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Correlation between cell attachment areas after 2 h of culture and osteogenic differentiation activity of rat mesenchymal stem cells on hydroxyapatite substrates with various surface properties

Kan Cheng [a], Motohiro Hirose [b,*], Xiupeng Wang [b], Yu Sogo [b], Atsushi Yamazaki [a], Atsuo Ito [b]

[a] Faculty of Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan
[b] Human Technology Research Institute (HTRI), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan

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ABSTRACT

The initial attachment of mesenchymal stem cells (MSCs) to substrates and osteogenic differentiation are supported by culture on a hydroxyapatite substrate. Cell attachment areas of rat MSCs after 2 h of culture on hydroxyapatite substrates with various microstructures and the osteogenic differentiation activity thereafter were measured. The perceived outcome was that, after 2 h of culture, rat MSCs with a small attachment area would have a high osteogenic differentiation activity, whereas those with a large attachment area would have a low osteogenic differentiation activity. Furthermore, rat MSCs with a small attachment area had many cytoplasmic processes, while those with a large attachment area revealed clear stress fibers and focal contacts. These results suggest that cell attachment area of rat MSCs after 2 h of culture has a strong effect on the osteogenic differentiation of rat MSCs. Thus, the measurement of cell attachment area after 2 h of culture could become valuable for estimating the osteogenic differentiation activity of rat MSCs thereafter.

1. Introduction

Cell adhesion to various types of substrates has received much attention in recent years owing to the effect on cellular functions, including proliferation, migration and differentiation [1–3]. Cell growth and viability are affected by cell attachment area on patterned surfaces [4–6]. These findings suggest that extracellular environments could also control cell attachment area, thus affecting cytoskeletal organization, proliferation, and differentiation. We have recently reported that human mesenchymal stem cells (MSCs) with a high proliferative activity are thick and those with a low proliferative activity are thin [7], and that rat MSCs with round-shape show a higher osteogenic differentiation activity on ceramics sheets than those with wide spread-shape on polymer sheets after 2 weeks of culture under osteogenic differentiation conditions [8]. However, the correlation between cell morphologies, particularly cell attachment area, for MSCs in the early stage of culture and later osteogenic differentiation activity remains unclear. Therefore, the aim of this study was to focus on investigation of the correlation between the attachment area of rat MSCs after 2 h of culture on hydroxyapatite substrates with slightly different surface morphologies and the osteogenic differentiation activity of rat MSCs thereafter.

As the cell culture substrates, hydroxyapatite (HAP) substrates with slightly different surface morphologies were prepared by sintering at various temperatures. We measured the attachment area of rat MSCs on the substrates 2 h after seeding and quantified the amount of osteocalcin secreted by rat MSCs after 3 weeks of culture under osteogenic differentiation conditions. In the present study, we report for the first time that rat MSCs having a smaller attachment area after 2 h of culture show a higher osteogenic differentiation activity thereafter.

2. Materials and methods

2.1. Reagents

Minimum essential medium (MEM) and dexamethasone from Sigma–Aldrich Co. were used. Fetal bovine serum (FBS) from Invitrogen Co. was used. The LIVE/DEAD viability/cytotoxicity assay kit from Invitrogen Co. was used to measure cell attachment area. Antibiotics (penicillin–streptomycin–amphotericin B suspension), β-glycerophosphate, phosphate-buffered saline (−) (PBS (−)), and L-ascorbic acid 2-phosphate magnesium salt *n*-hydrate (ascorbic acid-2-phosphate) from Becton Dickinson and Company were used. Trypsin–EDTA solution (0.25 w/v% trypsin-1 mmol/L EDTA·4Na solution with phenol red) from Becton, Dickinson and Company were used. Rat osteocalcin ELISA kit DS from DS Pharma

* Corresponding author. Fax: +81 29 861 6149.
E-mail address: motohiro-hirose@aist.go.jp (M. Hirose).

