台灣口腔醫學科學雜誌
ISSN 2072-2486

Cell adhesion of human bone marrow-derived mesenchymal stem (HMS0014) cells on titanium discs with different surface modifications

Running title: adhesion of HMS0014 cells on Ti discs

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Abstract

The modification of surface microtextures on the implant fixture increases direct bone-to-implant contact compatibility to benift primary stability and osseointegration of dental implant therapy. Cell proliferation and differentiation of HMS0014 cells, and mineralisation of the ECM in monolayer cell culture were preliminarily studied. Subsequently, the HMS0014 cells were GBR-engineered to initiate osteogenesis on either titanium (Ti) or Ti alloy discs modified with different surface substrates. Attachment onto the substratum, extension and intercellular contact of the HMS0014 cells under inducing condition were studied with fluorescent immunohistology and conventional scanning electron microscopy (SEM). The present study obtained results as follows: (i) The SEM demonstrated that the spherical-to-polygonal (d= 10-40 μm) HMS0014 cells proliferated and differentiated into flat polygonal (30×90-100×200 μm2) cells, showing prominent lamellipodia and dendritic filopodia, to employ cell-to-substrate and intercellular attachments on the Ti disc surface between 60 to 180 minutes of culture. (ii) The fluorescent immunohistochemistry demonstrated the co-expression of F-actin (actin filament, cytoskeleton protein) and CD51 (α V integrin, interfacing specific receptor protein) in the attached HMS0014 cells. (iii) The matured osteoblast-like HMS0014 cells initiated mineralisation on day 1 of culture; distribution of calcification loci in the ECM was prominently observed between days 7 and 14 of culture. We concluded that the present GBR method enhanced HMS0014 cells to initiate contact osteogenesis on Ti and Ti alloy discs subject to different surface modifications.

Key Words：cell adhesion, surface substrate, HMS0014 cells

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Submitted：June, 9, 2013
Accepted：May, 9, 2013

The Taiwan J Oral Med Sci 2013; 28:71-84

Introduction

The modification of surface microtextures on the implant (IP) fixture increases direct bone-to-implant contact (BIC) compatibility to offer better primary stability for immediate load bearing of dental IP therapy[1-6]. On the other hand, regulation of vital reactions occurring at the BIC interfaces to initiate contact osteogenesis followed with distant osteogenesis is essential for a successful dental IP occlusal rehabilitation therapy[6-13].

We have engineered rat and human dental pulp cells, and mouse bone marrow-derived cells in monolayer and 3-D cultures to initiate hard tissue deposition based on the guided bone regeneration (GBR) concept in previous studies[14-19]. Recently, we have reported that the immature human bone marrow-derived HMS0014 cells differentiated into bone matrix-producing cells exhibiting mature osteoblast phenotype within 2 days in either monolayer cultures or PuraMatrix (a self-assembling peptide hydrogel extra cellular matrix (ECM); RAD 16-1) 3-D cultures under inducing condition in vitro20. In the present study, chronological changes of ALP activity, Ca volume and osteoclacin (OCN) volume in monolayer cultures were performed for estimation of the properties of HMS0014 cells under inducing condition. Subsequently, proliferation and differentiation of the HMS0014 cells, and mineralisation of the ECM in the monolayer cultures were histologically studied. For elucidation of the first stage of osseointegration of GBR initiated on different substrate surfaces of titanium (Ti) discs, contact osteogenesis commenced by cell attachment, extension and intercellular contact was investigated with studying CD51 (α V integrin, an integrin molecule) and F-actin (a cytoskeleton protein) immunoactivities by the use of fluorescence light microscopy[20]. Furthermore, the proliferating BIC osteoblast-like HMS0014 cells adhered on the Ti discs under inducing condition were investigated with the conventional scanning electron microscopy. In the present study we evaluated the microenvironments of Ti discs having similar surface modifications as prevalent Ti dental IPs to optimize a direct bone-to-substrate contact of human bone marrow-derived HMS0014 mesenchymal cells, therefore contributed to a preliminary in vitro study on initial osseointegration of dental implant therapy.

