differentiation, a 3.1-fold increase of that of the controls, was found in the sample treated with PGA. The potencies of cell differentiation after 4 weeks of culture from the highest to the lowest were in the following order: PGA >> PLGA > PGCL > Control = DMSO > P(LA-CL)25 = PCL(Ti) >> fullerene C60 DMA. The increased cell differentiation with PGA and PLGA matrices are in agreement with our previous findings in a micromass culture system;[19] however, in our present study we included the matrix gene expression of these materials. The cell differentiation findings of PCL(Ti) and copolymers PGCL and P(LA-CL)25 could not be compared with other studies because we found no reports describing the effects of PCL and its associated polymers on chondrocyte differentiation. The recent discovery that fullerene C60 DMA can be produced in macroscopic quantities has sparked much interest in the chemistry of this unusual molecule, which did not cause acute toxic effects on mouse skin epidermis.[26] Increased cell proliferation and differentiation of rat embryonic limb bud cells by fullerene C60 were reported,[11] but the data of the present study showed that fullerene C60 DMA acted as a potent inhibitor of HAC differentiation.

As tissue engineering becomes increasingly complex, there is a need to understand how a specific biomaterial influences gene expression. Therefore, the matrices used in this study were evaluated with respect to their influence on the expression of collagen type II and aggrecan genes (Figs. 4 and 5). The increased expression of collagen type II and aggrecan genes in the PGA-, PGCL-, and PLGA-treated matrices was well correlated with their elevated level of cell differentiation values, as shown by alcian blue staining. The low expression of collagen type II and aggrecan genes in the fullerene C60 DMA-treated matrix paralleled the decreased level of cell differentiation, as shown by alcian blue. Therefore, low cell proliferation and differentiation values along with almost no expression of collagen type II and aggrecan genes in the fullerene C60 DMA-treated matrix completely exclude this matrix from use in ECM tissue engineering. The expression of collagen type II and aggrecan genes in the P(LA-CL)25-treated culture was consistent with its cell differentiation value. The data from this study showed that cultured chondrocytes also retained their phenotype throughout the experimental period, as indicated by expression of the type II collagen gene (Fig. 4A, 4B). To the best of our knowledge, this study is the first to show the bioactivity of PCL(Ti) and copolymers PGCL and P(LA-CL)25 in chondrogenic differentiation of HAC in a micromass culture system. Further, we know of no studies that have evaluated the matrix gene expression for PGA and PLGA matrices using HAC in a micromass culture system. Results of the present study confirmed PGA, PLGA, and PGCL as useful scaffolding matrices for cartilage tissue engineering, and information about the other matrices will further contribute to the development of improved cartilaginous constructs for future clinical implants.

The progression of chondrogenic differentiation can be followed by the expression of markers of cytodifferentiation. For example, precartilaginous condensations express type I collagen,[27] whereas the next phase of cartilage differentiation involves the expression of type II collagen, aggrecan, and link proteins, which form the cartilage matrix.[28] The mechanism of precartilaginous condensation is poorly understood, but cell–cell interactions are putative effectors for chondrocyte aggregation.[29] Chondrocytes in the primary culture can proceed through the same differentiation program as they do in the cartilaginous angle of the long bone, and the earliest morphological event on the way to overt differentiation is the formation of cell condensation.[17] The observed expression of Cx43 suggested that the process of condensation is in part caused by the interconnection of cells by means of gap junctions.[13] In this study, RT-PCR analysis showed that the mRNA level of Cx43 gene expression was consistent with chondrogenic differentiation in the presence of different biomaterials. Our findings on Cx43 expression by chondrocytes are in agreement with a previous study that reported expression of functional gap junctions by chondrocytes isolated from adult articular cartilage.[30] Gap junction-mediated intercellular communication is critically involved in the development of cartilage during differentiation.[31]

Conclusions

The analysis of three set of genes, namely collagen type II, aggrecan, and Cx43 was important to evaluate the effect of biodegradable polymers and other types of cartilaginous scaffolds on the chondrogenesis of HAC for tissue engineering.

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In vitro induction of polyploidy and chromatid exchanges by culture medium extracts of natural rubbers compounded with 2-mercaptobenzothiazole as a positive control candidate for genotoxicity tests

Atsuko Matsuoka, Kazuo Isama, Toshie Tsuchiya
Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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Abstract: We tested extracts of custom-made natural rubber samples for cytotoxicity using V79 cells and for chromosome aberration (CA) induction using CHL cells in compliance with the Japanese guidelines for basic biological tests of medical materials and devices. The samples were formulated with a high level of 2-mercaptobenzothiazole (MBT) (A); a low level of MBT (B); or zinc dibutyldithiocarbamate (ZDBC) (C). In the CA test, MBT induced mainly polyploidy, including endoreduplication, and ZDBC induced structural CAs. In the cytotoxicity test, culture medium extracts of A, B, and C suppressed colony formation to 50% of the control value at 53.1%, 94.3%, and >100%, respectively. Culture medium extracts of sample A induced polyploidy and structural CAs in the absence of an exogenous metabolic activation system (S9 mix), but at lower concentrations in its presence, indicating the existence of other leachable promutagens. The extracts of sample B induced structural CAs at the highest concentration and only with S9 mix. Sample C was negative. The facts suggest that sample A may be a candidate for a positive control for genotoxicity tests. The high frequency of polyploidy induced by sample A was not predicted by MBT, suggesting the usefulness of the test for safety evaluation of medical devices. Numerical CAs induced by MBT and sample A are discussed. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 75A: 439–444, 2005

Key words: cytotoxicity; chromosome aberrations; natural rubber; zinc dibutyldithiocarbamate; endoreduplication

INTRODUCTION

Safety evaluation of medical materials is an important step in the production and marketing of medical devices. The Japanese guidelines for basic biological tests of medical materials and devices[1] cover nine assay systems for the initial evaluation. Tests for medical materials are different from those for a single chemical substance in that the samples are extracts of the test material and contain a mixture of chemicals, thus, additive and/or compound effects are expected. We have been testing model materials to search for a positive control for genotoxicity tests because there are no standard positive materials for them.

In the present study, we investigated the culture medium extracts of natural rubber–based materials in the cytotoxicity test using V79 cells and in the *in vitro* chromosomal aberration test using CHL cells in compliance with the Japanese guidelines mentioned above. Rubber materials are widely used for surgical and household gloves and for urinary catheters, although they have induced strong cytotoxicity,[2] severe allergic reactions,[3–5] and urethral strictures.[6–10] The model rubber materials used in this study were originally prepared for sensitization tests.[11] They were custom made with low allergenicity. The only allergenic components are 2-mercaptobenzothiazole and zinc dibutyldithiocarbamate.

