The effect of substrate pore size on the differentiation of L929 fibroblast cells: Morphological observations and expression of type 1 collagen mRNA

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ABSTRACT

The purpose of this study was to investigate the behavior of L929 fibroblasts on substrates of different pore sizes and to find an appropriate pore size that allows them to differentiate into type 1 collagen synthesizing cells. The behavior of cultured fibroblasts on Millipore filters of 4 different pore sizes (1.2 μm , 3.0 μm , 5.0 μm and 8.0 μm) was compared morphologically. The expression of type 1 collagen mRNA was also analyzed by RT-PCR. The cell length was the greatest in the control group (without a filter) compared to the experimental groups cultured on filters at 3 days, however there were no significant differences among any of the groups at 7 days. At the ultrastructural level, small and fine cell processes had invaded the pores of filter membranes with pore sizes of 1.2 μm or 3.0 μm , while long and thick cell processes containing many organelles (some of them degenerated) had invaded the pores of filter membranes with pore sizes of 5.0 μm or 8.0 μm . The expression of type 1 collagen mRNA was the highest in cells cultured on membranes with 1.2 μm pores at 3 days but was not significantly changed among any of the groups at 7 days. This suggests that Millipore filters with a 1.2 μm pore size are the most suitable to allow L929 fibroblasts to express the phenotype for type 1 collagen synthesis at early time periods.

Dental implants in which biologic tissues and synthetic materials coexist, have become a requirement for modern dental science. Clinical success relies on the formation of a stable interface between the tissue and the artificial material (1). Osseointegration or fibrointegration refer to the integration between osseous or fibrous tissues, respectively, processes that are regulated by the implant surface geometry. Many previous studies have compared the influence of the surface geometry of materials and the characteristics of dental implants on cell behavior using *in vivo* and/or *in vitro* model systems to simulate the

implant surface *in situ* (2, 5, 15–17, 21–23). The characteristics of the dental implant surface influence the cell attachment, the orientation and the subsequent tissue integration *in vivo* (6–9, 12, 13, 24–28). Much information on interactions between cells and materials has been gained from *in vitro* studies examining the influence of surface characteristics on cell behavior using various approaches (3, 4, 11). Smooth and rough surfaces have been classified by the mean average surface roughness.

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 (14). For instance, type 1 collagen is known to be an essential protein for fibroblasts to anchor to a substrate (19).

The purpose of this study was to investigate the behavior of L929 fibroblasts on substrates of different pore sizes. In order to examine this, the cell

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length of attached fibroblasts was measured on Millipore filter (MF) substrates of different pore sizes by scanning electron microscopy (SEM) and morphological changes were observed by transmission electron microscopy (TEM). Further, the expression of type 1 collagen mRNA by fibroblasts growing on those substrates was analyzed by reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Substrates. Four types of MF (Millipore Co. Ltd., Tokyo, Japan) with different pore sizes (1.2 μm , 3.0 μm , 5.0 μm or 8 μm) were used in this study as substrates.

Cell culture. L929 mouse fibroblasts were obtained from the Japanese Cancer Research Resources Bank (Foundation for Promotion of Cancer Research, Tokyo, Japan). The cells were cultured in 75 cm^2 cell culture flasks in α -MEM (minimum essential medium; Gibco Laboratories, Carlsbad, CA, USA) with 10% fetal calf serum supplemented with 40 $\mu\text{g}/\text{mL}$ Gentamicin (Sigma Chemical, Co., St. Louis, MO, USA). Cells were incubated in 5% (v/v) carbon dioxide (CO_2) at 37°C for 5 days until the cells were confluent, with changes of medium every 24 h. Cells were observed every day by phase-contrast microscopy. When the cells became confluent, they were then 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 media were seeded on MF of each pore size in 35 mm culture dishes, or without a filter as a control. After 12 h incubation at 37°C, the cells were washed with phosphate-buffered saline (PBS) to remove any unattached cells, and the medium was then changed every 48 h after that. Cells cultured without a MF were used as controls.

