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ORIGINAL ARTICLE

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Changes in expression of genes related to cell proliferation in human mesenchymal stem cells during in vitro culture in comparison with cancer cells

Abstract We investigated the expression levels of several genes related to cell proliferation in human mesenchymal stem cells (hMSCs) during in vitro culture for use in clinical applications. In this study, we focused on the relationship between hMSC proliferation and transforming growth factor β (TGFβ) signaling during in vitro culture. The proliferation rate of hMSCs gradually decreased and marked changes in hMSC morphology were not observed in 3 months of in vitro culture. The mRNA expressions of TGFβ1, TGFβ2, and TGFβ receptor type I (TGFβRI) in hMSCs increased with the length of cell culture. There had been no change in the TGFβ3, TGFβRII, and TGFβRIII mRNA expressions by the 12th passage from the primary culture (at about 3 months). The mRNA expression of Smad3 increased, but those of c-myc and nucleostemin decreased with the length of hMSC in vitro culture. In addition, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of cancer cells. In conclusion, hMSCs derived from bone marrow seldom underwent spontaneous transformation during 1–2 months of in vitro culture for use in clinical applications. In hMSCs as well as in epithelial cells, growth might be controlled by the TGFβ family signaling.

Key words Stem cells · Cell proliferation · TGFβ signaling · TGFβ receptors

Introduction

Several recent studies demonstrated the potential of bioengineering using somatic stem cells in regenerative medicine.[1,2] Bone marrow includes both mesenchymal and hematopoietic stem cells. Adult human mesenchymal stem cells (hMSCs) derived from bone marrow have the pluripotency to differentiate into cells of mesodermal origin, e.g., bone, cartilage, adipose, and muscle cells.[1–5] Moreover hMSCs also have the capacity to differentiate into myocytes,[6,7] hepatocytes,[1,8] and neural cells.[3] In addition, because they are comparatively easy to expand ex vivo, hMSs have many potential clinical applications, not only in the field of orthopedic surgery but also for the treatment of cardiac infarction, cirrhosis, and diabetes. On the other hand, stem cells possess a self-renewal capability similar to that of cancer cells.[9] Recently Rubio et al.[10] reported spontaneous transformation of human adult stem cells derived from adipose tissue in long-term (4–5 months) in vitro culture. In practice, if hMSCs are to be used for clinical applications and tissue-engineered medical devices, they have to be expanded in vitro for about 1–2 months. The proliferation ability and the gene expression profile of hMSCs, however, might change during in vitro culture. In this study, we focused on the relationship between hMSC proliferation and transforming growth factor β (TGFβ) signaling during in vitro culture. TGFβ is a multifunctional protein that regulates cellular proliferation, differentiation, apoptosis, development, extracellular matrix formation, immunosuppression, and tumorigenesis. In humans, three TGFβ isomers have been identified: β1, β2, and β3. TGFβ signals through three high-affinity cell surface receptors: TGFβ type I (TGFβRI), type II (TGFβRII), and type III (TGFβRIII) receptors. TGFβRI and TGFβRII are serine-tyrosine kinases. TGFβRIII is known to be a betaglycan.[11] TGFβs are first bound to TGFβRII and TGFβRIII.[12] It has been considered that TGFβRIII regulates access to TGFβRII,[12–14] and then TGFβ signal transduction in the cellular pathway is started through stimulation of TGFβRI by TGFβRII. After that, activated TGFβRI phosphorylates Smad2 or Smad3, which are receptor-regulated Smads (R-Smad) activated by TGFβ and activin.[15,16] After Smad4, which is a common mediator Smad (C-Smad), is connected to phosphorylated R-Smads, the complex is transported to the cell nucleus and influences the transcription activity of TGFβ-dependent genes.[15,16] c-myc, which is one of the

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TGFβ-dependent genes, is regarded as an oncogene and regulates cellular proliferation. In the present study, therefore, we investigated whether the gene expression levels of three TGFβ isomers (TGFβ1, TGFβ2, and TGFβ3) and their receptors (TGFβRI, TGFβRII, and TGFβRIII), Smad3 and c-myc were changed in hMSCs during in vitro culture.

Wnt-8B is related to cell self-renewal and tumorigenesis,[9] and Wnt proteins can act as stem cell growth factors.[17] Wnt signaling activates the genes that promote proliferation (c-myc and others) by accumulating β-catenin in some kinds of stem cells and cancer cells.[9] Nucleostemin is involved in proliferation in both stem cells and cancer cells.[18] Therefore we also investigated the gene expression levels of Wnt-8B and nucleostemin in hMSCs.

In addition to investigating the expression of these genes relating to cellular proliferation in hMSCs during in vitro culture, we compared them with those in two kinds of cancer cell lines, HeLa S3 (a human cervical cancer cell line) and HepG2 (a human hepatoma cell line).

Materials and methods

Cell culture. Human mesenchymal stem cells (hMSCs) derived from bone marrow were purchased from Cambrex Bio Science (Walkersville, MD, USA). Their donor was an African American woman aged 19 years. The cells that we obtained from Cambrex Bio Science were second-passage cells. The hMSCs were cultured in mesenchymal stem cell basal medium (MSCBM; Cambrex Bio Science) supplemented with mesenchymal cell growth supplement (MCGS; Cambrex Bio Science), L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C under a 5% CO_2 atmosphere. The cells were seeded at a density of 6000 cells/cm^2 and were subcultured when they were just subconfluent (approximately 90% confluent) up to the 10th passage, corresponding to the 12th passage from when the hMSCs were collected from the donor. The human cervical carcinoma cell line HeLa S3 (JCRB Cell Bank, Osaka, Japan) was cultured using Ham's F-12 culture medium (Dainippon Pharmaceutical, Osaka, Japan) containing 10% fetal bovine serum (FBS) (Intergen, Purchase, NY, USA) and 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). The human hepatoma cell line HepG2 (Riken Bioresource Center, Tsukuba, Japan) was cultured using minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 0.1 mM nonessential amino acids (NEAA) (Invitrogen), 10% FBS (Intergen), and 100 U/ml penicillin-streptomycin (Invitrogen).

Preparation of total RNA. Because the purchased hMSCs had been expanded in the manufacturing process as described above, we express the 1st passage of the hMSCs in this study as the 3rd from the primary culture. For quantitative real time-polymerase chain reaction (RT-PCR), total RNA was extracted from hMSC cultures during the 3rd, 5th, 7th, and 12th passages from the donor with Isogen (Nippon Gene, Toyama, Japan). Total RNA was also extracted from HeLa S3 and HepG2 cells once only with Isogen (Nippon Gene).

Quantitative RT-PCR. RNA was then reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Basel, Switzerland). Primers and annealing temperatures for the c-myc oncogene, nucleostemin, Wnt-8B, transforming growth factor (TGF)β3, and TGFβRIII are summarized in Table 1. Amplifications were carried out for 10 s at 95°C, for 15 s at each annealing temperature, and for 12 s at 72°C for 40 cycles. Amplifications of TGFβ1, TGFβ2, TGFβRI, TGFβRII, and Smad3, plus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, were performed using Light Cycler Primer Sets (Roche Diagnostics). PCR was performed in Light Cycler Fast Start DNA Master SYBR Green I (Roche Diagnostics) in a Roche Light Cycler (software version 4.0).

Statistical analysis. All results are shown as means ± SD. The significance of the differences in mean values was evaluated by Student's *t* test.

Table 1. Primers and annealing temperatures used for real-time PCR

Gene name	GenBank accession number	Primer orientation	Nucleotide sequence	Starting sequence position	Size for the PCR amplicon(bp)	Annealing temp. (°C)
c-myc	V00568	Forward	5'- GCG AAC ACA CAA CGT C -3'	1626	315	50
		Reverse	5'- CAA GTT CAT AGG TGA TTG CT -3'	1940		
nucleostemin	X91940	Forward	5'- CCA TTC GGG TTG GAG TAA -3'	782	284	50
		Reverse	5'- CTG TCG AGC ATC AGC C -3'	1065		
Wnt-8B	NM_014366	Forward	5'- AGT GAC AAT GTG GGC T -3'	331	244	60
		Reverse	5'- CGT GGT ACT TCT CCT TCA G -3'	574		
TGFβ3	NM_003239	Forward	5'- AAA CAC CGA GTC GGA A -3'	535	284	60
		Reverse	5'- TGC CAC CGA TAT AGC G -3'	818		
TGFβRIII	NM_003243	Forward	5'- TCC CTA TCC CGC AAG C -3'	2369	189	60
		Reverse	5'- AGA TTA TCG AGG CGT CC -3'	2557		

PCR, polymerase chain reaction; TGFβ3, transfroming growth factor β3; TGFβRIII, TGFβ recepter type III

Results

The proliferation rate of hMSCs decreased with the length of in vitro culture (Fig. 1). The effects of the in vitro culture term on hMSC proliferation and the mRNA expressions of three TGFβ isomers (TGFβ1, β2, β3) and their receptors type I, II, and III (TGFβRI, RII, RIII) in hMSCs were investigated (Fig. 2). The mRNA expressions of TGFβ1, TGFβ2, and TGFβRI increased with the length of cell culture (Fig. 2A,B,D), but there had been no change in the TGFβ3, TGFβRII, and TGFβRIII mRNA expressions by the 12th passage (at about 3 months) (Fig. 2C,E,F). In addition, the mRNA expression of Smad3, which is one of the R-Smads activated by TGFβ and activin, in hMSCs was investigated. The mRNA expression of Smad3 decreased in the 5th and 7th passages of hMSCs but increased in the 12th passage (Fig. 3). The mRNA expressions of c-myc in hMSCs were higher in the 5th and 7th passages than in the 3rd and 12th passages (Fig. 4A). The mRNA expressions of nucleostemin in hMSCs decreased with the length of cell culture (Fig. 4B).

