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Three-dimensional growth of differentiating MC3T3-E1 pre-osteoblasts on porous titanium scaffolds

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Abstract

The present work assesses the potential of three-dimensional porous titanium scaffolds produced by a novel powder metallurgy process for applications in bone engineering through in vitro experimentation. Mouse MC3T3-E1 pre-osteoblasts were used to investigate the proliferation (DNA content), differentiation (alkaline phosphatase activity and osteocalcin release) and mineralisation (calcium content) processes of cells on titanium scaffolds with average pore sizes ranging from 336 to 557 μm , using mirror-polished titanium as reference material. Scanning electron microscopy was employed to qualitatively corroborate the results. Cells proliferate on all materials before reaching a plateau at day 9, with proliferation rates being significantly higher on foams (ranging from 123 to 163 percent per day) than on the reference material (80% per day). Alkaline phosphatase activity is also significantly elevated on porous scaffolds following the proliferation stage. However, cells on polished titanium exhibit greater osteocalcin release toward the end of the differentiation process, resulting in earlier mineralisation of the extracellular matrix. Nevertheless, the calcium content is similar on all materials at the end of the experimental period. Average pore size of the porous structures does not have a major effect on cells as determined by the various analyses, affecting only the proliferation stage. Thus, the microstructured titanium scaffolds direct the behaviour of pre-osteoblasts toward a mature state capable of mineralising the extracellular matrix.

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Keywords: Osteoblasts; Differentiation; Mineralisation; Three-dimensional scaffolds; Titanium

1. Introduction

Common among engineered implant materials used for the replacement of damaged bone tissue are limited survivability characteristics, with relatively few improvements demonstrated over the past 35 years [1]. Contributing to poor survivability are difficulties associated with the maintenance of a stable interface between the material and surrounding tissues, and poor blood diffusion through that interface. In addition, inadequate mechanical properties contribute to the

failure of replacement materials [1,2]. To address these issues, current attempts in implant design have shifted towards the development of biomaterials incorporating three-dimensional osteoconductive scaffolds for bone regeneration.

Osteoconductive materials guide bone ingrowth by providing cells with a microstructured scaffold that promotes sequential cell maturation. Commencing with the migration of pre-differentiated cells from the host tissue, cells proliferate and differentiate to create bone within the scaffold [3]. Biodegradable and bioresorbable polymers and ceramics have been studied extensively for such purposes [4–6]. These scaffolds generally have adequate properties for implantation in non-loading conditions or in applications as supports for in vitro

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bone maturation before the engineered tissue is implanted. However, the mechanical properties of polymers are insufficient to support bone growth under loading conditions, while ceramics are too brittle [7]. Metallic scaffolds provide an advantageous alternative for these load-bearing applications because they maintain sufficient mechanical strength and fatigue resistance over extended periods in vivo. As well, their elastic moduli can be adjusted to match that of trabecular bone to limit stress shielding, thereby preventing bone resorption at the implant interface.

Porous metallic scaffolds are fabricated using a variety of processes to provide a high degree of interconnected porosity to allow bone ingrowth. Fabrication technologies include chemical vapour infiltration to deposit tantalum onto vitreous carbon foams [7,8], solid freeform fabrication [9], self-propagating high-temperature synthesis [10], and powder metallurgy [2]. While these porous metals have been successful at encouraging bone ingrowth both in vivo and in clinical trials, the range of materials and microstructures available is still rather limited.

Recently, a novel powder metallurgy process for the fabrication of titanium foams with a unique microstructure has been developed [11]. This process consists of mixing a titanium powder with a polymeric binder and a foaming agent prior to a three-step thermal treatment. During the initial step of low-temperature heat cure, the binder is melted to create a suspension charged with the titanium powder, after which the foaming agent decomposes to release gas that forms the interconnected pores within the scaffold structure. The second phase incorporates the decomposition of the polymeric compound at high temperature. Finally, the remaining titanium particles are sintered to provide the necessary mechanical integrity to the foam.

