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Morphology and adhesion of mesenchymal stem cells on PLLA, apatite and apatite/collagen surfaces

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Abstract Biomimetic apatite/collagen composite coating, previously reported particularly with regard to its fabrication, characterization and interaction with osteoblast-like cells, has been investigated in this study to understand the response of human mesenchymal stem cells (hMSC) to such surface. PLLA films and PLLA films with apatite coating were compared with PLLA films with apatite/collagen composite coating. The hMSC morphology in response to such conditions was first observed using fluorescence microscopy. To further understand such cell-material interactions at a molecular level, integrin expression, actin assembly and vinculin-positive focal adhesion plaques were examined. Our results demonstrated that spreading of stem cells on the apatite/collagen composite surface was determined best among the three types of surfaces, followed by the apatite surface and then the PLLA control. Integrin expression on the apatite/collagen surface was higher than those on the apatite surface and PLLA surface. Immunostaining for vinculin and actin suggested that the composite coating on PLLA enhanced the formation of focal adhesion.

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Introduction

Surface characteristics such as chemical composition, surface energy, surface charge and wettability play an important role in mediating the interactions between the implant and the host tissue [1, 2]. Changes in the surface chemistry could influence cells attachment, spreading, differentiation and maturation. Several methods and techniques have been applied to modify the surface chemistry of implant materials, including ion implantation [3], inorganic coating [4], and organic components immobilization. In addition, biochemical factors such as growth factors [5], peptides [6] and ECM [7] proteins (e.g., collagen) are also likely to influence cell behaviors.

We have combined apatite coating with organic components to develop PLLA films and scaffolds coated, for example, with apatite/collagen composite. We have reported previously the biomimetic process and characterization of this composite coating [8, 9]. Osteoblast-like cells attachment and total activity on the composite coating were found to be higher compared to apatite coating and PLLA control [10], suggesting cell adhesion was enhanced by this composite surface. The present study was therefore undertaken to examine at a molecular level how the biomimetic coatings affect the behavior of mesenchymal stem cells cultured on them, including cell attachment, morphology, expression of integrins and vinculin in the focal adhesion plaques.

Materials and methods

Materials

PLLA with an inherent viscosity of approximately 7.11 dL/g was purchased from PURAC (Netherlands). Chloroform and all the analytical grade chemicals for making simulated

body fluid (SBF) were purchased from Acros (Belgium). Type-I Collagen was obtained from Tsinghua University in Beijing, China. Bone marrow-derived human mesenchymal stem cells (hMSCs) were obtained from the Tulane Center for Gene Therapy (New Orleans, LA). Based on the flow cytometry results, these cells showed negative staining for CD34, CD36, CD45, and CD117 markers (all less than 2%), and positive staining for CD44, CD90, CD166, CD29, CD49c, CD105, and CD147 markers (all more than 95%), indicating a minimal heterogeneity in the cell population.

Films and coating preparation

PLLA films were fabricated by the solvent-evaporation method [8]. Biomimetic apatite coating and apatite/collagen coating were formed on PLLA films as described elsewhere [9].

Cell culture

hMSCs were cultured in the complete medium that contains α-MEM (Invitrogen, Carlsbad, CA), supplemented L-glutamine, 10% fetal bovine serum (FBS), 1% antibiotics/antimycotics (final concentration; penicillin 100 units/mL, and streptomycin 100 μg/mL and amphotericin B 0.25 μg/mL). Cells were incubated in a culture flask in 5% CO_2 at 37 °C, and media was changed twice a week.

Cell morphology

Fluorescence images of hMSCs on PLLA, apatite coating and composite coating were recorded using a Nikon microscope (Eclipse E800). Illuminating light was focused on the sample through a 20 ×/ 0.75 NA objective or a 10 ×/ 0.25 NA objective and images were recorded using a CCD camera (CoolSnap fx, Roper Scientific, Tucson, AZ). The Live/Dead assay kit (Molecular Probes, OR) was used for fluorescence images. Live/Dead viability was expressed by the ratio of the number of live cells to that of dead cells. The shape of cells was quantitatively described by the eccentricity (e), which was defined as

$$e = \mathrm{sqrt}\{\{a^2 - b^2)/a^2\}$$

where a and b are the major and minor axes of a cell. Together with the surface density, the cellular characterization was quantitatively determined from fluorescence images using a MetaMorph (Universal Imaging, West Chester, PA) image processor.

