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Original article

Impacts of chemically different surfaces of implants on a biological activity of fibroblast growth factor-2–apatite composite layers formed on the implants

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ABSTRACT

Background. – Implants coated with fibroblast growth factor-2 (FGF-2)–apatite composite layers were previously reported to enhance soft-tissue formation, bone formation, and angiogenesis around the implants owing to the biological activity of FGF-2. However, it is unclear whether the chemistries of the material and surface of implants have some impact on the retention of the biological activity of FGF-2 in FGF-2 – apatite composite layers on them. Since magnitude of the impact should be evaluated for extensive application of the composite layer to coat various implants, following items were examined; (1) surface chemistries of six implants, (2) mitogenic activities of FGF-2 in FGF-2–apatite composite layers on the implants, and (3) improved synthesis method of the composite layer for retention of the mitogenic activity of FGF-2.
Hypothesis. – The biological activity of FGF-2 in the composite layer is affected by the chemistries of the material and surface of implants.
Materials and methods. – Six commercial products of pins and screws having different surface chemistries were coated with FGF-2–apatite composite layers. The composite layers were quantitatively analyzed for calcium (Ca), phosphorus (P) and FGF-2, and also evaluated the mitogenic activities of FGF-2. Improvement of the synthesis method was then attempted using two pin products.
Results. – Each commercial product had a chemically and morphologically characteristic surface. FGF-2–apatite composite layers were formed on all the commercial products. Although the Ca, P, and FGF-2 contents (4.7 ± 0.9 μg/mm, 2.2 ± 0.4 μg/mm, and 21.1 ± 3.7 ng/mm, respectively) and the Ca/P molar ratios (1.69 ± 0.01) of the composite layers were almost the same, rate of retention of the mitogenic activity of FGF-2 in the composite layers significantly decreased on some pin products (3/12–4/12). The decrease in rate of retention of the mitogenic activity of FGF-2 was prevented by a two-step synthesis method to form a composite layer on a precoating with calcium phosphate (9/12–12/12).
Discussion. – The chemistries of the implant surfaces had a significant impact on the retention of the mitogenic activity of FGF-2 in the composite layers formed on the implant. The two-step synthesis method was useful to retain mitogenic activity of FGF-2 regardless of the surface chemistries of the implants. The two-step synthesis method has potential to expand the applicability of FGF-2–apatite composite layers to a wider range of implants.
Level of evidence. – III, Case control in vitro study.

1. Introduction

Fixation and stabilization of pins and screws in bone and soft tissues are enhanced by coatings of calcium phosphates [1,2]. Pins and screws loaded with biologically active molecules including bone morphogenetic protein-2 and fibroblast growth factor-2 (FGF-2)

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are also promising for their fixation and stabilization [3,4] since appropriately immobilized growth factors on a material surface retain their biological activities [3–5]. FGF-2 accelerates wound healing and bone tissue regeneration [6–8]. The use of screws coated with the FGF-2 – apatite composite layer significantly reduces the risk of impaired bone apposition to the screws [9]. The use of pins coated with the composite layer notably tended to prevent pin tract infections in external fixation [3,10,11], whereas surface modification is generally applied for direct elimination of bacteria from the surface [12]. The enhanced fixation and stabilization of the pins and screws is partly due to the biological activity of FGF-2.

However, it is unclear whether the chemistries of the material and surface of pins and screws have some impact on the retention of the biological activity of FGF-2 in FGF-2–apatite composite layers on them. The mitogenic activity of FGF-2 is influenced by the chemistry of the calcium phosphate matrix in which FGF-2 is embedded [10]. Thus, it is natural that the biological activity of FGF-2 is influenced by the chemistries of the material and surface that are in contact with FGF-2. To expand the clinical applicability of FGF-2–apatite composite layers, it is necessary to understand the impact of the chemistries of the material and surface of commercial products on the biological activity of FGF-2 in the composite layers. Different pin and screw products have different material chemistries [13,14]. Thus, the questions that have driven the present study are as follows:

- do different commercial pin and screw products have the same surface chemistries if their bulk chemistry is the same?
- do differences in surface chemistry affect mitogenic activity of FGF-2 in the composite layer on the surface?
- how we modify the synthesis method of FGF-2–apatite composite layers if material and surface chemistry negatively impact on the mitogenic activity of FGF-2?