While the optimal range of pore size for bone ingrowth inside porous metals is often reported as 50–400 μm [12], structures characterised by pore sizes larger than 150 μm are generally preferred because they provide a suitable environment for bone reorganisation and vascularisation [13]. Furthermore, excellent bone ingrowth has been reported in structures with larger pore sizes than 400 μm , indicating that the optimal pore size for bone ingrowth is structure dependant [7,8]. The flexibility of the aforementioned process allows for adjustment of the average pore size within the range typically reported for porous metallic scaffolds in bone engineering. In addition, the process permits the control of microstructural properties such as the size of pore openings, surfaces properties, and the nature of the base metal. Titanium was selected for foam development based on its excellent overall biocompatibility, aided by the spontaneous formation of an oxide layer in both air and physiological environments, which limits ion release [14,15]. The shapable character of the material makes it

a potential candidate for applications such as craniofacial reconstruction scaffolds.

While characterisation of extracellular matrix (ECM) formation and mineralisation in vitro is well established in tissue culture plates [16–18], and for two-dimensional metallic scaffolds [19–21], the behaviour of osteoblasts within three-dimensional titanium scaffolds, and how mineralised ECM is formed, requires further investigation in order to optimise the design of porous metallic scaffolds. The impact of microstructural parameters such as surface topography, nanoscale roughness, and chemical composition on cell behaviour has yet to be understood for three-dimensional materials. The primary aim of this study is to assess the in vitro suitability of three-dimensional titanium scaffolds by analysing the differentiation processes of MC3T3-E1 mouse pre-osteoblastic cells and the formation of a mineralised ECM within the porous structure. Greater knowledge on the specifics of in vitro ECM formation and mineralisation in a porous metallic scaffold, as compared to conventional two-dimensional models, is also sought. To achieve this objective, cell culture studies on scaffolds having three different pore sizes are compared to a two-dimensional mirror-polished titanium reference material.

2. Materials and methods

2.1. Scaffold preparation

Titanium scaffolds with three pore sizes (A, B, and C from smaller to larger pore size), were prepared according to a novel powder metallurgy process. Briefly, irregularly shaped titanium powder with an average equivalent diameter of $39.7 \pm 15.5 \mu\text{m}$ (Reading Alloys Inc., USA) was dry-mixed with 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 blend composition. The homogenous mixture was then moulded and heated through a three step thermal treatment that included foaming at 210 °C for 2 h, decomposition of the polymeric binder at 425 °C for 4 h, and a pre-sintering step at 1200 °C for 2 h. Foams were then machined into discs (12.5 mm diameter, 2 mm thickness) and sintered at 1300 °C for 2 h. The density of each disc produced was obtained through gravimetric and volumetric measurements. An average pore size was measured for each of the three foam types through analysis of light microscopy images. Pore size and density parameters of titanium scaffolds are provided in Table 1. The unique microstructure of titanium foams is depicted in Fig. 1.

Polished titanium reference materials (referred to as P) were prepared by polishing dense titanium discs (Alfa Aesar, USA), having dimensions similar to the porous scaffolds, with an automatic polisher (Struers, UK) to ensure surface reproducibility. Samples were first wet-ground with 220 grit

Table 1
Microstructural parameters of titanium scaffolds produced through a novel powder metallurgy process

Scaffold identification	Density (g/cm ³)	Average pore size (μm)
A	1.23 ± 0.09	336.4 ± 48.4
B	1.02 ± 0.11	376.1 ± 49.0
C	0.82 ± 0.19	556.9 ± 196.9

Fig. 1. Scanning electron micrographs showing the microstructure of titanium scaffold C with an average pore size of 557 μm. (A) The porosity is interconnected and the structure is characterised by a high degree of surface topography. (B) The surface of each titanium particle is characterised by high levels of surface roughness (original magnifications 50 × and 2000 ×, respectively).

silicon carbide abrasive papers at 300 rpm with an applied pressure of 200 N. The discs were then polished with 9 and 0.04 μm diamond solutions on appropriate cloths (Struers, UK) at 150 rpm with applied pressures of 175 and 125 N, respectively.

Porous samples and polished titanium discs were cleaned through 15 min sonication cycles in soapy water, isopropanol and de-ionized water, interspersed by rinsing steps in distilled water. Titanium scaffolds were then autoclaved and allowed to dry overnight at 60 °C under sterile conditions.

