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Mesenchymal stem cell differentiation on microstructured Poly (methyl methacrylate) substrates

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Abstract

Recent studies on 2D substrates have revealed the importance of surface properties in affecting cell behavior. In particular, surface topography appears to influence and direct cell migration. The development of new technologies of hot embossing and micro-imprinting has made it possible to study cell interactions with controlled micro features and to determine how these features can affect cell behavior. Several studies have been carried out on the effect of microstructures on cell adhesion, cell guidance and cell proliferation. However, there is still a lack of knowledge on how these features affect mesenchymal stem cell differentiation. This study was designed to evaluate whether highly controlled microstructures on PMMA could induce rMSC differentiation into an osteogenic lineage. Structured PMMA was seeded with rMSC and cell number, cell morphology and cell differentiation were evaluated. Results confirm that microstructures not only affect cell proliferation and alignment but also have a synergistic effect with osteogenic medium on rMSC differentiation into mature osteoblasts.

Keywords: mesenchymal stem cells; osteoblasts; topography; microstructures.

Introduction

Research on stem cells is advancing the body of knowledge on how an organism develops from a single cell and how healthy cells replace damaged cells in adult organisms. This promising area of science is also leading researchers to examine the possibility of cell-based therapies for treatment of disease, which is often referred to as regenerative or reparative medicine.

Research using embryonic stem cells is growing in interest due to their potential medical applications. Despite this potential, the ethical issues surrounding these cells have created intense controversy. This concern has promoted in-depth research into adult stem cells. Adult stem cells are undifferentiated cells found in differentiated tissue that can renew themselves and differentiate (within certain limitations) to give rise to all the specialized cell types of the tissue from which they originate. Such adult stem cells have been discovered and characterized in a multitude of tissues, which suggests that autologous clinical implantation or genetically engineered stem cells for protein or drug delivery could be used without risk of immunorejection (the National Institute of Health resource for stem cell research).

In adult stem cell research, one of the main fields is the differentiation of stem cells into an osteogenic lineage for subsequent use in bone tissue engineering and reconstructive medicine. Interest in this field is related to the increased incidence of osteodegenerative diseases (i.e. osteoporosis and osteoarthritis) in the rapidly aging populations of developed countries. Bone is a mineralized tissue that confers multiple mechanical and metabolic functions to the skeleton. Bone contains two distinct cell types: osteoblasts

and osteoclasts. Osteoblasts are bone-forming cells of mesenchymal origin. Mesenchymal stem cells (MSC) have the potential to differentiate into osteoblasts, chondrocytes, adipocytes, fibroblasts, marrow stroma and other tissues of mesenchymal origin. These MSCs can be obtained from many sources (blood, adipose tissue, periosteum, muscle, dermis, etc.), but bone marrow aspirate is the most common supply. Due to the variety of cell types and the recently discovered plasticity of adult stem cells (stem cells from one tissue may give rise to cell types of a completely different tissue), there is a need for well-defined and efficient protocols for directing the differentiation of stem cells into the osteogenic lineage.

Cell behaviour depends on interactions with the environment. Consequently, the contact between cells and implantable materials will determine the success or failure of an implantable material or a medical device. It is well-known that the cell response is affected by the physicochemical parameters of the biomaterial surface, such as surface energy, surface charges or chemical composition. Topography is one of the most crucial physical cues for cells. Microtopography influences cell adhesion, proliferation and differentiation [Zinger 2005, Boyan 2002, Aparicio 2002]. More recently, it has become clear that nanotopography also guides cell behaviour [Dalby 2007]. Chemical cues in the form of various biomolecules, such as adhesive proteins (i.e. fibronectin and laminin) also influence cell behaviour in a crucial manner [Lan 2005].

Here we evaluate the effect of various surface modifications with the aim of directing rat mesenchymal stem cells to differentiate into osteoblast lineage.