2. Materials and methods

2.1. Formation of FGF-2–apatite composite layer or apatite layer

Four half pin products (Pin1, Pin2, Pin3, and Pin4 groups) and two pedicle screw products (Screw1 and Screw2 groups) (Table 1) were sterilized with ethylene dioxide. Then the pins and screws were coated with FGF-2–apatite composite layers in 100 mL of supersaturated calcium phosphate (sCaP) solutions containing FGF-2. In the one-step synthesis method [3,11], three pins or screws were immersed in the sCaP solution containing 400 μg of FGF-2 at 37 °C for 48 hours. In the two-step synthesis method, three pins were immersed in the sCaP solution free of FGF-2 at 37 °C for 3 hours, air-dried for 30 min, and immersed in the sCaP solution containing 400 μg of FGF-2 at 37 °C for another 48 hours. As the negative control, three half pins that were the same as the Pin2 group except the diameter (3 mm) were immersed in the sCaP solution free of FGF-2 at 37 °C for 48 hours to form an apatite layer.

2.2. Sample preparation for chemical analysis and cell proliferation assay

The FGF-2–apatite composite and the apatite layers were individually dissolved aseptically in 10 mM sodium citrate buffer (2 mL) at pH5.43. The resulting solutions, designated as the "FGF-2–apatite extract" and "apatite extract," respectively, were analyzed for FGF-2 by the Bradford method, for Ca and P by inductively coupled plasma atomic emission spectrometry (PS7800, Hitachi High-Tech Science Corporation, Japan) [10], and for mitogenic activity of FGF-2.

2.3. Cell proliferation assay

The mitogenic activity of FGF-2 was evaluated using mouse NIH3T3 cells that are frequently used in bioassay for FGF-2 [10,15,16]. NIH3T3 cells (RIKEN BioResource Research Center, Japan) were assessed for their FGF-2-sensitivity on mitogenic activity in every assay (Fig. 1). 2×10^3 or 3×10^3 of the cells were precultured in 70 μL of serum-free medium prepared by mixing reagents (Table 2) using a 96-well plate for 24 hours. Then, 30 μL of either FGF-2–apatite or apatite extract was added to the culture. After 72-hour culture, cell proliferation was determined using a cell counting kit (CCK-8 kit, Dojindo Laboratories, Japan) following the manufacturer's instruction. Relative proliferation, basically following the normal distribution, was defined as the ratio of the absorbance at 450 nm of the culture with the FGF-2–apatite extract to that with the apatite extract. When a relative proliferation was significantly higher than 1, FGF-2 in the extract was regarded to be mitogenically active. After the cell proliferation assay, the pins and screws were washed with 10 mM sodium citrate buffer for reuse. A series of experimental runs from 2.1 to 2.3 were repeated four times. Accordingly, the total number of pins and screws in each group was 12.

2.4. Surface analysis of pins and screws

Uncoated pins and screws were observed by scanning electron microscopy (SEM, XL30, FEI Company, Japan; or JSM-7400F, JEOL, Japan) with energy dispersive X-ray spectrometry (EDX, Genesis 2000; EDAX Japan).

2.5. Statistical analysis

The Ca, P, and FGF-2 content and the Ca/P molar ratios were analyzed by one-way ANOVA with the post hoc Tukey HSD test. The one-sample *t*-test was employed to compare the relative proliferation value with 1. The rate of retention of the mitogenic activity of FGF-2 was analyzed using the Chi^2 test of independence followed by the two-sided Fisher's exact test. The null hypothesis stated that

Table 1
Characteristics of pins and screws.