Correspondence to: A. Matsuoka; e-mail: matsuoka@nihs.go.jp
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MATERIALS AND METHODS

Cells

We obtained Chinese hamster fibroblast V79 cells (established by Elkind and Sutton[12]) from Japanese Collection of Research Bioresources (JCRB0603, Tokyo) and grew them in

TABLE I
Recipe for Model Materials

Component	Sample A	Sample B	Sample C
Natural rubber	100	100	100
Zinc oxide	5	5	5
Stearic acid	1	1	1
Filler	62	62	62
Black factice	5	5	5
Selected microcrystalline wax	1.3	1.3	1.3
Sulfur	2.0	2.0	2.0
MBT	2.0	0.2	0
ZDBC	0	0	0.5
Sum	178.3	176.5	176.8

Values: dry parts per hundred parts of rubber.

Eagle's minimum essential medium (MEM) (GIBCO 61100-061) supplemented with 10% heat-inactivated fetal calf serum (FCS) in a 37°C humidified atmosphere of 5% CO_2 in air.

We used CHL cells originally established from the lung of a female newborn Chinese hamster by Koyama and colleagues[13] and cloned by Ishidate and Odashima.[14] They were maintained in Eagle's MEM (GIBCO 11095-080) supplemented with 10% heat-inactivated FCS. The doubling time was around 13 h, and the modal chromosome number was 25.

Chemicals and model materials

2-Mercaptobenzothiazole (MBT, CAS No. 149-30-4) from Ouchi Shinko Chemical Industrial Co., Ltd. (Tokyo, Japan) and zinc dibutyldithiocarbamate (ZDBC, CAS No. 136-23-2) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) were dissolved in dimethyl sulfoxide.

Model materials of three rubber sheets (samples A, B, and C; thickness, 1 mm) were prepared with the components shown in Table I by Atom Co., Ltd. (Tokyo) and sterilized with ethylene oxide. Zinc oxide and stearic acid were compounded as vulcanizing accelerator activators. Black factice, selected microcrystalline wax, and sulfur were compounded as a softener, an antioxidant/antiozonant, and a crosslinking agent, respectively. MBT and ZDBC are vulcanizing accelerators. Sample A contained a high level of MBT and the level of MBT in sample B was lower than one tenth of that in sample A. Sample C contained ZDBC instead of MBT.

Cytotoxicity test

Materials were cut into approximately 2 × 15 mm pieces. The pieces (1 g) were put into a centrifuge tube, and 10 mL MEM supplemented with 5% FCS, nonessential amino acids, and 1 m*M* sodium pyruvate (5% FCS-GMNP) was added. After incubation at 37°C in a humidified atmosphere for 24 h, the extract, designated 100%, was decanted and serially diluted with 5% FCS-GMNP to give 80%, 64%, 51%, and 41% extracts.

V79 cells were seeded at 50/well in 24-well plates. After 24-h incubation, the medium was exchanged for 0.5 mL of the serially diluted medium extract or the medium without the extract (for control), and the cells were cultured for 6 days. The colonies formed were fixed with 10% formalin and stained with 5% Giemsa solution. The number of colonies on each well was counted, and the relative plating efficiency was calculated as the ratio of the number of colonies in the treated sample to the number in the control. The cytotoxic potential of the extracts was expressed as the concentration at which the relative plating efficiency was 50% of control (IC_{50}). The IC_{50} value was calculated by the probit method.

Chromosome aberration (CA) test

Materials were cut into approximately 2 × 15 mm pieces. The pieces (1 g) were put into a centrifuge tube, and 10 mL culture medium for CHL cells was added. After incubation at 37°C in a humidified atmosphere for 48 h, the extract, designated 100%, was decanted and diluted with the culture medium.

CHL cells were seeded at 1.5 × 10^5/plate (60 mm in diameter) and incubated for 17 h. They were then treated with extracts for 6 h in the presence or absence of S9 mix followed by expression cultivation with fresh medium for another 18 h. S9 mix was purchased from Kikkoman (Noda, Japan). The S9 fraction[15] was prepared from the livers of Sprague Dawley rats pretreated with phenobarbital and 5,6-benzoflavone. The final concentration of S9 was 5 v/v%. Colcemid (0.2 μg/mL) was added for the last 2 h. Chromosome preparations were made as follows: Cells were trypsinized and incubated in hypotonic KCl solution for 15 min and fixed three times with ice-cold fixative (glacial acetic acid:methanol, 1:3). Two drops of the fixed cell suspension were spread on a clean glass slide, air dried, and stained with Giemsa solution. All slides were coded, and the number of cells with structural or numerical CAs was counted on 100 well-spread metaphases with a modal chromosome number of 25 ± 2. The number of mitotic cells was counted on 1000 live cells and the mitotic index (MI) was used to express the cytotoxic potential of the treatment. The structural CAs were classified into 6 groups: chromatid and chromosome gap (ctg), chromatid break (ctb), chromatid exchange (cte), fragmentation (f), chromosome break (csb), and chromosome exchange (cte, mainly dicentrics and ring chromosomes). The mean and standard deviation (SD) for our historical negative controls of CHL cells are 1.03 ± 1.11 (without S9 mix) and 1.25 ± 1.16 (with S9 mix) for structural aberrations, 0.60 ± 0.93 (without S9 mix) and 0.84 ± 1.02 (with S9 mix) for polyploidy, and 0 for endoreduplication. The experimental groups were judged[16] as negative if the total CA frequency was less than 5.0%, inconclusive if it was 5.0 to up to 10.0%, and positive if it was 10.0% or more. Solvent-treated cells served as the negative control. Experiments were performed at least twice. A representative data from a single experiment are shown, unless otherwise stated.

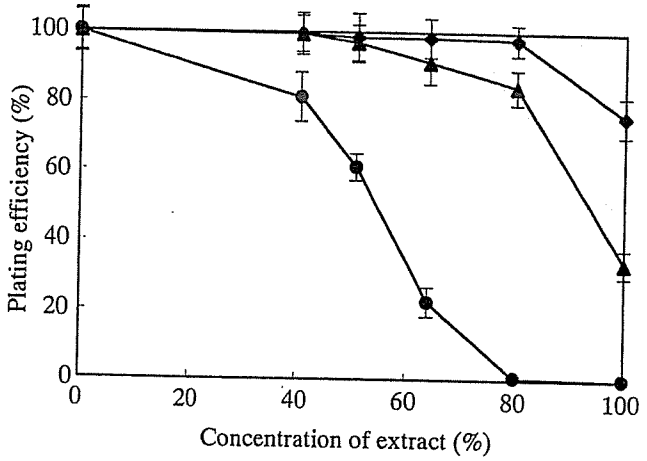

Figure 1. Plating efficiencies of V79 cells treated with medium extracts of samples A (●), B (▲), and C (♦). The samples were extracted with 5% FCS-GMNP for 24 h and the extracts were tested in the colony assay. Values are expressed as means ± SD for eight wells.

