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Publisher's version / Version de l'éditeur:
https://doi.org/10.1557/PROC-823-W12.9
Symposium W: Biological and Bioinspired Materials and Devices, 2004

Adhesion Kinetics of MC3T3-E1 Pre-Osteoblasts to Osteoconductive Porous Titanium Scaffolds

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ABSTRACT

Porous metallic scaffolds have recently gained recognition as a promising avenue toward the regeneration of damaged bone structures. Interest in these materials resides in their ability to provide guidance for bone growth by presenting a favorable structure for three-dimensional cellular adhesion and proliferation. Titanium foams with interesting microstructural parameters for applications in tissue engineering of bone have recently been developed. The aim of this study was to assess the potential of this novel material for applications as an osteoconductive scaffold. This was achieved through *in vitro* studies of cellular interactions with titanium foams characterized by three different pore sizes ranging from x to y µm. The viability of J774 mouse macrophages attached to these three types of foams was comparable to that observed on polished titanium controls, indicating that they do not have cytotoxic effects. Adhesion kinetics of MC3T3-E1 pre-osteoblasts showed that cells adhere to the material and proliferate in an attempt to cover the scaffold surface. Furthermore, the three-dimensional morphology of these cells at the surface of the scaffold suggested that the microstructural parameters of the foams had a biomimetic effect on their behaviour and could promote their differentiation and osteogenesis.

INTRODUCTION

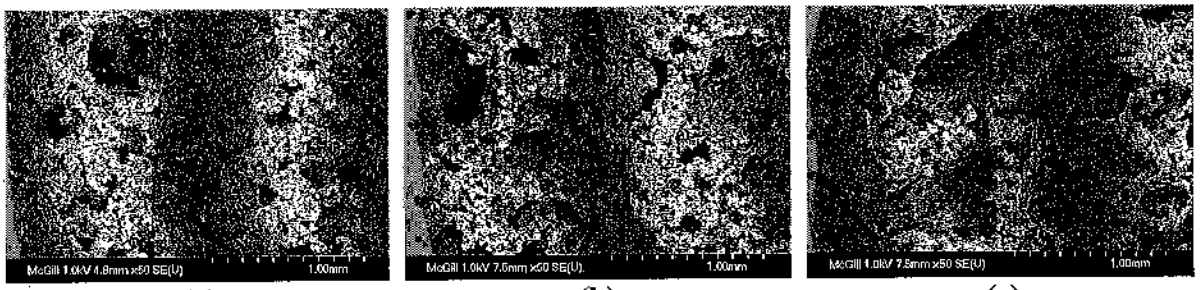
Porous metals have been employed for more than 30 years in the field of orthopedics as implant coatings to ensure a stable fixation through bone ingrowth. Conventional approaches to the fabrication of such porous coatings include bead and fiber sintering [1-2], and plasma-spray technologies [3]. Recently, a shift has been witnessed in the design of such biomaterials toward the fabrication of highly porous metals with microstructural parameters that facilitate bone regeneration [4,5]. In addition to conventional applications as porous coatings, these innovative materials have generated interest in related fields such as maxillofacial reconstruction. Highly porous titanium foams (up to 80% porous) with a unique and adjustable microstructure produced through a novel powder metallurgy process contribute to this trend [6]. The present study is aimed at assessing the biocompatibility of these titanium foams by an evaluation of the cellular viability and adhesion to the material.

EXPERIMENTAL METHODS

Scaffolds preparation and characterization

Titanium scaffolds with three different pore sizes, referred to as TiA, TiB and TiC (from the smaller to the larger pore size), were prepared. Briefly, titanium powder was dry-mixed with a low density polyethylene, a foaming agent (p,p'-oxybis[benzenesulfonyl hydrazide]) and a cross-linking agent (dicumyl peroxide), all in powdered form. The final pore size of titanium foams

was controlled through the composition of the blend. The homogenous mixture was then molded and heated in a three step thermal treatment that included foaming at 210°C for 2 hours, the decomposition of the polymeric binder at 425°C for 4 hours and a pre-sintering step at 1200°C for 2 hours. The foams were then machined into small discs (12.5 mm diameter and 2 mm thickness) and sintered at 1300°C for 2 hours. Figure 1 presents the microstructure of these titanium foams. Controls were prepared by polishing dense titanium and nickel discs (referred to as TiP and NiP respectively) of similar dimensions to that of foams to a mirror-finish (0.04 μm) with an automatic polisher (AbraPol, Struers). Before cell culture assays, porous samples and controls were cleaned through 15 minutes sonication cycles in soapy water, isopropanol and nanopure water, interspaced by rinsing steps in de-ionized water. The scaffolds were then autoclaved and allowed to dry at 60°C and under sterile conditions overnight.