Group	Material	Trademark (Producer)	Size		Elements in surface oxide layer
			Diameter	Length	
Pin1	Ti6Al4V	Apex Pin (Stryker)	5.0	80[a]	Ti, Al, V, O
Pin2	Pure Ti	SELDRILL™ (Synthes)	5.0	80[a]	Ti, O, P
Pin3	Ti6Al4V	JET-X™ (Smith & Nephew)	5.0	80[a]	Ti, Al, V, Na, Mg, Si, Ca, O
Pin4	Stainless steel	Apex Pin™ (Stryker)	5.0	80[a]	Fe, Cr, Ni, Mo, Si, O
Screw1	Ti6Al4V	Expedium™ (DePuy Synthes)	5.0[b]	35	Ti, Al, V, O
Screw2	Ti6Al4V	Solera™ (Medtronic)	5.5[b]	35	Ti, Al, V, Na, Mg, Si, Ca, O
Negative control	Pure Ti	SELDRILL™ (Synthes)	3.0	80[a]	Ti, O, P

[a] Cut into 80 mm length.
[b] Thread diameter.

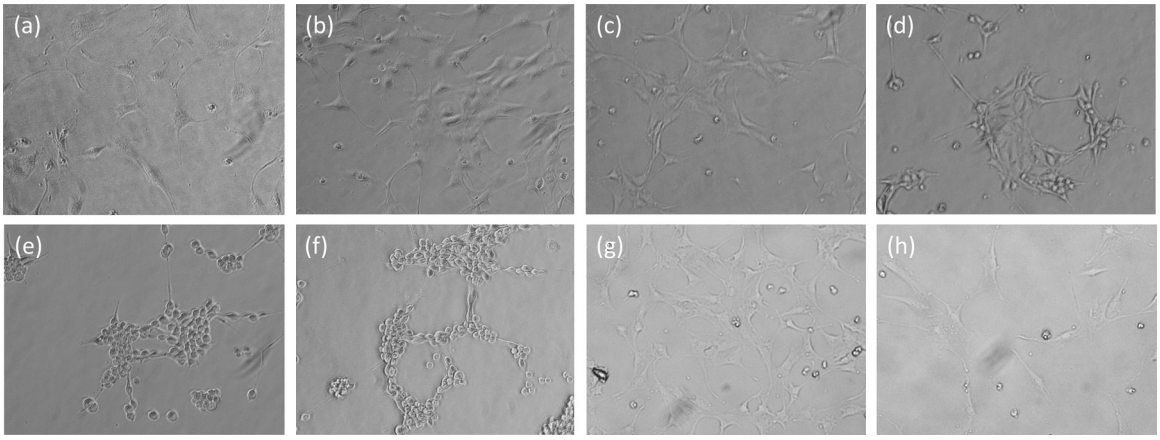

Fig. 1. Typical appearance of NIH3T3 cells; after FGF-2-sensitivity assessment at (a) 0, (b) 1, (c) 5, (d) 10, (e) 50 and (f) 100 FGF-2 ng/mL; after cell proliferation assay with (g) FGF-2–apatite and (h) apatite extract.

Table 2
Mixing ratio for preparation of serum-free medium.

Reagent	Manufacturer	Concentration	Mixing volume
Dulbecco's modified essential medium	Wako Pure Chemicals Industries, Japan	–	39.4 mL
l-glutamine	MP Biomedicals, USA	30 mg/mL	0.4 mL
bovine serum albumin	Thermo Fisher Scientific, USA	200 mg/mL	0.2 mL
insulin	Wako Pure Chemicals Industries, Japan	4 mg/mL	50 μL
transferrin	Merck Millipore Corporation, USA	4 mg/mL	10 μL

the mitogenic activity of FGF-2 is equally likely to be retained on the six commercial products. A value of $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Surface analysis of pins and screws prior to coating