Materials and methods

Polymer fabrication

Poly(methyl methacrylate) (PMMA, 125 μm thick) was purchased from Goodfellow (Goodfellow, UK) and used as received. A nanoimprint lithography apparatus (Obducat, AB, Sweden) was used to fabricate free-standing PMMA samples with surface features that had lateral dimensions ranging from 50 μm to 2 μm , following a previously reported hot embossing procedure [Mills 2007]. Briefly, this technique uses a microstructured silicon mould, covered by a grown silicon oxide layer, which is pressed into the bulk PMMA polymer at a temperature of 130°C and pressure of 30 bars. The moulds had been previously treated with a fluoroalkylsilane, (trichloro(tridecafluorooctyl)-silane, to prevent adhesion so that they could be reused after demoulding. The structural dimensions in the polymer replica were measured by an interferometric microscope (WYKO NT1100, Veeco, USA). After the nanoimprinting procedure, and prior to cell culturing, the transparent, free-standing PMMA samples were sterilized by ethanol.

Cell isolation and culture

Bone marrow stromal cells obtained from the femora of female Wistar rats aged 10 to 12 weeks were cultured as follows. Rats were killed and the femora were removed and placed in culture medium. The articular ends of each femur were excised at the epiphysis and the marrow expelled by injecting a stream of medium into the medullary cavity, using a syringe fitted with a 20-gauge needle. The expelled marrow was suspended in 10 ml medium (Advanced Modified Eagle Medium + 15% Foetal bovine serum + 1% penicillin/streptomycin + 1% glutamine) and heparin (10u/ml) and dispersed by repeated aspiration and expulsion through the needle. Cells obtained from

8 animals were seeded on T-75 tissue culture flasks. After 24h, non-adhered cells were removed by medium replacement. The medium was changed every 2 or 3 days. Once the cells were at 80% of confluence, they were split at a ratio of 1:2. Cells from passage 2 to 5 were used for all experiments. Cells from passage 2 were characterized for osteoblast lineage. Medium supplemented with β -glycerophosphate (10mM), dexamethasone (10^{-8} M) and ascorbic acid (50 μ g/ml) was used as the osteogenic medium (OM).

Alkaline phosphatase and von Kossa staining were used to analyze the osteogenic potential of the rMSC. Cells seeded on 24-well plates at a density of 10^3 cells/cm² were assayed on days 14 and 21 to detect alkaline phosphatase activity and mineralization nodules. For this purpose, cells were fixed in 10% formalin and a solution of Naphthol, AS-MX phosphate (70476 Sigma) and fast blue RR dissolved in Milli-Q water was poured into the wells, in accordance with the manufacturer's instructions (Sigma Kit #85). Cells were incubated in the dark for 30 min and rinsed in water. Cells were stained using the von Kossa method to detect calcium-phosphate-containing matrices. A solution of silver nitrate (2%) in water was added to the wells, which were then incubated for 15 min in the dark and rinsed twice in water. Finally, the samples were exposed to white light (15 min) and mounted in glycerine jelly. The samples were imaged using a microscope (Nikon Eclipse E600).

Cell morphology, proliferation and differentiation

rMSC from passage 4 were used to assay cell morphology, proliferation and differentiation on the different PMMA surfaces. Cell culture plates of polystyrene (PS) were used as a control.

Cell morphology

Cell morphology was analysed by confocal microscopy and scanning electron microscopy. Immunostaining was performed for the confocal microscopy. Cells were fixed (3% paraformaldehyde in phosphate buffer 0.1M and sucrose 60mM) and then permeabilised for 10 minutes in 1% Triton X-100 solution. Blockage was completed using 10mM PBS/20mM glycine/1% BSA for 20 min. Afterwards, primary antibody for actin was added and the samples were incubated for 1 h at 37°C in a humid atmosphere followed by a final incubation with secondary antibodies (goat anti-rabbit Alexa Fluor 568), phalloidin and Hoechst for nuclei staining in blocking solution at 37 °C for 1 h. Dried samples were mounted in Mowiol + antifade and imaged using a confocal microscope.