(a) (b) (c)

Figure 1: Scanning electron microscopy (SEM) images of TiA (a), TiB (b) and TiC (c).

The density of each porous titanium sample was calculated through measures of the weight and dimensions of each sample. An average pore size was also obtained for each of the three types of foams. Samples were embedded in epoxy resin (Struers, USA) under vacuum, mirror-polished and imaged with a metallograph (Olympus PMG3, Carsen Medical & Scientific Co.). The images were treated and processed with Visilog 5.4 to obtain the distribution of pore sizes.

Cell culture

J774 mouse macrophages (ATCC, USA) were cultured in RPMI 1640 medium (Gibco BRL, Canada) supplemented with 5% fetal bovine serum (ATCC, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Canada). Adherent monocyte-macrophages play an important role in the control of the early osseous wound healing process [7]. Thus, their viability in the presence of an implant material is essential to ensure faster recovery of patients. MC3T3-E1 subclone 14 mouse pre-osteoblastic cells (ATCC, USA) were cultured in α-MEM medium (Gibco BRL, Canada) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. This cell line is known to express alkaline phosphatase and to generate a mineralized extracellular matrix containing collagen type I and osteocalcin upon differentiation. It is also characterized by a similar morphology to the original stem cells [8]. Both cell lines were grown in T75 cell culture flasks at 37°C in an atmosphere with 100% humidity and 5% CO_2. The complete medium was replaced every 2 to 3 days and confluent cells were subcultured through scraping (J774) or trypsinization (MC3T3-E1).

Cell viability

J774 macrophages in their 8th passage were seeded on porous scaffolds and both controls contained in 24-well plates (Corning, USA) at a concentration of 100,000 cells/500 μl of complete medium and incubated under growing conditions for 48 hours. After the incubation period, the medium was replaced by fresh stock and a Vybrant MTT cell proliferation assay (Molecular Probes, USA) was performed. Briefly, an aliquot of 12 mM [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) in phosphate buffered solution corresponding to 10% of the medium volume was mixed to each well before incubating the cells at 37°C for 4 hours. This incubation allowed the cells to metabolize the MTT into insoluble formazan crystals. These crystals were then dissolved by the addition of a volume of sodium dodecyl sulfate-HCl solution equivalent to the medium volume and a subsequent incubation of 12 hours. The absorbance of the solution was then read at 570 nm with a spectrophotometer (μQuant, Bio-Tek Instruments). Three replicates were performed for each condition. Blanks consisted of scaffolds impregnated with complete medium without cells.

Cell adhesion

Pre-osteoblasts of passages ranging from x to y were seeded on porous scaffolds and both controls contained in 24-well plates at concentrations of 200,000 and 25,000 cells/200 µl of complete medium respectively. A third control consisting of tissue culture plates without scaffolds (TCP) was also investigated. After incubations of 30 minutes, 1, 2, 4, or 18 hours in growing conditions, samples were washed three times in Earle's balanced salt solution (EBSS) and transferred to new 24-well plates to prevent cells adhered to the wells from affecting the results. The number of cells was then measured through the Vybrant MTT cell proliferation assay described previously. Nine replicates were performed for each type of foams, while three replicates were performed for controls. A standard curve was also prepared with the pre-osteoblastic cell line to relate the number of viable cells to the absorbance readings.

Cell morphology

The cell morphology study was performed on titanium foams and TiP. Samples incubated with pre-osteoblasts for 18 hours according to the procedure employed for cell adhesion assays were washed three times in EBSS to remove unattached pre-osteoblastic cells and serum proteins, before adherent cells and their extracellular matrix were fixed for 21 hours with 2.5% Glutaraldehyde in 0.1M sodium cacodylate buffer, dehydrated in serial ethanol and amyl acetate baths, and critical point dried. Specimens were sputter-coated with Au-Pd and the cellular morphology was assessed through SEM (FE-SEM S-4700, Hitachi High-Technologies).

RESULTS AND DISCUSSION

Scaffolds characterization

Table I presents the pore sizes and porosity of the three types of titanium foams investigated. The porosity was calculated through a comparison of the measured densities of foams to the density of bulk titanium. These results highlight the fact that microstructural differences exist between these conditions. It must be stressed that SEM observations of the surface of titanium scaffolds confirmed the similarity of the three conditions at this level.

Table I: Porosity and pore size of the titanium scaffolds investigated.

Condition	Pore Size (μm)	Porosity[a] (% ± S.D.)
TiA		73.1 ± 1.6
TiB		76.6 ± 2.4
TiC		79.8 ± 2.6

[a] The porosity characterizing each condition is statistically different to that of other conditions, as determined by Student's t-test ($p<0.01$).