Each pin or screw group had chemically and morphologically different surfaces (Fig. 2, Table 1). Agreeing with description in their package inserts, Ti, Al, and V (Pin1, Pin3, Screw1, and Screw2), Ti (Pin2) and Fe, Cr, Ni, Mo, Si, and Mn (Pin4) were detected in their EDX spectra. An O Kα peak appeared in the spectra of all the products. Unexpected elements like P (Pin2), Na, Mg, Si, and Ca (Pin3 and Screw2), and Si (Screw1) were also detected. Their surface morphologies appeared characteristic as lamellar-like (Pin1), uneven (Pin2, Pin3, Screw1 and Screw2), and quite smooth (Pin4).

3.2. Quantitative analysis of calcium, phosphorus, and total protein

The FGF-2 contents and Ca/P molar ratios of FGF-2–apatite composite layers were similar among the pin or screw groups (Fig. 3a, b). The averaged Ca, P, and FGF-2 contents were proportional to

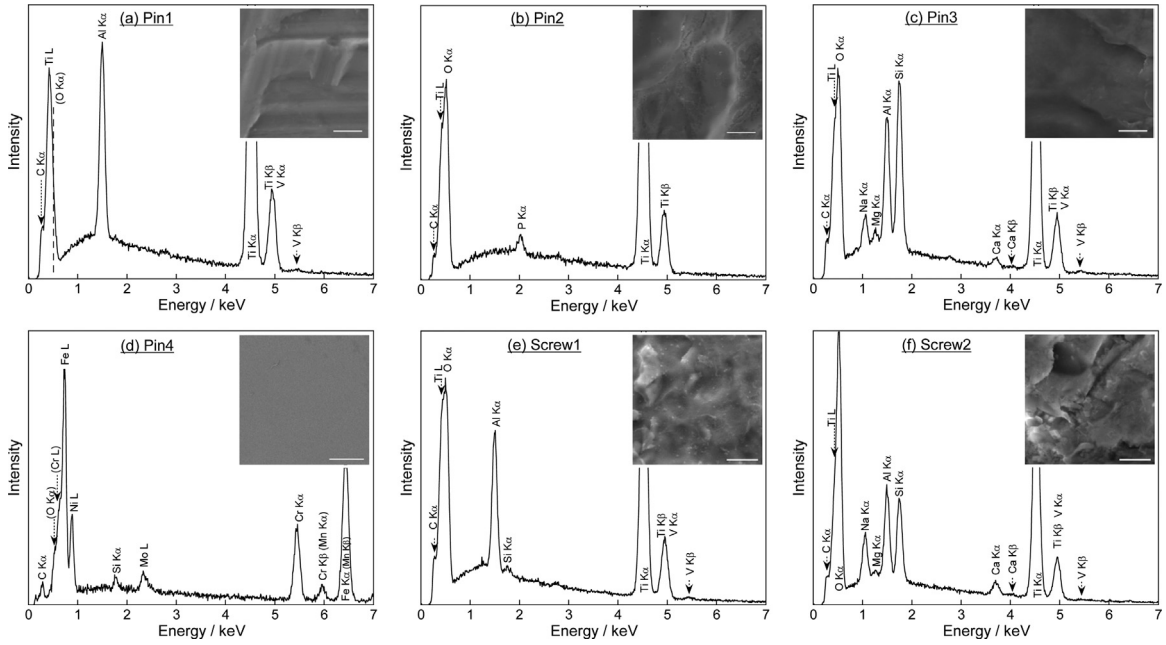

Fig. 2. EDX spectra and SEM photographs of the surfaces of (a) Pin1, (b) Pin2, (c) Pin3, (d) Pin4, (e) Screw1, and (f) Screw2. The white bar in SEM photo indicates 1 μm.

