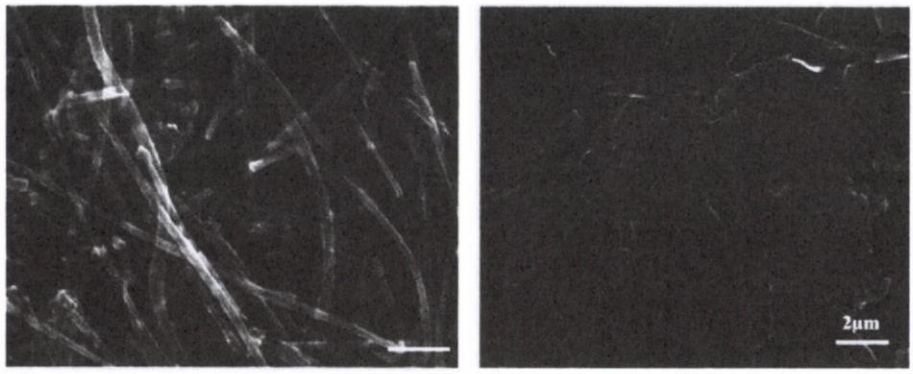

Figure 1. SEM images of the compacts: MWCNs (a); GP (b).

BIO-TEK automate microplate reader at 405 nm. For the standard curve, serial dilutions of p-Nitrophenol were made. Finally, the ALP content of cells was counted through the standard curve. The value was expressed as mean ± SD.

Total protein content was determined with the Quanti-Pro™ BCA Assay Kit (TaKaRa BIO INC). A total of 100 μL of each sample (n = 4) was added to the walls of a 96-wall and then 100 μL BCA solution was added. Then, the plate was continuously shaken for 2 h in dark at room temperature. Finally, the fluorescence was measured with a BIO-TEK automate microplate reader at 620 nm. The protein content, expressed as mean ± SD, was counted through a premade standard protein curve.

Cell culture on the samples after adsorbing FBS

At first, the samples were respectively immersed into culture medium containing 50% FBS for 24 h in an incubator at 37 C in a humidified atmosphere with 5% CO_2 and 95% air. Then, the FBS solution was completely removed and the discs were washed by the cultured medium of C2C12 with 1% FBS for three times. C2C12 was respectively cultured on the samples with a cell density of 4.0×10^4 per sample. After cell culture in culture medium with 1% FBS for certain time, DNA, ALP, and total protein content were examined with the methods mentioned above.

Statistical analysis

Statistical calculations were done with the SPSS (Chicago, IL) 12.0 software. Paired Student's t-test was used to analyze differences experiments results between different samples. $p < 0.05$ was regarded as significant difference.

RESULTS

The SEM images of the compacts were shown in Figure 1. The distinct difference in the structures between the MWNTs and GP compacts was exhibited. MWNTs formed a packed meshwork nanostructure, whereas GP compacts were formed with particles of about 4.5 μm. The ability to adsorb proteins of the compacts was showed in Figure 2, which showed that MWNTs compacts had much better ability to adsorb proteins than GP compacts. Although the mean value of the protein adsorption of GP compacts was greater than that of the culture plates (control), the statistical analysis showed that there was no significant difference between the two group values ($p > 0.05$).

Morphology of C2C12 cells cultured for 7 days on the compacts was shown in Figure 3. More cells could be observed on MWNTs than on GP compacts. Cells on MWNTs nearly grew to confluence after cultured for 7 days. For a comparison of cell attachment and proliferation at a quantitative stage, the results of DNA analysis of cells cultured on the different samples for 1, 4, and 7 days were shown in Figure 4. We used the slope of the curves in the

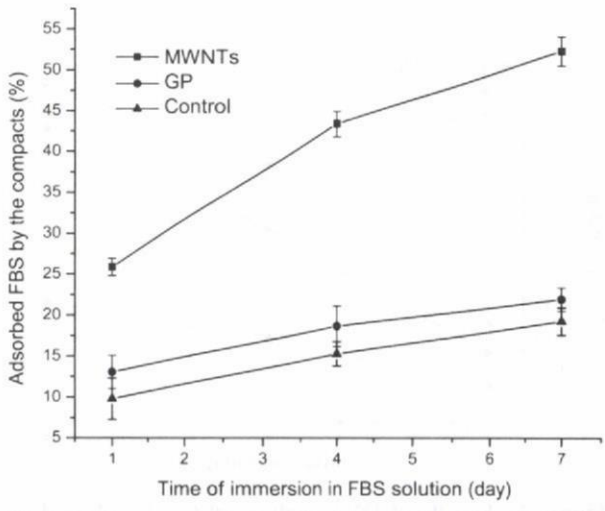

Figure 2. Protein adsorption of the samples (n = 5).