Materials and Methods Preliminary study of the HMS0014 cell

Immature HMS0014 cells (Human osteoblastic cell line, Bone Marrow(BM)-derived mesenchymal stem cells) in non-induction condition were distributed through Riken Bio Resource Center (RIKEN BioResource Center; Tsukuba, Japan). The immature osteogenic linage cells were filtrated, centrifuged, cultured and incubated in POWEREDBY10 (GP Biosciences Ltd., Tokyo, Japan) supplemented with 1% antibiotic-antimycotic agent (100 units/ml penicillin + 100 μ g/ml streptomycin; Nacalai Tesque, Kyoto, Japan) in 75 cm^2 cell culture flasks (TPP, Switzerland) at 37℃ in humid air with 5% Co^2.

The immature HMS0014 cells were monolayer-cultured in the POWEREDBY10 added with ascorbic acid (AA, 50 μ m/ml; Sigma-Aldrich Japan, Tokyo, Japan), β -glycerophosphate (β -GP, 10mM; Sigma-Aldrich, MO, USA) and dexamethazone (DEX, 100nM; Wako, Osaka, Japan) under inducing condition for 14 days. Proliferation and differentiation of the HMS0014 cells, and mineralisation of the ECM (alizarin red S: AR-S vital staining) under the inducing condition were investigated with an Olympus CKX41 phase contrast microscopy (PCM; Olympus, Tokyo, Japan).

On the other hand, proliferation rate and differentiation of the monolayer-cultured cells (day-1, -3, -7 & -14 specimens) were assayed and compared with a PicoGreen dsDNA Quantitation Kit (Invitrogen, CA, USA; excitation wave length:485 nm, emission wave length: 525 nm), Alkaline Phosphatase (ALP) Substrate Kit (Bio-Rad Labs., CA, USA) , Calcium (Ca) E-test-Wako (Wako) and the Gla type Osteocalcin (Gla-OCN) EIA Kit (TaKaRa Bio, Ohtsu, Japan). The intensity of visible light (wave length: 405 nm) emitted from the specimens was summarised and scored with a Softmax Pro5 Microplate Reader Analysis Software (Molecular Devices, CA, USA). ALPase activity expression was corrected for the total protein content (Alkaline Phosphatase Substrate Kit, Bio-Rad Labs., CA, USA) by the DNA amount, and was expressed as μ mol/ μ g DNA/min.

Tissue engineering of HMS0014 cells on discs with different surface modifications

Plates/discs (diameter : d=1cm, height: h=1mm; disc type; JAPAN MEDICAL MATERIALS (JMM) Physio OdontrumImplant (POI) system; JMM, Osaka, Japan) of primarily blasted α - β type titanium alloy (Ti-6Al-4V) those were secondarily processed with anodic oxidization (Disc-AO) or coated with hydroxyapatite (HA=$Ca_{10}(PO4)_6(OH)_2$, 20 μ m approx.; Disc-HA) and JIS type 4 (99% Ti) plates (diameter: d= 1 cm, height: h= 1 mm; disc type; THOMMEN medical/Morita, Osaka, Japan; SWISS PRECISION AND INNOVATION (SPI) system) finely blasted with Al_2O_3 sands (Disc-SPI) were supplied for the present in vitro study.

The isolated immature HMS0014 cells were seeded and cultured with the discs in the POWEREGBY10 and exposed to AA+β -GP+DEX at 1.0×10^5 cells/ml concentration in 12-well Petri dishes (IWAKI, Tokyo, Japan) and thereby the cells were induced to differentiate into mature osteogenic cells (osteoblast-like cells: Ob-like cells) in a humid microenvironment in 5% CO_2 at 37℃.

The histology of HMS0014 cells cultured to adhere onto Ti/Ti alloy plates

1.Scanning electron microscopy(SEM)

For investigation of the HMS0014 cells being attached and spreading to show distinct morphological changes during the initial osseointegration on the Ti/Ti alloy discs (i.e., Disc-AO, Disc-HA, Disc-SPI) under inducing condition, the specimens were prefixed with 1% glutaraldehyde (20 min), postfixed with 1% OsO_4 (30 min, 4℃) and prepared (Pt-Pd coating; Eiko IB-3 ion coater, Eiko engineering, Mito, Japan) for the conventional SEM. Secondary electron image SEM of the 60-min and 180-min specimens was particularly examined and photographed under a Hitachi S-4000 SEM (Hitachi, Tokyo, Japan) at accelerating voltage of 10 kV.