2.2. Cell culture

Newborn mouse calvaria-derived MC3T3-E1 subclone 14 pre-osteoblastic cells (ATCC, USA) were cultured in α-MEM medium (Gibco BRL, Canada) supplemented with 10% fetal bovine serum (ATCC, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL, Canada) at 37 °C in an atmosphere with 100% humidity and 5% CO₂. The complete medium was replaced every 2–3 days and confluent cells were subcultured through trypsinisation.

To study cell proliferation, differentiation, and mineralisation of pre-osteoblasts, cells were harvested and seeded at a density of 2.0×10^5 cells/well on the porous titanium scaffolds and polished titanium within 24-well plates. Cells were allowed to adhere to the discs for 24 h prior to samples being transferred to fresh 24-well plates in order to discard cells adhered to the plate rather than titanium. Differentiation was then induced through the addition of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate to the growth medium; this was considered day 0 of the experiment. Differentiation medium was subsequently replaced every 2 days, with sample analysis or preparation for qualitative assessment on days 0, 1, 5, 9, 13 and 17. As well, differentiation was induced on cells adhered to the tissue culture plate (TCP) as a positive control, with the inclusion of proper negative controls.

2.3. Proliferation

The number of cells adhering to titanium scaffolds, polished materials, and TCP was measured using a modified version of the CyQUANT cell proliferation assay (Molecular Probes, USA). Briefly, cells were washed twice with PBS before freezing at –80 °C until the performance of the assay, at which point cells were thawed at room temperature. Cell lysate was obtained by the addition of 500 μL of $1 \times$ CyQUANT cell-lysis buffer to each well followed by a 15 min incubation at room temperature. An aliquot of the lysate was then mixed with the same quantity of cell-lysis buffer supplemented with 360 mM NaCl, 2 mM EDTA and 1.5 Kunitz/ml DNase and protease free RNase A (Worthington Biochemical Corporation, USA) and incubated for 1 h at room temperature; lysate RNA was digested to ensure that potential fluctuations in RNA transcription at different stages of cell differentiation would not affect the data. A 100 μl volume of the RNA free solution was then mixed with the same quantity of $2 \times$ CyQUANT GR dye in cell-lysis buffer in fluorescence assay microplates and incubated for 5 min at room temperature, while shading from ambient light. After the incubation, plates were read with a FLx 800 microplate fluorescence reader (Bio-Tek Instruments Inc., USA) with a 485/20 excitation filter and a 528/20 emission filter. A standard curve was generated with λ DNA.

2.4. Alkaline phosphatase activity

The activity of intracellular alkaline phosphatase (ALP) was measured with a commercial phosphatase substrate kit (Pierce, USA). Aliquots of 100 μl of cell lysate were obtained according to the procedure described for proliferation measurements and mixed with an equal amount of 1 mg/ml *p*-nitrophenyl

phosphate in $1 \times$ diethanolamine buffer in microplates. Mixtures were then incubated for 30 min at 37°C . The reaction was stopped by the addition of $50\ \mu\text{L}$ of 2N NaOH to each well and the resulting optical densities were measured at 405nm with a μQuant spectrophotometer (Bio-Tek Instruments Inc., USA). Measurements were compared to p-Nitrophenol standards and normalised using the total protein amounts to account for differences in the number of cells on different scaffolds at individual time points.

2.5. Osteocalcin release

The production of osteocalcin by differentiating cells was measured as the release of the ECM protein in the culture medium over a period of 24 h using a commercial sandwich enzyme-linked immunosorbent assay (Biomedical Technologies, USA). The amount of osteocalcin released in the culture medium is correlated with the synthesis of the protein [22]. Mouse osteocalcin was used to generate a standard curve and results were normalised using the amount of total protein.

2.6. Total protein

Cellular protein content was measured with a BCA protein assay kit (Pierce, USA). Briefly, $25\ \mu\text{L}$ aliquots of cell lysate were mixed with $200\ \mu\text{L}$ volumes of BCA working reagent containing cupric sulfate and bicinchoninic acid in microplates and incubated for 30 min at 37°C . The resulting optical densities were measured at 562nm with a μQuant spectrophotometer. Bovine serum albumin was used to generate a standard curve.