The preparation for scanning electronic microscopy (SEM) was performed as follows. Cells were fixed in 2.5% glutaraldehyde solution, post-fixed in 1% OsO₄ solution and dehydrated at room temperature via immersion in increasing concentrations of ethanol. Finally, critical point drying was undertaken in liquid CO₂.

A time-lapse video recording was performed for 4 h, taking pictures every 10 minutes.

Cell proliferation

Cell proliferation was determined at 10 days of culture using the cell proliferation reagent WST-1 (Roche, Germany). Briefly, the overall activity of mitochondrial dehydrogenases in the sample was measured by adding 20 µl of reagent to each well and quantifying the formazan produced in a microplate ELISA reader at 450 nm and 600 nm as reference wavelengths. Measurements were completed in triplicate.

Cell differentiation

Quantification of alkaline phosphatase activity (Sigma Diagnostic Kit #104) and osteocalcin (MetraTM, DPC Dipesa, Barcelona) were carried out on PS samples, on flat PMMA and on structured PMMA (round and square structures) according to the

manufacturer's instructions. rMSC at a density of 10^4 /well in a 24-well plate were seeded for 7 days. The medium was replaced twice a week. After this period, the medium was replaced by OM in half of the samples and with regular medium in the other half, in order to evaluate the effect of the differentiation factors on the structures. Results were correlated to the number of cells.

Results

Cell isolation and culture

rMSC from bone marrow were isolated and passaged twice to evaluate their potential to become osteoprogenitors and mature osteoblasts. rMSC proliferated for 11 days. The cell population increased 5-fold. After 14 days of culture, 75% of the total cell population was ALP positive (Fig. 2A). After 21 days of culture, mineralization nodules were seen (Fig. 2B). Very few cells with adipocyte morphology were seen at the beginning of the cultures, and hardly any after 21 days.

Cell morphology, proliferation and differentiation

Cell morphology

rMSC acquired different morphologies, according to the structures on the PMMA (Fig. 1). Fig. 5 shows confocal images of rMSC on square and round structures, cultured for three days and stained for vinculin to visualize focal contacts, actin filaments and nuclei. Cells were more stressed on the square small structure. They spread out but were star-shaped. Several filopodia could be seen in the star shape. However, in some cases, cells had a spindle, needle-like appearance. Closer examination showed that the filopodia were already interacting, mostly with the microstructures. Each of these

filopodia was attached to one structure at the end (Fig 5), as shown by the SEM pictures (Fig.6). In addition, a certain orientation of the cells was observed on the small structure (Fig 5D, Fig 6B).

The rounded structures were wide enough for the cells to stay inside and even divide a couple of times (Fig. 5A). Cells were spread to the limit of the rounded structure. When rMSC were not inside the wells, they had a very well organized cytoskeleton, with a lot of vinculin expression, which indicates that there were many focal contacts (Fig. 5B). There were fewer focal contacts and less organized actin filaments on the rounded structures than on the small structure (Fig. 5C, D).

The time-lapse video (Fig. 7) recorded on the round structures for 4 h, showed that MSC tended to penetrate the structures. However, not all of them entered, some just bypassed the structures. When MSC were already inside the round features they did not come out.

Cell proliferation

Results of cell proliferation are shown on Figure 6. rMSC proliferated for 10 days on the flat and structured PMMA. Cells on flat PMMA proliferated extensively, at the same levels as on polystyrene with no osteogenic medium. When factors for osteoblast differentiation were added, proliferation decreased, both on flat PMMA and on polystyrene. Cells on the structured PMMA showed greater proliferation after 10 days on the samples without the osteogenic medium, compared to flat PMMA and PS. The behaviour when the osteogenic medium was added was similar to that on flat PMMA and PS.