Cell viability

Figure 2 shows the viability of J774 cells on titanium foams as a percentage of the positive control (TiP) after an incubation of 48 hours. The cellular viability obtained on titanium foams was comparable to that of TiP. In a previous study [9], it was determined that residuals from individual mixture components of the powder metallurgy process to produce titanium foams affected the nature of titanium surfaces. Nevertheless, these surface modifications did not affect the viability of U-937 human macrophages after an incubation of 48 hours. The present cellular viability assay allowed the assessment that the combined effect of the mixture components did not result in cytotoxic effects. Furthermore, the cellular viability was not affected significantly by the pore size of titanium scaffolds tested but was statistically lower on the negative control (NiP) as demonstrated by a Student t-test ($p<0.05$).

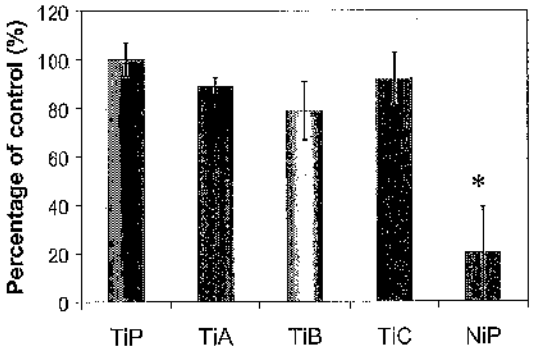
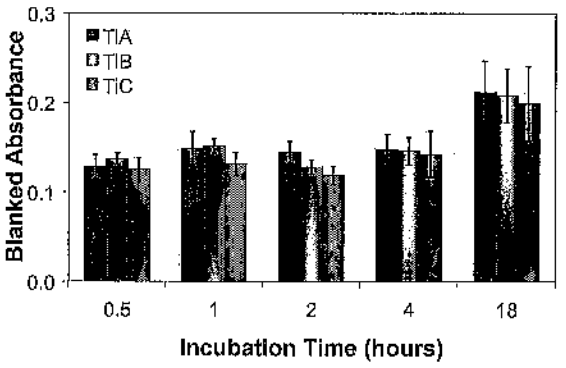


Figure 2: Viability of J774 macrophages on titanium foams as a percentage of the positive control. NiP had statistically lower results than the titanium foams and TiP ($p<0.05$).

Cell adhesion

Figure 3a shows the kinetics of adhesion for MC3T3-E1 cells on titanium foams. The data is presented as a blanked absorbance at 570 nm, which is directly proportional to the number of adherent cells (unpublished data). Cells seem to adhere well to the porous scaffold 30 minutes after seeding. However, a lower amount of cells are adhered to scaffolds after 2 hours than 1 hour. Furthermore, this trend is statistically significant (Student's t-test $p<0.05$) for the conditions TiB and TiC. This effect is not believed to be a response of cells to the porous material because the same trend was observed on TiP and TCP (see Figure 3b). It is more likely that this observation is provoked by the death of weaker cells due to their trypsination before

they are seeded on the test samples. The number of pre-osteoblasts attached on porous titanium scaffolds after 18 hours incubations was considerably increased as opposed to the shorter incubation times. Such results, combined with the fact that a decrease in the number of cells was observed after 2 hours allowed to conclude that cells have started their proliferation. The higher proliferation rate observed on TiP is in accordance with the literature and is caused by the lower surface roughness of these controls as opposed to porous titanium [10]. On NiP, the number of cells is lower after 18 hours than 4 hours of incubation, leading to believe that the smooth surface of this control allowed for an early proliferation but that the toxicity of the material affected the cellular viability on a longer time scale (see Figure 5b). The pore size of porous titanium did not have an effect on the adhesion kinetics of MC3T3-E1 cells.



(a) (b)

Figure 3: Kinetics of adhesion of MC3T3-E1 cells on titanium foams (a) and controls (b).

Cell morphology

The morphology of MC3T3-E1 pre-osteoblastic cells adhered to titanium foams with three different pore sizes was identical because the adhesion process is influenced by micro- and nano-scale surface features, which do not vary for the three conditions investigated. SEM images of cells adhered on TiA presented in this section are representative of the morphological features of cells on all types of titanium foams. MC3T3-E1 cells attached to the foams mainly exhibited a polygonal shape with lamellipodia extending in a three-dimensional fashion over the small spaces in between titanium particles of the scaffold structure to anchor themselves and create cell to cell interactions (see Figure 4a-b). Such morphological features have previously been reported for osteoblastic cells adhering to highly textured materials *in vitro* and are believed to encourage the differentiation of osteoblasts and thus osteogenesis [a-b]. It is hypothesized that the dimensions of titanium particles employed in the fabrication of the porous scaffolds investigated provide a desirable surface roughness to promote this type of three-dimensional adhesion. The morphology of pre-osteoblasts adhered on TiP was mainly spindle-like with long lamellipodia and numerous filopodia (see Figure 4c-d), although isolated cells showed rounded appearance few lamellipodia indicating a delayed migration due to a lack of signals (see Figure 4e). As well, MC3T3-E1 cells adhered on titanium foams were less flattened and spread than on polished titanium. This result can be associated to the surface roughness of titanium foams compared to mirror-polished controls [11]. Although cells did not form a confluent monolayer covering the surface of titanium foams after 18 hours incubation, observations indicate that the