2.7. Calcium content

Following incubation, cells were washed twice in PBS and incubated for 24 h in 1N HCl. The calcium content in each solution was then measured with an atomic absorption spectrometer (Perkin Elmer 3110, USA). Two measurements were averaged for each solution. A standard curve was obtained with Ca^{2+} solutions. Results were normalised using total proteins.

2.8. Scanning electron microscopy

Cells and ECM on titanium scaffolds and polished surfaces were washed twice in PBS and fixed 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 imaged with a Hitachi S-4700 field emission scanning electron microscope. Energy dispersive X-ray microanalysis (Oxford Instruments, USA) was also performed on samples prior to the application of the Au–Pd coating to verify the presence of calcium and phosphorus in the ECM produced by differentiated cells.

2.9. Statistics

Results are expressed as mean $\pm$ standard deviation with $n = 5$. Error bars in figures represent standard deviations. Differences between the experimental groups were analysed

according to a paired Student's *t*-test, with $p < 0.05$ considered statistically significant. Pore size effects were verified according to a single factor ANOVA.

3. Results

3.1. Proliferation

The number of MC3T3-E1 mouse pre-osteoblasts attached to scaffolds, reference materials and controls was measured through the cellular DNA content at different time points following the initiation of differentiation. Compared to porous scaffolds, DNA content is significantly higher on TCP at every time point and on polished titanium on days 0 and 1 (Fig. 2). DNA content is similar for titanium foams A and B at each time point investigated, but is significantly higher for scaffold C. The content increases significantly on all test materials at each time point until day 9, with the exception of day 1 for all porous scaffolds. Following day 9, DNA content remains statistically unchanged on the porous scaffolds, while attached cells are significantly lower, but constant, on both the polished material and the control. In considering the initial 9 day growth phase, proliferation rates measured on each material as the percent increase of DNA content per day indicate significantly higher rates on porous scaffolds than polished titanium and TCP, with an increasing trend in proliferation with smaller pore sizes (Fig. 3).

3.2. Alkaline phosphatase activity

Differentiation on the various substrates was characterised using ALP as an early phase marker. Cells adhering to titanium scaffolds exhibit significantly higher activity of the enzyme than on the polished material and the control on days 9 and 13, with the exception of scaffold A on day 13, though scaffold pore size does not affect the ALP activity of differentiating cells in a uniform fashion (Fig. 4). ALP kinetics show a significant increase in specific activity of the enzyme on all porous scaffolds at each time point up to day 13, followed by a significant decrease on day 17 for scaffolds A and C. In contrast, the pattern of ALP activity on polished titanium resembles that of TCP controls, which consists of a slow increase over the entire test period, with significantly greater activity on day 17 than on day 5.

3.3. Osteocalcin release

Late stage osteoblast differentiation was measured through osteocalcin release in the culture medium over a 24 h period. Osteocalcin release is significantly higher on TCP than any other substrate on day 13, while the same

Fig. 2. DNA content of mouse pre-osteoblasts after 0, 1, 5, 9, 13 and 17 days in culture. Foams (A, B and C with pore sizes of 336, 376 and 557 μm , respectively) have significantly lower DNA content than tissue culture plate controls (TCP) at every time point and than polished titanium (P) at days 0 and 1 ($*p < 0.05$). Foams with the highest pore size (C) have significantly higher DNA content than other foams ($**p < 0.05$).

Fig. 3. Overall proliferation rates of pre-osteoblasts over the initial nine days in culture is significantly higher on porous foams (A, B and C with pore sizes of 336, 376 and 557 μm , respectively) than on polished titanium (P) and tissue culture plate controls (TCP) ($*p < 0.05$).

is true for polished titanium on day 17, indicating that cells attached to the reference material and control achieve a mature osteoblastic state more rapidly than on porous substrates (Fig. 5). The pore size of titanium scaffolds does not impact osteocalcin release. Osteocalcin levels increase significantly from day 5 to day 9 on all materials, though a larger rise is observed on day 13. Finally, with the exception of cells grown on scaffold C and TCP, osteocalcin release remains unchanged following day 13.

