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Cellular responses of histatin-derived peptides immobilized titanium surface using a tresyl chloride-activated method

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Effects of histatin-derived peptides immobilization by tresyl chloride-activation technique for MC3T3-E1 cellular responses on titanium (Ti) were evaluated. MC3T3-E1 were cultured on sandblasted and acid-etched Ti disks immobilized with histatin-derived peptides, including histatin-1, JH8194, and mixed histatin-1 with JH8194. Surface topography and cellular morphology were examined using a scanning electron microscope. Elemental composition and conformational peptides on Ti surface were examined using energy dispersive X-ray and fourier transform infrared spectroscopy, respectively. Cellular adhesion, proliferation, osteogenesis-related genes, and alkaline phosphatase activity were evaluated. The results showed that peptides were successfully immobilized on Ti surface. Cell attachments on histatin-1 and mixed peptides coated groups are higher than control. Histatin-1 achieved the significantly highest cellular proliferation. Histatin-derived peptides improved the osteogenesis related-gene expression and alkaline phosphatase activity ($p<0.05$). This study suggested that histatin-1 immobilization by tresyl chloride-activation technique enhanced cellular responses and might be able to promote cellular activities around the dental implants.

Keywords: Osteoblast-liked cells, Histatin-derived peptide, Titanium

INTRODUCTION

Over the past 20 years, the number of patients treated with dental implants has dramatically increased because it gives the most natural-looking teeth and has shown more satisfaction than other prosthesis groups[1]. Although the success rate of implants was high, the early failure was still at 4%[2]. Therefore, the implant surface treatment has been developed to reduce the rate of early implant failure by improving the bone healing process, getting early fixation and direct attachment to bone[3,4]. Titanium is material of choice for dental implants due to its compatibility with body tissue and achieves anchoring within the bone tissue by direct contact from the bone to the implant, so-called osseointegration[5].

Biochemical surface treatment by immobilization of bioactive molecules is another way to improve the surface providing direct biochemical and biophysical signals, which can regulate the differentiation of a cell. Tresyl chloride-activated method has been proposed as an easy technique without the need for pretreatment. Tresyl chloride-activated method contributes to immobilize peptides even in polished Ti disks[6]. Moreover, tresyl chloride-activated method is a wet process. Therefore, we can immobilized the proteins or cytokines[4] on the any shape of titanium such as Ti disks[7], dental implant[8], Ti web, and Ti wire[9]. In several previous studies, tresyl chloride were used to immobilize proteins such as fibronectin (molecular weight=270–440 kDa). Fibronectin-immobilized Ti surface enhanced ALP activity of MC3T3-E1 cells after 3, 15, and 52 days[10]. Furthermore, fibronectin-derived peptides (GRGDSP, PHSRN, and mixed GRGDSP with PHSRN[11,12]) and recombinant human TGF-β2 proteins (molecular weight=25 kDa)[4] were immobilized on Ti surfaces with the same technique. They found that proteins and peptides immobilized with tresyl chloride on Ti surface increased cellular responses such as cell adhesion, proliferation, and differentiation[6,8-10,13]. From the reasons mentioned above, in this study, we used tresyl chloride to immobilize histatin-derived peptides (histatin-1 and JH8194 peptides).

Histatins are small histidine-rich polypeptides, which can be isolated from human parotid saliva[14]. Histatins have antimicrobial and antifungal properties[15] by the concentration of histatin peptides in the saliva at the range of 50–425 μg/mL and there are many natural histatin in saliva such as histatin-1, histatin-3, and histatin-5[16,17].

Histatin-1 structure consists of 38 amino acids and phosphorylated serine at number 2 residue, which contributes to indirect wound healing by stimulating the migration of the epithelium[18-21]. Recently, Van Dijk *et al.* immobilized histatin-1 peptide on Ti-surface and they found that histatin-1 improved osteoblast cellular attachment[22]. JH8194 are new histatin-derived

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peptides with alpha-helical structure and having similar amphipathic properties, which enhances osteoblast adhesion and differentiation[23]. Moreover, Makihira *et al*. also found that JH8194 peptide promote bone formation around the dental implants[24]. However, the comparison between histatin-1 and JH8194 peptides on osteoblast differentiation have not been studied. Therefore, we are the first group to demonstrate that histatin-1 is the most effective on promoting osteoblast cellular responses compared with JH8194 and mixed histatin-1 with JH8194.