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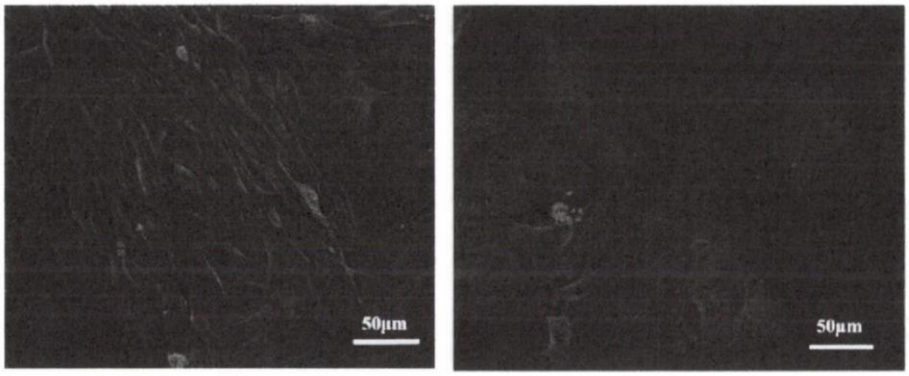

(a) (b)

Figure 3. SEM images of cells conventionally cultured for 7 days on: MWNTs (a) and GP (b).

Figure 4 to estimate the cell proliferation, and thought that the difference in the DNA value at day 1 was mainly due to the different cell attachment. As shown in Figure 4, both the values at day 1 and the slope of the two curves are significantly greater, suggesting that C2C12 attach and proliferate better on MWNTs than on GP compacts (slop of the two curves from day 1 to day 4: 0.558 ± 0.022 vs. 0.219 ± 0.0155, $p < 0.01 < 0.05$; from day 4 to day 7: 0.489 ± 0.096 vs. 0.166 ± 0.0115, $p < 0.01 < 0.05$). Cell attached better on the plates (control) than on MWNTs, but no significant difference was found in the cell proliferation between on the plates and on the MWNTs (slop of the two curves from day 1 to day 4: 0.558 ± 0.022 vs. 0.563 ± 0.021, $p > 0.05$; from day 4 to day 7: 0.489 ± 0.096 vs. 0.468 ± 0.0311, $p > 0.05$), which might be because of the influence of the composition of the materials and the proteins adsorbed on the materials together.

F5 Figure 5 showed the results of ALP/DNA (alkaline phosphatase per unit cell). ALP/DNA of C2C12 cultured on MWNTs compacts was significantly higher than that on GP compacts and on the plates at each culture time point of 1, 4, and 7 days, whereas this value for GP compacts and control had no significant difference at each time point, suggesting that C2C12 differentiate toward osteogenic better on MWNTs than on GP and the plates. At day 7, the value for MWNTs was as about four times as that for GP and the control.

Total-protein/DNA (total protein content per unit cell) was showed in Figure 6. Total-protein/DNA of F6 C2C12 cells on MMNTs was significantly higher than on GP and the culture plates at each culture time point of 1, 4, and 7 days. At day 7, the value for MWNTs was as about two times as that for GP. the Although the mean value of GP compacts was greater than that of the culture plates (control) at day 4 and 7, the statistical analysis showed that there was no significant difference between the two group values ($p > 0.05$).

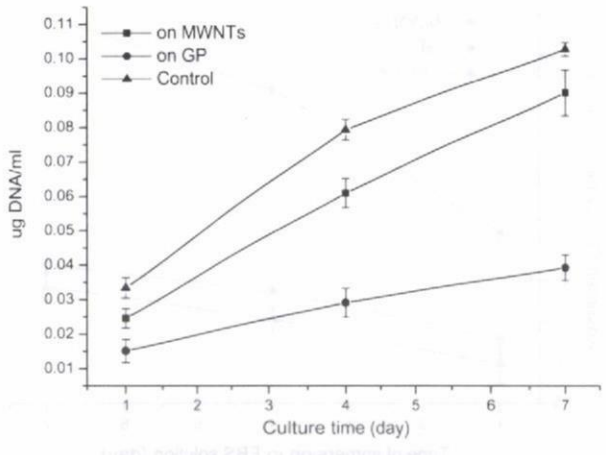

Figure 4. Results of DNA analysis of C2C12 cultured on samples ($n = 4$).

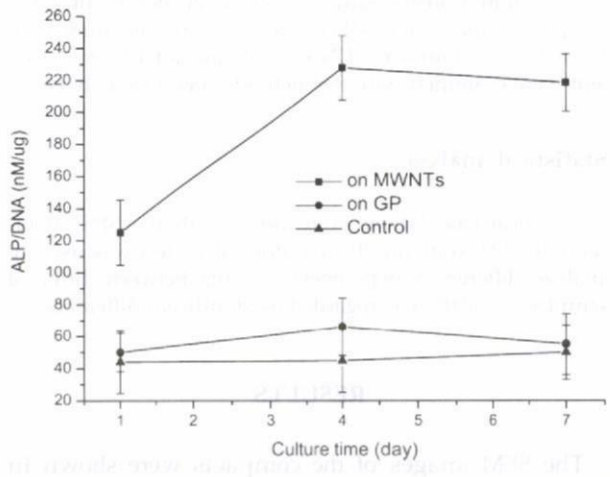

Figure 5. ALP/DNA of C2C12 cells conventionally cultured on samples ($n = 4$).