3.4. Calcium content

Mineralisation of the ECM by differentiated cells was measured through the ECM calcium content. Calcium

Fig. 4. Normalised ALP activity of cell lysates from pre-osteoblasts after 5–17 days in culture. The specific enzymatic activity is significantly higher on foams than on polished titanium (P) and tissue culture plate controls (TCP) at days 9 and 13, though pore size (A, B and C with pore sizes of 336, 376 and 557 μm , respectively) does not affect ALP activity ($*p < 0.05$).

content for both polished titanium and TCP is significantly higher on day 13 than for porous scaffolds, indicating a lesser degree of mineralisation for osteoblasts grown in porous scaffolds at this time point (Fig. 6). However, by day 17 there is no significant difference between scaffolds and the polished material, regardless of the pore size. A large standard error resulted from the calcium content analysis on polished titanium on day 13. Macroscopic analysis of these samples confirmed that some replicates had already started mineralising by this time, while cells on the other substrates had yet to reach this level of maturation; similar observations were made for the samples tested on day 17.

3.5. Scanning electron microscopy analysis

Evidence of continued cell proliferation on the titanium scaffolds and polished titanium could be detected up to day 9 through SEM analysis (Fig. 7). Cells appear to adhere to the scaffold through three-dimensional extensions, gradually filling in pores as proliferation proceeds. Continued progression of ECM development once proliferation is complete is evidenced by densification and mineralisation of the ECM on porous scaffolds, which was verified by EDX to confirm the presence of calcium and phosphorus at day 17. After 5 days, there are already indications of ECM bridging the scaffold pores (Fig. 7B), with continuing development of the ECM network within the porous structure at subsequent time points (Figs. 7C and D). Once the smaller pores are filled with ECM, subsequent growth results in a dense layer of cells covering the porous structure (Fig. 7D). While no correlations between pore size of titanium foams and growth traits were

Fig. 5. Normalised osteocalcin release from cells in medium over 24 h after 5–17 days in culture. Cells grown on tissue culture plates (TCP) on day 13 and on polished titanium (P) on day 17 have significantly higher osteocalcin release than porous scaffolds (* $p < 0.05$). Average pore size of titanium foams (A, B and C with pore sizes of 336, 376 and 557 μm , respectively) does not affect osteocalcin release.

Fig. 6. Normalised calcium content of the ECM deposited by cells after 5–17 days in culture indicate higher mineralisation on polished titanium (P) and tissue culture plate controls (TCP) on day 13, though no difference exists by day 17 regardless of pore size (A, B and C with pore sizes of 336, 376 and 557 μm , respectively; * $p < 0.05$).

qualitatively identified, cell growth within larger pores is limited by the ability of cells to bridge the pore with ECM, resulting in less dense, and in some locations, incomplete ECM formation (Fig. 8). In comparison, cell growth on polished titanium results in more flat, dense, and multi-layered structures with relatively little ECM (Fig. 9).

4. Discussion

In the present study, the proliferation and differentiation of mouse MC3T3-E1 pre-osteoblasts toward the

formation of a mineralised ECM were assessed on porous titanium scaffolds produced through a novel powder metallurgy process. Results obtained with porous scaffolds comprising three different pore sizes were compared to mirror-polished titanium. The clonal mouse pre-osteoblastic MC3T3-E1 cell line was selected for this study due to its high level of differentiation and its ability to form a well mineralised bone-like ECM containing bone markers such as collagen type I, ALP, and osteocalcin, making it an excellent model for differentiation [23,24]. Additionally, it has a distinct reproducibility advantage over primary cell culture systems.

The growth environment provided by the unique microstructure of titanium foams encourages cell proliferation compared to the polished material. The average pore size of titanium scaffolds does impact proliferation, though differentiation and mineralisation processes are not affected. Early-phase cell differentiation activity, as monitored by ALP activity, is also positively impacted by cell interactions with the three-dimensional scaffolds. In contrast, late-phase differentiation release of osteocalcin is lower for cells on titanium scaffolds than on polished titanium, resulting in a delayed transition toward mature osteoblasts capable of mineralising the ECM. Nevertheless, the calcium content on scaffolds was comparable to that obtained on polished surfaces by the end of the incubation period (17 days). SEM analysis reveals greater amounts of ECM laid down by differentiating cells on the porous structures, as compared to polished samples, with the three-dimensional structures providing a more voluminous environment for the deposition of ECM.

