Original Article

THE EFFECT OF SURFACE PORE SIZE ON THE DIFFERENTIATION OF RAT BONE MARROW CELLS: MORPHOLOGICAL OBSERVATIONS AND EXPRESSION OF BONE RELATED PROTEIN mRNA

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Abstract

The purpose of this study was to investigate the behavior of rat bone marrow cells (RBM) growing on surfaces with different pore sizes. RBM behavior on Millipore filters (MF-Millipore™ membrane filter) made from cellulose mixed esters with 5 different pore surfaces (0.45 μm , 1.2 μm , 3.0 μm , 5.0 μm and 8.0 μm) were compared in terms of morphological changes on the different pore sizes. Furthermore, the expressions of osteopontin and osteocalcin mRNAs were investigated. On the 1.2 μm and 3.0 μm pore surfaces, RBM attached to the substrate well, but cells on the 5.0 μm and 8.0 μm pore surfaces invaded deeply into the pores. Higher levels of both osteopontin and osteocalcin mRNA expression were always observed in cells cultured on the 1.2 μm filter. These results suggest that the 1.2 μm Millipore filter pore size is the most suitable for inducing RBM to differentiate into an osteoblastic phenotype among these surfaces and is probably related to production of the ECM but not to the phenomenon of cell spreading.

Key words: Pore surface—Osteopontin—Osteocalcin—RT-PCR—Surface geometry

INTRODUCTION

It is well known that osseointegration is the key to successful dental implantation¹⁾ and is

in part regulated by the implant surface geometry^{3,11)}. Many previous studies have compared the influence of the surface geometry of materials on cell behavior and the

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characteristics of dental implants using *in vivo* and/or *in vitro* model systems to simulate the implant surface *in situ*^{2,4,5,13,14,16,17,19,31}. The characteristics of the surface geometry influence cell attachment, orientation, and subsequent tissue integration *in vivo*^{10,11,13,14,32–35}. Much information on the interactions between cells and materials has been gained from *in vitro* studies using various approaches to examine the influence of surface characteristics on cell behavior; such research helps in understanding events that occur *in vivo*. Osteoblast proliferation, differentiation, and maturation around dental implants have all been widely studied both *in vivo* and *in vitro*^{7,27–29}, and it has been clarified that titanium implant surfaces modulate the phenotypic expression and metabolism of osteoblast cells^{3,6,8}. However, the data reported on the biological effects of surface properties on osteoblast proliferation and differentiation are rather contradictory and do not clarify the comparative efficacies of surfaces with different properties. This information is necessary because differentiation toward an osteoblastic phenotype is a multistep process regulated by several factors, including hormones, growth factors¹⁸ and mechanical stresses which affect cellular shape.

The study of mRNA expression is one of the most powerful tools for studying weak cell signals, such as physical or physicochemical stimulation by cell-substrate interactions^{15,30}. For instance, type I collagen is known to be an essential protein for pre-osteoblasts to anchor to a substrate²², and osteopontin and osteocalcin are known as bone-related proteins produced by differentiated osteoblasts. It is also known that blasting a smooth surface with Al₂O₃ particles of 25 μ m, 75 μ m and 250 μ m produces surfaces with roughness values of 1.16 μ m to 1.20 μ m, 1.43 μ m and 1.94 μ m to 2.20 μ m, respectively, and these roughness values are suitable for osseointegration¹⁴. Chemical etching creates pits of specific dimensions and shape, and these surfaces also function in the same way as the blasting surfaces. However, the effects of the pore size are not well understood.

The purpose of this study was to investigate the effects of surfaces of Millipore filters (MF-MilliporeTM membrane filter; Millipore Limited, Tokyo, Japan) with 5 different surface pore sizes on the differentiation of rat bone marrow cells (RBM). In order to examine this, the cell lengths of the attached cells were measured on substrates of different pore sizes by scanning electron microscopy. Further, the expression of osteopontin and osteocalcin mRNAs was analyzed by reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

The substrates used in this study were prepared with MF, which is made from cellulose mixed ester, with 5 different pore sizes; 0.45 μ m, 1.2 μ m, 3.0 μ m, 5.0 μ m and 8.0 μ m.

1. Preparation of RBM cells

Primary RBM cells were isolated from the femurs of Wistar rats weighing approximately 100 g/bw, according to the method described by Maniatopoulos *et al.*²⁰. Briefly, the proximal and distal ends of each femur were resected, and RBM were obtained from the diaphysis by flushing with the culture medium described below, using a stainless steel #26 hypodermic needle mounted on a 3 ml syringe. The cells were collected and centrifuged, and the pellets obtained were placed in culture dishes. These cells have been shown to exhibit an osteoblastic phenotype *in vitro*, including expression of alkaline phosphatase activity and synthesis of osteopontin and osteocalcin²⁰.