The purpose of this study was to examine the effects of the immobilization of histatin-derived peptides group, consisting of histatin-1, JH8194 and mixed histatin-1 with JH8194 peptides on adhesion, proliferation, and differentiation of osteoblast-like cells on a Ti surface.

MATERIALS AND METHODS

Titanium disks preparation

Commercially pure Ti disks ASTM grade 4 (diameters of 8 mm and 20 mm with thickness of 1 mm) (National Metal and Material Technology Center (MTEC), Pathum Thani, Thailand) were used as the substrate materials for surfaces modification. The samples were sandblasted by aluminum (Al_2O_3) particle (65 μm in size, the pressure of 5 bar), followed by completely immersed in sulfuric acid (H_2SO_4) and hydrochloric acid (HCl) for 90 s (PW PLUS, Nakhon Pathom, Thailand). As a control group, untreated Ti disks were employed. The disks were sterilized using ethylene oxide gas after sandblast and acid-etched.

Synthesis of peptides

Histatin-1 (DSpHEKRHHGYRRKFHEKHH SHREFPFYGDYGSNYLYDN)[25] and JH8194 (KRLFRRWQWRMKKY)[26] peptides were synthesized (Prima scientific company, Bangkok, Thailand).

Immobilization of histatin-derived peptides by tresyl chloride activation technique and surfaces analysis

The Ti disks were completely covered with tresyl chloride (Sigma Aldrich, St. Louis, MO, USA) in 37°C for 2 days, then washed with distilled water followed by water-acetone solution and dried with desiccator. Histatin-1, JH8194, and mixed histatin-1 with JH8194 peptides at ratio 1:1 were reconstituted as the manufacturer's advice. Tresylated Ti disks were immersed in 10 μM peptides solution at 37°C for 1 day[7]. Ti disks were washed twice with phosphate buffered saline (PBS) and used for entire experiments. The Ti surfaces were examined using a scanning electron microscopy (SEM) (S-3400N, HITACHI®, Tokyo, Japan)[27]. Elemental analysis of Carbon, Nitrogen, Oxygen, Aluminum, and Titanium on the surfaces after immobilization with histatin-derived peptides using an energy dispersive X-ray (EDX) (EDAX TEAM, EDAX, Tokyo, Japan).

FTIR study

The immobilization of peptides on Ti disks was analyzed using fourier transform infrared spectroscopy (FTIR)-attenuated total reflectance (ATR) (Nicolet iS5, Thermoscientific, Waltham, MA, USA). FTIR spectra from 400–4,000 cm^{-1} with a resolution of 4 cm^{-1} at room temperature were recorded. Peaks at 679 cm^{-1} and 1,590 cm^{-1} representing amino groups on the Ti disks[28, 29].

Cell culture

To evaluate the cellular response on Ti disks, MC3T3-E1 cells (CRL-2593, ATCC, Manassas, VA, USA) were cultured in Alpha Minimum Essential Medium; α-MEM (Sigma Aldrich) supplemented with 2 mM Glutamine, 10% Fetal Bovine Serum (FBS), 100 mg/mL penicillin/streptomycin, and 0.25 mg/mL amphotericin B (Sigma Aldrich). To analyze the differentiation of osteoblast, MC3T3-E1 cells were cultured in osteogenic medium consisted of 50 μg/mL ascorbic acid and 10 mM ß-glycerophosphate (Sigma Aldrich).

Cell adhesion assay

MC3T3-E1 cells were cultured on peptides immobilized Ti disks (5 mm in diameter) at the density of 10^5cells/cm^2 [30]. After 30 min, the disks were washed with PBS solution to remove the non-adherent cells. Total adhered cells were trypsinized and counted using a cell counter (Z-series Coulter Counter, Beckman Coulter, Brea, CA, USA). Untreated Ti disk was used as a control (n=3, for each group).