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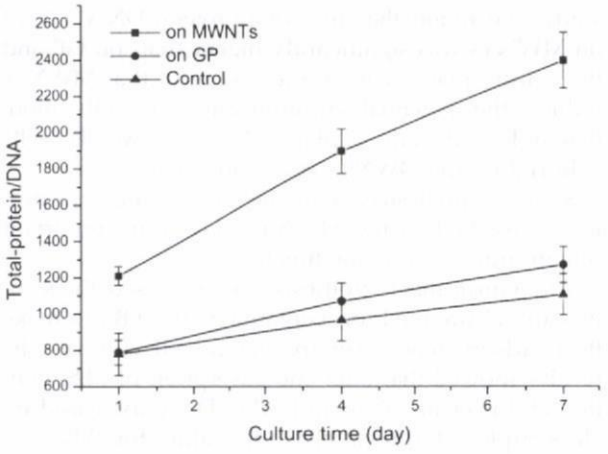

Figure 6. Total-protein/DNA of C2C12 cells conventionally cultured on samples ($n = 4$).

F7 Figure 7 showed ALP/DNA of C2C12 on the samples after adsorbing FBS at day 4 and day 7, compared with the results of the conventional cell culture. After the adsorption of FBS, ALP/DNA of cells on MWNTs increased significantly, while the value for GP increased slightly. The increased value for MWNTs was as about 18 times as that for GP and the control at both day 4 and day 7. The value for GP and the culture plates had no significant difference even after the adsorption of FBS ($p > 0.05$).

Total-protein/DNA of C2C12 on the samples after adsorbing FBS at day 4 and day 7, comparing with the results of the conventional cell culture, was F8 showed in Figure 8. Although the total-protein/DNA of cells on all the samples all increased after the adsorption of FBS, the value for MWNTs

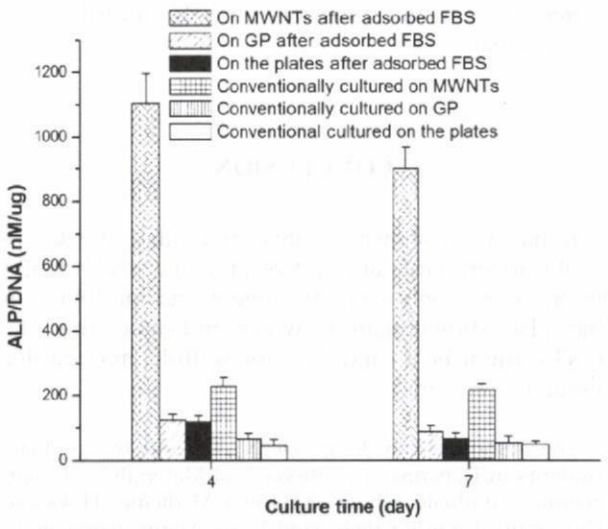

Figure 7. ALP/DNA of C2C12 cells cultured on samples with and without the adsorption of FBS in advance ($n = 4$).

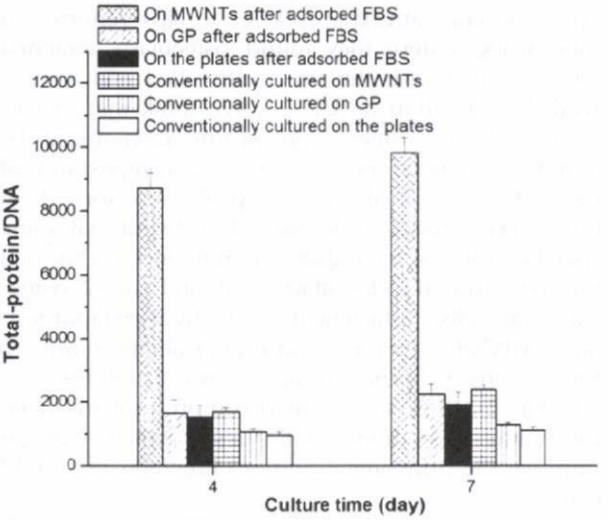

Figure 8. Total-protein/DNA of C2C12 cells cultured on samples with and without the adsorption of FBS in advance ($n = 4$).

increased most observably. The increased value for MWNTs was as about 11 times as that for GP and the control at both day 4 and day 7. The value for GP and the culture plates had no significant difference even after the adsorption of FBS ($p > 0.05$).