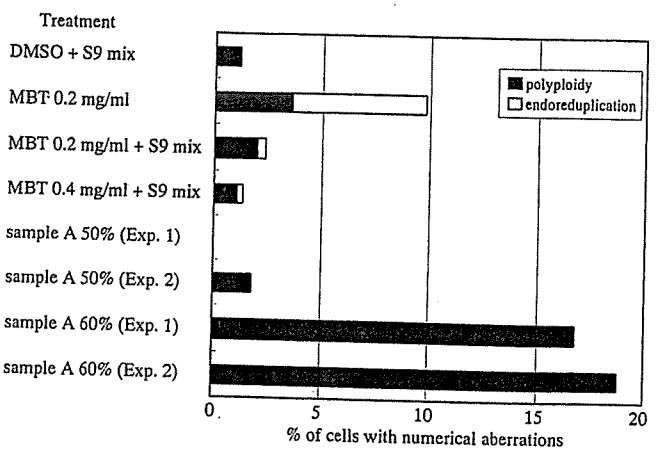

Figure 2. Numerical chromosome aberrations induced by MBT and sample A. Five hundred cells were examined.

RESULTS

In the cytotoxicity test performed with V79 cells, sample A showed the strongest response (Fig. 1). IC_{50} was 53.1%, 94.3%, and more than 100% for samples A, B, and C, respectively.

In the CA test performed with CHL cells, MBT induced polyploidy, including endoreduplication, in the absence and presence of S9 mix (Table II). The number of polyploid cells and endoreduplications was counted on another 500 metaphases for confirmation (Fig. 2). Frequency of polyploid cells and endoreduplications was 3.6% and 6.2% without S9 mix, and 2% and 0.4% with S9 mix, respectively. MBT showed inconclusive response in structural CA induction at 0.4 mg/mL with S9 mix. ZDBC induced structural CAs, mainly cte, in the presence of S9 mix and at lower concentrations in its absence.

Culture medium extracts of sample A induced numerical and structural CAs in the absence of S9 mix and structural CAs in its presence (Table III). The extracts were toxic at the higher concentrations. Structural CAs were induced at lower concentrations in the presence of S9 mix than in its absence. Interestingly, MI of the 40% extract remained high in the presence of S9 mix, although almost all the cells were dead. The numerical CAs were counted on another 500 metaphases for confirmation. The 60% extract induced 16.8% polyploidy and no endoreduplication without S9 mix (Fig. 2, Exp. 1). The 100% of extract of sample B induced 7% CAs in the presence of S9 mix. Sample C did not induce any CAs either with or without S9 mix.

TABLE II
Chromosome Aberration Test of MBT and ZDBC

Chemical	S9 mix	Conc.[a] (mg/mL)	Poly.[b] (%)	Cells with structural aberrations (%)							MI[c] (% of Control)
				ctg	ctb	cte	f	csb	cse	Total	
MBT	–	0	0	2	0	0	0	0	0	2	100
		0.2	8(3)	0	3	1	0	0	0	4	110
		0.4								Tox	12
	+	0	1	0	0	1	0	0	0	1	100
		0.2	5(3)	0	2	0	0	0	0	2	158
		0.4	2	1	2	4	0	0	0	7	87
		0.6								Tox	9
ZDBC	–	0	0	2	0	0	0	0	0	2	100
		0.002	1	0	0	0	0	0	0	0	99
		0.004	0	1	4	11	0	1	0	16	116
		0.006								Tox	10
	+	0	1	0	0	1	0	0	0	1	100
		0.006	0	1	0	2	0	0	0	3	120
		0.008	1	0	2	16	0	0	0	18	104
		0.010	2	2	3	12	0	0	0	16	55

[a]Concentration.
[b]Frequency of polyploidy. Figures in parentheses indicate the number of endoreduplication included.
[c]Mitotic index.

TABLE III
Chromosome Aberration Test of Culture Medium Extracts of Samples

Sample	S9 mix	Conc.[a] (%)	Poly.[b] (%)	Cells with Structural Aberrations (%)							MI[c] (% of Control)
				ctg	ctb	cte	f	csb	cse	total	
A	−	0	1	0	0	1	0	0	0	1	100
		50	1	1	0	8	0	0	0	9	107
		60	20	1	0	10	0	0	0	11	146
		70	26	1	3	7	0	0	0	11	20
	+	0	4	0	0	0	0	1	0	1	100
		10	0	0	0	0	0	0	0	0	84
		20	3	0	0	7	0	0	0	7	114
		30	3	1	3	12	0	0	0	14	139[d]
		40	0	2	6	10	0	0	0	16	93[e]
B	−	0	1	0	0	1	0	0	0	1	100
		60	0	0	0	0	0	0	0	0	92
		80	0	1	0	0	0	0	0	1	91
		100	0	0	0	0	0	0	0	0	101
	+	0	4	0	0	0	0	1	0	1	100
		60	2	1	0	1	0	1	0	3	86
		80	1	1	0	0	0	0	0	1	86
		100	2	0	0	7	0	0	0	7	85
C	−	0	1	0	0	1	0	0	0	1	100
		60	0	0	1	0	0	0	0	1	103
		80	1	0	0	0	0	0	0	0	107
		100	1	1	0	0	0	0	1	2	110
	+	0	4	0	0	0	0	1	0	1	100
		60	1	2	0	0	0	0	0	2	93
		80	1	0	0	0	0	0	1	1	99
		100	3	0	0	0	0	0	0	0	106

[a,b, and c] See the footnote in Table II.
[d]Around 50% cells on the preparation were dead.
[e]Around 90% cells on the preparation were dead.

DISCUSSION

In an earlier study, the IC_{50} for MBT and ZDBC in the cytotoxicity test was 49 and 5.4 μg/mL, respectively.[17] These values are compared with samples A to C in the present study and the lowest toxicity concentration in the CA test (Table IV). Similar cytotoxicity was shown in both tests.

