図1. 作製したハイドロキシアタパイトー
コラーゲン複合体膜。水分を含むと軟性を示す。

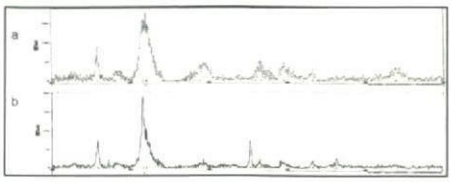


図2　nHAC のX線回析
a:EDC 架橋
b:アスコルビン酸

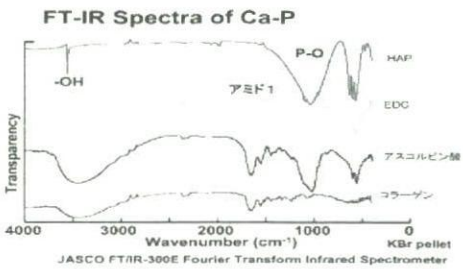
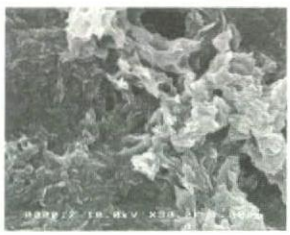


図3　nHAC の FTIR 分析



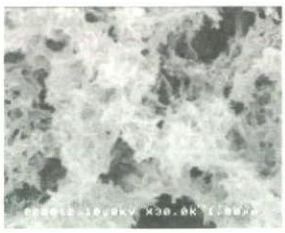


図4　SEM
EDC　　アスコルビン酸

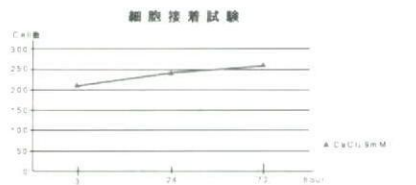
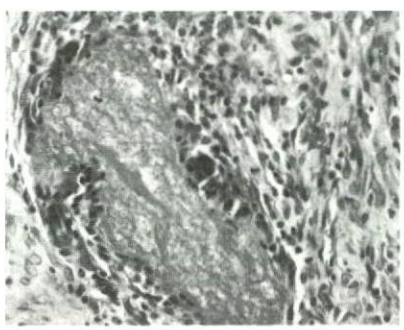


図5　細胞接着試験
(EDC)



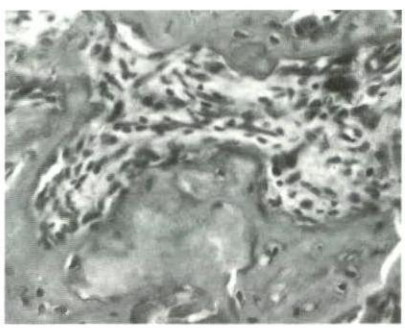
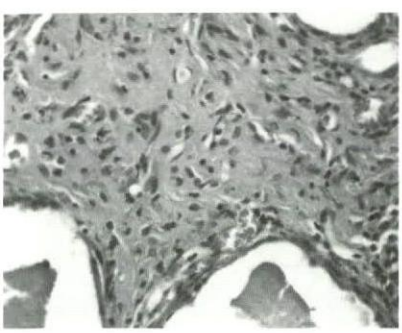


図6　2週後　筋内
a: EDC 架橋 BMP 群.

2週後　筋内
b:アスコルビン酸架橋ＢＭＰ群



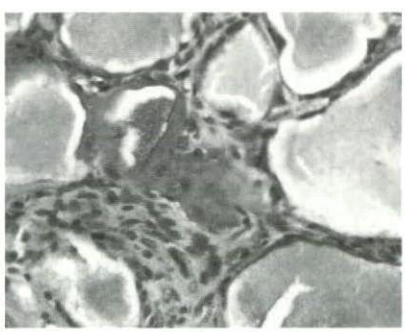


図7 結合組織内　2週後
a:EDC 架橋ＢＭＰ群

結合組織内　2週後
b:アスコルビン酸架橋 BMP 群

	Starting material			Product		abbreviation
	Acid-soluble Collagen aq	K_2HPO_4 /KH_2PO_4	$CaCl_2$	Hydroxapatite Collagen(%)		
A	3 mg/ml	9 mmol/l	5.4 mmol/l	70	30	70HA/30CG
B	3 mg/ml	6.78 mmol/l	4.06 mmol/l	66	34	66HA/34CG
C	3 mg/ml	4.5 mmol/l	2.7 mmol/l	56	44	56HA/44CG