DISCUSSION

It has been previously reported that the biological response to implanted material is determined not only by its chemistry, but also by surface energy and topography.[46,49,50] Cells in their natural environment interact with extracellular matrix (ECM) components in the nanometer scale.[51,52] Logically, nanoscaled biomaterials should have positive effect on the cell functions.

We believe our study is the first time that C2C12 line, a multipotent cell line able to differentiate toward different phenotypes under the action of specific proteins, some chemical or biological factors, was cultured on the CNTs, and that not only cell attachment and proliferation, but also cell differentiation and cell activity were investigated. CNTs and GP are both isomorphs of pure carbon, composed of the same grapheme sheet structure. However, only CNTs have nanostructures. So in this study, comparison of the influence of CNTs and GP on C2C12 cells *in vitro* was done to figure out the effect of CNTs on cellular functions. Meanwhile, we used cell culture on the culture plate, which was made of polystyrene and tissue-culture treated, as a control.

The results showed that C2C12 attached and proliferated better on MWNTs than on GP. These results

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are consistent with what Aoki et al. have reported in their work, where they found osteoblasts attached and proliferated better on multiwalled and single-walled CNTs than on GP.[17,53] We think that in this study cells attachment and proliferation might be mainly influenced not only by the composition of the materials, but also by the proteins adsorbed on the materials together. Kilpadi et al. found that some specific proteins have different influence on human marrow stromal cells attachment and saos-2 osteosarcoma cells attachments on hydroxylapatite.[54] Since MWNTs compacts had higher ability to adsorb the proteins or higher affinities toward proteins than GP (Fig. 2), they should adsorb more proteins from the culture medium and these proteins might improve the attachment and proliferation of C2C12 cells.

Besides cell attachment and proliferation, cell differentiation should be most important evaluation point for biomaterials because it may directly contribute to the tissue repair. So in this study our focus has been mostly on this. Evaluating the proteins in cells may be an effective method to evaluate the differentiation. In this study, we used total-protein/DNA to evaluate the effect of MWNTs on the potential of the differentiation of per unit C2C12 cell. Meanwhile, we used the change in ALP/DNA of C2C12 cells to reify this effect. The technique, we used in this study for lysing cells before ALP, DNA, and protein analyses, has been confirmed that only cells, no adsorbed proteins, could be lysed from the samples in advance. From the results of conventional cell culture, we found that the ALP/DNA of C2C12 on MWNTs was significantly higher than on GP and the culture plates, suggesting that MWNTs induce this kind of cell to differentiate into osteogentic cells more than GP and culture plates. So, CNTs may be osteoinductive under certain circumstances. Some publications have shown that although with the same chemical composition, some materials are osteoinductive, some others are not.[46,55–57] These osteoinductive materials all have specific structures, indicating larger surface area and therefore higher ability to adsorb proteins. The work of Ripamonti and coworkers demonstrated that osteoinductivity in hydroxyapatite was linked to the precise shape of surface concavities in implants, which indicated a larger surface area, and that by using immunolocalization they demonstrated that this osteoinductivity occurred as a result of a concentration of specific proteins within the surface concavities.[58,59]

ALP is one kind of protein and only account for small ratio of total proteins in cells. So analyzing total proteins in cells may be an evaluation of the potential of cell differentiation into the expected cells. From the conventional cell culture in this study, we found that the total-protein/DNA C2C12 on MWNTs was significantly higher than on GP and the culture plates, which might suggest that MWNTs induce the potential differentiation of cells more than GP and culture plates. In other words, cells cultured on the MWNTs were more active.

So we hypothesized that the large amount of proteins adsorbed on the MWNTs played an important role in inducing cellular functions.

To confirm this hypothesis, we immersed the samples in culture medium containing 50% FBS to make them adsorb more proteins before cell culture. The results showed that after the adsorption of FBS, both the total-protein/DNA and ALP/DNA increased on all samples. Impressively, the value for MWNTs increased significantly most. The increments of the total-protein/DNA and ALP/DNA for MWNTs was respectively as about 11 times and 18 times as the increments of those for GP and the control at both day 4 and day 7. Therefore, the results of cell culture after the adsorption of FBS might be an effective proof for our hypothesis.

Hing[60] has reported that competitive protein adsorption at a bioactive surface may vary in three ways: (i) the quantity of protein adsorbed, (ii) the species of protein adsorbed, or (iii) the confirmation of the adsorbed protein. Also he supposed that nanostructures might thus influence protein adsorption by providing a larger surface area, thereby increasing the quantity of adsorbed growth factors above a critical level for cell recruitment and activation. In our study, we substantiated the importance of the protein adsorption. CNTs might adsorb large amount of proteins due to their larger surface area, unique electronic, catalytic, and chemical properties. These proteins might not only improve cell attachment and proliferation, but also be helpful for cell differentiation and therefore directly contribute to tissue repair.