2.Immunochemical histology of HMS0014 cells on different surface modifications

On the other hand, the 60-min and 180-min specimens (i.e., Disc-AO, Disc-HA, Disc-SPI) were immersion-fixed with 4% formaldehyde (12 hrs, 4℃), washed with 0.1 % BSA-PBS and blocked with 3% goat serum. In this study, the specimens were immunochemically stained with primary Ab: anti-CD51 (1:1,000 Conc.; Enzo Life Sci, PA, USA) and secondary Abs: Alexa Fluor 488 goat anti-rabbit IgG (1:500 in 0.1% BSA-PBS;

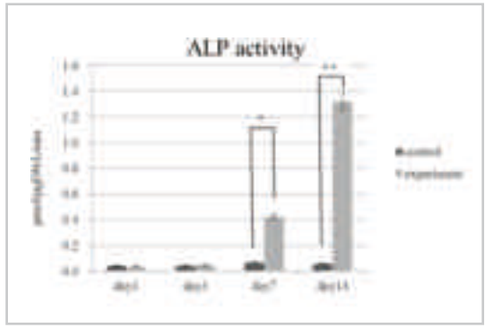
Molecular probes, OR, USA), and subsequently stained with Rhodamine phalloidin (1:1000 in 0.1% BSA-PBS; Molecular probes, OR, USA) and incubated (room temp., 1hr) following the conventional methods. Therefore, the expression of CD51 (α V integrin) and F-actin (actin filaments stained with Rhodamine-conjugated phalloidin) were examined with a BIOREVO BZ-9000 fluorescence microscope (KEYENCE, Osaka, Japan) for the evaluation of cell attachment and extension of the differentiating HMS0014 cells distributed on the Disc-AO, Disc-HA and Disc-SPI.

Results

The histochemical properties of HMS0014 cells in vitro

1.Chronological changes of ALPase activity, Ca volume and OCN volume in monolayer cultures of HMS0014 cells (Fig.1)

By comparison of data obtained from the experimental(inducing)- and control(non-inducing)-conditioned HMS0014 cells in monolayer cultures, we observed that the ALPase activity of the experimental group increased with elapsed time, and the activity was abruptly, markedly and significantly increased between days 7 (*p<0.05) and 14 (**p<0.01) of culture. Although increasing tendency of Ca volume seemed to be fluctuant and indistinctly occurred in either the control or experimental group, a significant difference between them was observed at 14 days culture (*p<0.05). OCN volume of the inducing-conditioned cells has found to be significantly higher than the non-inducing cells since day 1 of culture (**p<0.01).



*p<0.05
** p<0.01

Fig. 1.Monolayer cultures of HMS0014 cells in vitro
1)ALP activity was markedly increased between days 7 and 14 in the experimental group. A significant difference was in particular observed at 7 days (*p<0.05) and 14 days (** p<0.01) experiment.
2)A significant increase of Ca volume was evident in the 14th day of experiment (*p<0.05).
3)Significant differences of time-dependent increase of osteocalcin (OCN) volume were demonstrated (** p<0.01).

Fig. 1. Time-dependent changes of ALP activity, Ca volume and Osteocalcin (OCN) volume in monolayer cultures of HMS0014 cells in control (non-inducing condition) and experimental (inducing condition) groups

The results indicated that mineralisation of the ECM prominently progressed between days 7 and 14; in the day 14, more Ca and OCN volumes were significantly acquired in the experimental groups.