2. Culture conditions

Cells were cultured in α -MEM (minimum essential medium, Gibco Laboratories, Carlsbad, CA, USA) with 10% fetal calf serum supplemented with 40 μ g/ml penicillin G and 10,000 μ g/ml streptomycin sulfate. For RBM osteoblast-like cell culture, 10⁻⁸ M dexamethasone and 10 mM β -glycerophosphate were added to the medium. Cultures were incubated in 5% (v/v) carbon dioxide (CO₂).

at 37°C for five days until the cells were confluent, with changes of medium every 48 hours. Cells were observed every day by phase-contrast microscopy. When the cells became confluent, they were detached from the flasks using 0.1% trypsin. The cell number in each suspension was determined and adjusted to 2×10^4 cells/ml in the same culture medium. Approximately 5×10^4 cells in 5 ml of medium were seeded on the surface of each MF in a culture dish. After 12 hours of incubation at 37°C, the cells were washed with phosphate-buffered saline (PBS) to remove any unattached cells such as hemopoietic cells.

3. Scanning electron microscopic (SEM) evaluation

After 3 or 6 days of culture, cells cultured on MFs of each pore size were fixed with 2.5% glutaraldehyde in 0.1 mM sodium cacodylate buffer, pH7.2. They were then washed with PBS, dehydrated in graded ethanol, and critical point-dried from liquid CO₂ using a critical point-drier. Samples were sputter coated with Au-Pd for 2 min and then viewed using a HITACHI 8000 SEM.

4. Cell length

Both the long and short lengths of cells cultured on each size of MF at each time period were measured as viewed by SEM using an ultra scale and were compared by 1-way analysis of variance (ANOVA).

5. Quantitative RT-PCR using the Light Cycler™

Total RNA was extracted using the Isogen reagent (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Briefly, cultured osteoblast-like cells were homogenized in an Isogen/chloroform solution at 4°C. Supernatants were obtained by centrifugation at $12,000 \times g$ for 20 min at 4°C. The precipitates were obtained by decantation and washed with 75% ethanol. The RNA pellets were dissolved in RNAase free water and stored at -20°C until used. Using the extracted RNA as a template, reverse transcription reactions were carried out with an

RT-PCR kit (RNA PCR kit ver2.1, Takara Biomedicals, Tokyo, Japan) to synthesize cDNA. PCR was then carried out using osteocalcin and osteopontin primers. Primer sequences from 5' to 3' were: osteopontin (forward; CTC GGA GGA GAA GGC GCA TTA, reverse; CCA TCG TCA TCG TCG TCG TCA) and osteocalcin (forward; GGT GCA AAG CCC AGC GAC TCT, reverse; GGA AGC CAA TGT GGT CCG CTA). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control; its primer sequences from 5' to 3' were, forward; TGA ACG GGA AGC TCA CTG G, and reverse; TCC ACC ACC CTG TTG CTG TA. Thirty five reaction cycles were used for all of these primers.

To measure mRNA levels, a quantitative RT-PCR assay was conducted using a Light Cycler™ (Roch Diagnostics, Tokyo, Japan) with the double-stranded DNA dye SYBR Green I. The primers used for assays with the Light Cycler™ were the same as those described above for ordinary RT-PCR. Quantification was performed by comparing the mRNA levels obtained with standardized samples of GAPDH. The PCR conditions used in the Light Cycler™ were 40 cycles (95°C 10 sec, 60°C 5 sec and 72°C 12 sec). Melting curve analysis and agarose gel electrophoresis of PCR products were also performed after the PCR amplification to confirm the absence of the primer dimer in the PCR products.

RESULTS

1. Morphological observations of cells cultured on Millipore filters

At 3 days, the cells were characterized by polymorphous spread shapes without any microprocesses on any size of filter (Fig. 1a, 2a, 3a, 4a and 5a). However, cells cultured on 0.45 μ m filters were well spread, but cells cultured on the other pore sizes were elongated and attached to the substrates by cell processes.