CONCLUSION

It has been shown in this study that the CNTs could adsorb large amount of proteins, which might improve not only cell attachment and proliferation but also differentiation, which indicated that the CNTs might be a candidate for scaffold material for tissue engineering.

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Maturation of osteoblast-like SaoS2 induced by carbon nanotubes

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Abstract

Osteogenic maturation of the osteoblast is crucial for bone formation. In this study, multi-walled carbon nanotubes (MWCNTs) and graphite (GP) were pressed as compacts. The greater ability of carbon nanotubes to adsorb proteins, compared with graphite, was shown. Human osteoblast-like SaoS2 cells were cultured and the cell response to the two kinds of compacts was compared *in vitro*. Meanwhile, we used cell culture on the culture plate as a control. Assays for osteonectin, osteopontin and osteocalcin gene expression, total protein (TP) amount, alkaline phosphatase activity (ALP) and DNA of cells cultured on the samples were done. During the conventional culture, significantly higher osteonectin, osteopontin and osteocalcin gene expression level, ALP/DNA and TP/DNA on carbon nanotubes were found. To confirm the hypothesis that the larger amount of specific proteins adsorbed on the carbon nanotubes was crucial for this, the compacts were pre-soaked in culture medium having additional recombinant human bone morphogenetic protein-2 (rhBMP-2) before cell culture. Compared with GP, osteonectin, osteopontin and osteocalcin gene expression level, ALP/DNA and TP/DNA of the cells tested increased more on the MWCNTs after the compacts were pre-soaked in the culture medium with rhBMP-2. The results indicated that the carbon nanotubes might induce osteogenic maturation of the osteoblast by adsorbing more specific proteins.

Introduction

A scaffold is one of the key components in the tissue engineering paradigm in which it can function as a template to allow new tissue growth and also provide temporary structural support while serving as a delivery vehicle for cells and/or bioactive molecules [1–7]. Since nanotechnology embraces a system whose core of materials is in the range of nanometers (10^{-9} m), there are many similarities between nanophase materials and components of biological organs [8–10]. So, nanomaterials should logically have a significant impact on tissue engineering [11–14].

Carbon nanotubes (CNTs), one of the most representative nanomaterials, possess exceptional mechanical, thermal and electrical properties, facilitating their use as reinforcements or additives in various materials, such as plastics, metals and ceramics, to improve properties of the materials and introduce novel functionalities [15]. At present, carbon nanotubes have been extensively studied for use in biomaterial applications, and biomaterials using CNTs are expected to be developed for clinical use [14–27]. A lot of studies have been

[5] Corresponding author.

carried out on the interaction between CNTs and a variety of cells including osteoblasts, showing CNTs to be excellent substrates for cellular attachment and growth [28–36]. But few (if any) studies have elucidated the effect of CNTs on the differentiation and maturation of the attached cells, although these processes are even more important for tissue repair [37–39].

It has been suggested that nanostructures of the biomaterials are critical to start the differentiation of cells [2, 40–43]. In other words, the microenvironment around the cells may be crucial [2]. Park *et al* suggested that nanostructured Ca coating of Ti surfaces may be used as a potentially effective method for enhancing the osseointegration of the implants, by accelerating the proliferation and differentiation of osteoblast cells on their surfaces in the early bone healing phase [41]. Popat *et al* presented osteogenic differentiation of C57 BJ mice marrow stromal cells on nanoporous alumina surfaces, suggesting the ability of nanostructured biomaterials to enhance cell differentiation [44]. Yim *et al* reported that the nanopatterns, reproduced on poly(dimethylsiloxane) (PDMS) using soft lithography on the nanoimprinted poly(methyl methacrylate) (PMMA)-coated Si master mold, played an important role in directing differentiation of adult stem cells into neuronal lineage [45]. Ou *et al* reported that formation of nanohydrides by cathodization and oxidation by anodization are believed to promote biocompatibility and improve the bone-to-interface contact, accelerating initial osseointegration and re-osseointegration [46].

In this study, multi-walled carbon nanotubes (MWCNTs) and graphite (GP) were pressed as compacts. Human osteoblast SaoS2 cells were cultured on the two kinds of compacts with and without adsorbing recombinant human bone morphogenetic protein-2 (rhBMP-2). The cell differentiation and osteogenic maturation on the two kinds of compacts were respectively compared *in vitro*. Meanwhile, we used cell culture on the culture plate as a control.

Materials and methods

Materials

MWCNTs used in this study were obtained from NanoLab (Brighton, MA, USA). The MWCNTs of a curled shape and about 90 nm in diameter were produced by the chemical vapor deposition (CVD) method. The raw MWCNTs were refluxed in a hydrochloric solution and then washed thoroughly with deionized water and completely dried. The GP particles used in this study were about 4.5 μm in diameter.