表 1

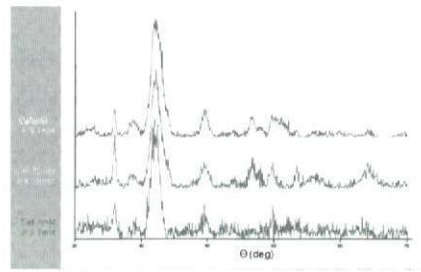


図 8　X 線回折

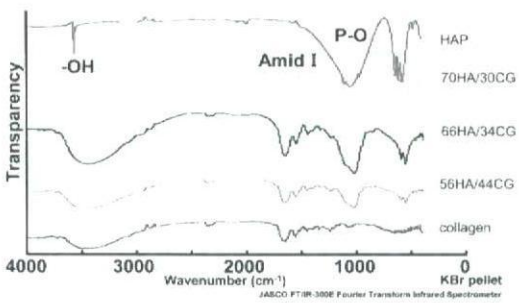


図 9　FTIR

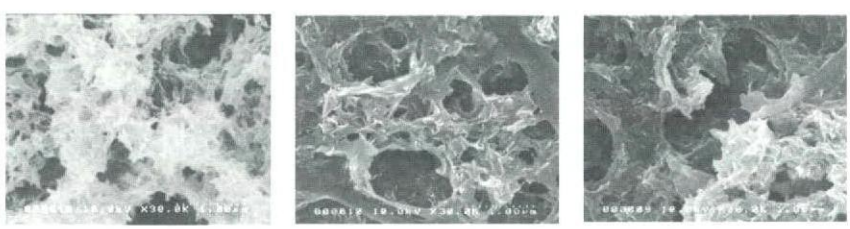


図 10SEM

a:70/30 群　　b:66/34 群　　c:56/44 群

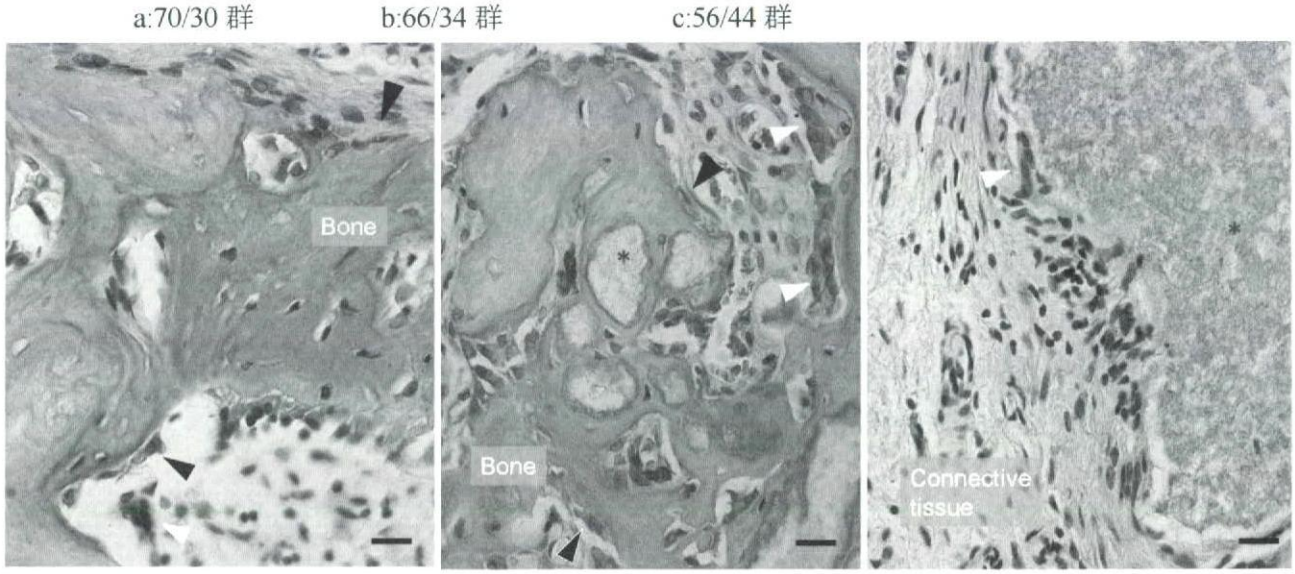


図 11　4 週　結合組織内

a:70/30 群　　b:66/34 群　　c:56/44 群

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分担研究報告書

Effect of carbon nanotubes on cellular functions in vitro

分担研究者	亘理文夫	北海道大学歯学研究科教授
研究協力者	**Xiaoming LI**	北海道大学歯学研究科 **JSPS** 研究員
研究協力者	宇尾基弘	北海道大学歯学研究科助教授
研究協力者	赤坂　司	北海道大学歯学研究科助手
研究協力者	阿部薫明	北海道大学歯学研究科助手

研究要旨　Carbon nanotubes have been shown to affect cell behaviour. But how and why the carbon nanotubes affect potential differentiation of the attached cells have not been largely known. In this study, multi-walled carbon nanotubes (MWNTs) and graphite (GP) were pressed as compacts. Higher ability of carbon nanotubes to adsorb proteins, comparing with graphite, 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 carbon nanotubes 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 carbon nanotubes might induce cellular functions by adsorbing more proteins, which indicated that the carbon nanotubes might be a candidate for scaffold material for tissue engineering.

A. 研究目的

Using tissue engineering to repair tissue damages is attracting more and more attention. Scaffolds, lying at the heart of all the new tissue engineering approaches, act as a substrate for cellular attachment, proliferation and differentiation. In the imminent ageing society of the 21st century, nanomaterials have been widely investigated for potential application in the medical field. 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. 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.

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. 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.

To start the differentiation of cells, it has been suggested that nanostructures of the biomaterials are critical . In other words, the microenvironment around the cells may be crucial. Fujibayashi et al suggested that even a non-soluble metal that contains no calcium or phosphorus can be an osteoinductive material when treated to form an appropriate nanostructure. Popat KC, 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. Yim EKF, 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.

In this study, myoblastic mouse cells (C2C12) were cultured on multi-walled CNTs (MWNTs) compacts, comparing on graphite compacts, with and without the adsorption of 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.

B. 研究方法

Materials

MWNTs used in this study were obtained from NanoLab (Brighton, MA USA). 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 approximately 500℃ for 90 min under atmospheric conditions. Next, the cooled MWNTs were transferred into a flask containing 6 M HCl and treated at 60℃ for 2 h, and then washed thoroughly with deionized water and completely dried. The graphite (GP) particles used in this study were about 4.5μm 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 (0.09GPa for 2min, then 0.22GPa for 3min, finally 0.36GPa for 3min) at room temperature. The compacts were washed ultrasonically with acetone, 70% ethanol and RX-water for 15minutes and then dry at 60□C. All the compacts were sterilized by ultraviolet radiation for 48 hours 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 (250ul FBS in 100ml 25ppm NaN3 solution) were sterilized with 0.22um filter. After immersing the compacts respectively for 1, 4 and 7days, the residual protein content (Pl) of the FBS solution (3ml 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 620nm. The adsorbed protein (Pa) was determined by the formula of (0.25%- Pl)/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 2.0×104 per sample. Then the samples were put into an incubator at 37□C in a humidified atmosphere with 5% CO2 and 95% air for 4 hours. 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 □g/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 non-adherent cells, fixed in a solution of 2% glutaraldehyde, and post-fixed 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 scanning electron microscopy (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 hours 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 0.5ml 0.2% triton was put into each wall with samples in the plates. The plates were shook gently for 45min. 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. 0.1ml of each sample (n=4) were diluted in TE to a final volume of 1.0ml test tubes. Then 1.0ml of aqueous working solution (dye) was added to each sample. After the tubes were incubated for about three minutes, 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, 20ul of each sample (n=4) was added to the walls of a 96-wall plate and then 100μl Paranitrophenylphosphate (PNP) solution was added. After shaken gently, the plate was incubated at 37□C for 15 min. After 80μl stop solution (0.2mol/L sodium hydroxide) was added, the plate was read with a BIO-TEK automate microplate reader at 405nm. 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 QuantiPro™ BCA Assay Kit (TaKaRa BIO INC). 100ul of each sample (n=4) was added to the walls of a 96-wall and then 100ul BCA solution was added. Then, the plate was continuously shaken for 2 hours in dark at room temperature. Finally, the fluorescence was measured with a BIO-TEK automate microplate reader at 620nm. 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 hours in an incubator at 37□C in a humidified atmosphere with 5% CO2 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 3 times. C2C12 was respectively cultured on the samples with a cell density of 4.0×104 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, USA) 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.

C. 研究結果

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, while 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 4 to estimate the cell proliferation, and thought that the difference in the DNA value at 1 day 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.

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 towards 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 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 7 showed ALP/DNA of C2C12 on the samples after adsorbing FBS at day 4 and day 7, comparing 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 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 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 ($p>0.05$).