Microscopic image of focal adhesion

Images on focal adhesion were acquired by a laser scanning multiphoton confocal microscope with 60× oil objective (NA = 1.40). The nucleus was visualized by using DAPI dye and multiphoton laser excitation at 700 nm and emission at 450 nm, for which the femosecond laser beam (80 MHz, 0.5 mW) was pumped from a mode-locked titanium:Sapphire laser (MaiTai, Spectra-Physics Inc., CA), and coupled to a visible laser (Bio-Rad, UK) into an inverted laser scanning confocal microscope (Nikon TE200-U, Japan).

To label integrin, cells were seeded on the substrates and cultured over night. Cells were then fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 min, and treated with 5% albumin for non-specific blocking, and incubated with R-Phycoerythrin conjugated monoclonal antibody against β_1-integrins (1:100 dilution) at room temperature [11] for 60 min. To visualize vinculin, hMSCs were fixed, permeabilized, and then treated with primary antibodies against vinculin, followed by FITC-conjugated secondary antibodies before imaging. To visualize microfilaments (actins), cells were fixed and permeabilized in cold acetone. After washing, cells were incubated with rhodamine-conjugated phalloidin (20 μM final concentration) for 30 min at room temperature.

Results

Cell morphology

Figure 1 shows images of hMSC morphology on the three surfaces after one day culture. The Live/Dead viability, cell shape and surface density were calculated from the fluorescence images (Table 1).

It appears that more cells were found attached on the composite coating and they exhibited an elongated shape, whereas fewer cells were observed on the PLLA substrate and their shape were nearly round. The cell shape on the apatite coating was similar to that on the composite coating; however, cell number on the apatite coating was lower than that on the composite coating. The Live/Dead viability of cells on the composite coating was highest among the results on the three surfaces.

Microscope image for integrin expression

Figure 2 shows images of integrin expressions on the three surfaces after 3 days cell culture. It could be seen that the integrin expression was highest in cells incubated on the apatite/collagen surface. In contrast, the integrin expression on PLLA was minimal.

Microscope image for actin and vinculin

Actin-associated contact sites were observed in Fig. 3. Cells plated on the apatite/collagen composite coating

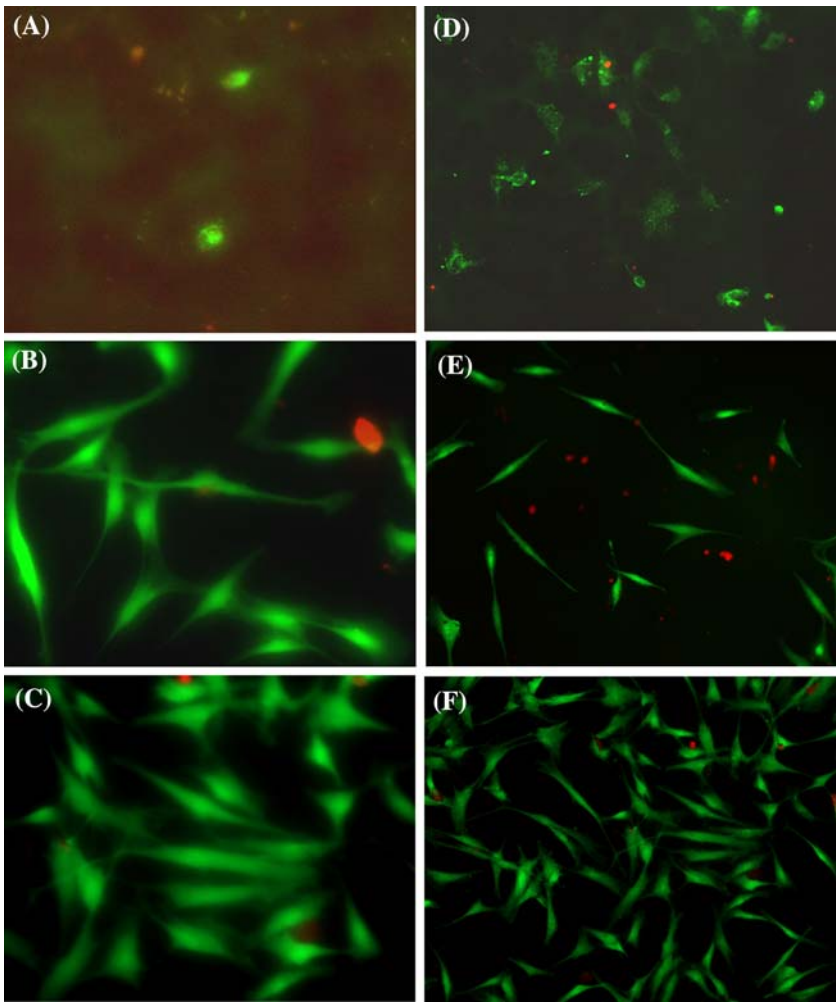

Fig. 1 Fluorescence images of hMSCs on PLLA films (**A**, **D**), PLLA films with apatite coating (**B**, **E**), and PLLA films with apatite/collagen coating (**C**, **F**) after one day stem cell culture. (**A–C**) were taken with a 10× microscope objective, and (**D–F**) were taken with a 20× microscope objective. The green and red indicate live and dead cells, respectively