ZDBC, strongly cytotoxic, is weakly positive in the CA test using human lymphocytes[18] and negative in the bacterial reverse mutation assay,[18–20] mouse lymphoma cell mutation assay,[18] and the *in vivo* micronucleus test.[18] In the present study, ZDBC induced structural CAs at relatively low concentrations both in the absence and presence of S9 mix, but at lower concentrations in its absence, indicating that ZDBC does not require S9 mix for activity. Sample C, which was compounded with ZDBC, showed a slight cytotoxicity and was negative in the CA test, perhaps due to its low extraction efficiency by culture medium.[11,18]

TABLE IV
Comparison of Cytotoxicity

	IC_{50}*	LTC**
MBT	49 μg/mL***	400 μg/mL
ZDBC	5.4 μg/mL***	6 μg/mL
Sample A	53.1%	70%
Sample B	94.3%	>100%
Sample C	>100%	>100%

*Inhibition concentration at which the relative plating efficiency is inhibited to 50% of the control value in the cytotoxicity test.
**Lowest toxic concentration in the CA test.
***Nakamura et al.[17]

On the contrary, sample A, which was compounded with MBT, showed strong cytotoxicity and induced structural CAs at lower concentrations in the presence of S9 mix than in its absence, suggesting the existence of promutagenic leachates other than MBT. In the present study, MBT yielded an inconclusive response in structural CA induction in the presence of S9 mix, while it yielded a positive response at similar concentrations in another Chinese hamster cell line, CHO.[21] Four percent cte-type structural CAs, however, suggest, based on our historical database, that MBT may show a biologically positive response in the presence of S9 mix.

Sample A showed an interesting phenomenon in that so many dead cells coincided with well living cells in the presence of S9 mix. In the present study the number of mitotic cells were counted on 1000 live cells. MI was 139% and 93% of control at 30% and 40%

extract of sample A, respectively, in the presence of S9 mix. Although the values of MI indicated no cytotoxicity, the ratio of live cells to total cells on the preparation was around 50% and 10%, respectively. Actually a few remaining live cells on the preparation of 40% extract of sample A were well and the chromosome morphology of the metaphase cells was fine.

Numerical chromosome aberrations consist of polyploidy and aneuploidy. Aneuploidy has been implicated in sterility, abortions, stillbirths, congenital abnormalities, and carcinogenesis.[22,23] The *in vitro* CA test is not routinely used to detect aneuploidy, although it could be,[24] but polyploid induction suggests the possibility of aneuploid induction. In the present study, sample A, which had a high concentration of MBT, induced a high frequency of polyploidy in the presence of S9 mix. MBT alone at the same concentration also induced polyploidy, but with a lower frequency and with accompanying endoreduplication. Thus, the induction of polyploidy by sample A did not seemed to be explained simply by the presence of MBT.

Endoreduplication, which shows an characteristic morphology (diplochromosomes), is an endomitotic chromosome duplication that occurs without mitosis-like events during interphase.[25] A typical endoreduplication event is characterized by two periods of DNA synthesis, S1 and S2, separated by a G period of variable duration.[26] Some chemicals, such as 4NQO, acridine yellow, cytoxan, captan,[27] and rotenone,[28] induce endoreduplication without S9 mix. The frequency of endoreduplication induced by those compounds was similar to the frequency induced by MBT in the present study.

The fact that sample A showed stronger cytotoxicity and induced a higher frequency of polyploidy than was predicted by MBT alone might have been due to the presence of other leachables in the sample. This suggests that sample A may be useful as a positive control for the safety evaluation of biomaterials and that the test might overcome the poor predictive value of individual components of materials.

In the Japanese guidelines for basic biological tests of medical materials and devices, the use of V79 cells is preferred in the cytotoxicity test. In the test the introduction of a metabolic activation system is not required. On the other hand, genotoxicity tests require the use of an exogenous metabolic activation system and of their methods following Japanese guidelines for drugs and chemicals, and OECD guidelines. CHL cells are popular in the CA test in Japan. In the present study each of the cytotoxicity test and the CA test followed the corresponding guideline independently. The difference in the cytotoxicty of sample A between with and without S9 mix suggests that the discussion of the introduction of an exogenous metabolic activation system into the cytotoxicity test may be needed.

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Enhancement of Gap Junctional Intercellular Communication of Normal Human Dermal Fibroblasts Cultured on Polystyrene Dishes Grafted with Poly-*N*-isopropylacrylamide

TSUTOMU NAGIRA, Ph.D.,[1,2] SUSAN BIJOO MATTHEW, Ph.D.,[1] YOKO YAMAKOSHI, Ph.D.,[3] and TOSHIE TSUCHIYA, Ph.D.[1]

ABSTRACT

Technology developed to allow recovery of cells without enzyme treatment, involving a dish grafted with a thermoreactive polymer gel of poly-*N*-isopropylacrylamide (PIPAAm), was found to significantly enhance gap junctional intercellular communication (GJIC) in normal human dermal fibroblasts (NHDF cells). NHDF cells were cultured for 4 days on PIPAAm-grafted dishes irradiated with various doses of electron beams, and GJIC was assayed by the scrape-loading dye transfer method. The area of dye transfer was greater in the PIPAAm-grafted dishes than in the control culture dishes, indicating that the PIPAAm-grafted dishes enhanced the GJIC of NHDF cells. Connexin-43 (Cx43) expression was analyzed because Cx43 is considered to be a main component of the gap junctional channel. PIPAAm-grafted dishes irradiated with 100, 250, or 500 kGy of electron beams showed significantly enhanced expression of Cx43-NP, Cx43-P1, and especially Cx43-P2. Enhanced expression of Cx43-P2, a functional transmembrane protein, may be related to the promotion of GJIC. These results suggest that the PIPAAm-grafted dish not only enables the enzyme-free recovery of a cell monolayer for use in the construction of a three-dimensional artificial tissue, but also significantly contributes to the enhancement of GJIC, which may partly promote tissue strength on the surface of the PIPAAm-grafted dish.

INTRODUCTION

GAP JUNCTIONS exist on the cell membrane and work as intercellular channels that allow the exchange of substances with molecular masses up to 1 kDa, such as ions, sugars, and amino acids, by the function called gap junctional intercellular communication (GJIC).[1–3] Gap junctions are constructed from transmembrane proteins, called connexins,[4,5] that form a hemichannel, called a connexon. GJIC is suggested to be well correlated with passage of metabolites,[6] cell proliferation,[7] and cell differentiation[8]; thus, enhancement of the function of the gap junction is supposed to be important in the differentiation of engineered tissue products, such as those involving heart cells.[9–11] Poly-*N*-isopropylacrylamide (PIPAAm)-grafted dishes, which were originally developed as a thermosensitive scaffold for cell culture, are useful to maintain the GJIC of tissues cultured on them because they do not require enzyme treatment, which destroys connexins.[12–14]

PIPAAm is a thermoresponsive polymer that has a low critical solution temperature of 32°C: hydrated PIPAAm

[1]Division of Medical Devices, National Institute of Health Sciences, Tokyo, Japan.
[2]Japan Association for the Advancement of Medical Equipment, Tokyo, Japan.
[3]Center for Polymers and Organic Solids, Department of Chemistry and Biochemistry, University of California, Santa Barbara, Santa Barbara, California.

has an extended chain conformation below 32°C and dehydrated PIPAAm has a collapsed chain conformation above 32°C.[15–27] This property of PIPAAm has been exploited in intelligent materials for drug delivery systems and chromatography technology.[16–23] The PIPAAm-grafted dish has been found to enable the recovery of cell monolayers easily without enzyme treatment because cells cannot adhere to a hydrophilic surface below 32°C.[24–26] Cell monolayers are the basic units used to construct three-dimensional tissues *in vitro*. Because a cell monolayer recovered without enzyme treatment maintains normal adhesive and junctional proteins, it can easily adhere to the other tissues or cell sheets to construct a three-dimensional artificial tissue.[27–29] Thus, the PIPAAm-grafted dish has the potential to enable the development of new techniques in tissue engineering.