D. 考察

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. Cells in their natural environment interact with extracellular matrix (ECM) components in the nanometer scale. 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 towards different phenotypes under the action of specific proteins, some chemical or biological factors, was cultured on the carbon nanotubes, and that not only cell attachment and proliferation, but also cell differentiation and cell activity were investigated. Carbon nanotubes and GP are both isomorphs of pure carbon, composed of the same grapheme sheet structure. However, only carbon nanotubes have nanostructures. So in this study, comparison of the influence of carbon nanotubes and GP on C2C12 cells in vitro was done to figure out the effect of carbon nanotubes 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 are consistent with what Aoki N, et al have reported in their work, where they found osteoblasts attached and proliferated better on multi-walled and single-walled carbon nanotubes than on GP. 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 KL, et al found that some specific proteins have different influence on

human marrow stromal cells attachment and saos-2 osteosarcoma cells attachments on hydroxylapatite. Since MWNTs compacts had higher ability to adsorb the proteins or higher affinities toward proteins than GP (figure 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 the ALP/DNA of C2C12 on MWNTs was significantly higher than on GP and the culture plates, suggesting 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. These osteoinductive materials all have specific structures, indicating larger surface area and therefore higher ability to adsorb proteins. The work of Ripamonti U and co-workers demonstrated that osteoinductivity in hydroxyapatite was linked to the precise shape of surface concavities in implants, which indicated a larger surface area, and that using immunolocalization, they demonstrated that this osteoinductivity occurred as a result of a concentration of specific proteins within the surface concavities.

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 suggested 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.

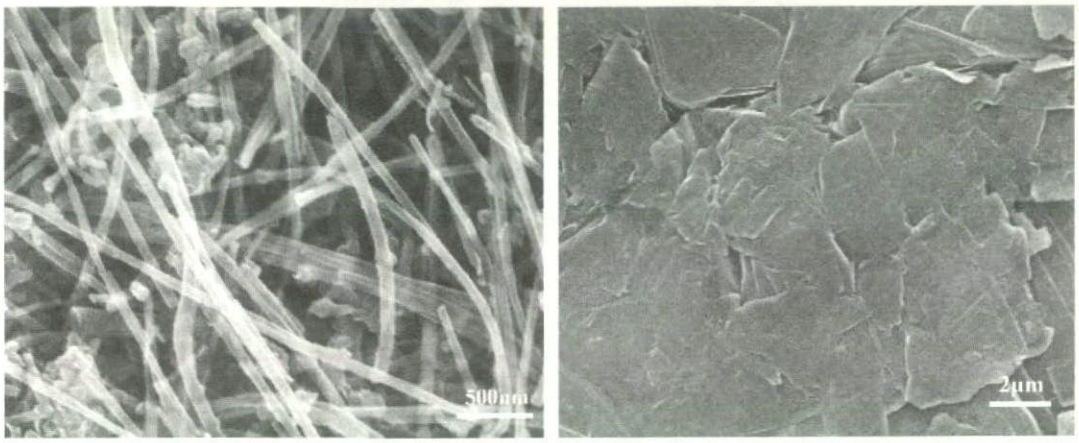
So we hypothesized that the large amount of proteins adsorbed on the MWNTs played an important role in inducing cellular functions.

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 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. Carbon nanotubes 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.

E. 結論

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



(a) (b)

Fig.1. SEM of the compacts: MWCNTs (a); GP (b)

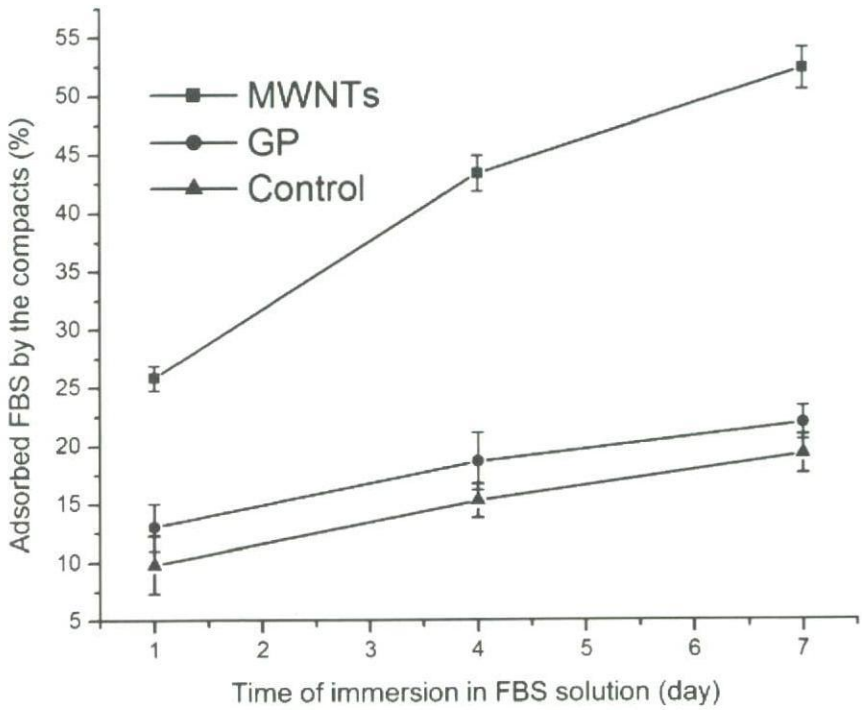
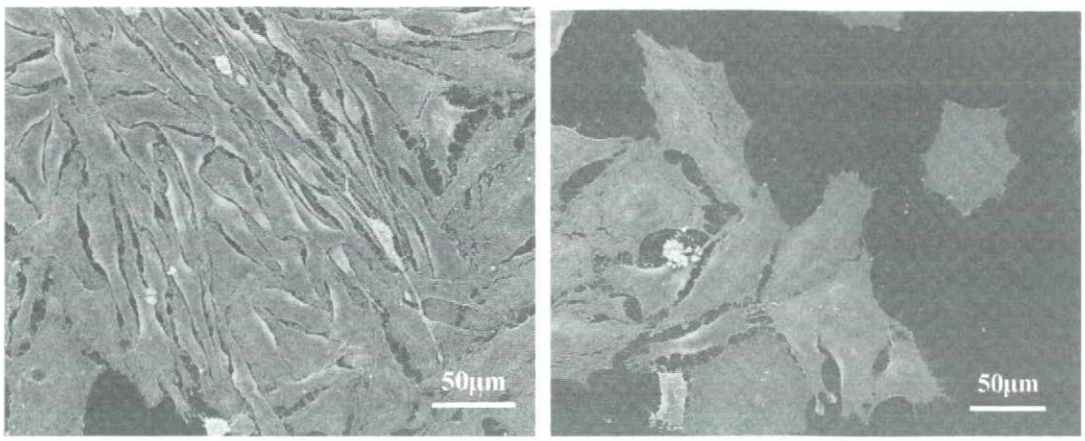


Fig. 2. Ability to adsorb proteins of the samples (n=5)



(a) (b)