Preparation of samples for SEM. Cells growing on MF of each pore size or on a culture dish without a MF were fixed after 3 and after 7 days of culture with 2.5% glutaraldehyde in 0.1 mM sodium cacodylate buffer, pH 7.2. Specimens were washed with PBS, dehydrated in a graded ethanol series and then dried in a critical point-dryer. The dried samples were coated with gold to a thickness of approximately 100 nm using sputter coating for 2 min and viewed using a HITACHI 8000 SEM.

Evaluation of cell length. In the experiments described herein, 20 cells at each of the time periods were selected randomly. The longest length of each attached cell was measured (mean $\pm$ SD) as viewed by SEM using an ultra scale. The data were analyzed via one-way analysis of variance (ANOVA) and were then compared by the Fisher PLSD (protected least significant difference) test.

Preparation of samples for TEM. Cells cultured on each size of MF for 7 days were fixed with Karnovsky's fixative solution (2.0% paraformaldehyde and 2.5% glutaraldehyde) in 0.12 M Sorensen's phosphate buffer, pH 7.2. The samples were then post-fixed with 4% osmium tetroxide before embedding in Epon 812. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and were observed using a JEOL 100CX-TEM.

Quantitative RT-PCR using the Light CyclerTM. Total RNA was extracted using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Briefly, cultured cells after 3 and 7 days were homogenized and supernatants were obtained by centrifugation at 13,200 rpm for 20 min at 4°C. The precipitates were obtained following decantation, and were washed with 75% ethanol. The RNA pellets were dissolved in RNAase-free water and were preserved at -20°C until use. Using the extracted RNA as a template, reverse transcription reactions were carried out with an RT-PCR kit (RNA PCR kit ver 2.1, Takara Biomedicals, Tokyo, Japan) to synthesize cDNA. PCR was then carried out using pro- $\alpha(1)$ collagen primers. The primer sequences used (from 5' to 3') were, forward; GAA CGG TCC ACG TTG CAT G, and reverse; GGC ATG TTG CTA GGC ACG AAG. To observe mRNA levels, quantitative RT-PCR assays were conducted with a LightCycler using the double-stranded DNA dye SYBR Green I (Roche Diagnostics, Tokyo, Japan). Quantification was performed by comparing the levels obtained with standardized samples. The PCR conditions used in the LightCycler were 40 cycles of 95°C for 10 sec, 60°C for 5 sec, and 72°C for 12 sec. Melting curve analysis was also performed after the PCR amplification to confirm the absence of the primer dimer in the PCR products. The PCR products were then separated on 2% agarose gels containing ethidium bromide.

RESULTS

SEM observations

Control group: After 3 days in culture, the cells were characterized by their spindle shapes with focal cytoplasmic extensions and a few small processes (Fig. 1a). After 7 days in culture, the cells were wider and had radically extended filopodia

(Fig. 1b).

Experimental groups: After 3 days in culture, the cells grown on the MF with 1.2 μm pores had a slight spreading of their cytoplasm with many small processes anchored to the substrate (Fig. 2a). The cells cultured on MFs of bigger pore sizes were rounded in shape and had many small processes anchored to the substrate (Fig. 3a). Microvilli were not

Fig. 1a SEM observations of the control group after 3 days in culture. A spindle-shaped cell has focal cytoplasmic extensions and a few small processes. ($\times 4,000$)

Fig. 1b SEM observations of the control group after 7 days in culture. Cells are wider and extend radically filopodia ($\times 4,000$)

Fig. 2a SEM observations of cells cultured on the 1.2 μm MF after 3 days in culture. A Cell has little spreading of their cytoplasm and have many small processes anchored to both the substrate and the spread cytoplasm. ($\times 1,500$)

Fig. 2b SEM observations of cells cultured on the 1.2 μm MF after 7 days in culture. Cells tend to become spindle shaped with many small processes. ($\times 4,000$)

Fig. 3a SEM observations of cells cultured on the 8.0 μm MF after 3 days in culture. Rounded cells have many small processes around the cells anchored to the substrate. ($\times 1,500$)

Fig. 3b SEM observations of cells cultured on the 8.0 μm MF after 7 days in culture. Cells became spindle shaped with many small processes. The cell surfaces are rough and are equipped with somewhat developed microvilli-like projections. ($\times 4,000$)

seen on any cells cultured on MFs of any pore size, and no spindle-shaped cells could be seen. After 7 days in culture, the cells grown on MFs of all pore sizes had a tendency to be spindle-shaped with many small processes (Fig. 2b, 3b). The cell surfaces appeared rough and showed inconspicuous microvillus-like projections (Fig. 3b). However, they had very conspicuous surface contacts with the substrate.