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Biomedical Co. was used to quantify osteocalcin in the culture medium of rat MSCs. Rhodamine phalloidin from Invitrogen Co. was used to obtain images of MSCs attached to the substrates. Quant-iT™ PicoGreen® ds DNA Reagent and Kits from Invitrogen Co. was used to measure DNA contents of rat MSCs.

2.2. Preparation of cell culture substrates

Four types of substrate were used for rat MSCs culture in this study. A pure stoichiometric HAP powder was supplemented with 3 wt.% poly(vinyl alcohol) and 1 wt.% poly(ethylene glycol), and then dried and filtered to a particle size below 75 μm. The obtained powder was molded into disk-shaped samples, which are pressed at 0.6 t for 1.5 min and then at 1.2 t for 3 min. The molded samples were sintered at 1100 °C, 1150 °C and 1200 °C for 1 h to prepare 1100 °C-HAP, 1150 °C-HAP and 1200 °C-HAP, respectively. The HAP substrates were sterilized by exposure to hot air at 160 °C for 2 h. The 1150 °C-HAP was soaked in 8.5 M acetic acid for 1 h to obtain 1150 °C-HAP treated with acidic solution (AC-1150 °C-HAP). Four types of substrate, i.e., 1100 °C-HAP, 1150 °C-HAP, AC-1150 °C-HAP and 1200 °C-HAP, were then rinsed with sterile water and immersed in sterile PBS(−) solution at 37 °C for 1 day prior to cell culture. The HAP samples were air-dried for 2 h at 25 °C. The air-dried samples were sputter-coated with gold and observed by scanning electron microscopy (SEM; Model XL30, FEI Japan Ltd.).

2.3. Preparation of suspension of rat MSCs

Rat MSCs were isolated and subcultured by the method described previously [9]. Briefly, rat bone marrow cells were obtained from the bone shafts of the femora of 7-week-old male Fisher 344 rats purchased from Japan SLC Co. MEM containing 10% FBS and 1% antibiotics (100 units/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotericin B) was used to culture the cells. Both ends of the femur epiphysis were cut, and bone marrow was flushed out with 10 mL of the medium through a 21-gauge needle. The cells from each femur were seeded onto individual T-75 flasks. The bone marrow cells were cultured for 1 day in a humidified atmosphere 95% of air and 5% $-CO_2$ at 37 °C. The medium was renewed every 3 days to wash out all floating cells. Then, cells adhering to the T-75 flasks were initially cultured up to 80% confluence in the same T-75 flasks. The adherent cells were harvested using trypsin–EDTA solution (P-1, passage 1) to prepare a cell suspension.

2.4. Cell attachment and osteogenic differentiation assays

Four types of culture substrate were placed on each well of a 12-well tissue culture polystyrene (TCPs) plate. To measure cell attachment area, rat MSCs in the medium without FBS were pre-stained using a LIVE/DEAD viability/cytotoxicity assay kit prior to seeding and then seeded on each cell culture substrate at a density of 4×10^4 cells/mL (1 mL/well). Cells attached to the surface of each culture substrate were observed 2 h after seeding under a fluorescence microscope (Olympus, Model BX51). Cytoplasmic areas of living cells stained with calcein appeared green owing to intracellular esterase activity. The cytoplasmic areas of living cells on each cell culture substrate were measured using Image-Pro® PLUS software (Media Cybernetics, Inc., Version 7.0). The green area was determined as the cell attachment area in this study. Cell attachment areas in the range between 350 μm^2 and 3500 μm^2 were used in the analysis. Areas less than 350 μm^2 were regarded as dust particulates and thus discarded, while areas larger than 3500 μm^2 were regarded as multiply connected cells and thus discarded.

For the osteogenic differentiation assay, MEM was supplemented with 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate, 0.28 mM ascorbic acid-2-phosphate and antibiotics. The cells were seeded on each cell culture substrate at a density of 5×10^4 cells/mL (1 mL/well). After the confirmation of cell adhesion to the culture substrate, the cell differentiation experiment was started (day 0). The cells that adhered to the culture substrate were cultured for 21 days. The medium was renewed three times a week. After 21 days of culture, the amount of osteocalcin secreted by the rat MSCs cultured on each substrate was measured. Fifteen microliters of the medium supernatant after the culture was collected from each well and diluted 100-fold with a dilution buffer in an appropriate vessel prior to the assay of intact osteocalcin using a rat osteocalcin ELISA kit DS. DNA content was measured by pulverizing MSCs on each substrate to evaluate the number of cells. Such DNA quantitation was carried out according to the manufacture's protocol using a fluorophotometer at an excitation wavelength of 520 nm.