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

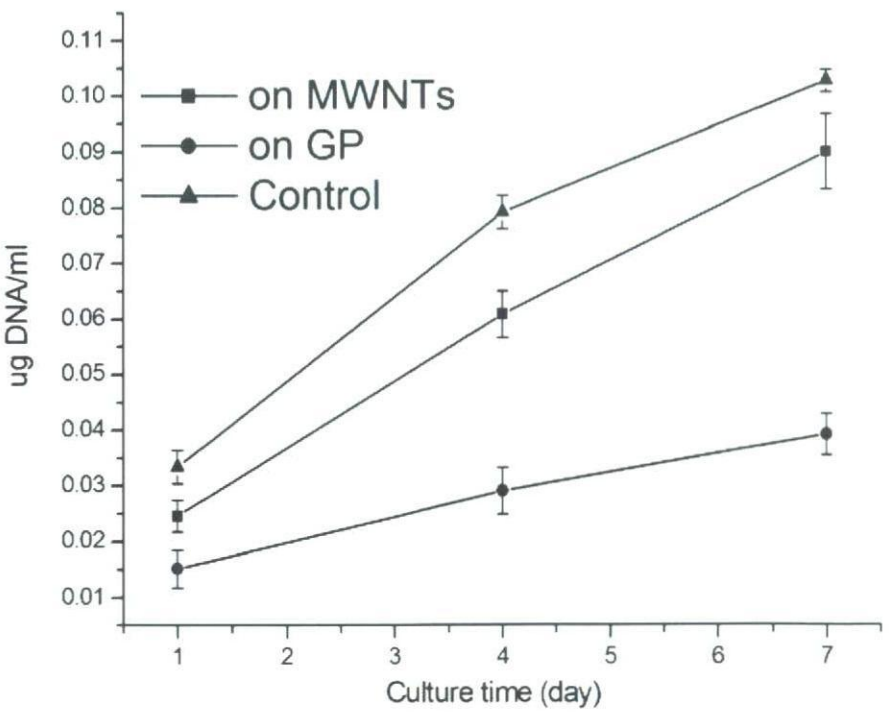


Figure 4. Results of DNA analysis of C2C12 cultured on samples (n=4)

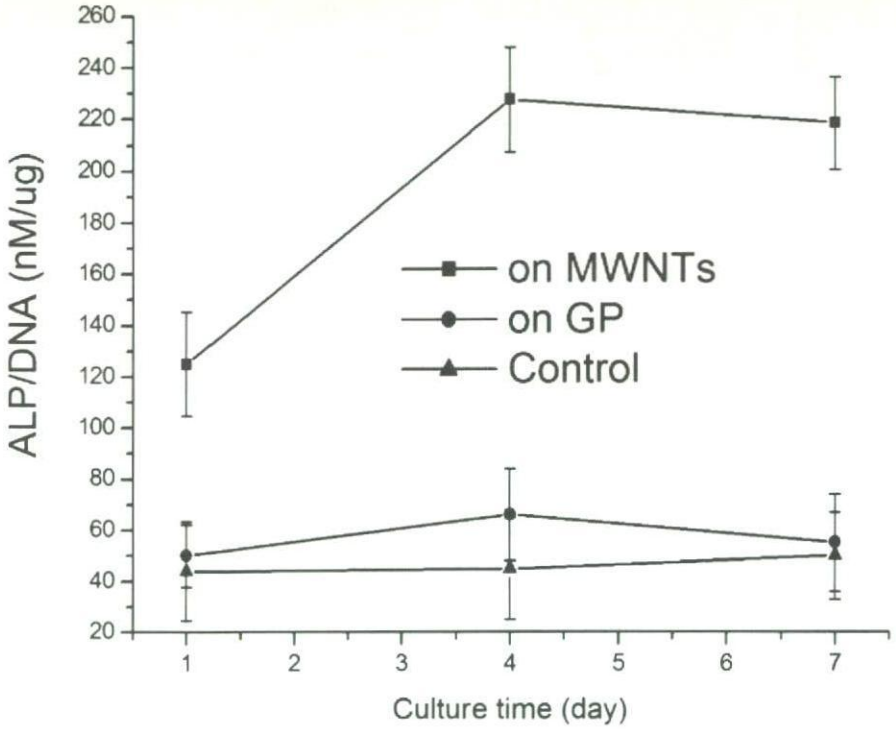


Figure 5. ALP/DNA of C2C12 cells conventionally cultured on samples (n=4)

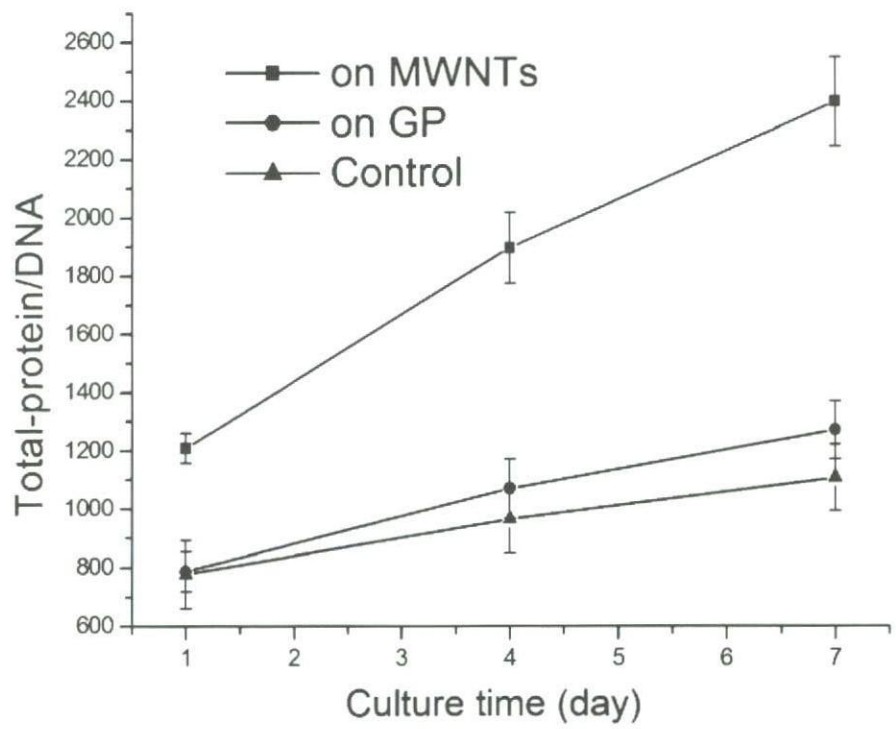


Figure 6. Total-protein/DNA of C2C12 cells conventionally cultured on samples (n=4)