Cell differentiation

In the absence of osteogenic medium, cells on flat PMMA substrates showed the same levels of alkaline phosphatase activity as on PS. When OM was added, alkaline phosphatase activity increased on flat PMMA, even though this result was not significant. In the absence of OM, levels of AP activity were lower on microstructured substrates than on flat PMMA and PS. This difference was enhanced for the rounded structures. In the presence of OM, rMSC on the square structures showed the same levels of AP activity as PS (Fig. 7A).

In terms of osteocalcin (Fig. 7B), low levels of this osteoblastic late marker were observed in the absence of OM in all samples. On structures, these levels were half those obtained for flat PMMA and PS. When OM was added, levels of osteocalcin increased 5-fold for the flat PMMA and PS and 6-fold for the structured PMMA. There were no differences among the samples.

Discussion

MSC are usually grown on PS treated with polylysine, to enhance cell adhesion and cell growth. The stability of these cultures on other 2D synthetic substrates has not been studied in depth. The most common polymers used for MSC cultures are natural polymers, such as collagen (mostly Type I), fibrin, alginates, hyaluronic acid, etc. However, synthetic polymers allow better control of physicochemical properties than natural polymers. In addition, the use of synthetic polymers reduces the risk of potential biohazardous complications associated with animal polymers [Cancedda 2003].

PMMA is a highly hydrophilic substrate that has shown good biocompatibility with cells and is being used in contact lenses. It is a common polymer, which is used for

micro and nanoimprinting processes. It is an amorphous, thermoplastic polymer with excellent optical transparency and a glass transition temperature $\sim 105^{\circ}\text{C}$ [Mills 2007].

Several studies have shown that cells react to topographical features, which affect cell adhesion, morphology and proliferation. The work done by the Boyan group [Batzner 1998] on titanium has had a great impact on the dental implant field. Their results show that a range of roughness of around $4\text{ }\mu\text{m}$ induces osteoblast differentiation. However, the establishment of a relationship between stem cell differentiation and microstructures has not been well studied. Only a few works [Zinger 2005, Dalby 2007, Popat 2008], have studied the effect of nano and microstructures on cell differentiation.

Cell differentiation is of major relevance in tissue engineering applications. The possibility of creating topography that is able to induce MSC differentiation would revolutionise the field of bone implants. In this sense, the results of this study support the idea that certain surface patterns can induce differentiation.

The cell proliferation results illustrate that rMSC tends to increase on patterned PMMA surfaces, in either large or small structures. These results were not significant, which could be due to the heterogeneity of rMSC [Derubeis 2004]. There was less cell proliferation on all substrates when differentiation was induced by OM. Nevertheless, there were still more cells on the structured PMMA than on the flat substrate.

When analyzing the activity of AP and osteocalcin as early and late markers for osteoblast differentiation in the absence of OM, both markers showed low levels on all surfaces, as expected. Levels on the structured surfaces were lower than those on PS and flat PMMA. When inducing osteogenic differentiation, a greater amount of both markers was quantified on PS and flat PMMA. The most interesting result was that levels of AP activity and osteocalcin secretion on structured surfaces were equivalent to those encountered on flat surfaces (PS and PMMA). This result was more marked for

osteocalcin, where no differences among the surfaces were detected. This means that the structures act synergistically with the osteogenic factors present in the medium. These results are in concordance with the results of Boyan's group [Batzner 1998] on titanium, and with the synergistic effect of roughness and $1\alpha,25\text{-(OH)}_2\text{D}_3$, a well known differentiation factor in osteoblasts cultures.