Cell proliferation assay

MC3T3-E1 cells were seeded on the peptides immobilized and untreated Ti disks (5 mm in diameter) at the density of 5×10^5 cells/cm^2. After 3 and 7 days of culture, cell proliferation was performed by MTT assay. Briefly, the cells were rinsed with PBS, the 3-[4,5-dimethylthiazol]-2, 5-diphenylterazolium bromide solution were added (Sigma Aldrich), and the cells were incubated at 37ºC for 5 h. Dimethyl sulfoxide and glycine buffer (Sigma Aldrich) were added. The optical density of cell proliferation was measured at 620 nm using a microplate reader (Sunrise™ Absorbance Reader, Tecan, Männedorf, Switzerland). After 2 days and 7 days of cell culture, Ti disks were rinsed thoroughly in PBS and fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4). Following fixation, disks were dehydrated through a graded series of ethanol (50, 60, 70, 80, 90, 95, and 100% respectively) and then dried with tetramethylsilane. After ion coating with gold, the morphology and the cell number were observed using a SEM (S4000, HITACHI®) (n=3, for each group).

Real time PCR analysis

The MC3T3-E1 cells were cultured on peptides immobilized and untreated Ti disks (20 mm in diameter) at the density of 5×10^5 cells/cm^2 for 7 and 14 days. Total RNA of adherent cells was extracted using Geneaid Total RNA Mini Kit (Geneaid Biotech, New Taipei City, Taiwan). The mRNA was reversed-transcribed to cDNA using the Primescript RT reagent kit (Takara Bio, Shiga, Japan). The gene expression of glyceraldehyde-

Table 1 Targets and housekeeping genes

Gene	Primer sequence (5'/3')	Amplicon size (base pairs)
GAPDH	(F) ATCACCATCTTCCAGGAG (R) ATCGACTGTGGTCATGAG	318
Runx2	(F) CTCAGTGATTTAGGGCGCATT (R) AGGGGTAAGACTGGTCATAGG	178
Col IA1	(F) CCCCAACCCTGGAAACAGAC (R) GGTCACGTTCAGTTGGTCAAAGG	453
BSP	(F) TACCGAGCTTATGAGGACGAA (S) GCATTTGCGGAAATCACTCTG	246
OC	(F) AGCTATCAGACCAGTATGGCT (R) TTTTGGAGCTGCTGTGACATC	180
ALP	(F) GATCATTCCCACGTTTTCAC (R) TGCGGGCTTGTGGGACCTGC	461

Forward (F) and reverse (R) primer sequences used in the real-time polymerase chain reaction (PCR) *GADPH*=glyceraldehyde-3-phosphate dehygrogenase; *Runx2*=Runt-related transcription factor 2; *Col-1*=type I collagen; *BSP*=bone sialoprotein; *OC*=osteocalcin; *ALP*=alkaline phosphatases

3-phosphate dehydrogenase (*GADPH*), Type I collagen (*Col-1*), runt-related transcription factor 2 (*Runx2*), osteocalcin (*OC*), bone sialoprotein (*BSP*), and alkaline phosphatase (*ALP*) were determined by a real time PCR machine (QuantStudio 3 Real-Time PCR System, Thermoscientific, Waltham, MA, USA) with KAPA Taq PCR Kit (Kapabiosystems, Wilmington, MA, USA). The primer sequences were listed in Table 1[11,31] (n=3, for each group).

Alkaline phosphatase activity

MC3T3-E1 cells were seeded and cultured on the Ti disks (20 mm in diameter) at the density of 5×10^5cells/cm^2. Cells were harvested after 7 and 14 days. After lysing the cells with radioimmunoprecipitation assay (RIPA) buffer (Sigma Aldrich), alkaline phosphatase (ALP) activity was determined using the ALP Kit (Elabscience, Houston, TX, USA) as per the manufacturer's protocol (n=3, for each group).

Statistical analysis

The values presented in the current study were mean±standard deviation of the mean. The analysis of variance (ANOVA) test with Bonferroni's posttest was performed using Prism GraphPad 8 software (Graphpad Software, La Jolla, CA, USA) to assess statistically significant differences (p<0.05) among the peptide immobilized and untreated groups.