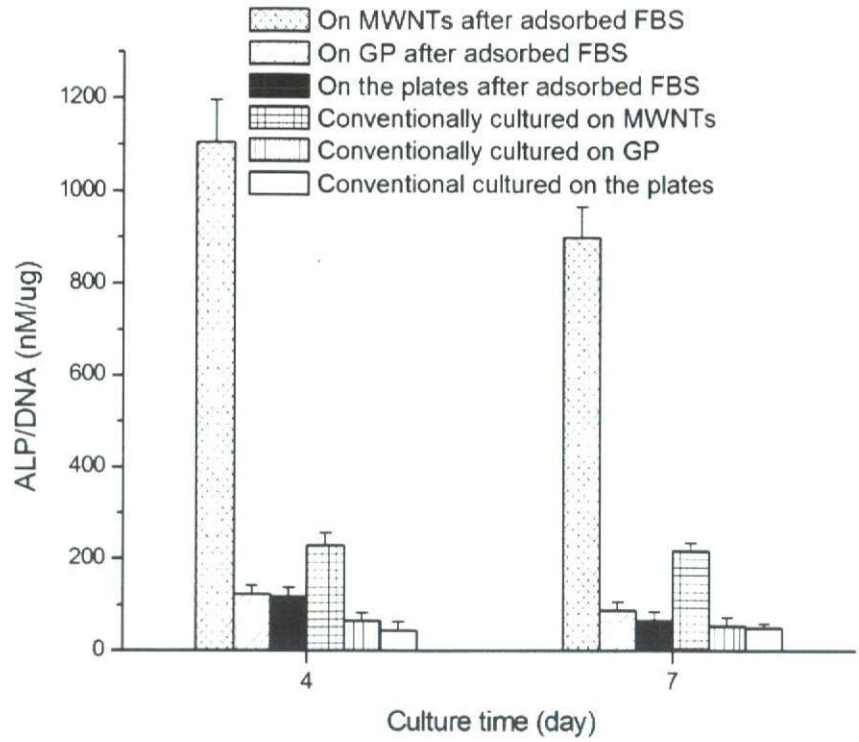


Figure 7. ALP/DNA of C2C12 cells cultured on samples with and without the adsorption of FBS in advance (n=4)

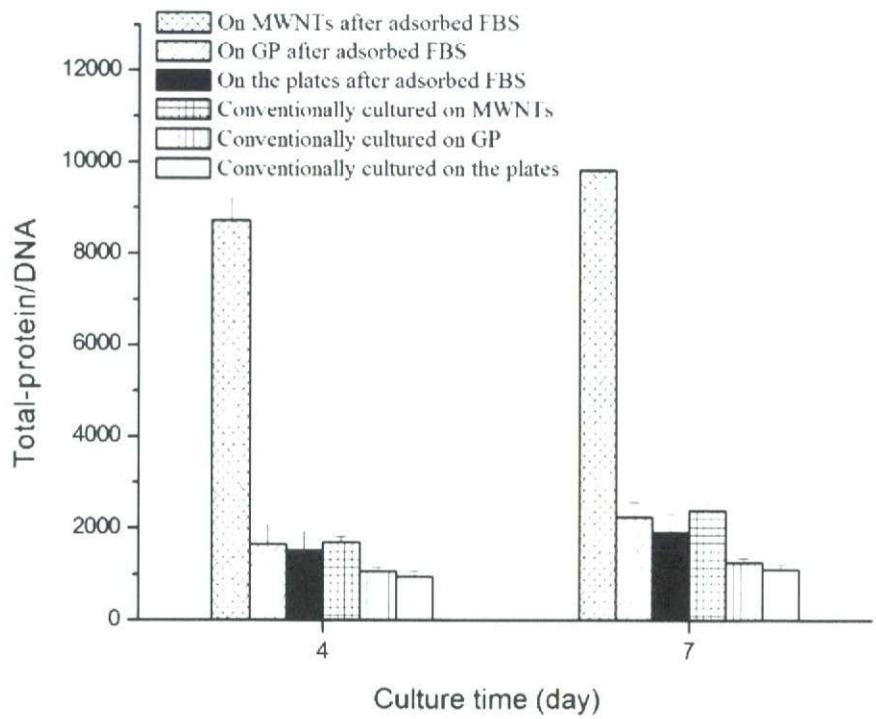


Figure 8. Total-protein/DNA of C2C12 cells cultured on samples with and without the adsorption of FBS in advance (n=4)

厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

正常および癌肝細胞培養における CNT 添加の影響

主任研究者　亘理文夫　　北海道大学歯学研究科教授
研究協力者　八若保孝　　北海道大学歯学研究科教授
研究協力者　伊藤佐智子　北海道大学大学院歯学研究科大学院生

研究要旨　本研究においては，CNT が各細胞へ及ぼす影響の違いを調べるため、ヒト正常肝細胞由来の HC 細胞とヒト癌肝細胞由来の HepG2 細胞、ラット歯根膜細胞由来の細胞株を用い CNT がそれぞれの細胞に及ぼす影響について観察、比較を行った。それぞれの細胞を 37℃、5%CO_2 環境下にて一定期間培養後、CNT を添加し、さらに培養した後位相差顕微鏡にて細胞を観察した。添加した CNT は、硝酸または過酸化水素水にて酸化処理を行ったものと未酸化処理の CNT の 3 種類を用いた。ヒト肝細胞では CNT 添加後に細胞数の著しい変化はみられなかったのに対し、ラット歯根膜細胞では CNT 添加群では CNT 無添加群に比較して RNA 量の増加がみとめられた。ラット歯根膜細胞においては CNT 添加群で数種のタンパク発現の低下がみられた。

A. 研究目的

CNT は細胞に対して様々な興味深い影響を与えることを見出しているが、本研究では細胞へ及ぼす影響と細胞の種類によってどのような違いがあるのかを調べるため、ヒト正常肝細胞由来の HC 細胞とヒト癌肝細胞由来の HepG2 細胞、ラット歯根膜細胞由来の細胞株を用い CNT がそれぞれの細胞に及ぼす影響について観察、比較を行った。

B. 研究方法

1)　MWCNTの添加による細胞への影響

CNTは500℃、90分で焼成処理を行い、その後硝酸、または過酸化水素水にて酸化処理を行った。添加するCNTは焼成処理のみのもの、硝酸にて酸化処理を行ったもの、過酸化水素水にて酸化処理を行ったものの3種類を用いた。

CNT はあらかじめオートクレーブにて滅菌を行った後に培養液中に 0. 02%の濃度で添加した。

使用細胞株　：ヒト正常肝細胞由来の HC 細胞、ヒト癌肝細胞由来の HepG2 細胞、ラット歯根膜細胞の 3 種類を用いた。

使用培地　　：DMEM 培地を用いた。血清はヒト正常、癌肝細胞では 10%、ラット歯根膜細胞では 20%の濃度で添加した。

培養環境　　：37℃、5%CO_2 環境下にて 7 日間培養後、CNT を添加し、さらに 2 日間培養した後位相差顕微鏡にて細胞を観察した。

2)　ラット歯根膜細胞遺伝子解析

PCR 法にて行った。

C. 研究結果

CNT を培養液添加 2 日後、正常細胞 HC 細胞、癌細胞 HepG2 細胞ともに位相差顕微鏡にて細胞核を取り囲むように CNT が細胞内に取り込まれている

様子がみとめられた（Fig.1）。走査型電子顕微鏡像ではCNTを添加した細胞群においてCNT無添加群に比べ細胞表面に多数の突起がみとめられた。Fig.2で示した透過型電子顕微鏡像では細胞表面に付着しているCNTと細胞質内に取り込まれているCNTがみとめられ、細胞質内ではCNTが全体に分散しているのではなく、局在している様子が観察された。