Fabrication of compacts

MWCNTs and GP were separately compacted serially in a steel-tool die via a uniaxial pressing cycle (0.09 GPa for 2 min, then 0.22 GPa for 3 min and finally 0.36 GPa for 3 min) at room temperature. The compacts were washed ultrasonically with acetone, 70% ethanol and deionized water for 15 min and were then dried at 60 °C. All the compacts were sterilized by ultraviolet radiation for 48 h prior to experiments with cells. The morphology of the compacts was examined by scanning electron microscopy (SEM; S-4000, Hitachi, Japan).

Evaluation of protein adsorption on the compacts

Before cell culture, the ability of protein adsorption of the compacts was evaluated. At first, 0.25% fetal bovine serum (FBS; Biowest) (250 μl FBS in a 100 ml 25 ppm NaN_3 solution) was sterilized with a 0.22 μm filter. After immersing the compacts respectively for 1, 4 and 7 days, the remaining protein content per cent (P_l) of the FBS solution (3 ml per sample, $n = 5$) was measured with the QuantiPro™ bicinchoninic acid (BCA) assay kit (TaKaRa Bio Inc, Japan), according to the guideline of the company. The adsorbed protein (P_a) was determined from the formula $(0.25\% - P_l)/0.25\%$.

Conventional cell culture on the samples

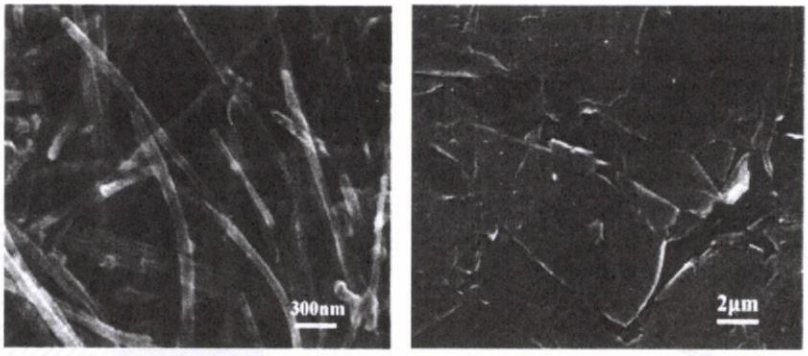
The compacts were placed in the cell culture plates. Then SaoS2 cells were respectively seeded on the compacts with a cell density of 3.0×10^4 per sample. The samples were then put into an incubator at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air for 4 h. Finally, 2.5 ml culture medium, Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% FBS and 1% penicillin/streptomycin (100 U ml^{-1} penicillin, 10 μg ml^{-1} streptomycin), was added to the wells of the plates and then the plates were put back in the incubator. The culture media were refreshed twice a week.

DNA, alkaline phosphatase (ALP) and total protein (TP) analyses

After cell culture, the samples with cells were washed by phosphate buffered saline (PBS) three times after the cultured medium was totally removed. Then the samples were stored in the freezer at −80 °C for at least 12 h for the biochemical analyses. As soon as the plates were taken out of the freezer, they were kept on ice, prepared in advance. Then 0.5 ml 0.2% triton was put into each well with samples in the plates. The plates were shaken gently for 45 min. Finally, the triton solutions with lysed cells were analyzed for DNA, ALP and protein content.

The DNA content was measured with the CyQuant Cell Proliferation Assay Kit (Invitrogen), according to the guideline of the company. 0.1 ml of each sample ($n = 4$) was diluted in the lysis buffer, which was included in the kit, to a final volume of 1.0 ml test tubes. Then 1.0 ml of an aqueous working solution (dye) was added to each sample. After the tubes were incubated for about 3 min, the fluorescence using instrument parameters was measured at an emission wavelength of 520 nm and an excitation of 480 nm. The DNA content of cells attached to the porous samples was counted through a pre-made standard DNA curve. The DNA content was expressed as mean ± SD.

For the determination of the ALP content, 20 μl of each sample ($n = 4$) was added to the wells of a 96-well plate and then a 100 μl paranitrophenylphosphate (PNP) solution was added. After being shaken gently, the plate was incubated at



(a) (b)

Figure 1. SEM images of the compacts: MWCNTs (*a*) and GP (*b*).

37 °C for 15 min. After an 80 μl stop solution (0.2 mol l^{-1} sodium hydroxide) was added, the plate was read with a BIO-TEK automatic microplate reader at 405 nm. For the standard curve, serial dilutions of p-nitrophenol were made. Finally, the ALP content of cells was counted through the standard curve. The value was expressed as mean ± SD.

For the determination of the protein content, 100 μl of each sample ($n = 4$) was added to the wells of a 96-well plate and then a 100 μl BCA solution was added. Then, the plate was continuously shaken for 2 h in the dark at room temperature. Finally, the protein content was measured with the QuantiPro™ BCA Assay Kit (TaKaRa Bio Inc, Japan), according to the guideline of the company. The protein content, expressed as mean ± SD, was counted through a pre-made standard protein curve.