Although the PIPAAm-grafted dish has made a new era in tissue engineering possible, its effects on connexin-43 (Cx43) expression and GJIC have not been studied well. These effects are important because Cx43 plays an important role in cell proliferation and cell differentiation.

In this study, GJIC and expression of Cx43 molecules were examined by scrape-loading dye transfer (SLDT) assay[30] and Western blotting, respectively, using NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams in order to clarify the safety and appropriateness of this material for the culture of artificial cultured tissues.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide monomer (NIPAAm) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Isopropyl alcohol was obtained from Dojindo (Kumamoto, Japan), and Lucifer yellow dye was from Molecular Probes (Eugene, OR).

Cell culture

Normal human dermal fibroblasts (NHDF cells; Sanko Junyaku, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (GIBCO DMEM; Invitrogen, San Diego, CA), supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen) and antibiotics (penicillin [100 units/mL]-streptomycin [100 units/mL]) (Invitrogen) at 37°C. NHDF cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air.

Preparation of PIPAAm-grafted culture dishes

One hundred microliters of 40% NIPAAm dissolved in isopropyl alcohol was added to 35-mm dishes and irradiated with various doses of electron beams (25, 100, 250, or 500 kGy), using an area electron beam-processing system (Nissin High Voltage, Kyoto, Japan). The PIPAAm-grafted dishes were then rinsed three times with ice-cold sterile water (2 ml) for 5 min, sealed, and dried under vacuum.

Cell morphology

NHDF cells were cultured on control and PIPAAm-grafted dishes. Confluent cells (after 4 days of culture) were fixed with formalin solution, stained with 3% Giemsa solution, and observed with an optical microscope.

Protein assay

The protein concentration of cells cultured on control and PIPAAm-grafted dishes was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). Ten-microliter cell samples were

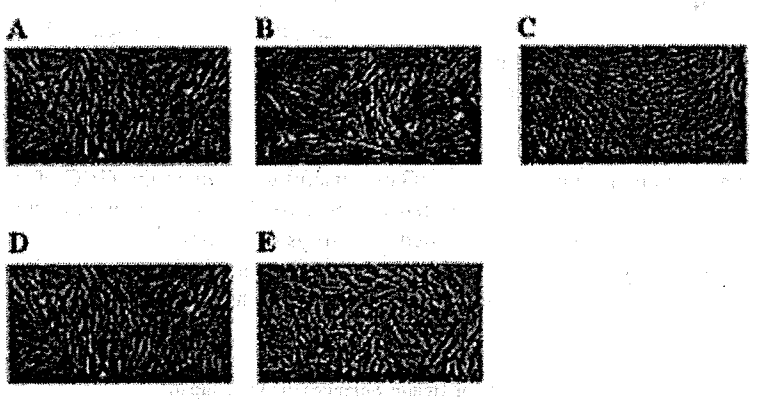

FIG. 1. Optical microscopy images of NHDF cells cultured on PIPAAm-grafted dishes. NHDF cells were cultured for 4 days on PIPAAm-grafted dishes prepared by irradiation with various doses of electron beams (0, 25, 100, 250, or 500 kGy). (A) Non-irradiated; (B) 25-kGy electron beam; (C) 100-kGy electron beam; (D) 250-kGy electron beam; (E) 500-kGy electron beam.

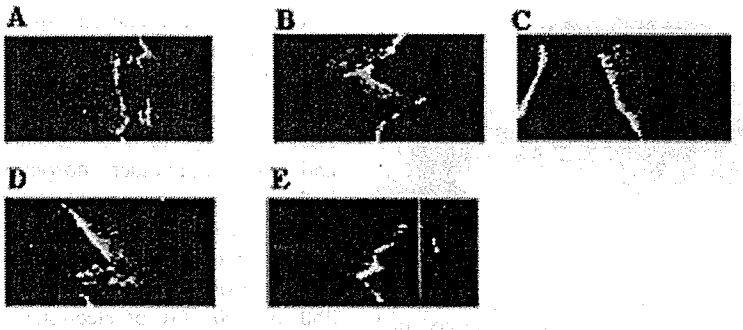

FIG. 2. Fluorescence of NHDF cells by SLDT assay. Transmission of Lucifer yellow into NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams was detected 5 min after scrape-loading. (**A**) Nonirradiated; (**B**) 25-kGy electron beam; (**C**) 100-kGy electron beam; (**D**) 250-kGy electron beam; (**E**) 500-kGy electron beam.

added to 200 μL of the working solution and incubated at 37°C for 30 min in a 96-well plate. Absorbance was then measured at 562 nm in accordance with the manufacturer's protocols.

Scrape-loading dye transfer assay

NHDF cells were seeded on control and PIPAAm-grafted dishes at a density of 1×10^5 cells/mL and cultured for 4 days to form a confluent monolayer. Confluent NHDF cells were washed three times with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} [PBS(+)], and the cell monolayer was scraped with a surgical blade. Fluorescent dye (Lucifer yellow; MW 457.2) at a concentration of 0.1% in PBS(+) was added.[30,31] Cells were exposed to the dye at 37°C for 5 min, and then the dye was discarded and the cells were washed four times with PBS(+). The distance that the dye had migrated was measured under a fluorescence microscope equipped with a type UFX-DXII CCD camera and super high-pressure mercury lamp power supply (Nikon, Tokyo, Japan). The dye migration was measured from the cut edge of the scrape to the edge of the dye front in the cells that were visually detectable.[30]

Western blotting

NHDF cells were cultured for 4 days. After being washed with ice-cold PBS(–) three times, the cells were lysed in 500 μL of lysis buffer (50 mM Tris-HCl [pH 6.8] containing 150 mM NaCl, 5 mM EDTA, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) for 30 min on ice with shaking. The cell lysates were centrifuged (10,000 rpm) at 4°C for 20 min, and the supernatants were collected. The protein concentrations of the lysates were determined by BCA assay.