Table 1 Live/Dead viability, cell shape parameter, and surface density of mesenchymal stem cells on PLLA films, PLLA films with apatite coating, and PLLA films with apatite/collagen composite coating

	Live/Dead viability (%)	Eccentricity	Surface density (/mm^2)
(A) PLLA films	39.8 ± 22.2	0.83 ± 0.18	6.18 ± 2.97
(B) PLLA films with apatite coating	87.8 ± 11.6	0.97 ± 0.02	88.40 ± 28.90
(C) PLLA films with apatite/collagen coating	97.3 ± 0.9	0.98 ± 0.02	119.11 ± 6.62

showed stress fibers of actin microfilaments with several clusters. The cells on the apatite coating demonstrated similar microfilaments organization. However, the actin filament density decreased. On the PLLA surface, the cells demonstrated a lack of such microfilament organization.

Images of the presence of vinculin-positive adhesion plaques at the periphery of cells cultured for 3 days on the three surfaces are also shown in Fig. 3. More adhesion plaques were found on the apatite/collagen surface compared to the control and apatite surfaces. Consistent with diminished microfilament organization, only a few adhesion plaques were observed on the PLLA control surface.

Discussion

Integrin binding and focal adhesion assemblies are critical to cell attachment to the biomaterial surfaces. We have examined the integrin expression, actin fibers and vinculins in hMSCs plated on the PLLA control films and with apatite or apatite/collagen coating.

Integrins are known to mediate the adhesion of anchorage-dependent cells to biomaterials and extracellular matrix (ECM). The substrate plays an important role in the integrin expression and the subsequent cellular behavior. The surface composition and topography are among the

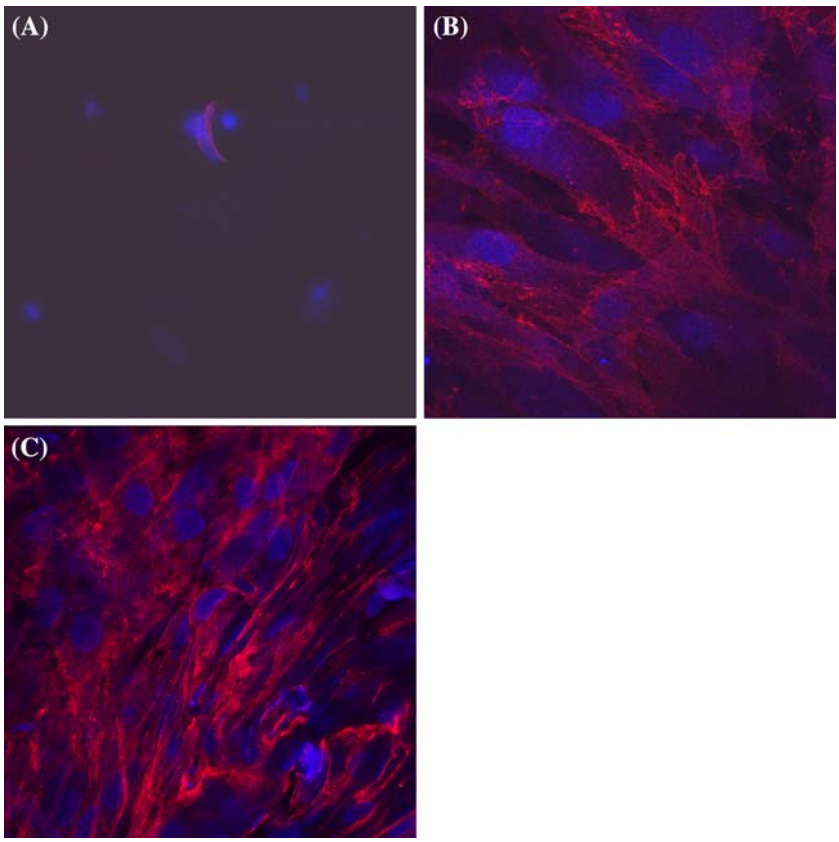

Fig. 2 Images of integrin expression (red) (**A**) PLLA films, (**B**) PLLA films with apatite coating, and (**C**) PLLA films with apatite/collagen coating after 3 days culture. These images were recorded using 60× objective