ラット歯根膜細胞でのPCRでは、多くのタンパク質でCNT添加群ではCNT無添加群と比較すると発現量の低下がみられたが、硝酸処理を行ったCNTを添加した群ではc-kitにおいて発現の増加がみられた。

D. 考察

TEM観察より、CNTを細胞質内にもつ細胞では形態の辺縁が無添加群と異なっていることから、CNTが細胞形態へ影響をおよぼすことが示唆された。添加直後からCNTは細胞に付着している様子がみられ、TEM観察よりその一部は細胞質内に入り込んでいる様子が観察されたが、CNTは細胞質内で凝集局在しており、細胞核内にはみとめられなかった。

また、ラット歯根膜細胞ではCNT無添加群に比べCNT添加群でのRNA量が増加しており、傾向は今回用いた3種類のCNTで同様であった。CNTが細胞増殖に与える傾向について解明していく必要があると思われる。

E. 結論

今回の結果より、CNTの細胞質内への侵入がみとめられ、CNTの細胞に及ぼす影響も細胞の種類によって異なることが示唆された。

F. 研究発表

1. 学会発表

1. 伊藤佐智子、八若保孝、赤坂司、亘理文夫、ナノ微粒子体内動態可視化法の開発研究会　2008
2. 伊藤佐智子、八若保孝、赤坂司、亘理文夫、歯科理工学会秋季大会　2008