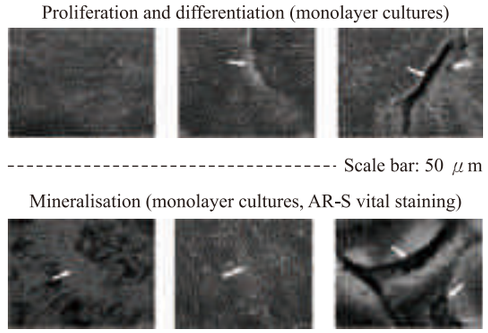


Fig.2.PCM studies on proliferation and differentiation, and mineralisation of HMS0014 cells (under inducing condition, monolayer cultures; arrow: calcification locus; scale bar: 50 μm)

The PCM observed that polygonal HMS0014 cells proliferated actively, and differentiated into cells secreting abundant ECM under induction condition. Alizarin red S (AR-S) staining indicated that mineralisation (arrows) initiated at day 1 and became markedly deposited in the ECM at 7 days experiment.
The data also indicated an abruptly increasing tendency of OCN volume between days 3 and 7 of culture, and significant differences were observed between the experimental and the control groups since day 1 of culture (**p<0.01)

2.Phase contrast microscopy (PCM) of HMS0014 cells in monolayer cultures (Fig.2)

Monolayer-cultured cells were induced to actively proliferate and differentiate into polygonal cells secreting ECM, which was mineralised since day 1 and markedly deposited with calcification loci (demonstrated by AR-S) in the day-7 experimental specimens under inducing condition.

Collectively, these results suggested that the proliferating HMS0014 cells were actively induced to differentiate into mature Ob-like cells which abundantly secreted mineralising ECM between days 3 and 7 of monolayer cultures; mineralisation of the ECM secreted by the inducing-conditioned HMS0014 cells prominently progressed in the day 7 and day 14 experimental cultures (Figs. 1 and 2).

Histology of HMS0014 cells cultured on discs with different substrate surfaces

1.Scanning electron microscopy (SEM) of HMS0014 cells cultured on Ti-alloy and Ti discs with different modifications (i.e., Disc-AO, Disc-HA, Disc-SPI)

At 60 minutes culture, the inducing-conditioned cells migrated towards depressions of the disc surface, loosely proliferated and differentiated to spherical (cells on Disc-HA: d= 20 μm , Disc-SPI: d= 10 μm; approx.) and flat, polygonal (cells on Disc-AO: d= 40 μm; approx.) cells with a bulging cell body sending out many minute filopodia on the Disc-SPI and some lamellipodia on the Disc-AO and Disc-HA to attach to the substrate in the concavities (Fig. 3a).

At 180 minutes culture, the HMS0014 cells actively proliferated and differentiated into large flat and polygonal shapes (cells on Disc-HA and Disc-SPI: size= 30×90 μm^2, cells on Disc-AO: size= 100×200 μm^2; approx.) cells, which extended lamellipodia

and many fine dendritic filopodia spreading on the disc surface to employ cell-to-substrate and intercellular attachments (Fig. 3b).

2.Fluorescent immunohistology of HMS0014 cells cultured on Ti-alloy and Ti discs with different modifications (i.e., Disc-AO, Disc-HA, Disc-SPI)

Co-expression of CD51 (α v integrin) and F-actin (actin filament) immunoreactivities of cells with massive extension to adhere on the Disc-AO, Disc-HA and Disc-SPI surfaces at 60 and 180 min under inducing condition were evident; the co-expression was more distinctly demonstrated in the 180-min specimens (Fig. 4).

Fig. 3a. SEM showing HMS0014 cells (*) were induced to attach to the substratum of Ti discs after 60 minutes of culture using the GBR method (×1,500).

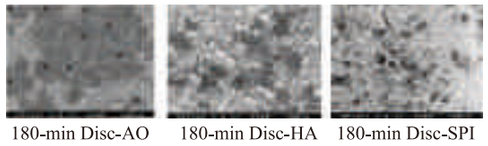


Fig. 3b. SEM showing HMS0014 cells (*) were induced to attach to the substratum of Ti discs after 180 minutes culture using the GBR method (×300).