RESULTS

Surfaces topography and roughness

Generally, after the surface treatment with the sandblasted followed by the acid-etched and the peptides coating, no differences in the surface morphology were observed on the control group (Fig. 1A) and the peptides-treated Ti disks (Figs. 1B–D).

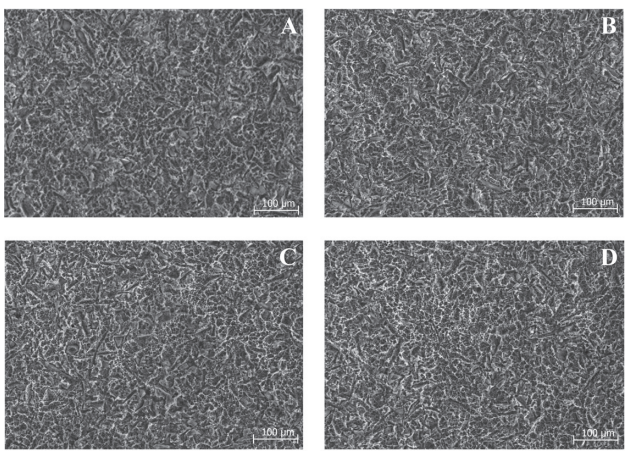

Fig. 1 Topographic analyses of differently treated Ti surfaces.
Scanning electron microscopy (SEM) of (A) control, (B) histatin-1, (C) JH8194, and (D) mixed peptides.

EDX surfaces analysis revealed the presence of essential elements on Ti surfaces for all of the groups. However, additional elements of C, O, and N were found in the histatin-derived peptides group, Table 2 (Table shows the element's weight and atomic percentages). Moreover, the weight percentage of the additional elements (C, O, and N) were increased after immobilized with histatin-derived peptides compared to the control group.

FTIR study

Peaks representing 679 cm^{-1} and 1,590 cm^{-1} which were derived from the N-H bond of the amino groups were

Table 2 Percentage of weight and atomic by element from Energy dispersive X-ray (EDX) on control, HST-1, JH8194, and mixed peptides

Peptides	Weight %					Atomic %				
	C	N	O	Al	Ti	C	N	O	Al	Ti
Control	1.6	3.6	17.3	4.7	72.8	6.5	9.9	33.7	4.1	45.8
HST-1	3.7	4.9	17.7	3.1	70.6	8.7	10.7	34	3.6	43
JH8194	3.1	4.7	17.5	2.5	72.2	8	10.6	33.2	2.9	45.3
Mixed peptides	3.4	4.4	18.1	3.7	70.4	8.5	10.3	34	3.1	4.1

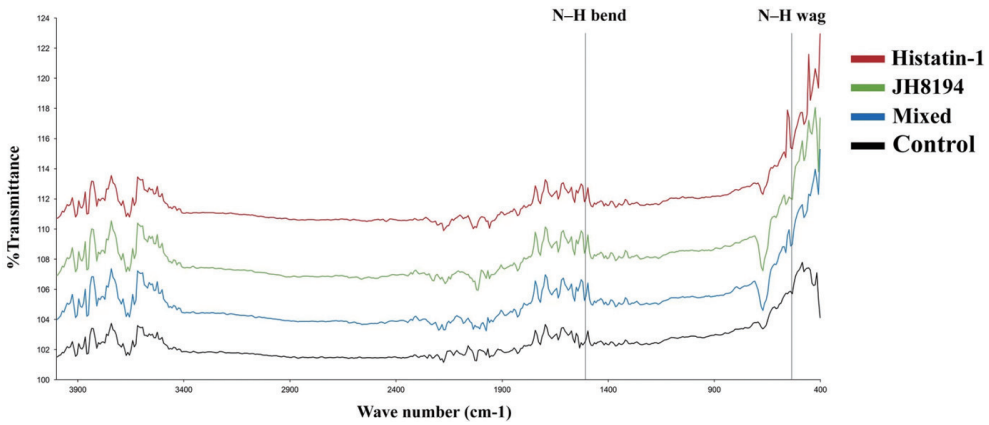

Fig. 2 FTIR spectra of differently treated Ti surfaces.
Control (black line), histatin-1 (red line), JH8194 (green line), and mixed (blue line). Characteristic assignments: N–H bend vibration located at 1,650–1,580 cm^{-1}. N–H wags located at 910–665 cm^{-1}.

detected in histatin-derived peptides groups. No peaks representing amino group were detected for uncoated disk (control group) (Fig. 2).