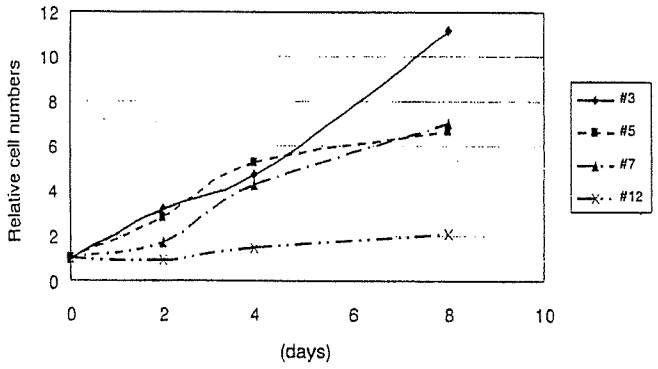


Fig. 1. Proliferation of human mesenchymal stem cells (hMSCs) in the 3rd, 5th, 7th, and 12th passages. hMSCs were seeded at 1.7×10^5 cells/F 60-mm dish (6000 cells/cm^2), and cells were counted after 2, 4, and 8 days. The initial cell number (0 days) is expressed as 1, and the other cell numbers (2, 4, and 8 days) are expressed relative to that of day 0. $n = 3$

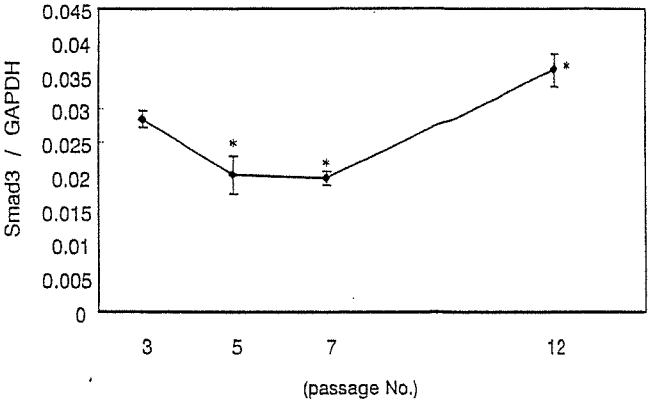
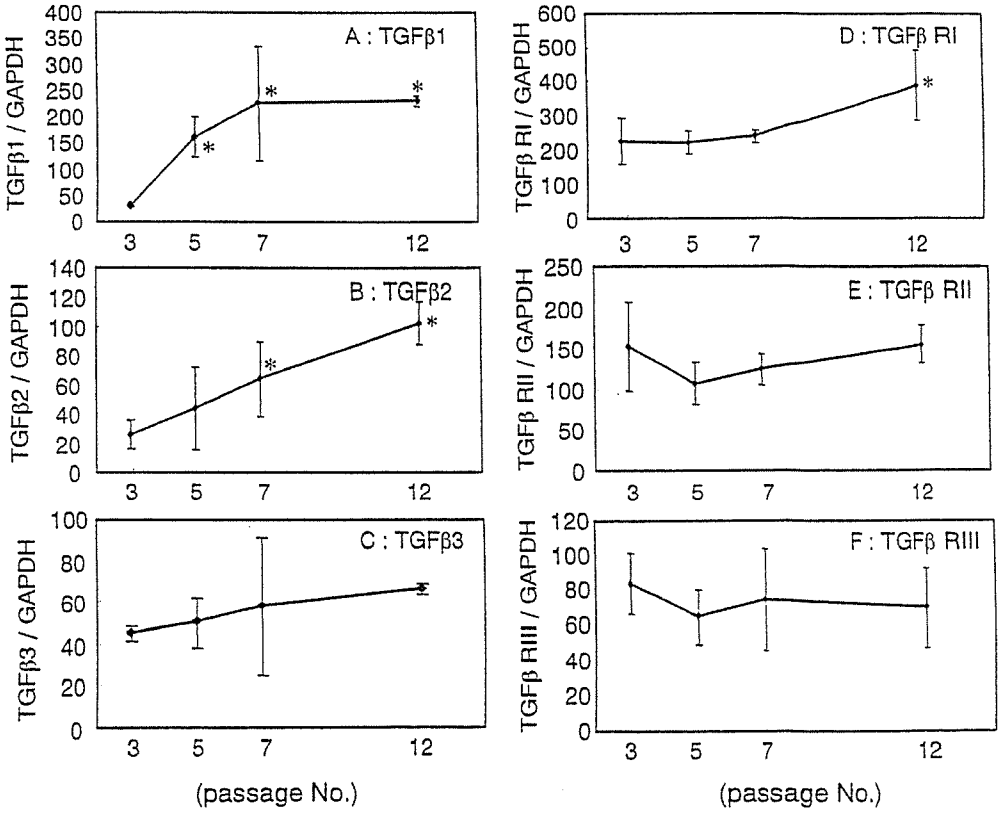


Fig. 3. Effect of in vitro culture length on mRNA expression of Smad3 in hMSCs. The expression of Smad3 relative to GAPDH in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages was investigated by quantitative RT-PCR. Mean values with SDs are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage (*$P < 0.05$)

Fig. 2. Effect of in vitro culture length on mRNA expressions of transforming growth factor β1 (*TGFβI*) (**A**), TGFβ2 (**B**), TGFβ3 (**C**), TGFβ receptor type I (*TGFβRI*) (**D**), TGFβRII (**E**), and TGFβRIII (**F**) in hMSCs. Expressions of the four genes, relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages were investigated by quantitative real time-polymerase chain reaction (RT-PCR). Mean values with SDs from three independent experiments are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage (*$P < 0.05$)



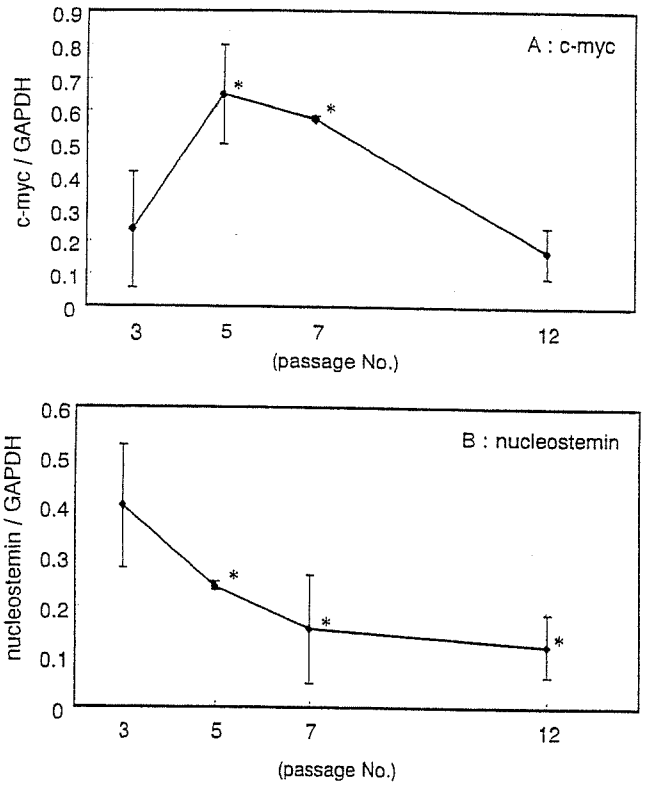


Fig. 4. Effect of in vitro culture length on the mRNA expressions of c-myc (**A**) and nucleostemin (**B**) in hMSCs. Expressions of the two genes relative to GAPDH in confluent cultures of hMSCs in the 3rd, 5th, 7th, and 12th passages were investigated by quantitative RT-PCR. Mean values with SDs from three independent experiments are presented. *Asterisks* denote statistically significant differences compared with the 3rd passage (*$P < 0.05$)

The mRNA expressions of TGFβs and TGFβ receptors in hMSCs of the fifth passage were compared with those of two kinds of cancer cells (HeLa S3 and HepG2) (Fig. 5). TGFβ1 mRNA levels in hMSCs and HepG2 cells were significantly higher than those in HeLa S3 cells (Fig. 5A). The mRNA expressions of TGFβ2, TGFβ3, TGFβRI, and TGFβRII in hMSCs were significantly higher than those in the cancer cells (HeLa S3 and HepG2) (Fig. 5B,C,D,E). TGFβRIII mRNA expression in hMSCs was significantly higher than that in HeLa S3, but lower than that in HepG2 (Fig. 5F). The expressions of several genes affecting cellular proliferation in all three cells were also investigated. The mRNA expressions of c-myc oncogene and nucleostemin in the cancer cells (HeLa S3 and HepG2) were significantly higher than those in hMSCs (Fig. 6A and B). Wnt-8B mRNA was expressed in the cancer cells (HeLa S3 and HepG2), but not in hMSCs (Fig. 6C). Wnt-8B mRNA was not expressed in any passage numbers of hMSCs (data not shown).

Discussion

In this study, we investigated the changes of gene expression profiles during in vitro culture of hMSCs to evaluate their safety for use in clinical applications and tissue-engineered medical devices. First, the time dependency of the growth speed of hMSCs derived from bone marrow up to the 12th passage (at about 3 months) was investigated. The proliferation rate of hMSCs decreased by degrees during 3 months of in vitro culture (Fig. 1). No marked changes of hMSC morphology in 3 months of in vitro culture were