Comparison of cell length

The lengths of cells cultured on MFs of 1.2 μm , 3 μm , 5 μm or 8 μm pore sizes were significantly shorter at 3 days than at 7 days (Fig. 4). However, the results of the control group were opposite, and control cell lengths at 3 days were significantly longer than at 7 days. The cell lengths of the control group at 3 days were significantly longer than the other experimental groups. However, there were no significant differences between the control group and any of the experimental groups at 7 days.

Ultrastructural observations at 7 days of culture

Control group: The cells were flattened in shape and/or contained small round nuclei in their centers, and a few mitochondria and some endoplasmic reticulum could be seen in the cytoplasm. The cells were in contact with the culture dish without any kind of intercellular junctions.

Fig. 4 Statistical analysis of average cell length at long axis. (**: $p < .01$)

Experimental groups: There were a few organelles, such as mitochondria and endoplasmic reticulum, in the cytoplasm and the fine cell processes had invaded the pores of the 1.2 μm or 3 μm MFs (Fig. 5). However, organelles could not be seen in the invading processes. In cells cultured on MFs of the 5 μm or 8 μm pore sizes, the thick cell processes had in-

Fig. 5 TEM observations of cells cultured on the 1.2 μm MF. At 7 days of culture on the 1.2 μm MF, there are few organelles such as mitochondria and endoplasmic reticulum in the cytoplasm. The cell processes invad into the pores. No organelles are seen in the invading processes. MF: Millipore filter, N: nucleus (original magnification $\times 9,000$)

vaded much deeper into the pores and a few organelles could be seen. Of particular note, the long and thick processes of cells grown on the 8 μm MF had deeply migrated and contained developed organelles but the distal ends were often degenerated (Fig. 6).

Expression of type 1 collagen mRNA

Estimation of type 1 collagen mRNA levels was carried out by RT-PCR. Fig. 7 shows the single melting peak at 88°C (Fig. 7a) and a single band at 167 bp (Fig. 7b) of the PCR products from each group at each of the time periods. The type 1 collagen

mRNA expression levels were higher in cells cultured on the MF with 1.2 μm pores than those cultured on the other MFs or on culture dishes without a MF at 3 days. Particularly, expression of type 1 collagen mRNA in cells cultured on the MF with 1.2 μm pores was 4 times higher than on the MF with 8.0 μm pores (Fig. 8). The mRNA expression levels at 7 days seem to be a little higher on the MF with 1.2 μm pores than those in other groups, however it was not a statistically significant difference (Fig. 9).

DISCUSSION

The implant surface geometry is considered to be an important factor for formation of implant-tissue interface, since it can significantly change cell behavior and differentiation (10–13), and many studies have emphasized the importance of surface characteristics (21–28). Inoue *et al.* (11) reported that cultured gingival fibroblasts orientated at right angles to a rough surface while they orientated parallel to a smooth surface *in vitro*, and concluded that the geometrical configuration of the surface of the implants could influence whether a capsule or an oriented fibrous attachment develop in association with implants *in vivo*. Martin *et al.* (18) 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 the matrix formation and the collagen synthesis were significantly elevated on a rough implant surface compared to cells cultured on a smooth surface. It is apparent from the literature that osteoblastic cells and other anchorage-dependent cells, such as fibroblasts, show similar morphologic behaviors in attachment studies. The cell attachment assay used in this study is similar to the technique used in previous studies to compare initial cellular responses to implants with different surface characteristics. This *in vitro* study using substrates of different pore sizes may be useful to understand events occurring in the peri-implant fibrous connective tissue interface *in vivo*.