Equivalent amounts of protein sample were applied to 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and then transferred to a nitrocellulose membrane at 120 V for 60 min. The membrane was blocked with Block Ace (Yukijirusi, Tokyo, Japan) overnight at 4°C. After being washed for 30 min in PBS with 0.05% Tween 20, the membrane was incubated for 2 h with anti-Cx43 polyclonal antibody [diluted 1:1000 in PBS(–) with 0.05% Tween 20; Zymed Laboratories, South San Francisco, CA], followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:5000; Zymed Laboratories). The image was visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences/GE Healthcare, Little Chalfont, UK).

Statistical analysis

Significant differences between groups were evaluated by Student *t* test. Mean differences were considered significant when $p < 0.05$.

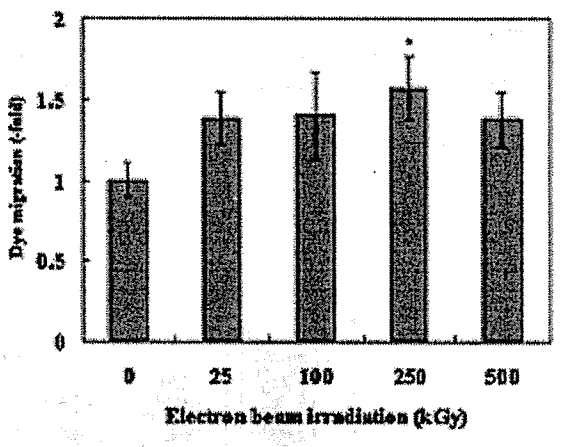

FIG. 3. Positive dye transfer in NHDF cells cultured on PIPAAm-grafted dishes. Transmission of Lucifer yellow was detected 5 min after scrape-loading in NHDF cells cultured on PIPAAm-grafted dishes irradiated with various electron beam doses (0, 25, 100, 250, or 500 kGy). Values represent means ± SD for three dishes. *Significant difference compared with control at $p < 0.05$ by *t* test.

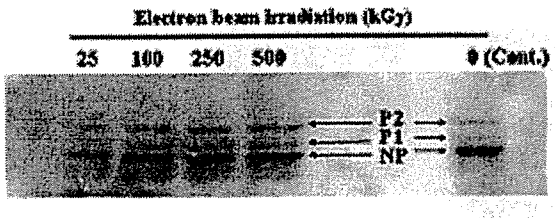

FIG. 4. Western blot of Cx43-NP, Cx43-P1, and Cx43-P2 expression; lysates of NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (0, 25, 100, 250, or 500 kGy) were applied to SDS–polyacrylamide gels. Fractionated proteins in the gels were transferred to nitrocellulose membrane and immunoblotted with anti-Cx43 polyclonal antibody as described in Material and Methods. Images of Cx43 on Western blot were captured with an Image scanner and analyzed with NIH Image software.

RESULTS

The appearance of NHDF cells grown on PIPAAm-grafted dishes irradiated with various doses of electron beams are shown in Fig. 1. No significant differences were observed by optical microscopy analysis between cells grown in dishes irradiated with various doses of electron beams. These results suggest that PIPAAm-grafted dishes are not toxic to NHDF cells.

The SLDT assay showed that dye migration in cells cultured on PIPAAm-grafted dishes irradiated with electron beams (25, 100, or 500 kGy) was enhanced by about 1.4-fold compared with that on control dishes. Interestingly, the dye migration in cells cultured on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam was particularly enhanced, about 1.6 times higher than that on control dishes (Figs. 2 and 3). These results suggested that the GJIC of NHDF cells cultured on PIPAAm-grafted dishes was enhanced and that the GJIC on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam was affected the most.

To further elucidate the effects of the PIPAAm grafting of culture dishes on GJIC, we analyzed the expression of Cx43, a transmembrane protein involved in GJIC. There are three forms of Cx43: Cx43-NP (nonphosphorylated Cx43), Cx43-P1 (monophosphorylated Cx43), and Cx43-P2 (another phosphorylated Cx43); Cx43-P2 is the most important and functional protein involved in GJIC. The results of Western blotting showed that the expression of Cx43-P1 and Cx43-P2 in NHDF cells cultured on PIPAAm-grafted dishes irradiated with 25, 100, 250, or 500 kGy of electron beams was considerably enhanced. Further, NHDF cells cultured on PIPAAm-grafted dishes irradiated with 100, 250, or 500 kGy of electron beams showed enhanced Cx43-NP expression (Figs. 4 and 5A). The Cx43-P2 expression of cells cultured on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam dose showed the highest value, about 46% higher than that of control dishes. Cells cultured on PIPAAm-grafted dishes irradiated with electron beam doses of 25, 100, and 500 kGy were shown to have enhanced total Cx43 expression. Cells cultured on PIPAAm-grafted dishes irradiated with 100- and 250-kGy electron beam doses showed the highest total Cx43 expression, about 36.6% higher than that of control dish (Fig. 5B).

The Cx43-P2 expression of NHDF cells cultured on PIPAAm-grafted dishes irradiated with 25, 100, 250, and 500 kGy correlated well with GJIC ($R^2 = 0.9398$).

DISCUSSION

Thermoresponsive PIPAAm-grafted dishes irradiated with electron beams have been used to culture cell monolayers because the monolayers can be recovered without enzyme treatment, making PIPAAm a useful material for tissue engineering.

It has been reported that junctional proteins, cellular adherence proteins on the cell membrane, interact via

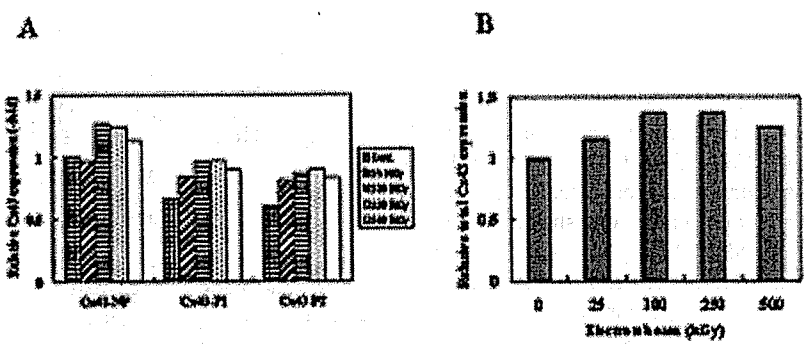

FIG. 5. Relative expression levels of Cx43-NP, Cx43-P1, and Cx43-P2 (A) and relative expression levels of total Cx43 (NP+P1+P2) (B) of NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (0, 25, 100, 250, or 500 kGy).