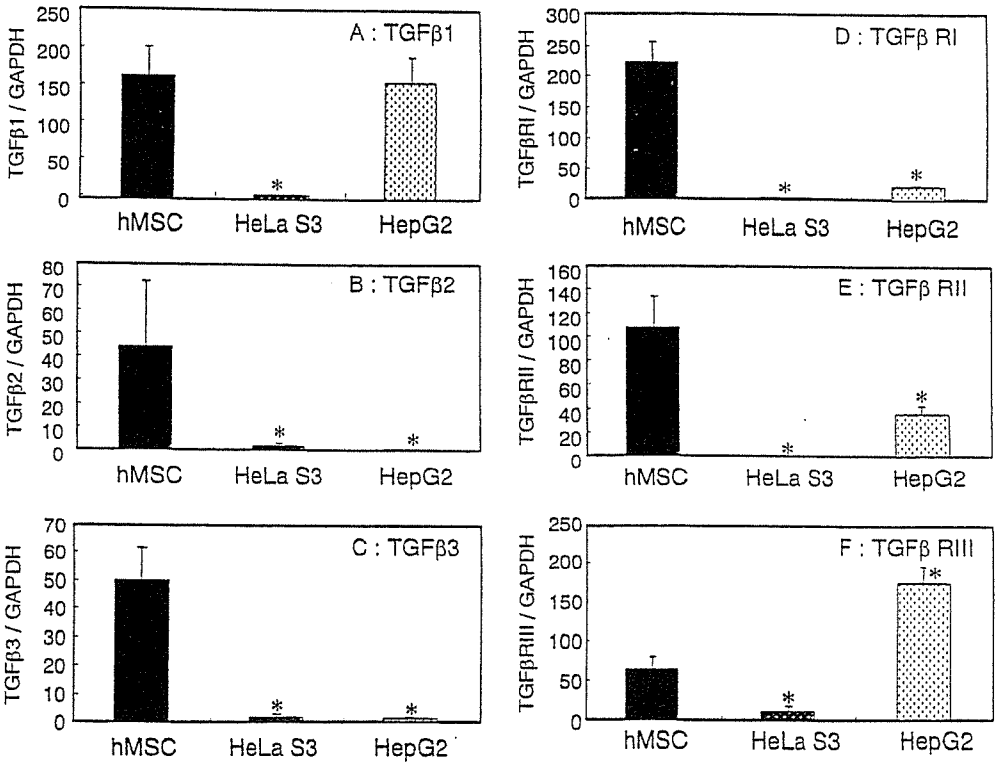


Fig. 5. mRNA expressions of TGFβ1 (**A**), TGFβ2 (**B**), TGFβ3 (**C**), TGFβRI (**D**), TGFβRII (**E**), and TGFβRIII (**F**) in hMSC, HeLa S3, and HepG2 cells. The expressions of the four genes relative to GAPDH in confluent cultures of hMSCs, HeLa S3, and HepG2 were investigated by quantitative RT-PCR. Mean values with SDs from three independent experiments are presented. *Asterisks* denote statistically significant differences from hMSCs (*$P < 0.05$)

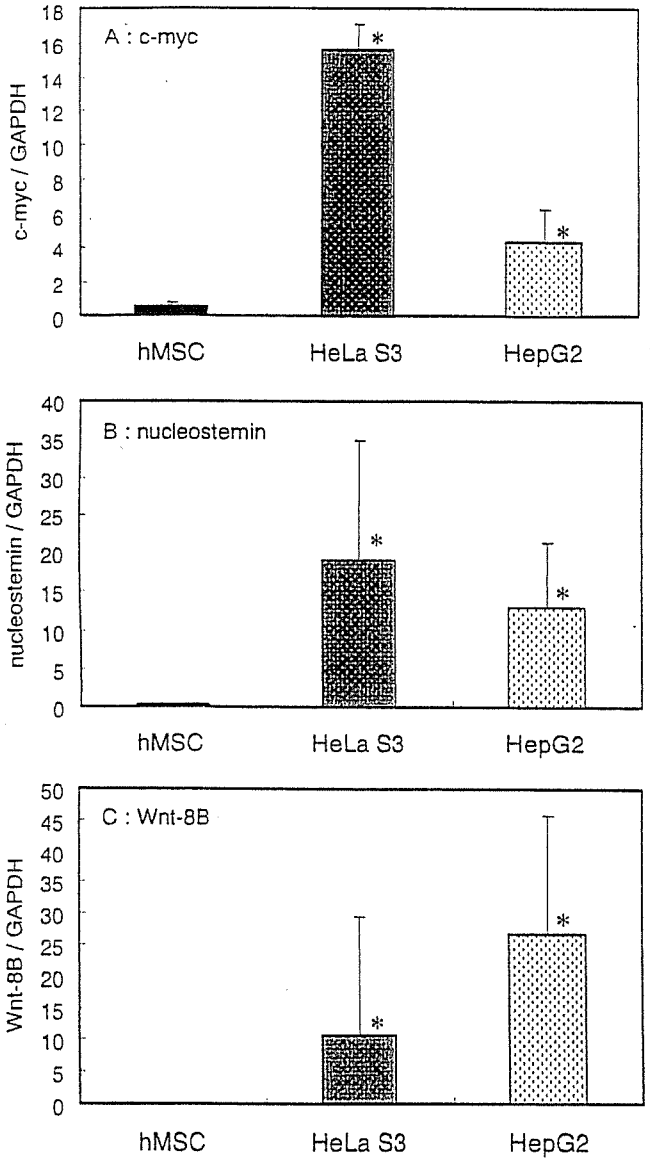


Fig. 6. mRNA expressions of c-myc (A), nucleostemin (B), and Wnt-8B (C) in hMSC, HeLa S3, and HepG2 cells. The expressions of the three genes relative to GAPDH in confluent cultures of hMSC, HeLa S3, and HepG2 cells were investigated by quantitative RT-PCR. Mean values with standard deviations from three independent experiments are presented. *Asterisks* denote statistically significant differences from hMSCs (*$P < 0.05$)

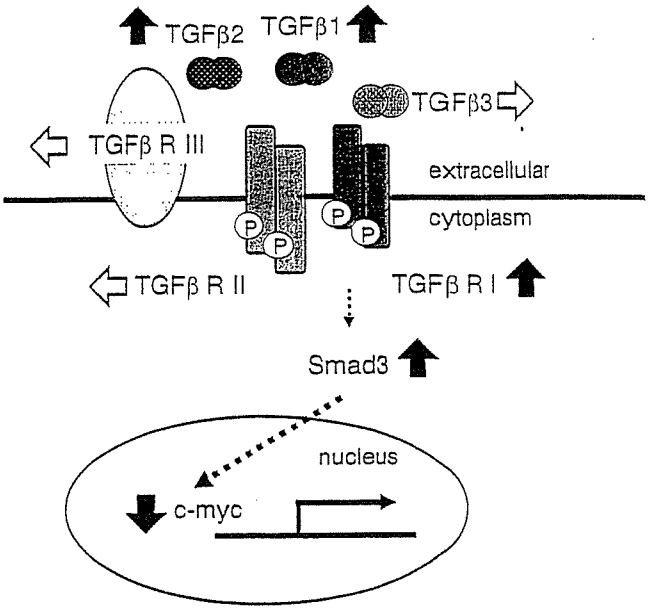


Fig. 7. Changes in the expressions of TGFβ signaling genes during hMSC in vitro culture for 3 months. The *dotted arrows* indicate the TGFβ signal pathway. *White arrows*, no changes; *black arrows*, up or down changes

observed. Several hMSCs derived from other donors' bone marrow did not undergo extraordinary proliferation either (data not shown). Adult stem cells have a self-renewal ability and undergo multilineage differentiation to maintain adult tissues.[9] In this study, however, hMSCs had more limited proliferative potential in in vitro culture. This phenomenon in hMSCs derived from bone marrow is the same result as that in hMSCs derived from adipose tissue reported by Rubio et al.[10] In addition, a decreasing cellular proliferation rate is often observed in several types of normal cells during in vitro culture. Consequently, these results suggest that hMSCs derived from bone marrow will seldom undergo spontaneous transformation during the 1–2 month period of in vitro culture necessary for use in clinical applications. But why does the proliferation of hMSCs decrease during in vitro culture? To focus on the proliferation mechanism of stem cells, we investigated whether the expressions of several genes related to cellular proliferation in hMSCs changed during in vitro culture. In the present study, we examined the expressions of TGFβs, their receptors, Smad3, c-myc, nucleostemin, and Wnt-8B. It has been proposed that the loss of TGFβRIII in renal cell carcinoma (RCC) is necessary for RCC carcinogenesis, and loss of TGFβRII leads to acquisition of the metastatic phenotype.[19] Therefore, the absence of changes in TGFβRII and TGFβRIII in hMSCs during in vitro culture might be important. The changes in mRNA expression levels during in vitro culture were different in each TGFβ isomer and receptor. TGFβ signal transduction in the cellular pathway is only possible through activation of TGFβRI. It was interesting that only TGFβRI mRNA expression increased with the length of cell culture among the three kinds of receptors (Fig. 2). The mRNA expressions of Smad3 increased (Fig. 3), but those of c-myc and nucleostemin decreased (Fig. 4) with the length of cell culture. We summarize the changes of TGFβ signaling gene expression during in vitro culture of hMSCs for 3 months in Fig. 7. TGFβ inhibits the growth of the many kinds of epithelial cells and hematopoietic, lymphoid, and endothelial cells.[20–23] In hMSCs as well as in the above-mentioned cells, hMSC growth might be controlled by TGFβ family signaling. As shown in Fig. 7, we hypothesized that the expressions of TGFβ1 and TGFβ2 in hMSCs increased during the period of in vitro culture, and then activated TGFβRI repressed the transcription of c-myc through Smad3; consequently, the cell cycle and cell growth might be arrested in hMSCs.

In addition, we compared the gene expression profiles of hMSCs with two kinds of cancer cell lines. One was HeLa S3 (a human cervical cancer cell line), which is markedly transformed, and the other was HepG2 (a human hepatoma cell line), which retains some hepatic functions. The mRNA expressions of TGFβs and their receptors in hMSCs were significantly higher than in the two types of cancer cells (HeLa S3 and HepG2) (Fig. 5). On the other hand, the mRNA expressions of c-myc and nucleostemin of the stem cells (hMSCs) were significantly lower than those of the two types of cancer cells (Fig. 6). Wnt signaling promotes self-renewal of hematopoietic, intestinal epithelial, and keratinocyte stem cells, among others;[9] however, Wnt-8B was not expressed in hMSCs derived from bone marrow (Fig. 6). These results suggest that expression of the genes that inhibit cellular proliferation and tumorigenesis were significantly higher and the genes that promote these processes were lower in hMSCs than in the cancer cells. Thus, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of cancer cells.

Conclusion

In the present study, we confirmed that spontaneous transformation seldom occurred in hMSCs derived from bone marrow during 1–2 months of in vitro culture for use in clinical applications. In hMSCs, as in epithelial cells, growth might be controlled by TGFβ family signaling. During the period of in vitro culture of hMSCs, the expressions of TGFβ1 and TGFβ2 increased, and then activated TGFβRI repressed the transcription of c-myc through Smad3; consequently, the cell cycle and cell growth might have been arrested in hMSCs. In addition, the expression profiles of the genes that regulate cellular proliferation in hMSCs were significantly different from those of the cancer cells.