Morphology of adherent cells

Cell morphologies on the treated Ti surfaces were observed. Most of the cells had a flat shape with cytoplasm and filopodial extension of lamellipodia, which was assumed to be microvilli structures. Immobilized histatin-derived peptides (Figs. 3B–D) did not affect the morphology of adherent cells as compared to the control group (Fig. 3A) but increased in the cell number on the peptides immobilized Ti disks (Figs. 4A–D).

Cell adhesion and proliferation

There was a significant improvement of histatin-1 and mixed peptides on the cell adhesion ($p<0.05$; Fig. 5A). A significant increase in cell proliferation was found when Ti was coated with histatin-1, followed by mixed peptides and JH8194 respectively in both 3 and 7 days ($p<0.05$; Fig. 5B).

Cell differentiation

The effect of the histatin-derived peptides on stimulating pre-osteoblast differentiation was evaluated. The expression of osteogenic genes includes *Runx2, Col-1,*

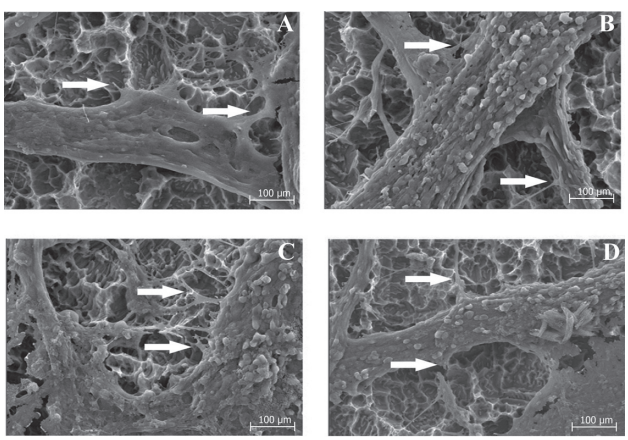

Fig. 3 Morphology of adherent osteoblasts on differently treated Ti surfaces after 2 days.
Scanning electron microscopy (SEM) of MC3T3-E1 cells cultured on (A) control, (B) histatin-1, (C) JH8194, and (D) mixed peptides. Attached cells showed cytoplasmic extensions, presumed to be filopodia-like structures (arrows).

and *ALP* were significantly upregulated by histatin-1 after 7 days ($p<0.05$). Nevertheless, after 14 days,