GJIC.[31] In this study, an SLDT assay demonstrated that dye migration in cultured NHDF cells was significantly enhanced in all PIPAAm-grafted dishes tested. Therefore, the chemical structure of the PIPAAm surface may stimulate junctional proteins on the cell membrane, and the stimulated junctional proteins may induce the enhancement of GJIC.

Cx43 expression of NHDF cells cultured on PIPAAm-grafted dishes irradiated with a 250-kGy electron beam changed significantly. Structural differences in PIPAAm triggered by the 250-kGy electron beam induced Cx43 protein expression by NHDF cells, probably by affecting the gene expression of NHDF cells. Further, total Cx43 expression was shown to be enhanced in cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (25, 100, 250, or 500 kGy). Differences due to the electron beam dose should be studied further.

Although the mechanism involved was not determined, it has been reported that basic fibroblast growth factor (bFGF) and keratinocyte growth factor (KGF) enhance GJIC activity and the expression of Cx43.[32–35] If bFGF and KGF in FCS are adsorbed onto the PIPAAm surface, cells can efficiently access these growth factors from the PIPAAm surface, and GJIC may be enhanced. It is also reported that bFGF activates protein kinase A (PKA),[36] an important regulator of Cx43, promoting the phosphorylation of Cx43 and enhancing GJIC.[37] Therefore, bFGF adsorbed onto the PIPAAm surface may bind its receptor and induce the activation of PKA, resulting in an enhancement of GJIC on NHDF cells caused by the increase in Cx43-P2 band protein.

In the process of posttranslational change, Cx43-P2 becomes insoluble in Triton X-100.[38] Thus, not all Cx43-P2 may be included in the lysate, and some Cx43-P2 may have been included in the pellet. More Cx43-P2 may have existed than was detected in the present results obtained by Western blotting.

In this study, it was shown that the use of PIPAAm-grafted dishes irradiated with various doses of electron beams enhanced GJIC and Cx43 expression in cultured NHDF cells. This suggests that PIPAAm-grafted dishes may promote efficient tissue regeneration, because GJIC plays an important role in increasing tissue strength.[39]

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Address reprint requests to:
Toshie Tsuchiya, Ph.D.
Division of Medical Devices
National Institute of Health Sciences
1-18-1 Kamiyoga
Setagaya-ku, Tokyo 158-8501, Japan

E-mail: tsuchiya@nihs.go.jp

F.Watari T.Akazawa M.Uo T.Akasaka

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In vitro study on the osteogenesis of normal human osteoblasts cultured on the discs of various kinds of calcium phosphate ceramics

Masato Tamai[1, a], Ryusuke Nakaoka[1,b] and Toshie Tsuchiya[1,c]

[1]Division of Medical Devices, National Institute of Health Science

1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501 Japan

[a]m-tamai@nihs.go.jp, [b]nakaoka@nihs.go.jp, [c]tsuchiya@nihs.go.jp

Keywords: Calcium phosphate ceramics, osteogenesis, cytotoxicity,

Abstract. We estimated effects of various CP ceramics on the properties of normal human osteoblasts (NHOst) as well as a viability of V79 fibroblasts. In the present study, five kinds of CP ceramics, namely, hydroxyapatite (HAp) flouroapatite (FAp), α-tricalcium phosphate (α-TCP), β-tricalcium phosphate (β-TCP) and tetracalcium phosphate (TTCP), were tested. Cytotoxicity test was carried out using V79 fibroblasts by colony assay system. The amounts differentiation level of NHOst was estimated from alkaline phosphatase (ALP) activity and osteocalcin.
From the results of colony assay, FAp and α-TCP showed strong cytotoxicities on V79 cells. The results from the proliferation studies of NHOst with CP ceramics were consistent with the results of colony assay. In addition, the ALP activities of NHOst with CP ceramics after 1week culture were significantly suppressed in comparison with that of NHOst alone. The osteocalcin amounts produced from NHOst cultured on β-TCP was the highest among five kinds of CP ceramics.

Introduction

Calcium phosphate (CP) ceramics have been studied to utilize as the scaffolds for repairing bone defects. For instance, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) or β-tricalcium phosphate (β-$Ca_3(PO_4)_2$, β-TCP), can be biologically bonded to natural bones and their porous materials are effective for restoration of bone defects [1]. 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 [2]. 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) or tetracalcium phosphate ($Ca_4(PO_4)_2O$, TTCP) are starting materials for the harden reaction of the bone cement [3].

To develop biomaterials for utilizing for bone tissue, various properties, e.g. biological, physical or chemical property, should be satisfied. Among them, biological safety and osteogenesis properties, e.g. proliferation and differentiation of the osteoblasts, should be important factors to provided to the biomaterials. However, understandings of the biological interaction between osteoblasts and various CP ceramics are few, since the interaction has not been studied under the same experimental condition in detail. Therefore, we estimated effects of various CP ceramics on the properties of normal human osteoblasts (NHOst) as well as a viability of V79 fibroblasts in this study.

Materials and Methods

Materials

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

Cytotoxicty test on CP ceramics

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

The method of cell seeding was shown below; At first, 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. Next, 50 cells/300μl of culture medium was added into each well and incubated for 4 h at 37°C. Finally, 400μl of culture medium was added and incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 7days.

In order to investigate the cell adhesive property on the CP ceramics, the culture medium was changed after cultivations for 4 h and incubated for 7days. The removed culture medium was transferred to another well of the 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 in 150rpm. The suspensions were centrifuged and the supernatants were collected to use as test extracts. The cytotoxicity test was carried out culturing 50 V79 cells in 1ml of the extracts and incubated at 37°C for 7days.

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

Osteogenesis evaluation of NHOst cultured on CP ceramics

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. 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. Similar to the method of the cytotoxicity test, each CP pellets were placed in 24-well culture plates (Corning Co. Ltd.) and 300μl of culture medium was added into each well, followed by addition of 1ml of cell suspension (4×10^4 cells/ml) into each well.

Proliferation of NHOst cells cultured on various kinds of CP ceramics 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 of 405 nm of glycine buffer was detected using μQuant spectrophotometer to evaluated alkaline phosphatase (ALP) activity of the test cells. The amount of Osteocalcin produced by NHOst was evaluated using Gla-type Osteocalcin EIT kit (Takara. Co., Ltd.). The structural change of CP before and after autoclave sterilization or 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

Cytotoxicity of various CP ceramics

The results of the cytotoxicity test of CPs are summarized in table 1. Notably, the colonies were hardly formed on FAp and α-TCP pellets and the ratios of the colony formation against V79-alone culture were 22.6% and 0.0%, respectively. In addition, the ratios of the colony formation on the HAp, β-TCP and TTCP pellets were 58.1%, 57.3% and 78.4%, respectively. From these results, it is suggested that V79 cells were viable and adhered on the pellet after for 4h after seeding despite of the type of CP ceramics, irrespective of the type of CP ceramics. On the other hand, the cytotoxicity test of extracts from CPs revealed that the tendency of their cytotoxicity was almost the same as that of the respective CP pellets themselves (table1).