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Novel Calcium Phosphate Ceramics : The Remarkable Promoting Action on the Differentiation of the Normal Human Osteoblasts

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Keywords: Hydroxyapatite, Niobium ion, Osteoblast, Alkaline phosphatase activity

Abstract.
To promote the activity of normal human osteoblasts (NHOst), the novel HAp ceramics containing Nb ions (NbHAp) were synthesized by wet chemical process, which reacting aqueous solution containing a mixture of $Ca(NO_3)_2$, $(NH_4)_2HPO_4$, and the Nb aqueous solution. X-ray diffraction patterns indicated that NbHAp had a monolithic apatitic structure, although crystallite decreased as Nb content increased. From inductively coupled plasma analysis, maximum amount of Nb ions in the sample was almost 8.2atom% of P ions. The NbHAps were presented as aggregates and composed of fine crystal of <1μm in diameter. Nb ions in NbHAp were uniformly distributed in the aggregates. Furthermore, high-resolution XPS spectra of Nb $3d_{5/2}$ indicated that Nb ions in the HAp were presented as Nb^{5+}. These results suggested that Nb ions were at PO_4 site in crystal structure of HAp. When NHOst were cultured with the NbHAp, their ALP activity were twice as much as that of NHOst cultured with HAp without Nb ions.

Introduction

Tissue engineering takes advantages of the combined use of cultured living cells and scaffolds to deliver vital cells to the damaged site of the patient. Some tissue engineering approaches have been devised to repair large bone defect. In developing of the scaffold for bone tissue, the interaction between osteoblasts cells and scaffolds are much important. To achieve the restoration the bone tissue at early stage, the scaffold is required to have the ability of promoting proliferation and mineralization.

It is well known that hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) ceramics can be biologically bonded to natural bones and have been studied to utilize as the scaffolds. In addition, the structure is very tolerant of ionic substitutions and Ca^{2+} ions, PO_4^{3-} ions and OH^- ions can be replaced by various cationic or anionic ions, partly or completely[1]. For example, K^+, Mg^{2+} and Sb^{3+}, can substituted for Ca ions and CO_3^{2-} and VO_4^{3-} can substituted for PO_4^{3-} ions, completely or partially. Thus various kinds of ion substitutions can be made to synthesize novel modified-HAps.

Recently, our co-workers reported that niobium (Nb) ions have the significant effect which promotes the proliferation and differentiation of normal human osteoblastic cells (NHOst)[2]. In the present study, therefore, we attempted to synthesize the novel HAp ceramics containing Nb ions (NbHAp) to promote the activity of NHOst and investigated the interaction between NbHAp and NHOst.

Materials and Methods

Synthesis of Nb containing HAp

The NbHAp was synthesized by wet chemical process, which reacting aqueous solution containing a mixture of $Ca(NO_3)_2$, $(NH_4)_2HPO_4$, and the Nb aqueous solution. The reagent grade $Ca(NO_3)_2$, $(NH_4)_2HPO_4$ and $NbCl_5$ (Wako Pure Chemical Industries, Ltd) were used without purification. The metal ion chemical reagent was completely dissolved in an exact amount of distilled water. The Nb aqueous solution was prepared by the mixing of distilled water and $NbCl_5$ solution, which dissolved in 5vol%-hydroxyaceton and 5vol%-2-aminoethanol[3].

aggregates. High-resolution XPS spectrum of Nb $3d_{5/2}$ of NbHAp-II annealed at 800°C is shown in Fig.3. The peak of XPS spectra due to $3d_{5/2}$ of Nb ions from annealed NbHAp-II is at 208.3eV. Since XPS peak of $3d_{5/2}$ due to Nb^{2+} from NbO and Nb^{5+} from Nb_2O_5 appears at 203.5eV and 207.2eV, respectively, the Nb ions in NbHAp can be identified as Nb^{5+}.

These results suggest that the NbHAp has apatitic structure containing Nb ions and the Nb ions are homogenously distributed in the grain. Generally, Nb^{5+} ions in the solution is not present as Nb^{5+} but as niobiumate acid, $H_xNb_6O_{19}^{(8-x)-}$ ions (X=0,1,2)[4]. The PO_4 in HAp can be replaced by anionic atomic group, e.g. CO_3^{2-}, VO_4^{3-} and AsO_4^{3-}. Therefore, it is probable that Nb ions are substituted in PO_4 site in HAp. However, measured Nb/(Nb+P) molar ratio in NbHAp-II was 0.082, despite their theoretical Nb/(Nb+P) ratio of 0.1667, suggesting that the value of the measured ratio might be the maximum amount of Nb ions in PO_4, practically.

Since Nb ions are expected to have an effect to promote the proliferation and ALP activity of osteoblastic cells, the NbHAps have a potential to promote the ALP activity of osteoblastic cells.

Figure 4 shows ALP activity of NHOst cultured with annealed NbHAps. As shown in Fig.4, NHOst cultured with the NbHAp expressed the ALP activities twice as much as that of NHOst cultured with HAp without Nb ions. It is well known that ALP is often expressed when fracture of bone is repaired *in vivo*. Furthermore, from the recent study, it has revealed that the ALP contributed to mineralization in bone formation[5]. Therefore, this enhancement in ALP activity of NHOst by NbHAp suggests that the NbHAp can promote the mineralization of bone formation.

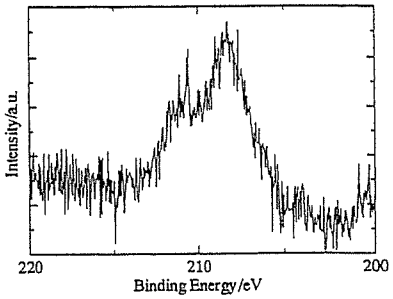

Fig.3. High-resolution XPS spectrum of Nb $3d_{5/2}$ of NbHAp-II annealed at 800°C.

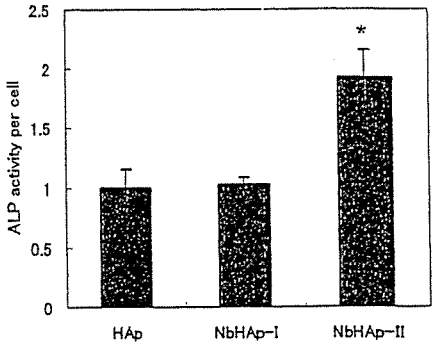

Fig.4. ALP activity of NHOst cultured with annealed NbHAp
*p<0.05 against HAp (without Nb ions)

Conclusion

We have succeeded to synthesize novel HAp containing Nb ions. The NbHAp would be a solid solution, which Nb ions were in PO_4 site in HAp and could enhance the ALP activity in NHOst.

Acknowledgment

This study was supported in part by a Grant-in-Aid for Scientific Research on Advanced Medical Technology from Ministry of Labour, Health and Welfare, Japan and a Grant-in-Aid from Japan Human Sciences Foundations.

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0.2M-$(NH_4)_2HPO_4$ and 0.01M $NbCl_5$ solutions were mixed and stirred with a magnetic bar. The Nb/(Nb+P) molar ratio of the mixing solution was set to 0.0000, 0.0167 and 0.1667. The pH of the mixing solution was adjusted to 10 using 1N-NaOH. 0.2M-$Ca(NO_3)_2$ was slowly dropped in the mixing solution (20ml/min) The ionic content of those starting solutions are shown in table 1. The pH was monitored and the reaction was terminated at pH 10.0. After the reaction, the suspension was stirred for 24h at room temperature. The precipitates were centrifuged at 3000rpm for 5min and washed with distilled water. The obtained apatites were annealed at 800°C for 2h (heating rate: 5°C/min). In this study, those precipitates obtained by reaction of $Ca(NO_3)_2$ solution and the mixing solution with different Nb/(Nb+P) molar ratio of 0.000, 0.0167 and 0.1667 are named HAp , NbHAp-I and NbHAp-II, respectively.

Characterization of NbHAp

The NbHAps were characterized by X-ray diffraction analysis (XRD, Rigaku, Rint2000). Ca, P and Nb ions concentrations in apatites are measured by inductively coupled plasma (ICP, Hewlett-Packard, HP4500). Microstructural evaluation was performed by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) (JEOL, LV5800). The chemical state of Nb ions in HAp was investigated by X-ray photon spectroscopy (XPS, Shimadzu, ESCA-3200).

Osteogenesis evaluation of NHOst cultured with NbHAp

NHOst were purchased from BioWhittaker Inc.(Walkersville,MD). The NHOst were maintained in alpha minimum essential medium (αMEM, Gibco, Grand Island, NY) containing 10%-FCS in incubators at 37°C in a humidified atmosphere with 5% CO_2. All assays were performed using αMEM containing 10%-FCS supplemented with 10mM beta-glycerophosphate. NHOst cells (4×10^4 cells/well/ml) were co-cultured with 5mg of the apatites for 7days to evaluated the effects of the apatites on NHOst.

Proliferation of NHOst cells cultured with the apatites was estimated by Tetracolor One assay (Seikagaku Co., Ltd. Tokyo, Japan), which incorporates an oxidation reduction indicator based on detection of metabolic activity. After 7-days incubation, 2%-TetraColor One/αMEM solution was added to each well, followed by 2h incubation. The absorbance of the supernatant at 450nm was estimated using μQuant spectrophotometer (Bio-tek Instrument, Inc., Winooski, VT). After estimating the proliferation, the cells were washed by phosphate-buffered saline (PBS(-)), followed by addition of 1ml of 0.1M glycine buffer (pH=10.5) containing 10mM $MgCl_2$, 0.1mM $ZnCl_2$ and 4mM p-nitrophenylphosphate sodium salt. After incubating at room temperature for 5min, the absorbance at 405 nm was detected using the μQuant spectrophotometer to evaluated alkaline phosphatase (ALP) activity of the test cells.