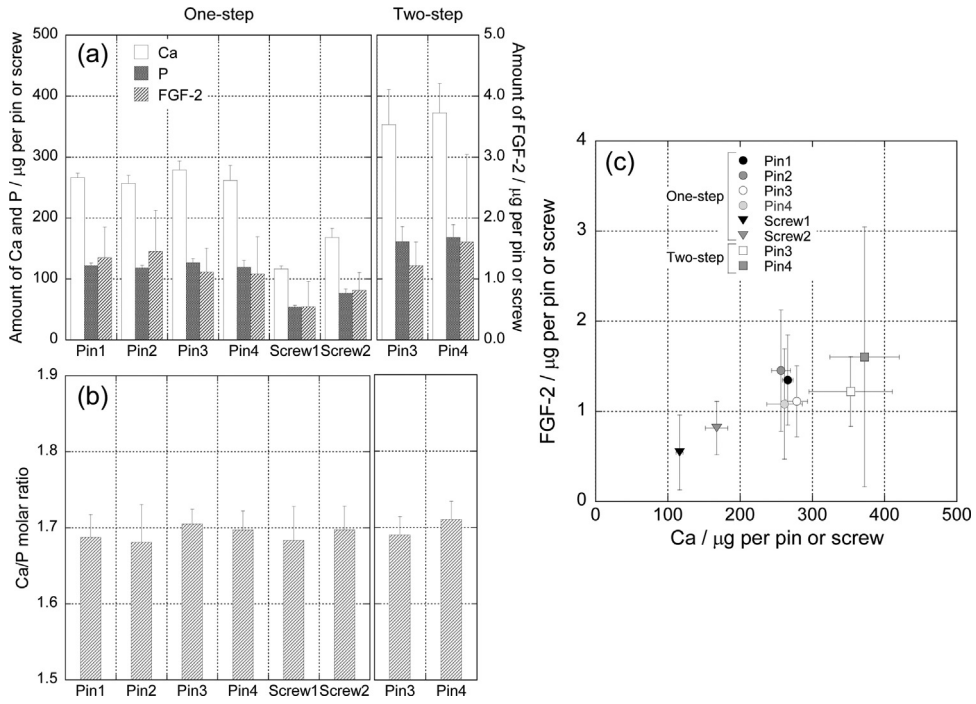

Fig. 3. Compositions of FGF-2–apatite composite layers, (a) Ca, P, and FGF-2 contents, (b) Ca/P molar ratio, and (c) overall relationship between the Ca and FGF-2 contents.

the coated lengths of the pins and screws (60 and 35 mm, respectively), or 4.7 ± 0.9 μg/mm, 2.2 ± 0.4 μg/mm, and 21.1 ± 3.7 ng/mm, respectively. Although positive correlation was observed between the Ca and FGF-2 contents (Fig. 3c), the FGF-2 contents showed no significant differences within the pin or screw groups. The Ca/P molar ratios of the FGF-2–apatite composite layers of all the groups (1.69 ± 0.01) corresponded to that of hydroxyapatite (1.67, Fig. 3b).

3.3. Cell proliferation assay

NIH3T3 cells changed their shape and number after culturing with not apatite but FGF-2–apatite extract (Fig. 1g, h). Twelve relative proliferation values were obtained for each pin and screw group without missing data (Table 3). The rate of retention of the mitogenic activity of FGF-2 significantly varied ($p < 0.0001$ in the chi-square test) among the pin and screw groups in the one-step synthesis method (from 3/12 to 12/12 [Fig. 4]). The rates of retention of the mitogenic activity of FGF-2 of Pin3 (4/12) and Pin4 (3/12) were significantly lower than those of Pin2 and Screw2 (12/12). By the two-step synthesis method, those rates rose to 9/12 and 12/12, respectively (Table 3).

4. Discussion

The six commercial products of pins and screws had different surface chemistries even if their bulk chemistry was the same. The differences in surface chemistry affected mitogenic activity of FGF-2 in the composite layer on the surface of the products. The composite layers synthesized by the two-step synthesis method demonstrated the high rates of retention of the mitogenic activity of FGF-2 even though the material and surface chemistry had negative impacts on the mitogenic activity of FGF-2.