It is known that cells change shape over time depending on the surface geometry of the matrix. Based on phase contrast microscopy, the morphological changes undergone by cells during attachment and flattening onto a glass surface have been reported by Brunette *et al.* (4). In fibroblasts cultured on glass, many microvilli appear and a thin layer plate is established between the matrix and the

Fig. 6 TEM observations of cells cultured on the 8.0 μm MF. At 7 days of the culture on 8.0 μm MF, the cell processes invaded much deeper into the pores. The distal end of the process had degenerated but the proximal part was preserved. Many organelles, such as mitochondria, are seen in the processes. MF: Millipore filter, N: nucleus (original magnification $\times 4,600$)

Fig.7a

Fig.7b

Fig. 7 Estimation of mRNA levels of Type 1 collagen carried out by RT-PCR. The single melting peaks at 88°C (Fig. 7a) and the single band at 167 bp (Fig. 7b) of the PCR products of each of the groups at each of the time periods are shown.

cells. The cytoplasm then radiates in all directions, and becomes gradually smoother as time elapses. Finally, the cells become polarized and slender, and at this stage, the cells and the matrix do not completely adhere to each other. In this study, we found that cells growing on MFs of all pore sizes (except 1.2 μm) were round or cuboidal in shape at 3 days and then all cells became spindle-shaped at 7 days. However, cells at each time period had many small processes anchored to the substrate. MFs have irregular shapes, similar to granular or blasted surfaces which

have gaps or spaces, where there is no area of contact for cytoplasmic extensions similar to glass surfaces as described above, possibly because of the steep angle of the adjacent surface, small pits or sharp edges. This suggests that MFs might disrupt cell spreading (except for the 1.2 μm MF) which is probably the proper surface geometry for fibroblasts to avoid the disruption.

The long axes of cells cultured on MF were not significantly different at any of the time periods examined. However, in the TEM observations of

Fig.8

Fig.9

Fig. 8 The mRNA expression of type 1 collagen levels is higher in cells cultured on the 1.2 μm MF than those cultured on MFs of other pore sizes and on the control surface at 3 days.

Fig. 9 The mRNA expression levels at 7 days seem to be a little higher on the 1.2 μm MF than those in other groups, however it was not a statistically significant difference.

this study, many types of attachment could be seen. Brunette (3) reported that 3 types of gaps exist between cells and the matrix when observed by TEM. The first type is the focal contact, which is approximately 10 nm wide. The second type is a focal adhesion with a 30–50 nm gap, in which microfibrillar bundles containing actin filaments exist in the cytoplasm. The third type is the extracellular matrix, which is more than 100 nm and contains fibronectin and integrin. In this study, a few contact areas and many extracellular matrix areas were frequently seen. This is probably because the cell processes invaded deep into the pore but did not contact the pore walls with either focal contacts or focal adhesions which created wide gaps between the cell and the substrate. It is possible that the sharp angularity

of this type of surface hinders the anchorage of cell processes on adjacent regions and thus slows down the process of attachment.

Salter *et al.* (20) demonstrated that integrins, which link components of the extracellular matrix with actin filaments in the cytoplasm of bone cells, function as mechanoreceptors. This suggests that the extracellular matrix when cultured on the 1.2 μm MF is more abundant than when cultured on the other MFs and implicates the mechanoreceptors of cultured cells. This may be one reason why the expression of collagen type 1 mRNA, a protein important for fibroblast anchoring, was greatest in cells cultured on the MF with 1.2 μm pores at 3 days.

Initial cell attachment and spreading is affected predominantly by the physical characteristics of the

surface, such as the stability and the overall topography. These properties include the surface free energy, the wettability, and the zeta potential, which in turn influence the adsorption of serum proteins to which the cells attach. This suggests that a surface with 1.2 μm pores is crucial for regulating cell differentiation compared with the control surface, and is the best surface for cells to express essential proteins. In conclusion, a surface with 1.2 μm pores is a proper substrate for fibroblasts to differentiate into protein synthesizing cells at early time periods.

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