At 6 days, cells cultured on the 0.45 μ m filters were much more spread than at 3 days

(Fig. 1b). Cells cultured on the 1.2 μ m and 3.0 μ m filters were characterized by polymorphic shapes and were attached to the substrate with small processes, but not microprocesses (Fig. 2b, 3b). Cells cultured on the 5.0 μ m and 8.0 μ m filters had invaded the

large pores (Fig. 4b, 5b) and had some small processes anchored to the substrate. Some cells cultured on 8.0 μ m filters exhibited a small nodule-like appearance (Fig. 5c). Microvilli were not seen on any of the cells cultured on MFs of any pore size, and no

Fig. 1a-5a SEM observations of cells cultured on Millipore filters after 3 days

1a-0.45µm, 2a-1.2µm, 3a-3.0µm, 4a-5.0µm, 5a-8.0µm
Cells on 0.45µm filters were well spread, but cells on the other pore size surfaces were elongated and attached to the substrate by cell processes. bar: 10µm

Fig. 1b-5b and 5c SEM observations of cells cultured on Millipore filters after 6 days

1b-0.45µm, 2b-1.2µm, 3b-3.0µm, 4b-5.0µm, 5b-8.0µm, 5c-8.0µm

Cells on 0.45µm MF were much more spread than they were after 3 days (1b). Cells on 1.2µm (2b) and 3.0µm (3b) filters were characterized by small processes on the filters. They had a tendency to be spindle-shaped with many small processes and were attached to the substrate. Cells on 5.0µm (4b) and 8.0µm (5b) filters were spindle-shaped or spread, but most of the cell bodies had invaded the large pores and had some small processes anchored to the substrate. Some cells on the 8.0µm MF showed a small nodule-like appearance (Fig. 5c). bar: 10µm

spindle-shaped cells were seen.

The average cell length cultured on 1.2µm filters at 3 days tended to be longer than that of cells cultured on 3.0µm and 5.0µm filters

but was not different from that of cells cultured on 8.0µm filters. The average cell length cultured on 0.45µm filters at 6 days tended to be longer than that of cells cultured

Fig. 6 Statistical analysis of average cell length after 3 days in culture

- a: The long axes of cells cultured on $0.45\mu\text{m}$ MF for 3 days were significantly longer than cells cultured on the other pore-sized MF ($p < 0.01$). The long axes of cells cultured on $1.2\mu\text{m}$ filters at 3 days were significantly longer than cells cultured on $3.0\mu\text{m}$ ($p < 0.05$) and on $5.0\mu\text{m}$ ($p < 0.01$) filters but were not significantly different compared to cells cultured on $8.0\mu\text{m}$ MF.
- b: The short axes of cells cultured on $0.45\mu\text{m}$ filters for 3 days were significantly longer than cells cultured on the other pore-sized MF ($p < 0.01$). The short axes of cells cultured on $1.2\mu\text{m}$ at 3 days were significantly longer than cells cultured on $5.0\mu\text{m}$ ($p < 0.05$) MF but not significantly different compared to cells cultured on $3.0\mu\text{m}$ and $8.0\mu\text{m}$ MF.

on the other pore sizes. On the other hand, the average cell length cultured on $1.2\mu\text{m}$ filters at 6 days was tended to be shorter than that of cells cultured on the other pore sizes.

2. Cell length

The long axis lengths of cells at 3 days were 81.3 ± 15.3 on $0.45\mu\text{m}$, 64.9 ± 20.8 on $1.2\mu\text{m}$, 51.0 ± 17.0 on $3.0\mu\text{m}$, 47.8 ± 13.0 on $5.0\mu\text{m}$ and 57.7 ± 16.0 on $8.0\mu\text{m}$ filters. The long axes of cells cultured on $0.45\mu\text{m}$ filters at 3 days were significantly longer than cells cultured on the other pore sizes ($p < 0.01$). The long axes of cells cultured on $1.2\mu\text{m}$ filters at 3 days were significantly longer than cells cultured on $3.0\mu\text{m}$ ($p < 0.05$) and $5.0\mu\text{m}$ ($p < 0.01$) filters, but were not significantly different from cells cultured on $8.0\mu\text{m}$ filters (Fig. 6a).

The short axis lengths of cells at 3 days were

44.7 ± 10.4 on $0.45\mu\text{m}$, 36.8 ± 16.1 on $1.2\mu\text{m}$, 29.6 ± 12.4 on $3.0\mu\text{m}$, 27.7 ± 9.8 on $5.0\mu\text{m}$ and 30.24 ± 17.0 on $8.0\mu\text{m}$ filters. The short axes of cells cultured on $0.45\mu\text{m}$ filters at 3 days were significantly longer than cells cultured on the other pore sizes ($p < 0.01$). The short axes of cells cultured on $1.2\mu\text{m}$ at 3 days were significantly longer than cells cultured on $5.0\mu\text{m}$ filters ($p < 0.05$) but were not significantly different compared to cells cultured on $3.0\mu\text{m}$ or $8.0\mu\text{m}$ filters (Fig. 6b).