Fig. 3. SEM showing time-dependent (Fig. 3a: 60-min specimens, Fig. 3b: 180-min specimens) morphological changes of HMS0014 cells (*), which were induced to proliferate and differentiate towards spreading mature Ob-like cells on the substratum of titanium discs (i.e., Disc-AO, Disc-HA and Disc-SPI)

The loosely distributed spherical-to-polygonal HMS0014 cells (Disc-AO: d= 40 μm, Disc-HA: d= 20 μm, Disc-SPI: d=10 μm; avg., 60-min specimens) subsequently differentiated into flat, large polygonal cells with prominent lamellipodia and dendritic filopodia (Disc-HA & Disc-SPI: 30×90 μm2, Disc-AO: 100×200 μm^2; approx., 180-min specimens).

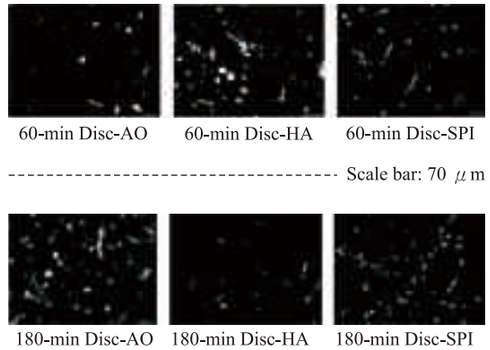


Fig. 4. Fluorescent immunohistology demonstrating the co-expression of CD51 (α v integrin) and F-actin (actin filament) immunoreactivities of the 60-min and 180-min specimens (i.e., Disc-AO, -HA and -SPI specimens (arrows: cells with CD51/F-actin co-expression, arrow heads: polygonal cell with massive extension; scale bar: 70 μm)

Discussion

Many previous studies on the endosseous dental implantation have demonstrated that micro-textured implant(IP) surface substrates evolve the immediate mechanical stability to enhance cell attachment, as well as improving the host-to-IP response and thereby optimizing osseointegration of the dental IP therapy[1,2,5,22-26]. Many histological studies have elucidated that osseointegration began with recruitment and migration of osteogenic lineage cells, which adhered and spread, proliferated, and subsequently differentiated into mature

osteoblast(Ob)-like cells on the substrates. Furthermore, the osteocalcin (OCN) gene was found to be expressed in the mature Ob-like cells, which changed in shape concomitantly with extension of cell processes and hard tissue deposition onto the IP surface in the mineralising phase of osseointegration[1,7,26-29]. On the other hand, interfacing specific receptor proteins (e.g., integrins) and cytoskeleton proteins (e.g., actin filaments) have been elucidated to be proteins closely related with the development of focal contacts, which play important roles in signal transduction and thereby induce adhesion, spreading, cell migration and consequently regulate cell growth and differentiation of the Ob-like cells attached to the IP substratum[21,28,29].

Earlier we studied 3-D cultured rat JCRB 119:KUSA/A1 cells with a collagen gel scaffold (Cellmatrix Type I-A) on dental IPs (IP-AO, IP-HA & IP-SPI) to develop a microenvironment conducting contact and distant osteogenesis since the first three days of culture by using the GBR tissue-engineering methods[19]. By using the similar methods, in this study we performed monolayer culture of human osteogenic HMS0014 cells on the Disc-AO, Disc-HA and Disc-SPI, and demonstrated that the HMS0014 cells grew and differentiated into flat, polygonal cells having long filopodia (cytoplasm supported with long F-actin bundles) and defined wide-spreading lamellipodia (extended cytoplasm supported with a F-actin meshwork) to facilitate cell motility on the disc surface[21,30]. Previous studies show that α v integrin and F-actin proteins were closely related with cell adhesion, signal transduction, cell spreading and migration of osteoblasts on certain biomaterials[21,28,29]. The present fluorescent immunehistology of the expression of α v integrin and F-actin indicated that we engineered actin-rich HMS0014 cells to employ focal adhesions on the discs (i.e., Disc-AO, Disc-HA and Disc-SPI). The proliferating cells actively migrated and dispersed along the bioactive interface with participation to bind, initiate and establish cell attachment with the modified substratum within 180 minutes of culture[26,29,30-32].