Osteonectin, osteopontin and osteocalcin gene expression

Cell lysate was collected after week 1, and the total RNA was isolated and purified with RNeasy columns (Qiagen, Basel). The isolated RNA was reverse-transcripted to cDNA with the StratScript enzyme (Stratagene, San Diego, CA). Quantitative real-time RT-PCR (Taq Man ABI Prism 7700, Applied Biosystem, Foster City, CA) was used to measure the gene expression of osteonectin, osteopontin, osteocalcin and a housekeeping gene (18S) using the amplifluor universal detection system (Intergen, Purchase, NY). Primers were designed with the Software Primer Expresst (Applied Biosystem). Primers were purchased from Integrated DNA Technologies (Coralville, IA). Use of 18S allowed the different samples to be normalized and compared between experiments.

Cell culture on the samples after adsorbing rhBMP-2

At first, the samples were respectively immersed in rhBMP-2 (Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan) solutions (rhBMP-2 in the cell culture medium) with a concentration of 500 ng ml^{-1} for 24 h in an incubator at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. Then, the solution was completely removed and the samples were washed by the cultured medium of SaoS2 with 1% FBS three times. And then, the cells were respectively cultured on the samples with a cell density of 5.0×10^4 per sample. After cell culture in the culture medium with 1% FBS for a certain time, DNA, ALP and the total protein content were examined with the methods mentioned above.

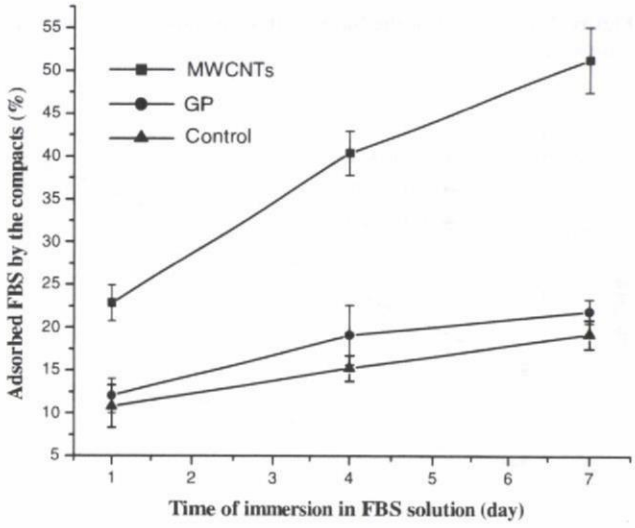


Figure 2. Ability to adsorb proteins of the samples ($n = 5$).

Statistical analysis

Data obtained were statistically analyzed with one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was regarded as significant difference.

Results

Figure 1 shows the SEM images of the compacts. The distinct difference in the structures of the MWCNTs and GP compacts was exhibited. MWCNTs formed a packed meshwork nanostructure, while GP compacts were formed with particles of about 4.5 μm. The ability of protein adsorption of the compacts is shown in figure 2, which shows that MWCNT compacts had much greater ability to adsorb protein than GP compacts. Although the mean value of the protein adsorption of GP compacts was higher than that of

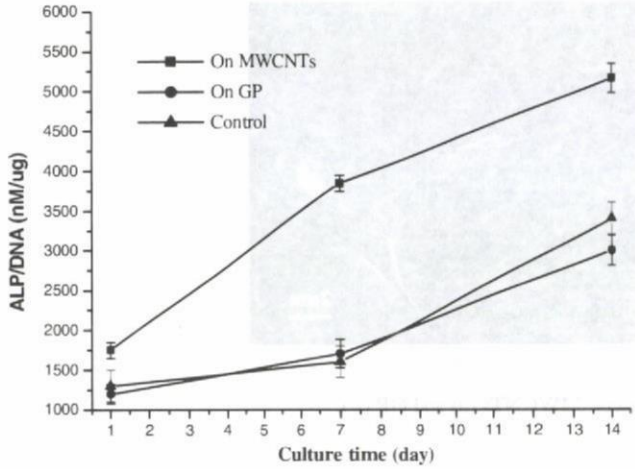


Figure 3. ALP/DNA of the SaoS2 cells conventionally cultured on samples ($n = 4$).

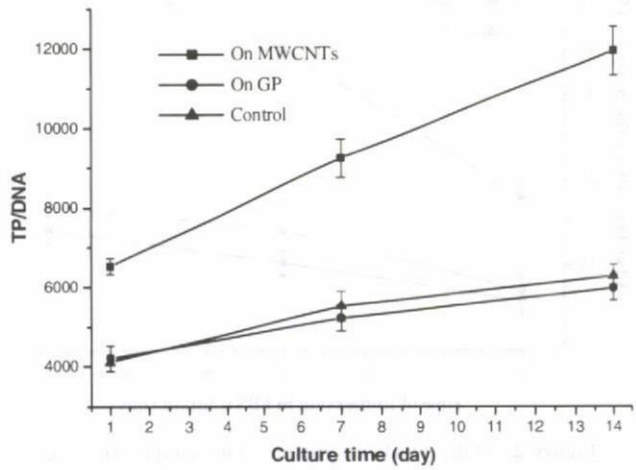


Figure 4. Protein/DNA of the SaoS2 cells conventionally cultured on samples ($n = 4$).

the culture plates (control), the statistical analysis showed that there was no significant difference between the two group values ($P > 0.05$).