Results and Discussion

XRD patterns of NbHAps prepared by wet chemical process are shown in Fig.1(a). Irrespective of Nb/(Nb+P) molar ratio in starting solution, the precipitates were identified as monolithic HAp.

Table1. The ionic content of starting solution and the composition of the obtained precipitates.

Samples	Ionic content of Starting Solution*,			Theoretical Ca/(Nb+P)**	Nb/(Nb+P)**		Color of Precipitates
	Ca	PO_4	Nb		Theoretical	Measured***	
HAp	60.0	36.0	0.0	1.67	0.0000	-	White
NbHAp-I	60.0	35.4	0.6	1.67	0.0167	0.015	Pale yellow
NbHAp-II	60.0	30.0	6.0	1.67	0.1667	0.082	Buff yellow

*mmol, **Molar ratio, ***The precipitates were dissolved with HCl and the ionic concentration of HCl solutions were measured by ICP.

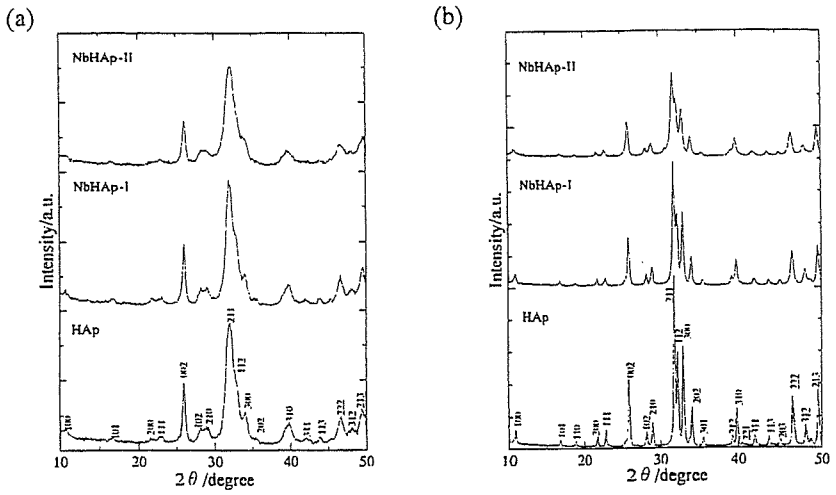

Fig.1. XRD patterns of HAp and NbHAp-I and NbHAp-II before (a) and after (b) annealing(800°C, 2h).

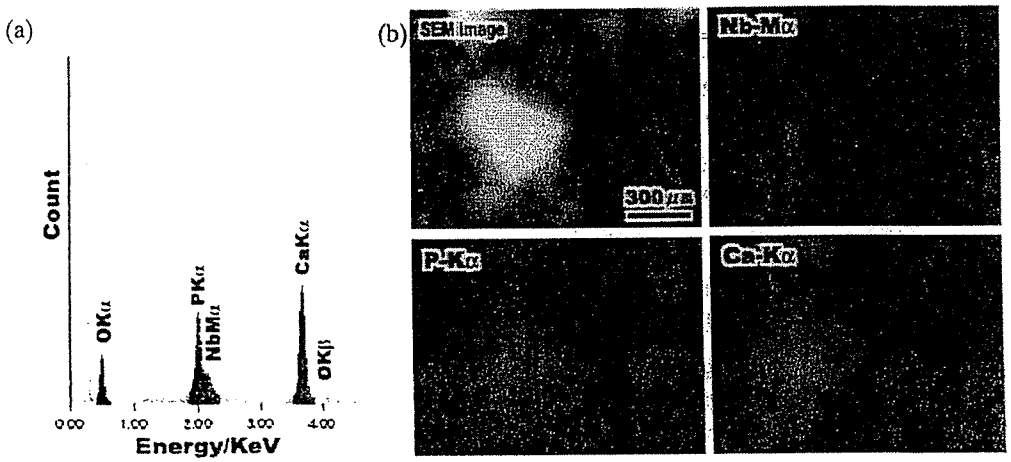

Fig.2. SEM-EDX analysis of NbHAp-II. ((a) An EDX spectrum and (b) SEM image and element mapping images of Nb, Ca and P).

As shown in Table1, the Nb/(Nb+P) molar ratio of NbHAp-I and NbHAp-II were 0.015 and 0.082, respectively. SEM observation revealed that the precipitates were present as aggregates composed of primary particles of less than 1μm in diameter.

XRD patterns of NbHAps annealed at 800°C are shown in Fig.1(b). The crystalline of the precipitates become high by the annealing and XRD patterns of all annealed NbHAps could be identified as monolithic apatitic structure. It is noted that the crystallite of the NbHAp decreased as Nb content increased. Figure 2(a) shows an EDX spectra of the whole region of SEM image in Fig.2(b). The EDX spectrum from Nb M_α was separated from P K_α line and could be observed at 2.17KeV, although the intensity of the spectra was weak. The mapping image of Nb, Ca and P ions are shown in Fig.2(b). As shown in Fig.2(b), Nb ions were present at the same site of Ca and P ions. Based on these observations, Nb ions are suggested to be uniformly distributed in the

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Cytotoxicity of Various Calcium Phosphate Ceramics

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Keywords: Calcium phosphate ceramics, Cytotoxicity,

Abstract. The cytotoxicity of five calcium phosphate ceramics, hydroxyapatite (HAp), flouroapatite (FAp), α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP) and tetracalcium phosphate (TTCP), was investigated. Based on the guidelines of biological test for medical devices in Japan, a cytotoxicity test of these calcium phosphates was carried out using Chinese hamster V79 lung fibroblasts. The cytotoxic study revealed that FAp and α-TCP showed high cytotoxicities. From various analyses, it was considered that the cytotoxicity of the FAp was due to fluorine ions extracted in a culture medium and the cytotoxicity of α-TCP resulted from a decrease in pH of the medium by the phosphoric acid, which produced by hydrolysis of the α-TCP.

Introduction

From the view point of biological affinity to bone, calcium phosphate (CP) ceramics have been studied to utilize for many purposes in a medical field. For instance, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) and β-tricalcium phosphate (β-$Ca_3(PO_4)_2$, ß-TCP), are known to be biologically bonded to natural bones and their porous materials have been studied for effective restoration of bone defects.[1,2] Fluoroapatite ($Ca_{10}(PO_4)_6F_2$, FAp) has been reported to have a potential of novel bone repairing materials with high stability *in vivo*, since solubility of FAp is lower than that of HAp.[3,4] In addition, CP cement is also promising for bone repair and it is well known that α-tricalcium phosphate (α-$Ca_3(PO_4)_2$, α-TCP) and tetracalcium phosphate ($Ca_4(PO_4)_2O$, TTCP) are starting materials for the harden reaction of the bone cement.[5,6]

To develop biomaterials for utilizing for bone tissue, various properties, e.g. biological, physical and chemical property, should be satisfied. Among them, biological safety is important for the biomaterials. Since only a few studies which discuss the cytotoxicity of calcium phosphate ceramics have been reported, the cytotoxicity of CP ceramics is worthy to be investigated in order to design bioceramics with good biological safety for medical application. Therefore, the cytotoxicities of five calcium phosphate ceramics, hydroxyapatite (HAp), flouroapatite (FAp), α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP) and tetracalcium phosphate (TTCP) were investigated.

Materials and Methods

Materials

Five kinds of CP ceramics, HAp, FAp, α-TCP, β-TCP and TTCP were purchased from Wako chem. Co. Ltd. CP powders (0.25 g) was put into stainless mold and uniaxially pressed at 30MPa for 1 min to form a pellet. The dimensions of the obtained CP pellet were 1mm in thickness and 12mm in diameter. CP pellets were sterilized by an autoclave at 121°C for 20 min.

Cytotoxicty test on CP ceramics

Cytotoxicity test was carried out using Chinese hamster V79 lung fibroblasts by a colony assay system. V79 cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co. Ltd.) with 10% fetal calf serum (FCS, Intergen Co. Ltd.) and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

The method of cell seeding in the cytotoxicity test of CP ceramics was shown below; each CP pellets were placed in each culture wells of 24 well culture plates (Corning Co. Ltd.) and 300μl of culture medium was added into each well. Then, 50 cells/300μl of the cell suspension in the

that calcium phosphates convert to HAp in aqueous solution with high pH value. Since the solubility of α-TCP is higher than that of other calcium phosphates, α-TCP rapidly converts to HAp as follows;

$$10Ca_3(PO_4)_2+6H_2O \rightarrow 3Ca_{10}(PO_4)_6(OH)_2+2H_3PO_4$$

According to the report of this conversion [7], HAp produced by the above reaction has whisker-like morphology. Therefore, the whisker-like precipitates in Fig.3 (d) can be regarded as HAp, so that it is considered that the above conversion occurs at the surface of the α-TCP during incubation.

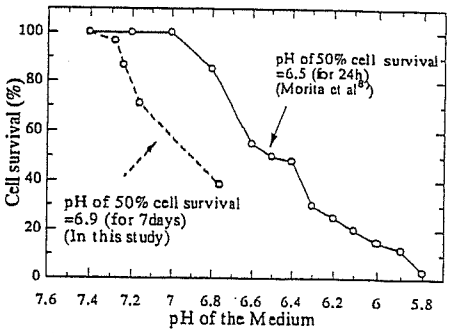

Fig.4. V79 cell survival in the medium with various pH values.

In this case, phosphoric acid is produced as a byproduct in the conversion reaction and the phosphoric acid causes the decrease in pH of the solution. As shown in Fig.4, Morita and co-workers[8] have reported that low pH itself could be clastogenic to mammalian cells and the pH of 50% V79 cell survival was 6.5 for 24h incubation. In the present colony assay system, the pH of 50% V79 cell survival was 6.9 for 7-days incubation. In addition, we confirmed that phosphoric acid showed no or weak cytotoxicities under our present experimental conditions. Therefore, it is suggested that the cytotoxicity of α-TCP is mainly due to the pH decrease resulting from an increase of the phosphoric acid ion by the hydrolysis conversion from α-TCP to HAp.