The long axis lengths of cells at 6 days were 127.9 ± 27.5 on $0.45\mu\text{m}$, 47.0 ± 14.5 on $1.2\mu\text{m}$, 68.6 ± 22.4 on $3.0\mu\text{m}$, 65.8 ± 20.0 on $5.0\mu\text{m}$ and 74.4 ± 20.8 on $8.0\mu\text{m}$ filters. The long axes of cells cultured on $0.45\mu\text{m}$ filters at 6 days were significantly longer than cells cultured on the other pore sizes ($p < 0.01$). The long axes of cells cultured on $1.2\mu\text{m}$ filters at 6 days were significantly shorter than cells

Fig. 7 Statistical analysis of average cell length after 6 days in culture

- a: The long axes of cells cultured on 0.45 μm MF for 6 days were significantly longer than cells cultured on the other pore-sized MF ($p < 0.01$). The long axes of cells cultured on 1.2 μm filters at 7 days were significantly shorter than cells cultured on the other pore-sized MF ($p < 0.01$).
- b: The short axes of cells cultured on 0.45 μm filters for 6 days were significantly longer than cells on the other pore-sized MF ($p < 0.01$). The short axes of cells cultured on 1.2 μm filters for 7 days were significantly shorter than cells cultured on 5.0 μm ($p < 0.05$) and 8.0 μm ($p < 0.01$) MF but were not significantly different compared to cells cultured on 3.0 μm filters.

cultured on the other pore sizes ($p < 0.01$) (Fig. 7a).

The short axis lengths of cells at 6 days were 65.1 ± 20.5 on 0.45 μm , 25.4 ± 10.0 on 1.2 μm , 33.4 ± 12.1 on 3.0 μm , 33.6 ± 10.3 on 5.0 μm and 36.2 ± 10.9 on 8.0 μm filters. The short axes of cells cultured on 0.45 μm filters at 6 days were significantly longer than cells cultured on the other pore sizes ($p < 0.01$). The short axes of cells cultured on 1.2 μm filters at 6 days were significantly shorter than cells cultured on 5.0 μm ($p < 0.05$) or 8 μm filters ($p < 0.01$) but were not significantly different from cells cultured on 3.0 μm filters (Fig. 7b).

3. Expression of osteopontin and osteocalcin mRNA

Expression levels of osteopontin mRNA at 3 days seemed to be greater in cells cultured on 1.2 μm and 3.0 μm filters than in cultured

on 0.45 μm , 8.0 μm or 5.0 μm filters (Fig. 8a). The expression level of osteopontin mRNA at 6 days in cells cultured on the 1.2 μm surface was still the greatest, and the levels in cells cultured on 0.45 μm , 5.0 μm and 8.0 μm filters were greater than those of cells cultured on 3.0 μm filters (Fig. 8b).

The expression levels of osteocalcin mRNA at 3 days also seemed to be larger in cells cultured on the 1.2 μm and 3.0 μm filters than in those cultured on 0.45 μm , 5.0 μm and 8.0 μm filters (Fig. 9a). The expression level of osteocalcin mRNA at 6 days in cells cultured on the 1.2 μm surface was still the greatest, and the levels in cells cultured on 0.45 μm , 5.0 μm or 8.0 μm filters were greater than in cells cultured on the 3.0 μm filter (Fig. 9b).

Higher levels of both osteopontin and osteocalcin mRNA expression were always observed in cells cultured on the 1.2 μm filter.

3 days RBM-OPN

a

6 days RBM-OPN

b

Fig. 8 Levels of expression of osteopontin mRNA (Results are typical of three independent experiments)

- a: Expression levels of osteopontin mRNA at 3 days seemed to be greater in cells cultured on 1.2 μm or 3.0 μm MF than in cells cultured on 0.45 μm , 8.0 μm or 5.0 μm MF.
 b: The expression level of osteopontin mRNA at 6 days in cells cultured on the 1.2 μm surface was still the greatest, and the levels in cells cultured on 0.45 μm , 5.0 μm or 8.0 μm MF were greater than those in cells cultured on the 3.0 μm MF.

3 days RBM-OCN

a

6 days RBM-OCN

b

Fig. 9 Levels of expression of osteocalcin mRNA (Results are typical of three independent experiments)

- a: Expression levels of osteocalcin mRNA at 3 days also seemed to be greater in cells cultured on 1.2 μm or 3.0 μm MF than in cells cultured on 0.45 μm , 5.0 μm or 8.0 μm MF.
 b: The expression level of osteocalcin mRNA at 7 days in cells cultured on the 1.2 μm surface was still the greatest, and the levels in cells cultured on 0.45 μm , 5.0 μm or 8.0 μm MF were greater than those in cells cultured on the 3.0 μm MF or controls.