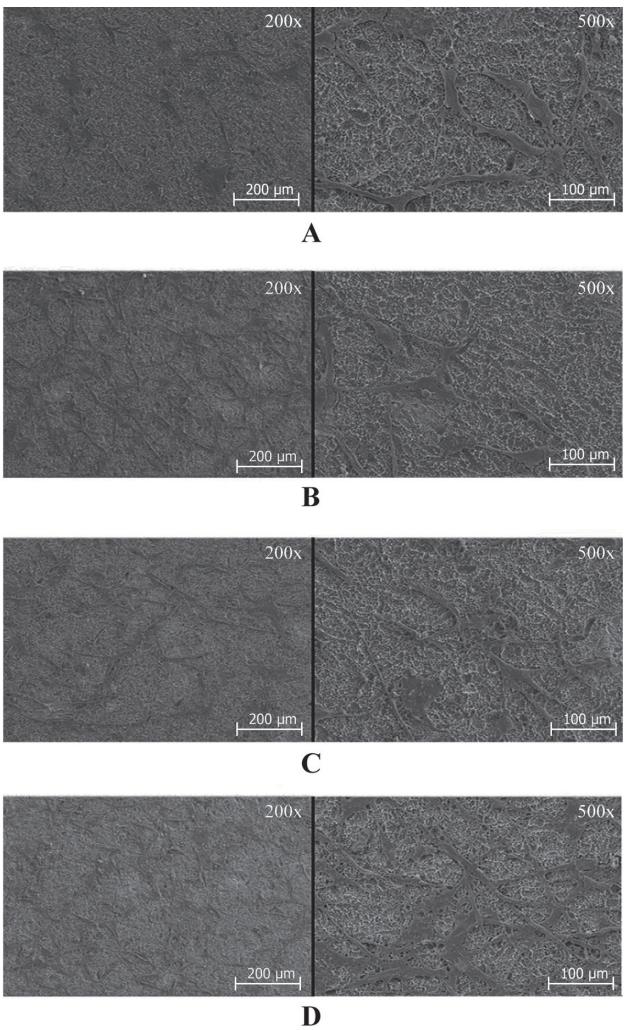

Fig. 4 Densities of adherent osteoblasts on differently treated Ti surfaces after 7 days.
Scanning electron microscopy (SEM) of MC3T3-E1 cells cultured on (A) control, (B) histatin-1, (C) JH8194, and (D) mixed peptides.

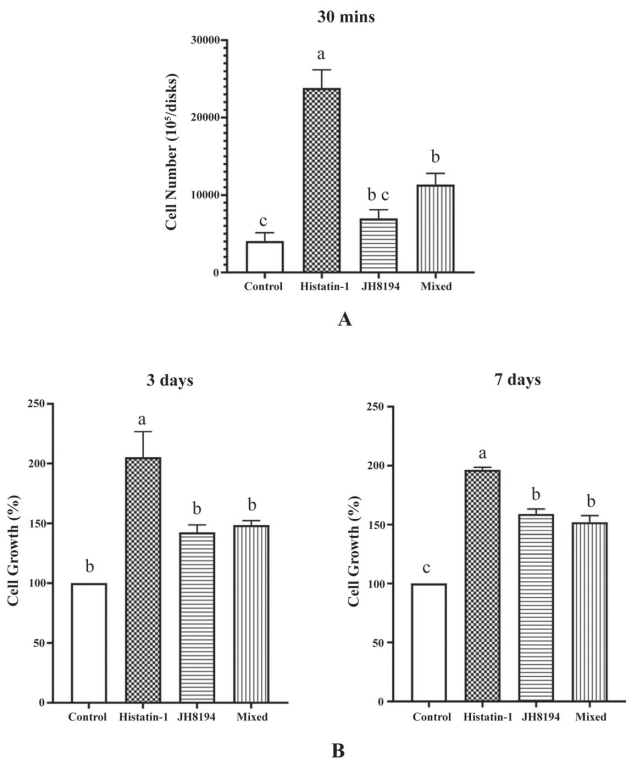

Fig. 5 Cell adhesion and cell proliferation of adherent osteoblasts on differently treated Ti surfaces.
(A) Adhesion of MC3T3-E1 cells on treated Ti surfaces after 30 min. (B) Cell proliferation of MC3T3-E1 cells determined by an MTT assay after 3 and 7 days. Differences between groups were determined by the Bonferroni test. Different alphabet: significant $p<0.05$ (top of bar).

higher *ALP*, *BSP*, and *OC* expression were observed on histatin-1 and mixed peptides groups (Figs. 6A–B).

Alkaline phosphatase activity

After 7 days, ALP activity levels were significantly increased in cells on Ti immobilized with histatin-1 ($p<0.05$; Fig. 7A). The histatin-derived peptides groups exhibited a significantly highest ALP activities at day 14 ($p<0.05$; Fig. 7B).

DISCUSSION

The implant surface is an important factor by increasing the response of cells, such as adhesion, proliferation, and differentiation. Sandblasting is a general method used for surface treatment. It is made by projecting particles of silica, hydroxyapatite, or alumina in sizes from 25–75 μm on Ti surfaces. Acid etching is a common chemical method used for etching Ti surfaces. Sandblasted-large grit and acid-etched implant surface (SLA) are applied to commercially available dental implant. Some of the dental implant systems have surfaces similar to those used in this study[32]. Currently, implant surface modification using a bioactive molecule was introduced to reduced implant failure, especially in patients with poor bone quality.

Nowadays, various techniques were developed to produce a thin layer of proteins or peptides[33]. Proteins were incorporated onto to dental implants as biologic coatings to improve its osteoconductive and osteoinductive properties[34,35]. The bioactive modification with proteins, such as collagen-1, showing survival rates at 100%[33,35,36]. Nevertheless, proteins have several important limitations such as the high cost of synthesis, antigen formation, and molecular instability[37,38], thus peptides derived from main proteins were introduced to overcome these problems[39].