three-dimensional adhesion occurring could lead to its formation. Such an organization of cells could lead to the formation of a uniform bone matrix (see Figure 4).

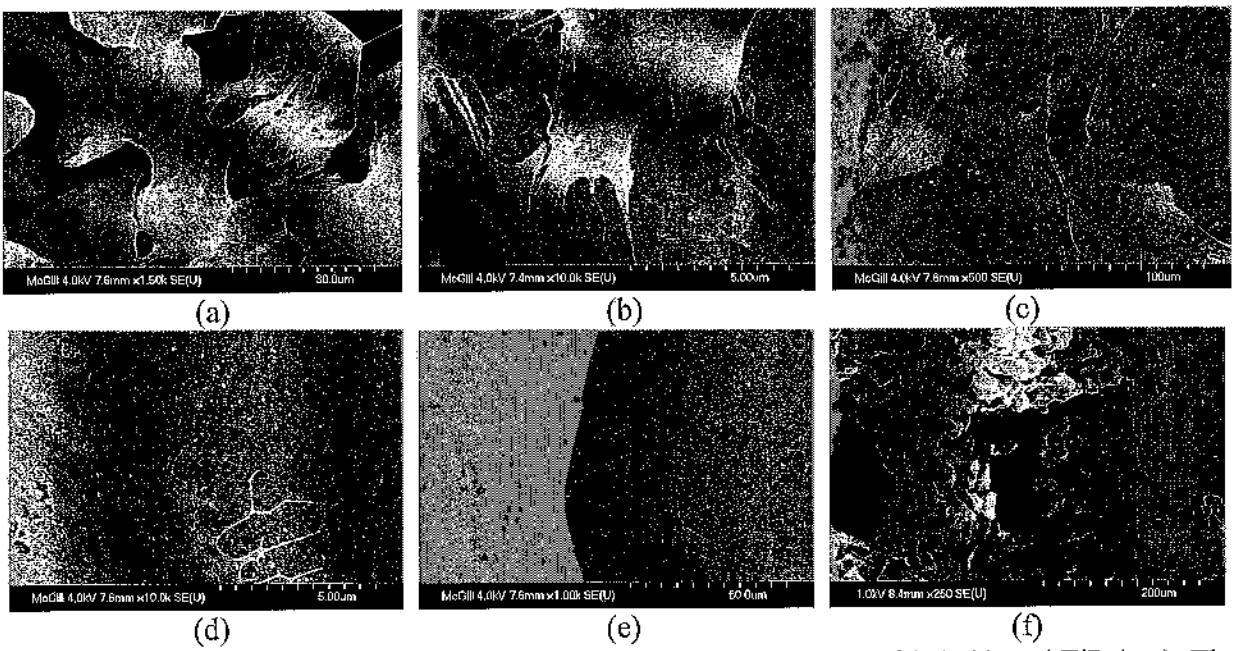


Figure 4: SEM images of MC3T3-E1 cells cultured for 18 hours on TiA (a-b) and TiP (c-e). The lower magnification image shows a cell monolayer at the surface of TiA. It was obtained prior to coating the sample with Au-Pd to obtain a better contrast between cells (dark gray) and the scaffold (light gray) (f).

CONCLUSIONS

The early mechanisms by which cells adhere on bone regeneration scaffolds and interact with them provide useful insight on the biocompatibility of biomaterials, as well as their capability to promote cellular differentiation. Hence, adhesion kinetics and cellular morphology on novel highly porous titanium foams was investigated. Results indicated that the foams provided a proper support for pre-osteoblasts attachment and proliferation. Furthermore, the scaffolds have a biomimetic microstructure that allows cells to assume a three-dimensional morphology, which is believed to be the basis for a proper cellular differentiation and extracellular matrix mineralization. This hypothesis will be investigated further in future work.

ACKNOWLEDGMENTS

Financial support for this research was provided by the Natural Sciences and Engineering Research Council of Canada. The authors are also grateful to Dr. Alain Petit, Prof. Mary Kaartinen, Dr. Stéphane Ruggeri and Lyne Mongeon for their assistance.

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