The initial cell growth profile, as measured by cellular DNA content on porous titanium scaffolds, exhibits differences compared to polished titanium and TCP (Fig. 2). During the initial phases of proliferation, fewer cells adhere to the scaffolds than to the reference material or the control. This can be attributed to the permeability of porous scaffolds, which provides an interconnected structure for cell penetration but allows some cells to attach to the underlying wells rather than the scaffolds upon seeding. By day 9, proliferation peaks for all test materials, as determined by both the quantification of the DNA content (Fig. 2) and qualitative SEM analysis (Fig. 7). Though cell counts indicate greater numbers of cells on polished titanium and TCP, the rates of proliferation measured up to day 9 are significantly greater for porous foams, likely due to the three-dimensional proliferation environment provided by the porous scaffolds (Fig. 3) [25]. Subsequently, DNA content remains constant on porous scaffolds, resulting in typical sigmoidal curves for all three porosities having a plateau beginning on day 9. However, while polished surfaces and TCP also reach a maximum on day 9, a

Fig. 7. Scanning electron micrographs depicting cell proliferation of pre-osteoblasts within scaffold A (336 μm pore size) up to day 9. (A) Day 1. (B) Day 5. (C) Day 9. (D) Day 13 (original magnification 500 $\times$). Observations on days 0 and 17 are comparable to those on days 1 and 13, respectively.

significant decrease in measured DNA content occurs by day 13. This reduction is consistent with previous reports documenting diminishing DNA content for osteoblasts grown on implant materials [26]. These investigators propose that the dense ECM network produced by differentiating osteoblasts could prevent a complete release of DNA content during cell lysis, thereby lowering proliferation measurements. SEM observations support this justification given the density of the cell layer observed on polished titanium after 13 and 17 days (Fig. 9), which is considerably more dense than is observed on day 9 or on porous scaffolds.

The culmination of proliferation on day 9, with an ensuing shift in cell maturation level towards early differentiation stages, is confirmed by a significant increase in ALP activity on porous scaffolds. Following the proliferation stage, the number of cells on titanium scaffolds is comparable to that on polished samples, even though the foams provide substantially higher surface area and volume for cell growth (Fig. 2). This can be explained by SEM observations indicating that cells are not as densely packed in the ECM network laid down inside pores of the titanium foams than on the surface of polished titanium. However, the greater early phase differentiation activity on porous

scaffolds noted by ALP activity at days 9 and 13 indicates that scaffolds may provide adhered pre-osteoblasts with a more robust foundation to commence differentiation (Fig. 4). Proliferation and ALP activity were expected to follow trends of lower proliferation and increased ALP production for porous scaffolds, due to their unique microstructure, based on published reports from two-dimensional models linking increasing surface roughness and topography of titanium substrates with the promotion of osteoblasts towards a differentiated phenotype [27–29]. The ALP kinetics obtained in this study are in agreement with the literature, as the enzymatic activity significantly increases on the foams following the proliferation stage, as compared to the polished material (Fig. 4). Furthermore, the profile of ALP activity for cells on porous scaffolds is typical of the expression of that enzyme during the early stages of differentiation, as the activity increase is followed by a drop in activity that coincides with the initiation of the mineralisation process [30]. The discrepancy between the noted increased proliferation found on scaffolds and published findings may be attributed to the greater available space for cell growth provided by the porous three-dimensional surface topography.

Fig. 8. (A) Scanning electron micrographs showing the development of extensive ECM networks inside pores of scaffold A (336 μm pore size) after 9 days in culture. (B) Cells grow on the walls of a larger pore inside scaffold A, failing to form a dense ECM network within the pore after 17 days due to its size. This micrograph also provides evidence of cell penetration inside the three-dimensional structure (original magnification 100 ×).