Different pins and screws have different chemistries of their material and surface (Fig. 2). Pin1 and Pin4 had naturally formed and relatively thin oxide layers, as evidenced by presence of a weak O Kα peak in the EDX spectra. Pin2 and Screw1 had intentionally formed thick oxide layers, as evidenced by their interference colors and a relatively intense O Kα peak. The thicknesses of the intentionally and naturally formed titanium oxide layers are more than 40 nm and less than 10 nm [17], respectively. The thickness of a passive oxide layer on stainless steel was about 3 nm [14]. Pin3 and Screw2 had thick bioactive glass-like oxide layers that contained O, Na, Mg, Si, and Ca [18].

This study suggests that the chemistries of the materials and surfaces have impacts on the mitogenic activity of FGF-2 in the FGF-2–apatite composite layers. In this study, the Ca, P, and FGF-2 contents in the composite layers had no relation to the rate of retention of the mitogenic activity of FGF-2. Although the mitogenic activity of FGF-2 is known to vary according to the Ca/P molar ratio of the FGF-2–apatite composite layer [10], the Ca/P molar ratios of the composite layers were essentially the same among the groups. Thus, the major difference among the groups that can impact on the mitogenic activity of FGF-2 was the chemistries of the material and surface oxide layer. The oxide layer on titanium and titanium alloy (Pin1, Pin2, and Screw1) showed a higher level of retention of the mitogenic activity of FGF-2 than the oxide layer on stainless steel (Pin4) despite the comparable FGF-2 contents. Previously, FGF-2–apatite composite layers on pure titanium or titanium alloy having surface titanium oxide layers containing P successfully promoted cell proliferation *in vitro* [3,10,19,20]. Although titanium oxide layers induce apatite layer formation in simulated body fluid [21,22], it is unclear whether this apatite-forming ability is related to the higher rate of retention of the mitogenic activity of FGF-2 compared with the oxide layer on stainless steel. A complex interplay of growth factors as well as adhesion and structural proteins enables optimal cellular adhesion and proliferation [5]. The bioactive glass-like oxide layers on titanium alloy (Pin3 and Screw2) showed divergent impacts on the retention of the mitogenic activity of FGF-2. This diversion of impacts could result from the differences in Na/Si and Na/Ti ratios between their surfaces.

The two-step synthesis method is effective for preventing the reduction of the biological activity of FGF-2. The difference between the two-step and one-step synthesis methods is the presence and absence, respectively, of a precoating on the implants with calcium phosphate, which was inspired by previous studies [19,23,24], prior to coating with an FGF-2–apatite composite layer. The composite

Table 3
Results of cell proliferation assays for FGF-2–apatite composite layers.