Furthermore, we confirmed that the E CM of the osteogenic linage HMS0014 cells in experimental (under inducing condition) monolayer cultures showed an increase of OCN activity through the experimental term, and the cells actively proliferated and differentiated into Obs with an abrupt and significant increase of ALP activity and Ca volume in particular at 14 days experiment. In accordance with the assay of the osteoinductive property of the experimental HMS0014 cells, the present PCM observed that polygonal Ob-like cells had actively proliferated and differentiated to secrete abundant ECM with sedimentation and aggregation of calcification loci since day 1 of culture; subsequently, the ECM became markedly mineralised since day 7 of experiment. To sum up, the histochemical results indicated that HMS0014 cells were condition-induced to differentiate into mature Ob-like cells to secrete ECM which was prominently mineralised between day 7 and day 14 in the monolayer cultures.

We elucidated that the present GBR procedure engineered HMS0014 cells to obtain extensive cell attachment within 180 minutes, thereby we surmised that the Obs might be further regulated to initiate peri-Ti osteogenesis on different micro-textured Ti discs since day 1 of culture. There have some studies on GBR of osteogeneic cells on 3-D scaffolds which enhanced cell-biomaterial interactions to foster growth and differentiation of cultured

mesenchymal cells[19,33-37]. Hence, we propose that to acquire an en-bloc peri-Ti osteoid tissue by culturing human osteogenic cells on 3-D woven scaffolds to benefit optimal osseointegration should be our next steps in programming of GBR tissues for the dental IP therapy in edentulous cases with massive bone defect.

Acknowledgements

The morphological research, tissue culture facilities and dental bioscience facility I of the Institute of Dental Research, Osaka Dental University, were used to perform this study. The authors gratefully acknowledge to Dr. Akihiro Umezawa, national Institute for Child Health and Development, for the supply of HMS0014 cells in the present study. This study was supported by Oral Implantology Research Grand at the Osaka Dental University (No. 11-04. Application of human tooth as a 3-D scaffold in the dental implant therapy by the GBR treatment concept).

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The Taiwan J Oral Med Sci 2013; 28:71-84

HMS0014骨髓衍生間質細胞在不同表面處理的鈦金屬板上的細胞附著

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摘要

人工牙根的表面處理可以增加人工牙根與周圍骨組織的結合，以利於人工牙根的初期穩定性及骨整合(osseointegration)。我們先前對人類骨髓由來的間葉幹細胞(HMS0014)的單層培養，細胞的繁衍及分化，與細胞外物質(ECM)的礦化先作了初步研討。接著，我們以骨再生誘導法(GBR)，將HMS0014 細胞培養在不同表面處理的鈦或鈦合金的圓板上，並啟始其骨化作用。然後，以免疫螢光組織學的方法及掃瞄電子顯微鏡探討在inducing condition下，附著、貼附於鈦金屬圓板的表面基質上的HMS0014 細胞，並觀察細胞的伸展以及細胞間鍵結的發生。

本研究得到以下三個結果:

1. 掃瞄電子顯微鏡顯觀察到，許多圓形或多角形（直徑約10-40 μm）的HMS0014 細胞分佈於鈦金屬圓板上。在60-180分鐘的培養後，繁衍並分化為扁平多角形（大小約30×90-100×20 μm2）的細胞；此細胞具有板狀偽足及髮絲樹突狀偽足，用以作為細胞間以及細胞與鈦金屬圓板表面基質間的結合。

2. 免疫螢光組織學顯示， 附著於鈦金屬板的HMS0014細胞有F-actin(actin filament)和CD51(α V integrin)的共同存在。

3. 從第一天的培養後，成熟的類骨母細胞樣(osteoblast-like)HMS0014細胞的ECM已有礦化現象，而到第7至第14天，在ECM內可明顯地觀察到點狀、塊狀的鈣化物的分佈。

我們得到一個結論，GBR可促使HMS0014細胞在經過不同表面處理的鈦或鈦合金表層上產生骨化現象。

關鍵字：細胞附著、鈦金屬板、HMS0014細胞

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受文日期：民國102年1月9日
接受刊載：民國102年3月9日