The increased activity of the early differentiation stage marker (ALP) on titanium foams is not indicative of the late-phase osteocalcin release. While production of the late differentiation stage marker is similar for foams and the polished control up to day 13, significantly higher production results were obtained for cells grown on polished titanium by day 17 (Fig. 5). The increased production is associated with an earlier shift toward a mature state of osteoblasts capable of mineralising the ECM, as was confirmed by calcium content measurements (Fig. 6). This result suggests that the two differentiation markers investigated in this study are expressed through independent control pathways, and is consistent with previous observations reported for human osteoblasts grown on various metallic substrates [21]. These investigators attributed this phenomenon to different levels of transforming growth factor- β (TGF- β), which is known to have different patterns of expression by osteoblasts on implant materials of

Fig. 9. Scanning electron micrograph showing the multilayered growth of pre-osteoblasts with minimal ECM production after 13 days on mirror-polished titanium. This micrograph demonstrates that the tissue grown on the flat surface is dense and could prevent proper extraction of DNA from adhered cells, thus affecting results for the proliferation study (original magnification 100 ×).

different chemical compositions. This growth factor has been shown to act as an up-regulator of ALP expression by osteoblasts, while down-regulating the expression of osteocalcin [31]. Since surface roughness of metallic implants promotes the release of TGF- β by osteoblasts [29,32], the microstructure typical of titanium foams could promote release relative to cells grown on polished surfaces, thus affecting the expression patterns of differentiation markers.

In the final stage of osteoblast maturation, mineralisation of the ECM, calcium content measurements indicate equivalent performance by three-dimensional scaffolds and the polished samples. The similarity in mineralisation on day 17 is presumed to result from the deposition of a more extensive ECM within the porous structures than on the polished material, as observed through SEM analysis (Fig. 8A). It is worth noting that macroscopic examinations of the scaffold undersides indicate that ECM production does not occur through the entire depth of scaffolds over the experimental period, likely due to diffusive limitations on nutrients through the scaffolds and matrix [26,33]. It remains to be determined whether or not the increased amounts of ECM found in scaffolds would promote the deposition of minerals beyond day 17, potentially leading to enhanced bone formation relative to controls in a longer term analysis.

Many investigators have examined the effects of pore size on bone ingrowth in porous metals using in vivo models [12,34,35]. While they have identified a range of pore size within which bone ingrowth occurs, no consensus on optimal pore size exists due to the range of materials investigated and their respective microstructural impacts. Similar in vitro studies indicate that pore size is an important microstructural parameter to

control in the design of three-dimensional osteoconductive scaffolds [36]. In the present study, the average pore size of titanium scaffolds was found to significantly affect only the proliferation stage, leaving the differentiation and subsequent mineralisation processes unaffected. Given that the pore size distributions for the three scaffolds overlapped significantly and differed primarily due to the size of larger pores present, it is possible that differences in the cellular response on the three scaffolds were too small to measure significant differences with the selected tests. Though this study demonstrates the suitability of these foams for osteoblast proliferation, differentiation, and mineralisation, additional work is required to differentiate the individual impacts of pore size from microstructural properties. Results from these additional studies would optimise the design of the titanium scaffolds, allowing studies to proceed toward in vivo characterisation of these titanium scaffolds to ascertain their suitability for bone tissue regeneration applications.

5. Conclusions

The microstructure of porous titanium scaffolds produced through a novel powder metallurgy process promotes MC3T3-E1 pre-osteoblast proliferation and early differentiation compared to polished surfaces. The microstructural properties of foams appear to slow the shift to later stages of cell differentiation toward mature osteoblasts. At the culmination of the investigation period on day 17, cells grown on titanium scaffolds and on polished titanium produced similar levels of ECM mineralisation. These results indicate that the microstructure of the three-dimensional titanium scaffolds investigated promotes proliferation, differentiation, and mineralisation processes required to characterise the osteoconductive behaviour of porous metallic scaffolds in vitro. Furthermore, the average pore size of the structures investigated does not have a major effect on the differentiation and mineralisation processes. Based on quantitative and qualitative analyses, the smaller pore size structure is more favourable than the other two porous structures investigated because of its narrower pore size distribution and fewer large pores, which are more difficult to bridge and fill with ECM. However, cell culture experiments provide a simplified model of the phenomena being studied. A true measure of the osteoconductive utility of these titanium scaffolds requires confirmation of these results through additional characterisation and in vivo experimentation.

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