Although EDS is not the specific technique for detecting peptides coated on specific surfaces. But our assumption to confirm the presence of immobilized peptides came from the combination of the results

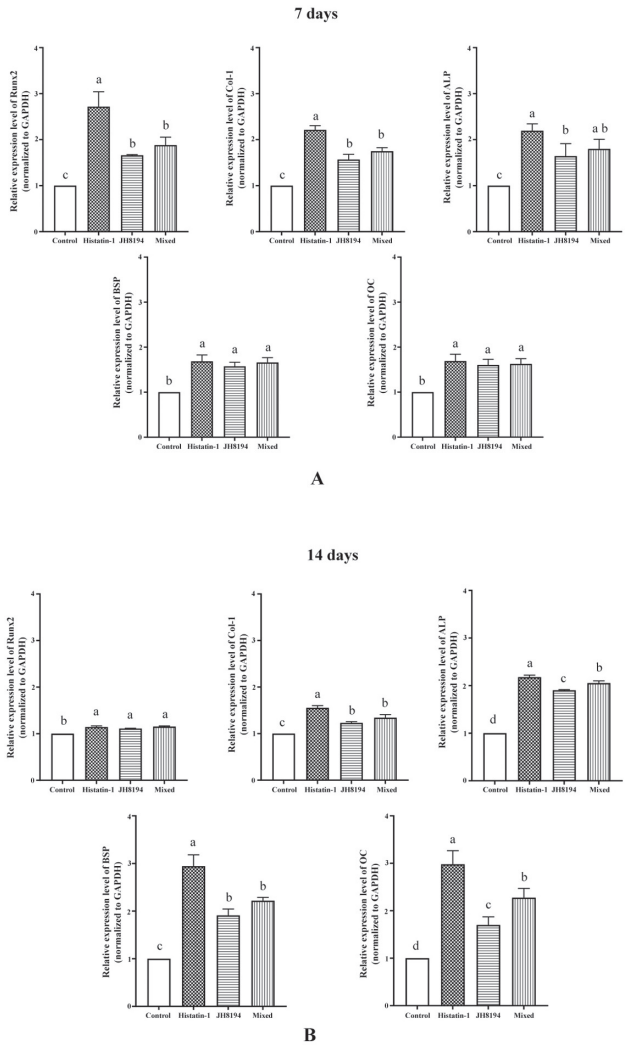

Fig. 6 Gene expression of *Runx2*, Type I collagen (*Col-1*), Alkaline phosphatase (*ALP*), bone sialoprotein (*BSP*), and osteocalcin (*OC*) in MC3T3-E1 cells at 7 (A) and 14 days (B) on differently treated Ti surfaces.
Gene expression was determined by real-time polymerase chain reaction (PCR). Ratios of target genes were normalized to the housekeeping gene (*GAPDH*). Relative expression levels represent fold changes of gene expression in MC3T3-E1 cells on coated Ti surfaces. Different alphabet: significant $p<0.05$ (top of bar).

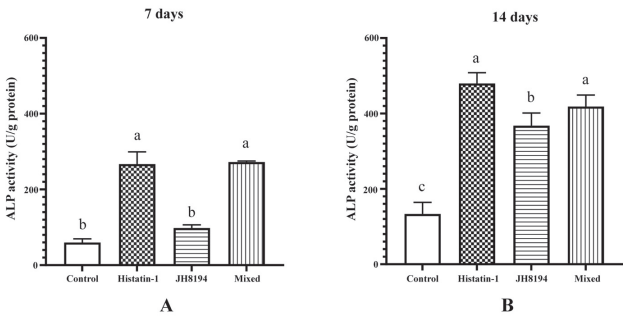

Fig. 7 Alkaline phosphatase (ALP) activity of MC3T3-E1 on differently treated Ti surfaces.
MC3T3-E1 cells were cultured on differently treated Ti disks for 7 (A), and 14 days (B). ALP activity was determined in cell lysates at indicated time points. Different alphabet: significant $p<0.05$ (top of bar).