On the other hand, FAp has the same crystal structure of HAp but the hydroxyl ions in HAp substituted by fluorine ions. Since it is probable that difference of the colony formation on various CP ceramics are due to eluted substances from CP as described above, the cytotoxicity of FAp would be due to eluted fluoride ions from FAp. In conclusion, this study has revealed that FAp and α-TCP have a cytotoxicity, while TTCP has lower cytotoxicity than other calcium phosphates. To develop biomaterials made from calcium phosphate, further studies are necessary to clarify their cytotoxic mechanisms.

Acknowledgment

This study was supported in part by a Grant-in-Aid for Scientific Research on Advanced Medical Technology from Ministry of Labour, Health and Welfare, Japan and a Grant-in-Aid from Japan Human Sciences Foundations.

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The fact that less formation of colonies was observed on FAp and α-TCP pellets suggests that they are highly cytotoxic. In addition to results shown in Fig.2, it is suggested that the differences in the colony formation ratio on various CP pellets are ascribed to difference in extract properties from the CP, which may be related with the composition or crystal structure. As shown in Table 1, the pH of culture medium after incubation with FAp pellets is almost the same as that of HAp, while the pH of the α-TCP-incubated medium is much lower than that of the other CP ceramics-incubated media.
In order to consider the reason of the low pH of the a-TCP-incubated medium, a surface structural change of α-TCP before and after incubation was analyzed by SEM. SEM images of α-TCP before and after extraction treatment are shown in Fig.3. Before extraction, a particle size of α-TCP was about 10μm and its surface was smooth (Fig.3(a) and (b)). However, whisker-like precipitates of 1-2μm in length and 2-300nm in width were observed at the surface of α-TCP after the extraction, although there was no change in its particle size (Fig.3(c) and (d)). It is well known

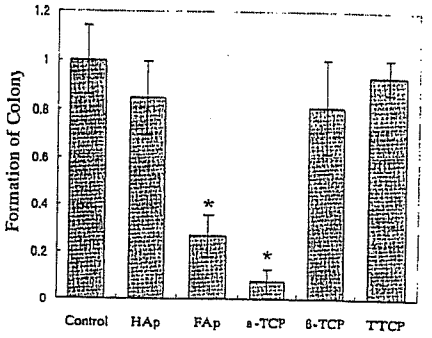

Fig.2. Formation of colony cultured in extract from various CP ceramics. (*p<0.01 against for V79 alone)

Table 1. The pH and Ca concentration of culture medium after incubation.

Samples	pH of medium after culturing	Ca concentration /ppm
V79 alone	7.12	-
HAp	7.24	0.19
FAp	7.20	0.17
α-TCP	6.76	72.62
β-TCP	7.40	1.27
TTCP	7.65	0.58

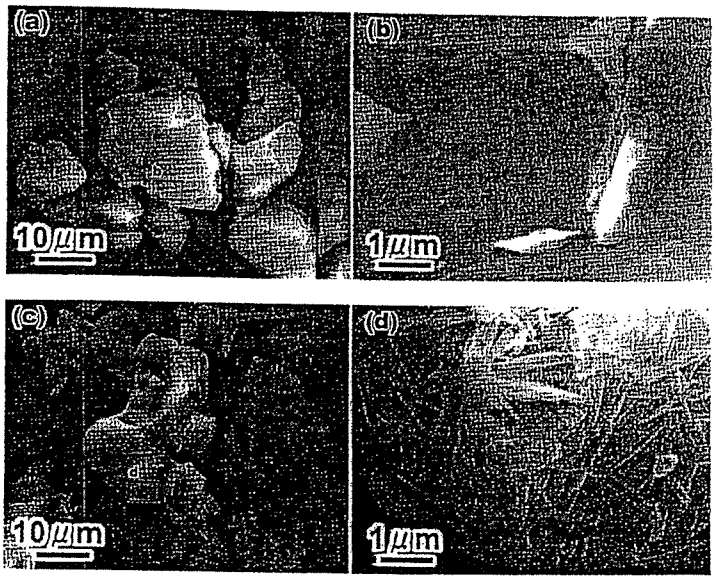

Fig.3. SEM images of α-TCP before (a), (b) and after extract treatment (c), (d). (a) and (c) are whole image of before and after extract treatment, respectively. (b) and (d) are enlarged image of the area enclosed by a rectangle in (a) and (c), respectively.

culture medium were added into each well and incubated at 37°C for 4 h. Finally, 400μl of the culture medium was added into each well and the plates were incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 7days. In order to investigate a cell adhesive property on the CP ceramics, the culture medium was changed after 4 h and further incubated for 7days. The removed culture medium was transferred to another well of a new plate and incubated for 7days as well.

Cytotoxicity of extracts from CP ceramics was also investigated in this study. Suspensions of CP ceramics in the culture medium (100mg/mL) were stirred at 37°C for 3days under the rotation condition at 150rpm. The suspensions were centrifuged and the supernatants were collected as test extracts. In addition, media with various pH values were prepared using HCl solution to investigate an effect of pH on cell survival. Fifty V79 cells in 1ml of the extracts or the medium with different pH value were incubated at 37 °C for 7days.

After 7-day incubation, the cells were fixed in methanol and the number of the V79 colonies was counted after staining cells with 5%-Giemsa solution to estimate the cytotoxicity of the test sample. In addition, the pH of the medium after 7-days culture was measured to estimate the effect of the pH of the medium on the cytotoxicity test.

Characterization of CP ceramics

The structural changes of CP before and after an autoclave-sterilization or an incubation at 37°C culture were investigated by powder X-ray diffraction (XRD) analysis and scanning electron microscopy (SEM). XRD analysis was carried out (Rigaku Co., Ltd. / RINT 2000) with the CuK_α radiation at 40kV, 50mA. SEM observations were performed (JEOL / JSM-5800LV) with an accelerating voltage of 25kV.

Results and Discussion

Cytotoxicity of various CP ceramics

From XRD analysis, no structural changes of CPs were observed after an autoclave sterilization. After staining CP pellets, it was observed that cell colonies were formed on various CP ceramics pellets (Fig.1(a)). The results of the cytotoxicity test of CPs are shown in Fig.1(b). The cell colonies were hardly formed on FAp and α-TCP pellets and the ratios of the colonies formed on these pellets against V79-alone culture were 22.6% and 0.0%, respectively. In addition, the ratios of the colonies on the HAp, β-TCP and TTCP pellets were 58.1%, 57.3% and 78.4%, respectively. As no colonies were observed after 7-day culture of the removed medium in cell adhesion studies of CP ceramics, these results suggested that V79 cells can adhere and be viable on these pellets, irrespective of the type of CP ceramics. Figure 2 shows the formation of colonies cultured in extract from CP ceramics. The cytotoxicity test of extracts from CPs revealed that the tendency of their cytotoxicities was similar to that of the cytotoxicities on the respective CP pellets themselves (Fig.1(b)).

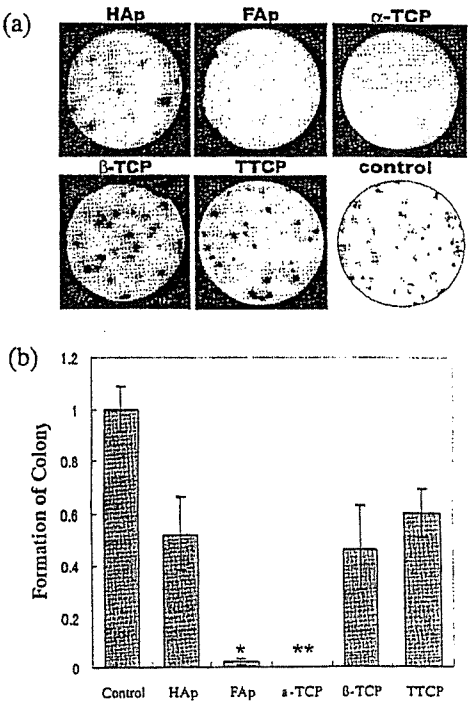

Fig.1. The appearance of colonies on various CP pellets (a) and their colony formation ratios (b). (*p<0.05 against for V79 alone, **p<0.01 against for V79 alone)

座談会

バイオマテリアルと未来社会

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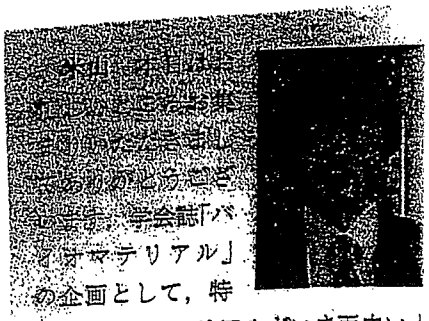
（発言順）

この座談会は2005年10月4日(火)東京医科歯科大学生体材料工学研究所にて収録致しました．



米山　本日は，「バイオマテリアルがいま面白い」ということで，学会誌「バイオマテリアル」の企画として，特集「バイオマテリアルがいま面白い」を編みました．特に未来に着目して，若手の研究者あるいは他分野の先生方の参考になるような忌憚のないご意見をお聞かせいただければと思います．

注目のバイオマテリアル技術

米山　まずは，現在この領域で研究されている，あるいは実際の応用を進めている先生方が，いま注目されているバイオマテリアルの技術あるいは研究内容，テーマというのはどのようなものでしょうか．

岡野　ウイルスも含めて，いままである生物がわれわれの体内に入ってきたときには，古くから免疫系が関与することが知られ，その研究が非常に進みました．しかし，バイオマテリアルという新しい人工材料が体内に入ったらどんなことが起きるのかということは，まさにここ30～40年の間に一気に進んだ研究分野です．われわれの体のなかに人工物を使って治療することが多数出てきていますし，診断，治療が大変な勢いで進んでいます．そういう局面がますます増えてくるなかで，本当にバイオマテリアルの研究あるいはバイオマテリアルが重要となる時代になったと思います．ある材料を使ってなにかをやるという時代から，界面で起こる問題を予知し，それを制御して材料を設計して使っていくという時代に突入してきています．そのなかでバイオマテリアルの役割の重要さがますます大きくなってきております．

この日本バイオマテリアル学会はそういったことにチャレンジする研究者の集団で，つぎの時代の新しい局面をつくっていくと私は信じています．いま注目される技術や研究でどんなことがあるかという具体的な問題について，私が注目しているタイプのものをお話します．