Proliferation and differentiation of NHOst cultured on CP ceramics

The effects of various kinds of CP ceramics on the osteogenesis of NHOst are represented in table2. The effects of the CPs on proliferation were consistent with those on the colony formation. Similar to the cytotoxicity test, the proliferation of NHOst was inhibited on FAp and α-TCP pellets. ALP activities of NHOst on CP ceramics after 7-days culture were significantly suppressed in comparison with that of NHOst alone. On the other hand, the osteocalcin amounts produced from NHOst were influenced by the type of CP ceramics. NHOst on β-TCP showed the highest Osteocalcin production among five kinds of CP ceramics.

4. Discussion

The fact that less formation of colonies was observed on FAp and α-TCP pellets suggests that they are strongly cytotoxic. It is suggested that the differences in the colony formation on various CP pellets are ascribed to difference in extract properties from the CP related with the composition or crystal structure (table1). In addition, proliferation of NHOst also was inhibited on FAp and α-TCP. The pH values of culture medium after incubation for 7 days are shown in table1. As shown in the table, 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 medium is much lower than other CP ceramics. In order to considering the reason of the low pH of the culture medium with α-TCP pellet, a surface structural change of α-TCP before and after incubation was analyzed by SEM. SEM images of α-TCP after extraction treatment are shown in Fig.1.

Table1. Cytotoxicity test of various CP ceramics.

Samples	Composition	Formation of colony		pH of medium after culturing	Ca concentration[1] /ppm
		On pellets /%	Extraction / %		
V79 alone	-	100.0±4.5	100.0±13.9	7.12	-
HAp	$Ca_{10}(PO_4)_6(OH)_2$	58.1±12.8	84.6±15.1	7.24	0.19
FAp	$Ca_{10}(PO_4)_6F_2$	22.6±20.9**	26.9±8.6*	7.20	0.17
α-TCP	α-$Ca_3(PO_4)_2$	0.0*	7.6±5.1*	6.76	72.62
β-TCP	β-$Ca_3(PO_4)_2$	57.3±6.9	81.1±19.3	7.40	1.27
TTCP	$Ca_4(PO_4)_2O$	78.2±5.0	93.7±6.8	7.65	0.58

*p<0.01 against V79 alone, **p<0.05 against V79 alone, 1)The Ca ions concentration was extracted Ca ions from CP-ceramics in PBS(-), which were measured by inductivity coupled plasma-atomic emission spectroscopy.

Table2. Osteogenesis of NHOst cultured on various kinds of CP ceramics.

Samples	Proliferation / %	Differentiation level	
		ALP activity / %	Osteocalcin / %
NHOst alone	100±7.9	100±4.4	100±46.2
HAp	63.2±3.5	20.4±1.8*	81.1±31.0
FAp	42.9±19.5	1.24±0.3*	47.2±20.7
α-TCP	18.3*±2.5	17.9±3.8*	110.7±18.8
β-TCP	56.0±4.7	6.3±3.2*	177.1±78.4**
TTCP	82.3±27.3	17.5±4.6*	114.8±4.0

*p<0.01 against NHOst alone, **p<0.01 against HAp

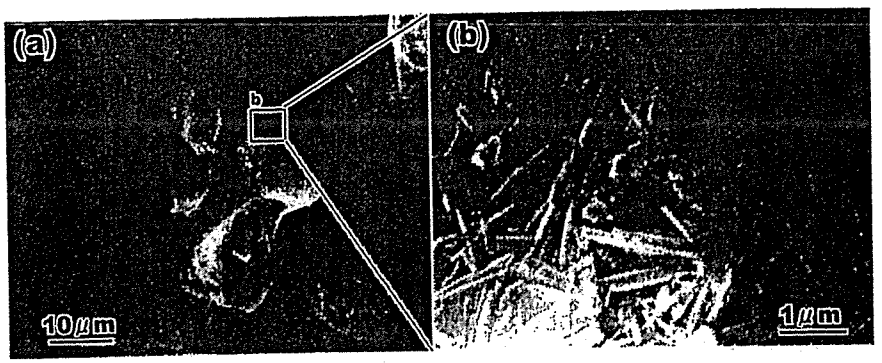

Fig.1. SEM images of α-TCP after extract treatment. (a) whole image and (b) enlarged image of the area enclosed by a rectangle in (a)

Before the extraction, a particle size of α-TCP was about 10μm and its surface was smooth. However, as shown in Fig.1, whisker-like precipitates of 1-2μm in length and 2-300nm in width are observed at the surface of α-TCP after the extraction, although there is no change in its particle size. It is well known that slightly water-soluble 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, the α-TCP converts to HAp rapidly as following the reaction.

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

According to the report of this conversion [4], HAp produced by the above reaction has whisker-like morphology. Therefore, the whisker-like precipitates can be regarded as HAp, so that it is considered that the above conversion occurs at the surface of the α-TCP during incubation. In this case, phosphoric acid is produced as a byproduct in the conversion reaction and the phosphoric acid caused the decrease in the pH of solution. Morita and co-workers [5] 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. Therefore, it is suggested that the cytotoxicity of α-TCP was mainly due to the pH decreasing resulting from an increase of the phosphoric acid ion produced 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 difference of the colony formation on various CP ceramics would be due to eluted substances from CP as described above, the cytotoxicity of FAp would be due to eluted fluoride ions from FAp.

Effects of CP ceramics on osteogenesis function of NHOst are shown in table2. As shown in the table, ALP activities of NHOst were significantly suppressed on CP ceramics irrespective of their type and the amount of osteocalcin on β-TCP was the highest among five kinds of CP ceramics. Since it is well known that osteocalcin express in maturated stage of differentiation level of NHOst, these results suggest that maturation of NHOst proceeds on β-TCP. . The differences of maturation of NHOst on various kinds of CP ceramics may be related with the amount of extracted Ca^{2+} and/or PO_4^{3-} ions.

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

Masato Tamai[1a], Ryusuke Nakaoka[1b], Kazuo Isama [1c] and Toshie Tsuchiya[1d]
[1]Division of Medical Devices, National Institute of Health Sciences,
1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501 Japan
[a]m-tamai@nihs.go.jp, [b]nakaoka@nihs.go.jp, [c]isama@nihs.go.jp, [d]tsuchiya@nihs.go.jp

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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].