Synthesis method	Substrate		Assay								Rate of retention of the mitogenic activity of FGF-2
			1st		2nd		3rd		4th		
	Group	No.	Relative proliferation	*p*-value*	Relative proliferation	*p*-value*	Relative proliferation	*p*-value	Relative proliferation	*p*-value	
One-step	Pin1	#1	0.71 ± 0.20	0.9991	1.23 ± 0.44	0.0641	3.91 ± 1.76	0.0003	3.12 ± 1.04	0.0001	7/12
		#2	0.69 ± 0.16	0.9999	1.32 ± 0.58	0.0550	3.33 ± 1.51	0.0004	2.90 ± 1.05	0.0001	
		#3	0.85 ± 0.20	0.9791	1.57 ± 0.58	0.0063	2.13 ± 0.98	0.0026	2.50 ± 0.85	0.0002	
	Pin2	#1	1.67 ± 0.68	0.0064	1.66 ± 0.73	0.0095	3.85 ± 2.07	0.0009	2.41 ± 0.88	0.0004	12/12
		#2	1.49 ± 0.52	0.0079	2.62 ± 1.78	0.0090	2.07 ± 1.43	0.0209	2.15 ± 0.86	0.0011	
		#3	1.30 ± 0.34	0.0106	3.04 ± 1.75	0.0025	1.88 ± 1.17	0.0208	2.34 ± 0.86	0.0004	
	Pin3	#1	1.20 ± 0.35	0.0539	1.07 ± 0.30	0.2403	1.42 ± 1.07	0.1235	3.22 ± 1.29	0.0002	4/12
		#2	1.10 ± 0.23	0.1007	1.20 ± 0.41	0.0768	1.49 ± 0.74	0.0319	2.79 ± 1.17	0.0005	
		#3	1.02 ± 0.22	0.3772	1.02 ± 0.33	0.3969	1.05 ± 0.44	0.3644	2.41 ± 0.98	0.0007	
	Pin4	#1	0.88 ± 0.17	0.9664	1.14 ± 0.19	0.0212	0.92 ± 0.10	0.9753	1.00 ± 0.12	0.4671	3/12
		#2	1.00 ± 0.21	0.4842	1.22 ± 0.21	0.0051	1.00 ± 0.17	0.4421	1.07 ± 0.16	0.0944	
		#3	0.84 ± 0.20	0.9806	1.11 ± 0.18	0.0342	0.97 ± 0.09	0.8204	0.96 ± 0.17	0.7487	
	Screw1	#1	1.20 ± 0.22	0.0077	1.51 ± 0.16	0.0000	1.62 ± 1.43	0.0997	2.50 ± 0.57	0.0000	10/12
		#2	1.14 ± 0.19	0.0206	1.60 ± 0.23	0.0000	1.58 ± 1.44	0.1167	2.70 ± 0.55	0.0000	
		#3	1.34 ± 0.28	0.0017	1.46 ± 0.22	0.0001	2.18 ± 1.92	0.0412	2.75 ± 0.52	0.0000	
	Screw2	#1	1.24 ± 0.13	0.0001	1.54 ± 0.13	0.0000	2.64 ± 0.90	0.0001	2.51 ± 0.57	0.0000	12/12
		#2	1.62 ± 0.20	0.0000	1.63 ± 0.18	0.0000	1.80 ± 0.51	0.0004	2.71 ± 0.56	0.0000	
		#3	1.31 ± 0.14	0.0000	2.35 ± 0.29	0.0000	3.07 ± 0.89	0.0000	2.76 ± 0.53	0.0000	
Two-step	Pin3	#1	1.54 ± 0.27	0.0001	1.62 ± 0.26	0.0000	1.38 ± 0.20	0.0001	0.77 ± 0.30	0.9774	9/12
		#2	1.71 ± 0.23	0.0000	1.67 ± 0.30	0.0000	1.42 ± 0.19	0.0000	0.61 ± 0.14	1.0000	
		#3	1.52 ± 0.20	0.0000	1.75 ± 0.30	0.0000	1.48 ± 0.20	0.0000	0.85 ± 0.21	0.9709	
	Pin4	#1	1.52 ± 0.12	0.0000	1.30 ± 0.10	0.0000	1.19 ± 0.27	0.0268	1.34 ± 0.25	0.0012	12/12
		#2	1.70 ± 0.17	0.0000	1.41 ± 0.12	0.0000	1.26 ± 0.43	0.0439	1.40 ± 0.26	0.0005	
		#3	1.61 ± 0.13	0.0000	1.39 ± 0.11	0.0000	1.28 ± 0.39	0.0257	1.36 ± 0.26	0.0009	