However, the expression levels of both osteopontin and osteocalcin mRNA in cells cultured on the 3 μm filters contrasted with those in cells cultured on 0.45 μm , 5.0 μm and 8.0 μm filters at 3 and 6 days.

DISCUSSION

Implant surface geometry is considered to be an important factor for the osseointegration of osteoblast-like cells into bone tissue or bone marrow, because rough surfaces can

change cell behavior and differentiation, and many studies have emphasized the importance of surface characteristics in this process^{2,4,12-14,19,31}. Martin *et al.*²¹ assessed the proliferation and differentiation of osteoblastic cells and reported that both the regularity and the roughness of the surface geometry influenced cell differentiation and calcification and that matrix formation and collagen synthesis were significantly elevated in cells grown on rough implant surfaces compared to cells on smooth surfaces. Shiigai *et al.*³⁰ suggested that 1.2 μm is the most appropriate substrate for accelerating the collagen synthesis function of fibroblasts, but they did not comment about even smaller sizes of MF. In the present study, we added 0.45 μm MF and investigated the influence of 5 substrates with different pore sizes.

It is known that cells change shape over time depending on the surface geometry and that cells spread well with many microprocesses on a culture dish. The cells cultured on the MFs were also spread, but no microprocesses were observed. MFs have irregular shapes which have gaps or pores of different sizes where there is an area of contact for cytoplasmic extensions but only a few areas of contact of microprocess attachment. Shiigai *et al.*³⁰ reported that an extracellular matrix (ECM), which is more than 100 nm wide and contains fibronectin and integrin, might be created between the wall of an MF and an L-929 fibroblast. Several studies have suggested that differentiation toward a differentiated osteoblast correlates with an increase in alkaline phosphatase activity⁹, bone related protein and ECM production^{18,24}. Owen *et al.*²³ reported that type 1 collagen, fibronectin, vitronectin and tenascin are among the components of the ECM produced by differentiated osteoblasts, and Postiglione *et al.*²⁵ demonstrated that the ECM was easily produced, depending on the type of surface. Rough surfaces induced higher levels of ECM synthesis and organization than smooth surfaces, proving to be optimal for ECM production by osteoblast-like cells. Salter *et al.*²⁶ demonstrated that integrins, which link

components of the ECM with actin filaments in the cytoplasm of bone cells, function as mechanoreceptors and lead cells to become differentiated bone cells.

Cells cultured on 1.2 μm or 3.0 μm filters contact the substrate and probably produce ECM much more readily than do cells cultured on 5.0 μm or 8.0 μm filters. Because the construction of 1.2 μm and 3.0 μm filters is more complicated than that of 5.0 μm or 8.0 μm filters, it is easier for RBM to attach to the substrate. In contrast, the cell bodies invaded deep into the pores of 5.0 μm and 8.0 μm filters, where the construction was simpler and not much ECM was found between the cells and the substrate³⁰.

Lumbikanonda and Sammons¹⁹ (2001) reported that cells span over the surface, creating spaces or gaps underneath. Such results imply that cells spread relatively more slowly on smooth titanium than on total plasma spraying (TPS) surfaces. However, on a smooth titanium surface, the cells eventually formed very close contacts with the surface, adapting to the underlying topography. This contrasts with the observations of Bowers and coworkers², who reported that significantly higher numbers of cells attached to irregular, sandblasted surfaces than to regular ones. In our study, the results of the 0.45 μm filter surfaces were similar to smooth titanium surface and 1.2 μm and 3.0 μm filters were probably similar to the blasting surface. The cells on 1.2 μm and 3.0 μm filters attached to the substrate of the MF with ECM instead of spreading, and the cells cultured on the 5.0 μm and 8.0 μm pore size MF invaded the pores and did not spread. However, after 6 days of culture, cells cultured on 5.0 μm or 8.0 μm filters (with large pores) probably start to produce ECM on the wall of the MFs, and the expression of osteopontin and osteocalcin, which are also important for osteoblast-like cell anchoring, overtakes that of cells cultured on filters with smaller pore sizes. Furthermore, cells cultured on a 0.45 μm filter exceed the other MF groups after 6 days culture in terms of the degree of production of ECM and expression of bone-related protein mRNA.

These results suggest that the expression of bone-related protein mRNA depends on the surface geometry and is related to production of the ECM but not to morphological changes such as the phenomenon of cell spreading with microprocesses.

Taken together, the results suggest that the 1.2 μ m MF is the most appropriate substrate for RBM to differentiate into osteoblasts and promotes the expression of bone related protein mRNA.

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