一つは，血管ステントです．狭窄していた血管のなかに持っていってぱっと開くものは，やはり再狭窄の問題が起きてきます．その表面から薬を徐放させることで，圧倒的に再狭窄の少ないステントができるようになってきました．すると，マテリアルと薬と界面の問題をどこまで制御できるのかというテクノロジーの重要さをこの例が示していて，このデバイス治療はどんどん売り上げも上がっています．そういう点では，薬の放出を制御するというDDSテクノロジーとバイオマテリアルというのは切っても切れません．武田薬品社のリューブリンはバイオマテリアルとDDS設計の成功例です．バイオデグラダブルな高分子の200 μのビーズのなかに，ペプタイドLH-RHのアナログ，リュープロライドを封入し，徐放させることによってがんの縮小，特に前立腺のがん治療に使われるような製剤が開発されて，1商品で1,200億円もの売上げをするヒット商品になっているのです．バイオマテリアルと薬が完全に一体化した新しいシステムとして使われているので，こういう使い方がやはりバイオマテリアルの一つの重要なポイントだと思います．

さらに，薬の点でいうと，最近，東大の片岡一則先生や，いまは神奈川科学技術アカデミーに移った横山昌幸先生と一緒に，ポリエチレングリコールと疎水性連鎖でブロック共重合体を利用すると高分子のミセルで人工のウイルスのような小さな粒子をつくることができることを示しました．そのなかに抗がん剤を入れることで，がんのところへ集積できるタキソールのミセル製剤というものが，いまphase Ⅰがようやく終わるところまで研究が進んでいまして，かなりがん治療に効果があるとのことが臨床的にわかりつつあります．

薬と材料，バイオマテリアルを合わせていって，いままでの薬でもデバイスでもないような，新しいタイプの新しいフィールドがどんどん切り拓かれてこようとしています．私は，埋め込み型のデバイスあるいはDDSのフィールドにバイオマテリアルは必須だと思っています．

明石　いま岡野先生がご指摘になった，バイオマテリアルの分野でどういうところがポイントで，なにを目指していくかということについてはまったく同感です．たんなるデバイスだけではやはり面白みもないし，展開もできないだろうし，将来性もないし，それこそ産業基盤をつくるようなことにもならない．といって，創薬の分野とは違うと思う．そのあたりにポイントを絞って開発していくと，企業がよりさまざまなことができるでしょうし，われわれ研究者もやっていける気がします．たんなる組み合わせだけのデバイスではなく，といって合成化学の粋を駆使するような創薬分野ではないようなところに，新しい，いまも進んでいると思いますが，バイオマテリアルの充分に将来展望可能な領域がはっきりみえてきたような気がしています．

それが，岡野先生がご指摘になったような高分子ミセル系の話でしょうし，私も今日CRESTのヒアリングを受けてきたところですが，実際に樹状細胞のなかに組み立てた純合成ではないですが，工夫して組み立てたような免疫賦活剤を開発して送り込むようなところは，けっして製薬メーカーからは出ないような，マテリアルをやっている人でないと絶対出てこないような発想がどうもあるようです．いま，岡野先生の話を伺っていて，同じようなことを感じながら研究をされていると思いました．

米山　材料単独ではなくさらにそれにつけ加える機能，特にドラッグデリバリーという方面で最近大きく展開している点と，岡野先生がはじめのほうでご指摘になった材料と界面のところがよくわかってきたという点，マテリアルの複合化に関して二つの話が出てまいりました．

土屋　複合化という点で，DES（ドラッグエリューティングステント）が医療機器として，厚生労働省では医療材料部会で審議され，承認されています．ですからこれからは医療材料の分野のなかでかなり複合化されたものが評価される時代になる．というのは，医療機器・医療材料をよく知っている人でないと，薬が組み込まれたものの評価もなかなか難しいということが，先行きみえてくるのです．

実際，骨や血管といった物理的な補強，そういうものを埋め込む従来型の人工物や人工血管といわれているものを使用される患者さんの骨や血管は，通常病的な状態となっており，薬を飲みながら治療をしているのです．薬というものは，それを治療すべき部位に持っていくまでに多くのテクノロジーが要ります．薬の場合，一般的に効き目が遠い代わりに副作用も強い．そういった場合に，直接治療すべき部位にその薬を局在させる，まさしくデバイスが必要な部位に薬を同時に存在させることは，経口あるいは静注による全身への副作用を低減化させることが可能となる．巧みなドラッグリリースのシステム設計が材料屋の技術レベルとして求められるところです．このようなタイプの医療機器は，治療器としての効率的な治療効果からも，これからはまさしく医療側の医師，それから患者も望む医療機器であると思います．大量の薬を飲まなくても治療すべき部位に薬が存在し，不具合を低減化でき，かつ有効性が高い治療法となる．再生医療の分野でも効果が早く，よく効き目のあるものがこれからはどんどんつくられていくと思います．

再生医療の場合，ただスキャフォールドがあって，細胞を組み込んで*in vitro*で培養物ができればそれでよいというものではありません．材料そのものがよくなければ，いくらよい細胞をそこにせっかく分離して，きれいにして，培養しても，結局その細胞の機能を低下させることがあるわけです．ですから，すぐれたスキャフォールドの開発は，再生医療品の効果の開発をも促進することになります．

米山　次世代の先進医療機器のなかで，材料の果たす重要性が明確になるようなお話がつぎつぎに出てきますね．

堤　バイオマテリアルのそういう生物医学的・科学的・薬学的性能が非常に向上して，それが患者さんに非常に貢献をしているのです．全体としての最期の寿命を支える一つの大事な要因として，やはり物理的なことも忘れてはならないと思います．生体そのものが生体材料から受ける影響と，材料に与える影響を，物理的な面にも若い人たちに大いに参加してもらって，総合的な性能を向上させる研究がより必要になってくると思います．

その一つとして，私たちは体のなかに入った生体材料の長期の状況を予測したり，不具合症例を再現する生体力学的シミュレーションを研究しています．動物実験も一つのシミュレーションですから，計算シミュレーションだけではなく体のなかでの現象を追究できるシミュレーターを実現したく思います．もっともっと開発しなければならないのですが，技術がかなり発展してきましたので，これから応用が大いに期待されています．予測技術，また先端からの改善技術をどんどん開発していくことによって，よりよい医療具に発展することを期待します．

米山　薬剤などと組み合わせて，局所で特に効果を発揮するお話，また違って，生体全体を構成している硬組織を再建するという応用に関する，最近の注目技術エリアについてお話しいただきました．

堤　硬組織だけではなくて，やわらかい組織，それから血管や血液の流れなどもシミュレーションの対象です．

岡野　こういう人工材料が空気中，室温気下１気圧で外に置かれているのと，体のなかに入れられるのとでは，まったく違う環境です．生体内の環境でどのようなことが起きるかというと，いままでわれわれが予測しなかったようなことが起きるわけです．それは体のなかにイオンがあったり，脂質があったり，蛋白があったり，

まざまな細胞との関わりのなかで材料が機能を果たしていくわけですから，それをいま堤先生がおっしゃったように，かなり正確にシミュレートできるようなテクノロジーがつくれれば，われわれはそういう材料やデバイスが体のなかでどんな寿命を持っているかとか，あるいは体がどんな影響を受けるかということがしっかり描けるわけです．そうすると，やはり長期治療や，埋め込みでなにか治療していくような局面では非常に大事なテクノロジーになります．

バイオマテリアルは材料が高分子とか金属とかセラミックスとかそういうものだけではなくて，堤先生のように機械工学的な立場からみたり，もう少し生物細胞との病理学的な側面からみたり，解剖学的な面からみたり，さまざまな総合学問として捉えなくてはいけないのではないでしょうか．

米山　実際の製品をつくっていらっしゃる立場からはいかがでしょうか．

松下　たとえば一つの関節をシステムとして使うときは，総合工学的あるいは科学的でなければいけないというのはそのとおりだと思います．

一方で，先ほど岡野先生がおっしゃった，界面がどこまで設計できるかとか，あるいはどこまでコントロールができるかという点については，たとえばインプラントと硬組織を考えたときに，従来はアパタイトなどの生体活性材料は骨と引っつくということで捉えられました．しかし私は金属屋ですから，たとえばチタン材料を骨と引っつけるためにはどうしたらよいか，あるいはバイオイナートセラミックスを引っつけるためにはどうしたらよいかと考えて取り組みました．

チタン材料については中部大の小久保 正先生のアルカリ処理技術があります．チタン表面に最終的にOH基が出来ると，それをベースにしてアパタイトが生体内で出来て骨と引っつくというものです．それが公表されて以降，その発想をさまざまな材料に適用していくと，結構チタン以外の金属とも引っつくようになりました．一つの現象がわかると基本的な情報として，それが波及的にさまざまなところへ広がっていきます．

また，骨は微細な空隙に対して侵入する特性を持っているという情報もあります．結果的には数年かかっていますが，バイオイナートなジルコニアセラミクスと骨は引っつかないと思われますが，表面をミクロ凸凹構造にすると結果的にはちゃんと引っつくんです．従来は骨セメントを使って引っつけていたものを，今度はそういうものを使わずに，表面を微細構造にするだけで骨がそのなかに入り，結合します．そうするとセメントレスのインプラントとして実際に使えるようになる．そういう意味で，界面の現象を追求すること自体が，バイオマテリアルの本質を引き出してくる重要な課題になっていると考えます．

明石　材料工学かなにかの学会で，もともと冶金，金属工学の阪大の馬越先生・中野先生のお話を聞きました．ストレスのかからないところには骨が出来ない．これはわれわれが材料を一方的な方向からみていると出てこないような発想です．実験結果をとると，ストレスがかかるようなところにテープを巻く．応力緩和みたいな感じです．そのようなことで骨は出来ていくのだという結果を出されていました．マテリアルの分野にも，物それ自体でもない，界面でもないこのような考え方があるのだと思いました．