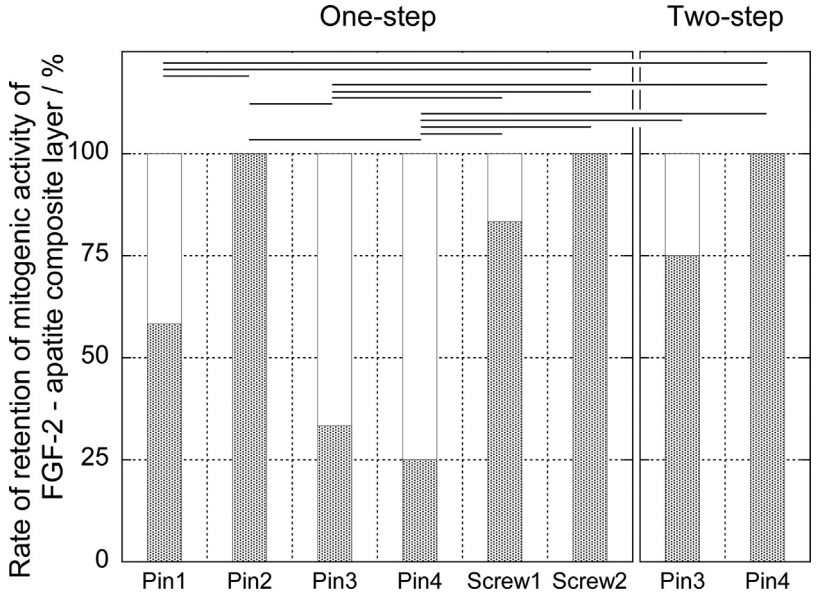

Fig. 4. Rate of retention of mitogenic activity of FGF-2 in FGF-2–apatite composite layers (shaded bars). Horizontal solid lines indicate significant differences between the groups.

layers formed on hydroxyapatite retained the biological activity of FGF-2 *in vivo* [25]. Thus, the improvement in the rate of retention of the mitogenic activity of FGF-2 in the two-step synthesis method is attributed to the presence of the interfacial calcium phosphate layer between the implant surface and the composite layer. The two-step synthesis method expands the applicability of FGF-2–apatite composite layers to a wider range of implants.

Further studies are required including those on osseointegration activity of FGF-2, those using direct cell culture on the composite layers, and those on the mechanism. The use of only cell proliferation assay is a limitation. Impacts of chemically different surfaces on osseointegration activity of FGF-2 are yet to be clarified in *in vivo* studies. Cell proliferation assay directly on the FGF-2-apatite composite layers could be closer to *in vivo* conditions. The mechanisms how chemistries of the materials and surfaces influence the mitogenic activity of FGF-2 is yet to be clarified.

5. Conclusion

FGF-2–apatite composite layers were formed on the six commercial products having different chemistries of their material and surface. The varied rates of retention of mitogenic activities of

FGF-2 suggested influence of the difference in chemistries of the product surfaces. When the two-step synthesis method was appropriately applied, the rate of retention of the mitogenic activity of FGF-2 was improved. The two-step synthesis method has potential to expand the applicability of FGF-2–apatite composite layers to a wider range of implants with various surface chemistries.

Disclosure of interest

The authors declare that they have no competing interest. Outside the current study, Yu Sogo, Fumiko Kobayashi and Atsuo Ito received grants and other from Cell-Medicine Inc. Japan, and have patents application number 2019-015509 and PCT/JP2020/003285 pending. Outside the current study, Kengo Fujii, Yohei Yanagisawa, Shinji Murai, Hirotaka Mutsuzaki, Yuki Hara and Masashi Yamazaki received grants from Cell-Medicine Inc. Japan

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Contributions

Yu Sogo, Hirotaka Mutsuzaki, Yuki Hara, Masashi Yamazaki and Atsuo Ito were responsible for the study design and conception. Each experimental procedure in this study was established by Yu Sogo, Kengo Fujii, Yohei Yanagisawa, Fumiko Kobayashi, Shinji Murai, and Atsuo Ito. Kengo Fujii, Yohei Yanagisawa, Fumiko Kobayashi, and Shinji Murai performed all the experiments. Yu Sogo, Fumiko Kobayashi, and Atsuo Ito analyzed the data. All authors have read and approved the final submitted manuscript.

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