Several studies indicate that chemical surface modifications have the greatest impact on osteoblast behaviour [Lan 2005, Keselowsky 2005]. However, topographical modifications also promote chemical modifications. The wettability of a surface is known to be affected by its topography [Navarro 2006]. Previous results [Mills 2007] on PMMA contact-angle measurements have shown that the embossed, nonstructured PMMA surface is originally hydrophilic but becomes hydrophobic when patterned with microstructures. The surface also becomes increasingly hydrophobic as the dimensions of the structures are reduced. These facts may explain why the cells are thinner and more elongated, producing a more spherical cross section, than those on the nonstructured surface, where the cell is forced to interact with a flat surface. Cells seem to prefer hydrophilic surfaces. We have observed this trend in adhesion (data not shown) to the structured surfaces (the most hydrophobic) during the first 24h. However, the number of cells was equivalent after 10 days of culture. This is due to the different protein adsorption associated with hydrophobicity and hydrophilicity, which affects the initial adhesion. However, after 2 or 3 days, cells have produced their own extracellular matrix and have developed a microenvironment that is feasible for cell growth.

The differential reaction to the two types of structures is in accordance with the cell morphology. As seen in Figure 5, cells on the structures showed stress fibres. These were more evident in the small structure and in large structures when cells were in contact with the feature. It has also been reported that cell shape regulates the switch in

lineage commitment of human mesenchymal stem cells (hMSC), by modulating endogenous RhoA activity. When hMSC are allowed to adhere, flatten and spread, they undergo osteogenesis, whereas unspread, round cells become adipocytes [McBeath 2004]. Apparently, changes in cell spreading alter RhoA-mediated cytoskeletal contractility, focal adhesion assembly and downstream integrin signaling. In contrast, we could not observe adipocytes in our cultures. This could be due to the different type of structures and the cell origin.

In conclusion, our results confirm the relevance of surface topography to MSC behaviour. Structures enhanced MSC proliferation and gave good levels of differentiation in presence of osteogenic medium. Surface topography is one of the main parameters used to control the design of medical devices and implants. Moreover, the design of microtextured tissue engineering matrices for specific tissues can be used to facilitate experiments on cultured cells *in vitro* and to provide more physiological conditions under which to maintain cells.

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Figures

Figure 1. Interferometry images of the microstructured PMMA. A) is the rounded structure of 50 μm width and 1 μm height and B) is the square structure of 2 μm width and 1 μm height.

Figure 2. rMSC cultured for 14 days (A) and 21 days (B). Positive alkaline phosphatase staining could be observed in all wells after 14 days. After 21 days, staining of mineralisation nodules could be seen. Fig. 2B shows a zoom of the mineralization nodules.

Figure 3. rMSC proliferation after 11 days in culture. Cells were cultured on all surfaces, including PS, flat PMMA and structured PMMA, either with and without OM.

Figure 4. rMSC differentiation. Cells cultured for 11 days in the presence or absence of OM. A) Alkaline phosphatase activity correlated to cell number. B) Osteocalcin amount correlated to cell number.

Figure 5. Immunohistochemical staining of actin (red), vinculin (green) and cell nuclei (blue). (A) rMSC on round structures. Three cells are located inside the structures and are connected to one another among the structures. (B) rMSC on round structures are well spread, with many stress fibres and a high number of focal contacts (vinculin). (C) rMSC on square structures have two types of morphologies: one more elongated, with no spreading at all, and another that is star-shaped. (D) rMSC on square structures show stress fibres but fewer focal contacts, due to the lower spreading. Notice the alignment of the cytoskeleton.

Figure 6. SEM images of rounded structures (A), square structures (B) and flat PMMA. rMSC on round structures can also get around these features. On square (small) structures, cells are aligned and several prolongations or filopodia can be observed that attach preferentially to the structures. On flat PMMA, the cells are randomly oriented and several morphologies can be observed, depending on the cell activity.

Figure 7. Pictures taken by time lapse video recording show how cells get inside the round structures (pointed with arrows) and how the ones that were already inside these structures do not get out (pointed by arrow heads).

Fig 1 A and B.

Fig 2 A and B.

Fig. 3.

Fig. 4.

Fig. 5.

Fig 6.

Fig 7 A and B.

A

B