Figure 3 shows the results of ALP/DNA (alkaline phosphatase per unit cell). ALP/DNA of the SaoS2 cells cultured on MWCNT compacts was significantly higher than that on GP compacts and on the plates at each culture time point of 1, 7 and 14 days, whereas this value for GP compacts and the control had no significant difference at each time point.

TP/DNA (total protein content per unit cell) is shown in figure 4. TP/DNA of the SaoS2 cells on MWCNTs was significantly higher than on GP and the culture plates at each culture time point of 1, 7 and 14 days. At day 14, the value for MWCNTs was about twice the value for GP. Although the mean value for GP compacts was higher than that for the control at days 7 and 14, the statistical analysis showed that there was no significant difference between the two group values ($P > 0.05$).

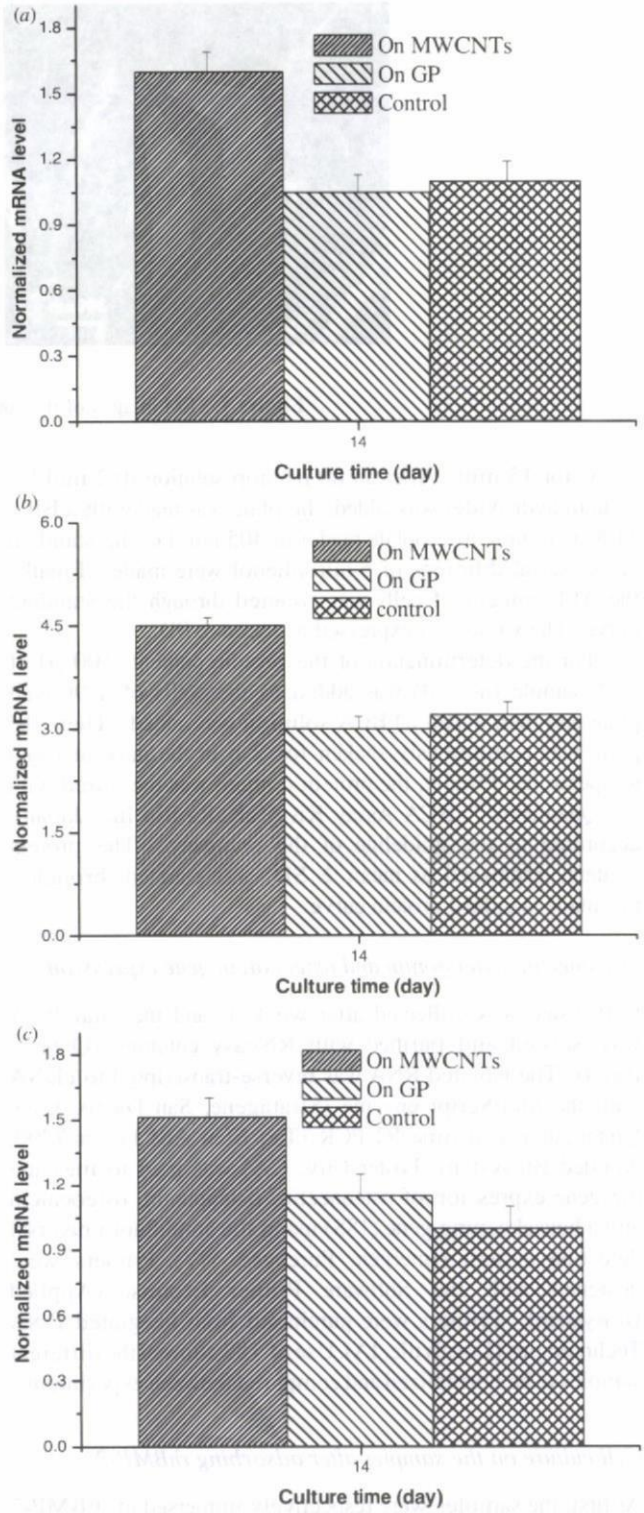


Figure 5. Gene expression of (*a*) osteonectin, (*b*) osteocalcin and (*c*) osteopontin normalized by the gene expression of 18S of cells conventionally cultured on different samples ($n = 4$).

As is shown in figure 5, statistically higher osteonectin, osteopontin and osteocalcin gene expression of SaoS2 at day 14 on MWCNTs than on GP scaffolds was found ($P < 0.05$). There was no significant difference in the osteonectin,