岡野　古くから知られた現象で，われわれの骨というのは，やはり圧力をかけていかないと，カルシウムが抜けていってしまうのです．

明石　私が疑問だったのは，たんにサイトカインを出してアパタイト系のものをつくっても，陥没したところに骨ができるのは当然ですが，上に骨を接ぐことができるかどうかという点です．その結論からすると出来ないのです．ですから歯科インプラントをしたいときには，土台の骨をしっかりつくりたい．本来はその人の必要としないところにはやはり骨は出来ません．そうするとどうなるのですか．

堤　歯が抜けてしまった跡に人工歯根を植えなければ歯槽骨はどんどん減っていきます．骨を維持するためには咬合力の刺激が必要です．

明石　骨を高く積むということですね．

堤　力学的刺激をなんらかの形で与えれば骨は維持できます．

明石　たとえば，もっといいますと，背が高くなるのでしょうか．

堤　その治療法をイリザロフ法骨延長術といいます．骨に切れ目を入れて，そこにピンを打ち込んで，毎日少しずつ引き離していくと，1日0.1 mm 程度で，延べ 15 cm ぐらいは伸ばせます．

明石　ここで先ほどの話です．力のかかっていないところに自然に骨は出来るのですか．

堤　カルシウムや生化学的な要因で再生することはできるけれども，力学的な刺激がなければやがて消えてしまいます．

土屋　それはいま再生医療で，力学的刺激を導入した培養法などさまざまところで研究されています．軟骨も力学的刺激がないと分化や強度，

微細構造などがどうかなるとかいった研究も進められています．

明石　騙してつくることはできるけれども，結局，出来ないということですか．

岡野　吸収されて安定性がわるいということです．

米山　離れたままですと骨は出来ませんが，ちょうどよい具合に離しつづけると，そこの間がなくなったら困ると認識されて骨が添加されていきます．

明石　なくなったら困るという場所では出来るので，本来盛り上げることというのはきわめて難しい．

米山　はい．盛り上げることは難しいです．

医療技術とサイエンス

米山　いま，エリアの違う松下先生のお話でも，以前の研究，たとえばミクロのレベルの材料研究から一歩進んで，徐放性のものや，表面に何基が出ているかというような微細構造の話になりますと，いまはやりのナノエリアの研究に入ってきていると思います．やはり界面と，徐放性のような機能性の分子という２方面がメインでしょうが，先生方のご意見はいかがでしょうか．

岡野　物理的な接着の強さ・弱さというのが，表面の上で反応基があって反応するかしないといったことはもちろん起きます．一方，やはり生体の側のレスポンスというのは代謝を使って変化します．ですから細胞が表面をみて形態変化していきますから，異物として認識するのかとか，表面上でどういう接着をしていくかというような現象に関し，代謝が関与する世界だとかなり通常の物理化学的現象と違います．それがバイオマテリアルの非常に面白いところです．これがコントロールできるようになれば，表面で生体を刺激したり，細胞を刺激したりできるので，これまでは受身的に材料の安定がどうかといった面ばかりが強調されてきましたが，今度は逆に，表面を使って生体を刺激して病気を治すことに使うような話までがいま出てきているのです．そういう意味では，この表面がどのくらい強い相互作用があるのかとか，スペシックなシグナルが入るかとか，そういった問題が非常にホットになってきています．これに関連し，さまざまなことがわかってきて，応用が広がっていくのではないかと思います．

土屋　そういう意味で界面の反応として，細胞側の生体適合性評価指標としてギャップジャンクションが一つの指標になるのではないかということで数年以上研究をつづけています．アメリカの規格協会ASTMからギャップ結合細胞間連絡に関する標準化文書をつくってくださいと依頼されており，会議に出席している外資系企業の質問などを受け関心の高さがわかりました．

細胞間情報伝達はギャップジャンクション構成蛋白分子のみがキーとなり行われるわけではないのです．エキストラセルラーマトリクスなどが絡んでギャップジャンクション機能が上がるわけです．いくつもの候補因子があるなかで，やはりなにが生体適合性や安全性においてキーポイントになるかということを効率よく適切に絞らないと非常に時間と費用がかかると思います．そういう意味ではギャップジャンクションは一つのマーカーになるのではないかと思います．

岡野先生の再生医療で，心筋再生ではこのギャップジャンクション蛋白質の発現や局在性機能を心筋再生の指標としての評価においても発表されています．これからは細胞と材料の相互作用として，細胞・組織の形態のみでなく細胞間・細胞内の情報伝達・シグナル伝達を考慮した設計に基づく医療機器開発の重要性が明らかになり，国際的な開発戦略としてさまざまなところに広がっていくと思います．

堤　わかるという意味では，特に再生医療などで，計測技術や計測パラメーターを抽出して，体のなかに入れてしまった細胞や組織がどのように反応していくのかを追跡する技術というのが，これからは非常に望まれてくるだろうと思います．私は先ほどからシミュレーションが必要と言っていますが，シミュレーションも嘘であってはいけないので，バリデーションが非常に大切です．信頼性を上げるために，体内でなにが起こっているかを追跡できる計測技術が，もっと必要になってくるだろうし，望まれる技術だと思います．

土屋　堤先生のおっしゃられるシミュレーション技術ですが，すでに米国では，審査のなかでの自主基準ですけれども，ごく一部分は承認申請書に使用されはじめています．

米国の不具合の40%強は設計上に原因があるといわれています．新技術が科学的な根拠に基づいて適切に導入される必要があります．わが国もそういう意味でも，堤先生がリードしてこられたものを，より育てて，耐腐蝕性，精密化，寿命，デザインとしての適切性などが的確に数字として出せるような技術レベルになるよう大いに期待します．それをやっていただかないと安全性を担保し，コスト面で合理性を追究するメーカーも困ると思います．

堤　そのときに必要な基本のデータベースがまだ不十分ですので，皆の力で構築していかなければなりません．よい例は当然データベースとして客観的に洗い直しされる例は

もっと大事ですから，さらに慎重に調べて蓄積していくのです．そこから重要な因子を絞り出していきます．シミュレーションのよいところは因子を抽出して，純化できる点ですので，特定の因子だけを追及すればどうなるかとか，分類できるというのは大事な特色の一つであろうと思います．審査基準や標準化にもっていくためには，計測技術と臨床データベースの構築が必須ですから，これから学会をあげての取り組みを望みたいと思います．

明石　エリアのどういうサイエンス，どういうテクノロジーと組み合わせるかということを整理する必要が出てきているのではないのでしょうか．そうしないとマテリアル研究に焦点が当たらずに，たんなる組み合わせだけになってしまって，マテリアル研究に反映されないようなことになる可能性があるように思います．

松下　先ほどのシミュレーションのお話ですが，われわれメーカーの立場ですと，構造解析や変形解析に数値シミュレーション技術を活用し，データを蓄積していますので，申請書類にシミュレーションの結果を，これは工学的にできることですから，それなりの経験に基づいた数値を付けているのですけれども，なかなか信用してもらえないのです．それはおそらく，先ほど堤先生がおっしゃった臨床成績とその数値シミュレーションの結果が合っているかというところが充分理解されていないのではないかと思います．結果的には，実験したデータをも付けています．それを何回もやることではじめて，このシミュレーションも信用できるということになるようです．

土屋　そこはまさしく，バイオマテリアル学会や医療機器フォーラムで主題に取り上げています．審査官側の方に活用していただけるような整理，蓄積，そういったものを目にみえる形で行ったほうがよいと思います．

松下　現状ではそれでかなりの回数，書類のやり取りを行っています．

岡野　結局それはサイエンスだと思います．たとえばシリコンみたいなものですと，脂質がじわじわ入っていって，屈折のところが弱くなります．しかし，親水性のものだとそんなに入っていきません．ところが今度はカルシウムが入ってきて，石灰化が起きて固くなってしまう．それは材料にもよります．そうするとシミュレーションといいながらも，そのような影響を勝手にないようにしてしまったり，勝手にあるようにしたりということが行われないとも限りません．どこまでリアリティーがあるのか．そういう意味では堤先生がおっしゃるように，ある仮定のもとでおいたシミュレーションは生体のなかの現象をこれだけ反映している，というデータが必要です．

しかしいま，そこがないから必ずしも信用できないので，そこをみんなで集めていく必要があります．界面でどういうことが起きていて，この現象はこの材料に限っては無視できるけれど，この材料に関してはこういう成分の影響は考えなくてはいけないというのを，もっときれいに整理していくことが重要だと思います．結局は界面のサイエンスをわれわれがどこまできちんと把握できるかで，やはり企業の人たちも含めた会員のなかで，そういうデータベースを全員で構築していくという雰囲気が重要ではないでしょうか．

松下　重要だと思います．シミュレーションの前提がなにであるかを明確にしてデータを蓄積する必要があります．そこが曖昧であれば結果の信用度がぐらついてしまいますから．

岡野　結局日本のバイオマテリアルのレベルを上げるためにも，バイオマテリアル学会できちんとそういうことをやっていくというのは重要です．

土屋　昨年5月のASTMで，有限要素法のWGがありましたが，40～50人の米国系や海外の企業の人が参加していました．非常に注目されていると思いました．しかし日本からは私以外誰も参加していなかったのです．1メーカーが大変優秀で全部できるのならよいですけれども，そうでなければ，やはりトップと常に一緒になって情報を収集するというのが非常に重要だと思います．彼らは，すでに数社以上でvalidation studyを行っており，結果について活発に議論していました．われわれ厚生労働省の国立医薬品食品衛生研究所としましては，再生医療をどうやって早くするかとなると，結局はそのなかで一番トップの人をよんでやっていかないと，とてもではありませんが早く進みません．その分野の人がいればよい，数が集まればよい，そういったことではなくて，先端医療となるとやはりポイントとなる人を集めてつくっていくことが非常に重要だと思います．

新技術とこれからの医療

米山　実際に応用される医療用具の開発のところまでサイエンスは必要であるというお話で進んできましたが，ではそういう新しい技術が，実際に社会の未来にどのような貢献をするかという観点からお話しいただけますか．

土屋　従来の医療機器に関するイメージが非常に変わると思います．薬以上に劇的に効く，例外はありますが，従来型の医療機器ですと，使用