2.5. Fluorescence images of cell cytoskeletons in attachment area stained for F-actin stress fibers

To image the cytoskeleton in the cell attachment area, the cells were stained using a rhodamine phalloidin solution. After the cells were cultured for 2 h on each substrate, the cells were fixed with 2% glutaraldehyde solution. The cells were stained for 20 min at 25 °C with the rhodamine phalloidin solution in the dark, and washed with PBS(−) solution and then observed for focal contacts and F-actin stress fibers under a fluorescence microscope.

2.6. Statistical analysis

Cell attachment area, the amounts of osteocalcin and total DNA are presented as average values with standard deviations. The Student's *t*-test was used to evaluate statistical significances among groups. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Surface characterization of HAP substrates

Depending on sintering temperature, the surfaces of 1100 °C-HAP, 1150 °C-HAP and 1200 °C-HAP were formed with slightly different surface morphologies. The particles observed at the surface of 1100 °C-HAP were less than 1 μm in diameter and those at the surface of 1150 °C-HAP were near 1 μm in diameter, those at the surface of 1200 °C-HAP were more than 2 μm in diameter (Fig. 1-A).

3.2. Attachment area of MSCs

Rat MSCs attached on different substrates were shown in Fig. 1-B. By fluorescence microscopy, small cytoplasmic areas of the cells (i.e., small cell attachment areas) with many cytoplasmic processes on the surfaces of 1100 °C-HAP, 1150 °C-HAP, and AC-1150 °C-HAP were observed (Figs. 1-B(A)–(C)). The cells on the surface of 1200 °C-HAP were widely-spread shapes (i.e., large cell attachment areas) with many focal contacts (Fig. 1-B(D)).

3.3. Attachment areas, DNA content and osteogenic differentiation of MSCs

We measured the attachment area of rat MSCs by image analysis. The cell attachment areas on 1200 °C-HAP were the largest, while those on the surface of 1100 °C-HAP were the smallest