G. 知的所有権の取得状況

なし

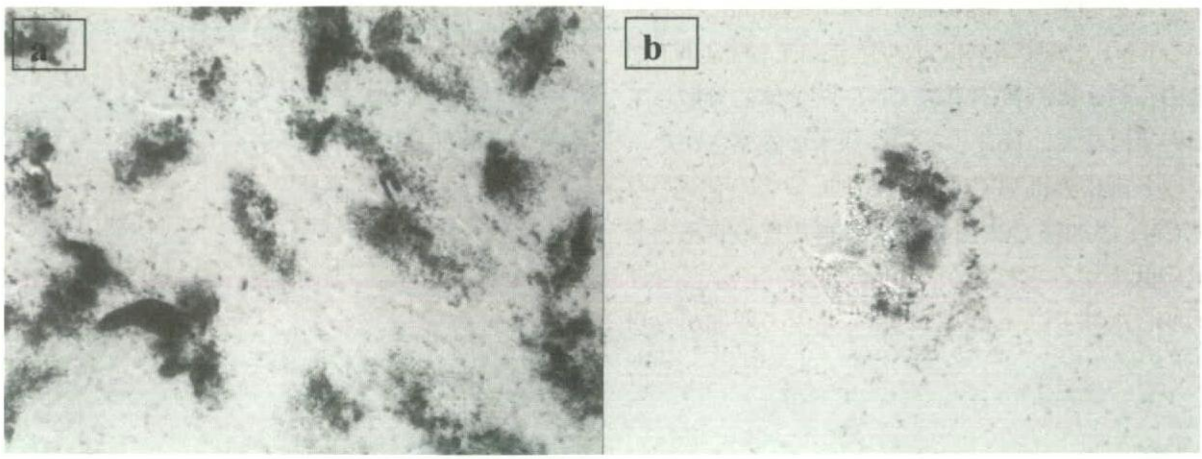


Fig.1 ： CNT 添加後培養 2 日後の位相差顕微鏡像
a:正常細胞
b:癌細胞像

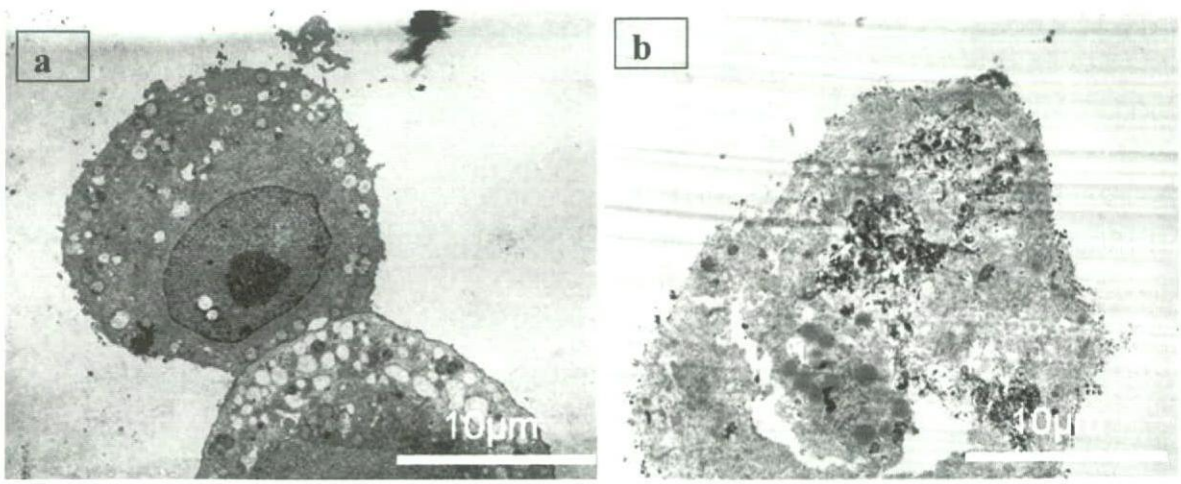


Fig.2 ： 正常肝細胞の透過型電子顕微鏡像
a：CNT 無添加群
b：CNT 添加群

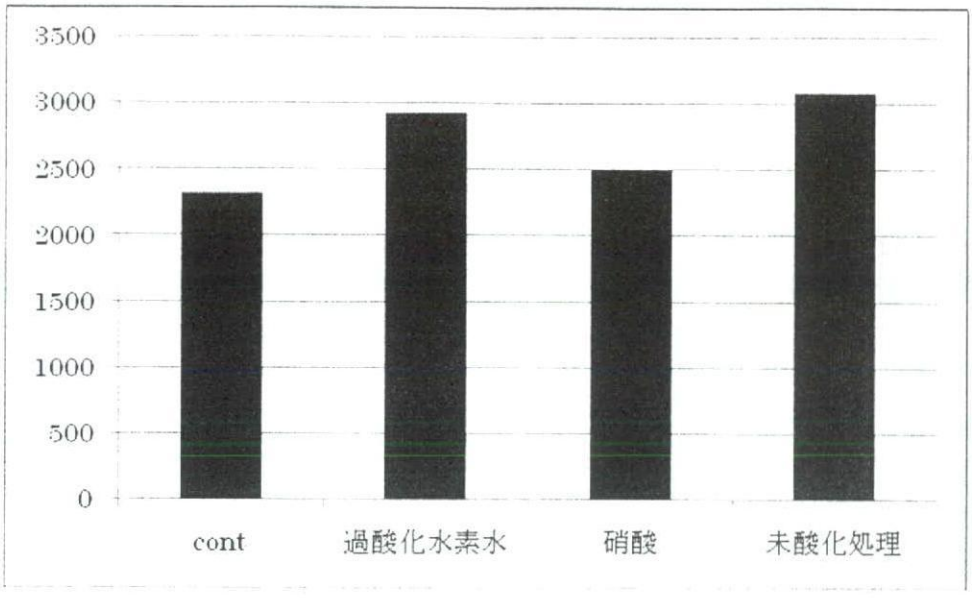


Fig.3 : ラット歯根膜細胞の RNA 量

厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

Imogoliteスキャホールドが細胞機能へ与える影響

主任研究者　亘理文夫　　北海道大学歯学研究科教授
研究協力者　八若保孝　　北海道大学歯学研究科教授
研究協力者　石川紘佑　　北海道大学歯学研究科大学院生

研究要旨　本研究では、Imogoliteのバイオ応用を検討した。
Imogoliteは、自己組織化能を有する粘土鉱物の1つであり、およそ外径2nm、内径1nm、長さ数μmの白色ナノチューブである。

今研究では、Imogolite分散液を作製し、Imogoliteをcell culture dish上に直接コートしスキャホールドとした。作製したスキャホールド上で14日間、骨芽細胞様細胞（Saos2）を培養後、細胞数計測、SEMによる形態観察及びカルシウム・リン酸塩定量を行った。比較対象として、形態の似通った、Single wall carbon nano tubes（SWCNTs）を使用した。

Imogolite上で細胞は良好に増殖した。細胞数、形態ではSWCNTsとほぼ同様の結果を示した。cell culture dishと比較すると、ナノチューブ上の細胞は形態が多様で、分化に有利であると考えられた。

A. 研究目的

Imogoliteは高い比表面積及び保水性を有するアルミノシリケートであり、柔軟性および自己組織化能を持った直径約2nmの白色ナノチューブである。本研究では、Imogolite及びSWCNTsをcell culture dish上にコートし、スキャホールドとした。さらに、作製したスキャホールド上で細胞培養を行い、細胞成長に対するスキャホールドの影響を検討した。

B. 研究方法

Imogoliteは、天然産出量が極わずかであり、鉄を主とした不純物を含むため、今研究では合成物（産業技術総合研究所　地圏資源環境研究部門　地下環境機能研究グループ　鈴木正哉）を使用した。SWCNTsは、名城ナノカーボン社製FH-Pを使用した。

まず、1000ppmImogolite及び5ppmSWCNTs分散液を作製し、cell culture dish上にコートしスキャホールドとした。作製したスキャホールドの表面粗さ及び、ぬれ性を測定した。さらに、スキャホールドより溶出されるSi、Al量をICPにて検索した。

得られたスキャホールド上でヒト骨肉腫由来骨芽細胞様細胞（Saos2）を所定期間培養し、固定、乾燥の後、細胞数計測、SEMにて細胞形態観察及びカルシウム・リン酸塩定量を行った。

さらに、蛍光ラベルされたアルブミンを用い、蛋白質吸着試験及び、9000ppmNaF溶液を用い、フッ素イオン吸着試験も行った。イオン計測にはフッ素複合電極（Thermo Orion）を使用した。

C. 研究結果

スキャホールド上にて、14日間ヒト骨芽細胞様細胞の培養を行ったところ、強い細胞接着および伸展形態が観察された。

また、コントロールであるcell culture dishと比較したところ、ほぼ同等な良好な細胞増殖が認めら

れた。

一方で、Imogolite及びSWCNTs上では細胞表面に多くの生成物が観察される傾向があり、分化に有利である可能性も考えられた。

カルシウム・リン酸塩定量においては、ポリスチレンディッシュと比較し、Imogolite上の細胞においては形成カルシウム量の増加傾向が認められた。

また、ImogoliteはCNTsと同様に高いタンパク質吸着能があり、細胞の初期接着に有効と考えられる。

ICPによるSi、Al溶出試験において、微量ではあるものの、培養液中にSi、Alの存在が確認された。特にSiは骨芽細胞の成長促進効果が確認されており、CNTスキャホールドとImogoliteスキャホールドの大きな違いと考えられる。しかし、現時点ではSi、Alともにイオン化しているか、もしくは微粒子であるかは明らかとなっておらず、今後の課題の一つである。

イオン吸着試験では、SWCNTsがほとんど吸着しなかったのに対し、Imogoliteは、フッ素イオンを、多量に吸着した（自重のおよそ75%）。この性質は、Imogoliteをフッ素にて修飾し、歯科充填材料に導入するなど種々の用途に応用可能と考えられる。

D. 考察

Imogolite スキャホールド上の多様な細胞形態及び、生成物、形成カルシウム・リン酸塩量より分化に有利であることが推測される。しかし、今研究での、**SWCNTs** との比較から、**Imogolite** のどの要素が細胞培養に有利に働いたのか解明できなかった。今後、**ALP** 活性等種々の生化学的アッセイを行い、その有効性を検討してゆく必要があると考えられた。

E. 結論

Imogolite は細胞培養に、有効な材料であると考えられる。

F. 研究発表

1. 論文発表

無し

2. 学会発表

1. 石川紘佑、赤坂　司、八若保孝、亘理文夫 "イモゴライト上での細胞培養"、第51回粘土科学討論会講演要旨集、P144 北海道、札幌市　2007

2. 石川紘佑、赤坂　司、八若保孝、亘理文夫 "ケイ酸塩無機ナノチューブ（イモゴライト）上での細胞培養"、第52回日本歯科理工学会、大阪、豊中市　2008

G. 知的所有権の取得状況

1. 特許取得

無し

2. 実用新案取得

無し

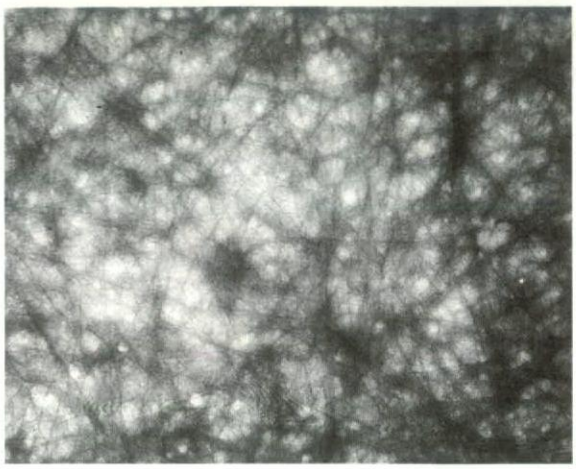


図 1　Imogolite TEM 像(×50000)