from EDS and FTIR analyses. Wei-Jen Chang *et al.* showed that the carbon, oxygen, and nitrogen elements originated from type I collagen were detected on Ti-surface using EDS[40]. The presence of elements carbon, oxygen, and nitrogen was identified in the amino peptide on graphene-based materials[41]. In addition, FTIR spectra showed the presence of the amino groups on the Ti surface after coated with peptides, the spectra appearing at 680 cm^{-1} were assigned to the wagging mode of N–H (primary and secondary amines) and the bending mode of N-H (primary amines) also was observed at 1,590 cm^{-1} [28,29]. Moreover, Chen Li *et al.* combined EDS and FTIR analyses to confirm the peptide structures[42].

For the concentrations of histatin-1, 5–10 μM was the most suitable for cell adhesion and proliferation. Moreover, the histatin-1 concentrations mentioned earlier, are also the concentration ranges found in the human saliva[43,44]. Although the immobilized histatin-derived peptides did not directly affect the shape of the cells attached to the Ti disks, but rather increased the number of cells to control group after 7 days, which corresponded to the previous study[45].

In the previous studies, histatin-1 and JH8194 peptides promoted pre-osteoblast cells spreading on SLA Ti surfaces[21,23]. Interestingly, this is the first study reported the effect of histatin-1 on osteoblast differentiation. The expression of *Runx2* and *Col-1* were upregulated by histatin-derived peptides, which considered as early markers for pre-osteoblast differentiation[46,47]. *BSP* and *OC* were specific marker genes for late-stage osteoblast cell differentiation[48]. This result suggests that the expression of *BSP* and *OC* by histatin-derived peptides were important for the osteoblast differentiations[49]. Furthermore, ALP activity was crucial for the mineralization and also indicative for the maturation of the osteoblasts[50]. Hayakawa *et al.* also reported change in ALP activity of MC3T3-E1 cells cultured on fibronectin-immobilized Ti surface after 3, 15, and 52 days[7].

Pramono *et al.* reported that the combination of fibronectin-derived peptides (GRGDSP and PHSRN) improved osteoblast cell adhesion, proliferation, and differentiation when compared with a single peptide (GRGDSP or PHSRN alone)[11]. In contrast, Chen *et al.* reported that there was no difference between the mixtures of BMP-7-derived peptides (SNVILKKYRN, KPSSAPTQLN, and KAISVLYFDDS) and single BMP-7-derived peptide on the production of collagen and proteins synthesized by osteoblasts after 7, 14, and 21 days[51]. Although, JH8194 has been reported to enhance

the biological modifications of Ti surfaces by upregulating the mRNA expressions of *Runx2* and *OPN*, and ALP activity of osteoblast-liked cells along with increasing new bone formation around dental implants[24,52].

Histatin-1 could activate p38 signaling pathway[53] by stimulating through the ERK signaling pathway and play crucial role in oral epithelial cell migration[54]. The combination of the mixed peptides that act through the same pathway would be able to stimulate pre-osteoblasts response *via* p38 MAPK signaling pathway[55]. On the other hand, the molecular mechanism of JH8194 remained unclear. We suspect that mixing JH8194 might dilute the concentration of histatin-1 until it minimized the effect of histatin-1 on osteoblast responses. Therefore, the mixed peptides group did not show the most effective result. Moreover, our study was consistent with Chen *et al.* which demonstrated that the combination of peptides derived from BMP-7 was not different from the use of a BMP-7 peptide alone in term of the amounts of total collagen synthesized by osteoblasts[51]. Marquis *et al.* demonstrated that choosing an inappropriate concentration of a peptide derived from BMP-9 could inhibit pre-osteoblasts MC3T3-E1 cell proliferation[56].

CONCLUSION

Tresyl chloride-activated technique can be used for immobilizing peptides on Ti surfaces. This study demonstrated the effectiveness of histatin-1 for improving cellular attachment, proliferation, and differentiation. Taken together, the findings here indicate that histatin-derived peptides may be another technique for surface modification on the dental implants to promote new bone formation and increase the success rate of the implant surgery. Nevertheless, further studies would be required to confirm these findings.

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