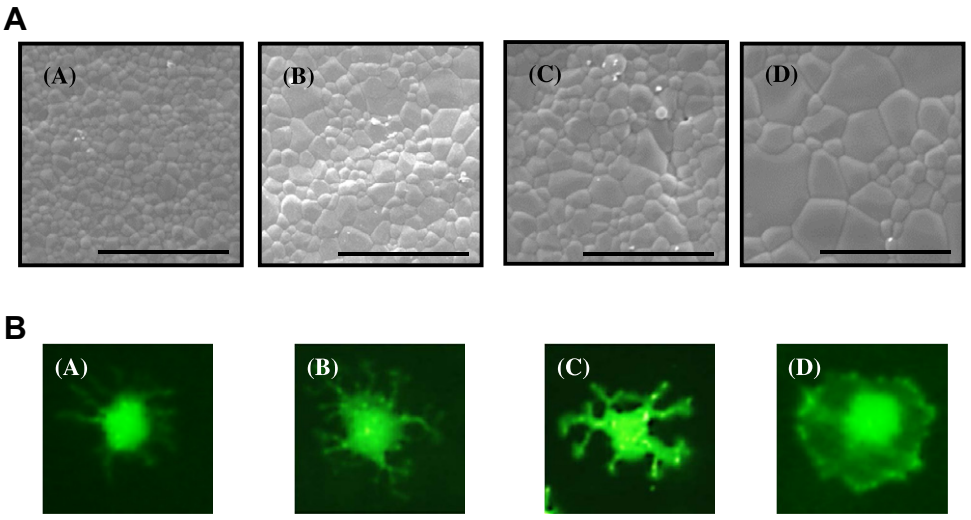

Fig. 1. HAP substrates and cell attachment of rat MSCs to the HAP substrates. A. Scanning electron micrographs of surfaces of (A) 1100 °C-HAp, (B) AC-1150 °C-HAp, (C) 1150 °C-HAp, and (D) 1200 °C-HAp. Bar: 5 μm. B. Fluorescence objective microscopy images of rat MSCs after 2 h of culture on (A) 1100 °C-HAP, (B) AC-1150 °C-HAP, (C) 1150 °C-HAP, and (D) 1200 °C-HAP. A typical cell on each HAP substrates is shown.

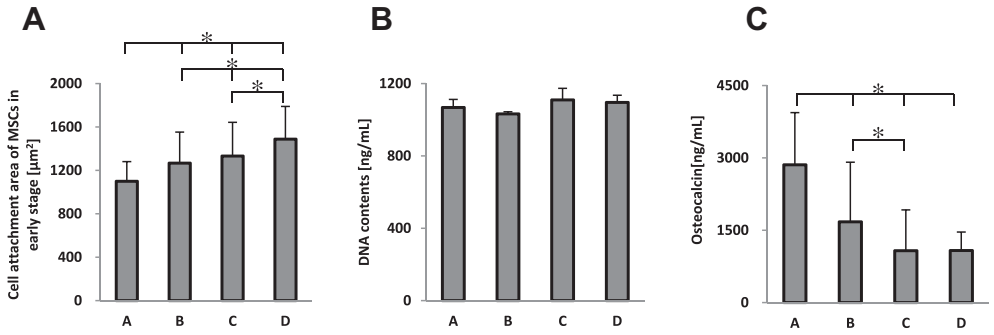

Fig. 2. Attachment areas, DNA content and osteogenic differentiation of rat MSCs. A. Cell attachment area of rat MSCs after 2 h of culture on (A) 1100 °C-HAP, (B) AC-1150 °C-HAP, (C) 1150 °C-HAP, and (D) 1200 °C-HAP. Two hundred cells in the range between 350 μm^2 and 3500 μm^2 on each HAP substrate were measured. B. DNA contents of rat MSCs after 3 weeks of culture on (A) 1100 °C-HAP, (B) AC-1150 °C-HAP, (C) 1150 °C-HAP, and (D) 1200 °C-HAP ($N = 3$). C. Osteocalcin contents of MSCs after 3 weeks of culture on (A) 1100 °C-HAP, (B) AC-1150 °C-HAP, (C) 1150 °C-HAP, and (D) 1200 °C-HAP ($N = 6$). Cell attachment area, the amounts of osteocalcin and total DNA are presented as average values with standard deviations. The Student's *t*-test was used to evaluate statistical significances among groups. *P* values less than 0.05 were considered statistically significant.

among the cell culture substrates tested (Fig. 2A). To measure the number of cells, the DNA content of the cells cultured on the four substrates was measured. As shown in Fig. 2B, the DNA contents were not significantly different among all the substrates after 3 weeks of culture. As shown in Fig. 2C, the osteogenic differentiation activity of the rat MSCs on 1200 °C-HAP was lowest, while that on 1100 °C-HAP was the highest among the cell culture substrates.

3.4. Correlation between osteogenic differentiation activity and attachment area of rat MSCs

The cell attachment areas of rat MSCs measured after 2 h of culture were as follows: 1100 ± 181 μm^2 (1100 °C-HAP), 1267 ± 286 μm^2 (AC-1150 °C-HAP), 1332 ± 310 μm^2 (1150 °C-HAP), and 1488 ± 301 μm^2 (1200 °C-HAP) (Fig. 2A). To compare osteogenic differentiation activity as a single cell on each substrate, values of the amounts of osteocalcin (Fig. 2C) divided by the DNA contents (Fig. 2B) after 3 weeks of culture were given as follows: 2.68 ± 1.02 (1100 °C-HAP), 1.62 ± 1.20 (AC-1150 °C-HAP), 0.97 ± 0.76 (1150 °C-HAP), and 0.99 ± 0.35 (1200 °C-HAP) (Fig. 3A). The square of the coefficient of correlation between cell attachment area (Fig. 2A) and osteocalcin amount divided DNA content (Fig. 3A) was 0.8316. The results strongly suggest that there is a negative correlation between the attachment area and osteogenic differentiation activity of rat MSCs (Fig. 3B).