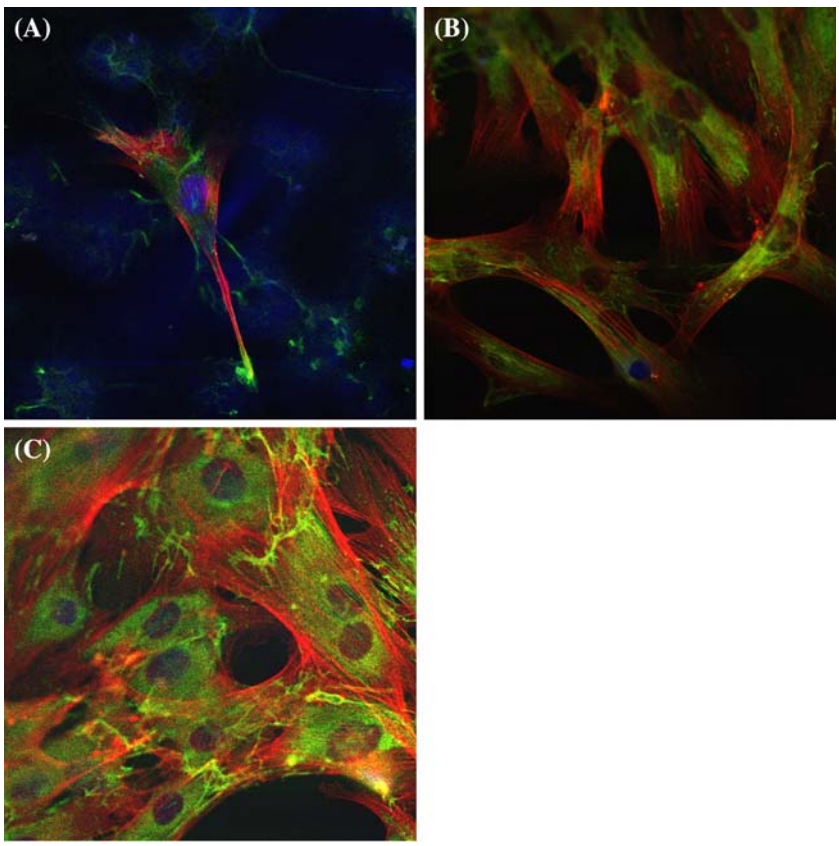

Fig. 3 Images(60×) of actin (red) and vinculin (green) expression of hMSCs on (**A**) PLLA films, (**B**) PLLA films with apatite coating, and (**C**) PLLA films with apatite/ collagen coating after 3 days culture

main surface characteristics that could affect integrin expression [12]. It is reported that integrin function is dependent on the concentration of divalent cations [13]. It is possible that Ca^{2+} ions contained in the apatite and apatite/collagen coatings might enhance ligand binding of the integrin receptors. Integrin was more specifically and significantly upregulated on the apatite/collagen surface than apatite surface. This indicated that Type-I collagen contained in the surface upregulated integrin expression. Other studies also reported that coating of titanium alloy and polystyrene with type I collagen increased the adhesion, spreading and proliferation of osteoblasts [14, 15] because collagen could bind its specific motif, GFOGER, to integrin [16].

Besides chemical compositions, the surface topography could also influence the interaction between integrins and the substrate. Studies have shown that an increase in roughness of titanium substrate may increase the adhesion of Saos-2 cells, rat osteoblasts, and U2OS cells [17–19]. In our previous study, we reported that the contact angle of PLLA substrate with apatite/collagen coating was lower than those of PLLA with apatite coating and PLLA control films [Y. Chen et al. Submitted]. As the apatite coating and apatite/collagen coating tend to increase the surface roughness of the substrate, such an increase in surface roughness may contribute to the increase of integrin expression.

Both microfilaments and vinculins are used as marks to identify the focal adhesion plague. In this study it is demonstrated that the biomimetic composite coating supports the formation of focal adhesions. Compared to the PLLA control substrate and apatite coating, the apatite/collagen composite surface is found to mediate stronger focal adhesion and more actin-associated contact sites.

Conclusion

Spreading of mesenchymal stem cells on the apatite/collagen composite surface was determined the best followed by the apatite surface and then PLLA control. Integrin expression and focal adhesion on the apatite/collagen surface was higher than apatite surface and PLLA surface. Based on these findings, it is suggested that the PLLA substrate coated with an apatite/collagen layer is a suitable scaffold for the development of MSC-derived tissue engineering constructs.

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