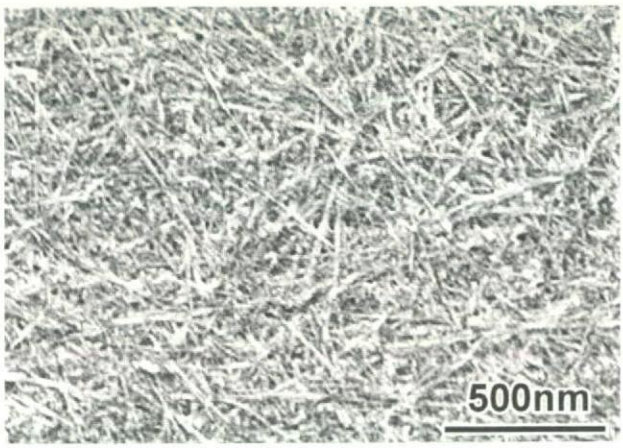


図 2　Imogolite スキャホールド SEM 像

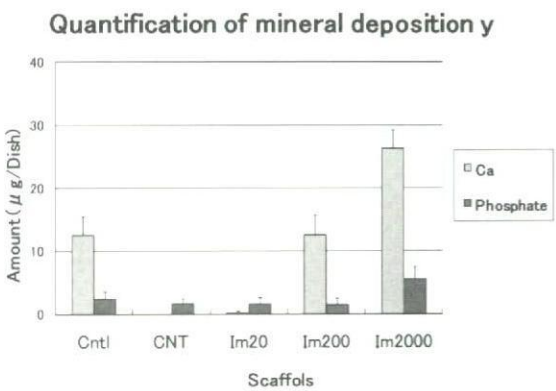


図 3

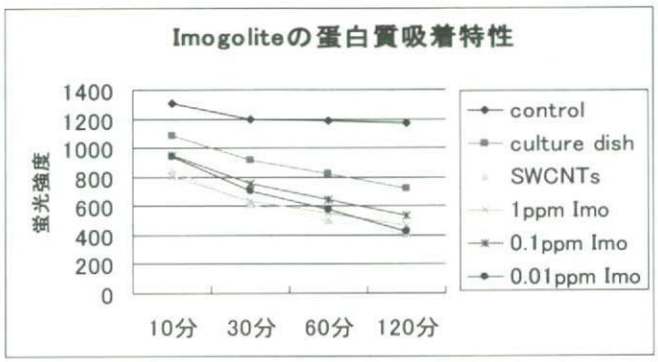


図 4

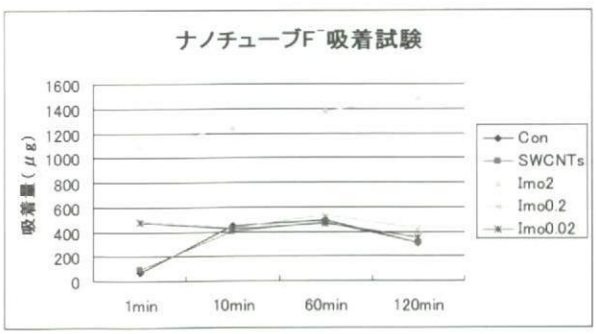


図 5

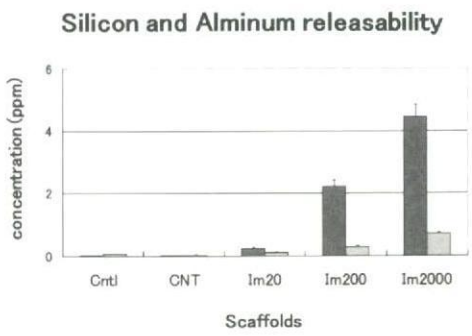


図 6

厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

生体と無機ナノ粒子のかかわりと応用に関する研究

主任研究者　亘理文夫　　北海道大学大学院歯学研究科教授
研究協力者　米澤　徹　　東京大学大学院理学系研究科 准教授

研究要旨　本研究においては，生体と無機ナノ粒子のかかわりとその応用について検証することを目標にした。ナノ粒子の形状異方性について検証を行うため、自ら金属ナノ粒子の合成を行った。無機ナノ粒子と生体のかかわりについては、その粒子の材質、表面に吸着している分子、大きさ、形状、表面電位など、様々なファクターが関与することが考えられる。本年度は、生体関連物質の質量分析を行い、将来のマスマッピングに寄与すべく、ナノ粒子を用いた表面支援レーザー脱離イオン化法の検証を行った。上記で見出した異方性ナノ粒子が生体関連物質のイオン化に非常に良好であることが見出された。そのたき、良好な質量分析のために、金属表面を保護剤などの有機分子でコートせずに安定に水中に分散させた金属ナノ粒子の調製について試験・検討を行った。

A. 研究目的

これまでも粉体や微粒子の材料化は世の中の大きな興味であった。近年、ナノ材料に対する関心が近年高まってきており、金属や半導体のみならず、有機物･無機物、様々な物質のナノ化が行われてきた。ナノ物質は、バルク状態とも原子・分子状態とも異なる物性を示しうる新奇材料として非常に期待される。こうしたナノ粒子は、そうした新物性と、高い比表面積とによって、触媒、色材、蛍光材料、バイオセンシング、抗菌作用、磁性材料、電子材料、電極材料、配線材料、担体、光学材料などに幅広い応用が期待されている。特に、化学法によって容易に合成できる金属や金属酸化物のナノ粒子に関する研究が膨大になっており、世の中の期待を背負っている。

このように材料化への期待が強いナノ粒子であるが、一方で、その生体とのかかわりについての研究も目立ってきている。とはいえ、金ナノ粒子は電子顕微鏡の染色剤などに用いられてきたり、プラズモン吸収をもつ金や銀ナノ粒子がバイオセンサーとして利用されてきている。また、金属光沢をもった表面を形成するための色材としてアルミホイール表面への塗布などへも応用展開されている。さらに、白金族のナノ粒子はこれまで様々な化学反応のための触媒として利用されてきており、燃料電池触媒としても非常に興味が持たれている。

また最近では、白金ナノ粉末に活性酸素除去能があることが示され、より生体とかかわりの深い場面で使用され始めている。さらに、様々な金属において、ナノ粒子に異方性をもたせることが容易であって、棒状、花弁状の白金ナノ粒子などが構築可能である。

一方、金属酸化物のナノ粒子は、よく形状・粒径の揃ったシリカの集積体がフォトニック結晶として興味が持たれているほか、化粧品用途はもちろんのこと、チタニアは、光触媒や白色顔料として多用されている。また、マグネタイトやFePtなどの磁性粒子は、ハイパーサーミアなどの磁気医療分野や、垂直時期記録方式の記憶メディアなどへの展開も図られている。