3.5. Fluorescence images of cytoskeletons of MSCs in attachment area after 2 h of culture

The cytoskeletal organization of rat MSCs on the substrates was characterized. The cells were stained with rhodamine phalloidin which is known as a specific fluorescent material against F-actin to observe F-actin stress fibers (Red) and focal contacts (Fig. 4). The cells on 1100 °C-HAP, 1150 °C-HAP and AC-1150 °C-HAP showed small cytoplasmic areas with many cytoplasmic processes. Clear focal contacts were observed in the terminal regions of each cytoplasmic process (Figs. 4(A)–(C)). In contrast, the cells on 1200 °C-HAP showed widely-spread shape and large cytoplasmic areas with clear stress fibers. Many focal contacts were found at the cell periphery (Fig. 4(D)).

4. Discussion

In this study, we confirmed the correlation between the cell attachment area after 2 h of culture and the osteogenic

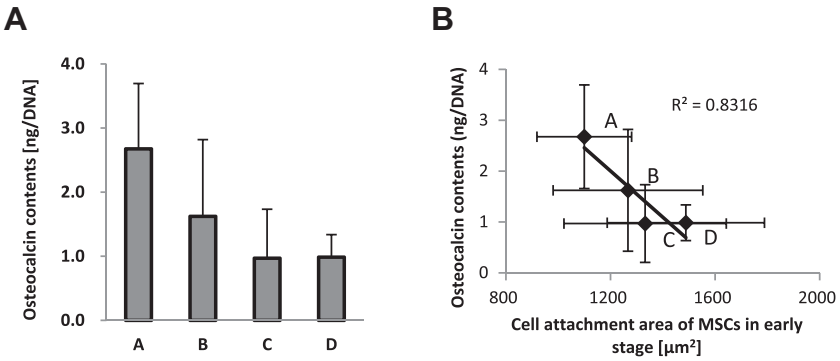

Fig. 3. Correlation between the cell attachment area and the osteogenic differentiation activity of rat MSCs. A. Amounts of osteocalcin shown in Fig. 2C were divided by corresponding DNA contents shown in Fig. 2-B of MSCs after 3 weeks of culture. (A), (B), (C) and (D) indicate 1100 °C-HAP, AC-1150 °C-HAP, 1150 °C-HAP, and 1200 °C-HAP, respectively. B. Correlation between the cell attachment area of rat MSCs after 2 h of culture and amounts of osteocalcin divided by DNA contents of those after 3 weeks of culture on (A) 1100 °C-HAP, (B) AC-1150 °C-HAP, (C) 1150 °C-HAP, and (D) 1200 °C-HAP. The square of the coefficient of correlation between cell attachment area (Fig. 2-A) and osteocalcin amount divided by DNA content (Fig. 3-A) was 0.8316.

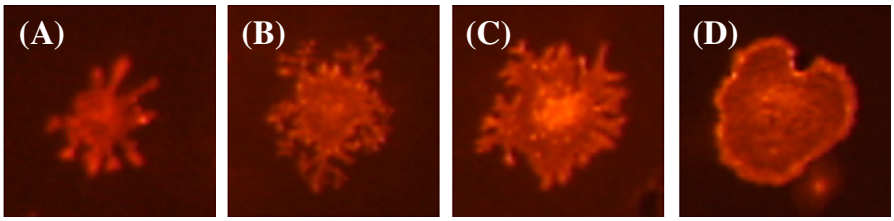

Fig. 4. Fluorescence objective microscopy images of cytoskeletons in attachment area of rat MSCs. Rat MSCs were cultured on (A) 1100 °C-HAP, (B) AC-1150 °C-HAP, (C) 1150 °C-HAP, and (D) 1200 °C-HAP for 2 h, and then the cells were stained using a rhodamine phalloidin solution as described in materials and methods. A typical cell on each HAP substrates is shown.

differentiation activity after 3 weeks of culture of rat MSCs on hydroxyapatite substrates with slightly different surface morphologies.

The attachment areas of rat MSCs in the early stage were different among those on the HAP substrates with slightly different surface morphologies (Fig. 1). In particular, the surface morphologies of the HAP substrates were modified owing to the size of particles appearing at the HAP surface changing when the sintering temperature of HAP was varied. These results, i.e., the differences in cell attachment area, could be caused by dissimilar particle faces on the surface of HAP substrates that absorb various types of extracellular adhesion proteins, including acidic and basic proteins [10–13]. The different size of particles on the surface of HAP substrates could affect protein absorption, i.e., in terms of amount and structure. In general, integrins have been shown to bind to a protein domain within their respective ligands that contain an arginine–glycine–aspartate (RGD) amino acid sequence and Asp–Gly–Glu–Ala (DGEA). These RGD and DGEA affect cell attachment area through integrin-mediated mechanisms.

The difference in cell attachment areas in the early stage were measured on four kinds of HAP substrate with slightly different surface morphologies (Fig. 2A). From Fig. 1-A, we observed larger cell attachment areas after 2 h of culture, i.e., larger particles on the HAP surface when the sintering temperatures were higher. The mechanism of cell morphology control has been widely studied, but not well understood. In rat MSCs, Tanaka et al. found by SEM that small cells have high osteogenic differentiation activity [8]. Recently, Go et al. have reported that human MSCs can maintain their expansion and osteogenic differentiation capabilities by the forced expression of Sox2 or Nanog [14]. Sox2-expressing cells in the presence of basic FGF had a higher potential for differentiation into osteoblasts and were smaller than control cells. These reports suggest that the correlation between cell size and osteogenic differentiation might be a generalized phenomenon in adult stem cells such as MSCs.

Differences in osteogenic differentiation activity were observed for rat MSCs cultured on the four kinds of HAP substrate with slightly different surface morphologies (Fig. 3A). Tanaka et al. have reported that the osteogenic differentiation activity of rat MSCs differs depending on their morphology on different substrates such as ceramic sheets and polymer sheets [8]. There is as yet no previous study showing different osteogenic differentiation activities of MSCs depending on MSCs morphology on similar substrates. In the present study, the different osteogenic differentiation activities of MSCs on similar substrates depending on MSCs morphology after 2 h of culture were demonstrated.

In addition, the geometry of the microenvironment of MSCs has begun to emerge as a critical parameter for regulating MSCs differentiation [8,15]. Differences in cell morphologies were observed in the study of the attachment areas of rat MSCs after 2 h of culture on HAP substrates with similar surface morphologies (Fig. 4). It has been shown that MSCs with large attachment areas and many focal contacts on 1200 °C-HAP after 2 h of culture had a lowest osteogenic differentiation activity (Fig. 1-B(D), Fig. 4(D)) whereas those with small attachment areas and many cytoplasmic processes on 1100 °C-HAP had a highest osteogenic differentiation activity (Fig. 1-B(A), Fig. 4(A)). Therefore, the smaller the attachment areas of rat MSCs after 2 h of culture, the higher the osteogenic differentiation activity (Fig. 3B).

MSCs are attractive candidates for applications in regenerative medicine because of their lots of differentiation potential. Currently, in regenerative medicine, only autologous and noncryopreserved MSCs are used in the clinical applications of MSCs in bone regeneration. Unfortunately, some cultures of regenerative bone tissues could not be implanted in patients because of low osteogenic differentiation levels. For these reasons, the estimation of

the osteogenic differentiation activity of MSCs in advance of clinical application is essential to determine whether or not the MSCs could be suitable for implantation. Our present findings indicate that cell morphologies, especially the cell attachment area in a very early stage of culture, might become an important tool in estimating the osteogenic differentiation activity of MSCs.

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