TISSUE ENGINEERING
Volume 9, Number 2, 2003

Evaluation of Various Seeding Techniques for Culturing Osteogenic Cells on Titanium Fiber Mesh

JULIETTE VAN DEN DOLDER, Ph.D.,[1] PAUL H.M. SPAUWEN, M.D., Ph.D.,[2] and JOHN A. JANSEN, D.D.S., Ph.D.[1]

ABSTRACT

The objective of the present study was to learn more about the effect of seeding and loading techniques on the osteogenic differentiation *in vitro* of rat bone marrow cells into titanium fiber mesh. This material was used as received or subjected to glow discharge treatment (RFGD). The seeding methods that were used included a so-called droplet, cell suspension (high and low cell density), and rotating plate method. Osteogenic cells were cultured for 4, 8, and 16 days into titanium fiber mesh. DNA, osteocalcin, scanning electron microscopy (SEM) analysis, and calcium measurements were used to determine cellular proliferation and differentiation. DNA analysis of the differently seeded specimens showed that proliferation proceeded faster in the first versus second run for droplet and cell suspension samples. No clear and distinct additional effect was found when RFGD treatment was used. Statistical analyses revealed that high cell density and low rotational speed resulted always in a significantly higher DNA content. Calcium measurements and osteocalcin analysis showed that using high cell densities during inoculation of the scaffolds prevented the occurrence of differences between experimental runs. SEM examination showed that for droplet and cell suspension samples cells were present at only one side of the mesh. The mesh side where the cell sheet was observed depended on the additional use of glow discharge treatment. On these materials, the cells had penetrated through the meshes and formed a cell sheet at the bottom side. When rotation was used, no cell sheet was formed and cells had invaded the meshes and were growing around the titanium fibers. On the basis of our results, we conclude that (1) titanium fiber mesh is indeed suitable to support the osteogenic expression of bone marrow cells, and (2) changing the initial cell density as well as the use of dynamic seeding methods can influence the osteogenic capacity of the scaffold.

INTRODUCTION

ENGINEERED BONE CONSTRUCTS can form the basis for novel therapies for patients with large skeletal defects. To create such a construct a porous scaffold material is used, to which osteogenic cells and/or systemic factors are added. For this purpose a wide variety of scaffold materials can be used. In a series of consecutive studies, we investigated the applicability of porous sintered titanium fiber mesh. The advantage of this material lies in the combination of excellent tissue characteristics, both in soft and hard body tissues, with typical characteristics of metallic fiber products such as flexibility and stiffness.[1] On the other hand, a disadvantage is that the material is not degradable. However, this is no hindrance for many bone reconstructive purposes.

[1]Department of Biomaterials, College of Dental Science, University Medical Center Nijmegen, Nijmegen, The Netherlands.
[2]Department of Plastic and Reconstructive Surgery, University Medical Center Nijmegen, Nijmegen, The Netherlands.

In one of our previous studies, we investigated the osteogenic activity of porous titanium mesh loaded with cultured rat bone marrow cells in a syngeneic rat ectopic assay model.[2] For this study, the titanium fiber meshes were seeded by a dynamic seeding method (2 rpm) and a high cell density. The experiment showed that the combination of titanium fiber mesh with bone marrow cells could indeed generate bone formation. Nevertheless, the amount of newly formed bone was significantly less compared with experiments in which the titanium mesh was loaded with bone morphogenetic protein instead of osteogenic cells.[3] In view of this, we know that the method used to seed and culture marrow cells into the porous scaffold is essential to achieve a successful three-dimensional bone graft. Several research groups have already used different techniques to optimize the bone-generating properties of scaffold materials. In general, the methods focus on improvement of the seeding or loading efficacy of the scaffold. Various so-called static and dynamic systems have been created to inoculate cells in a three-dimensional scaffold material. Examples of static methods are droplet[4,5] and cell suspension[6] seeding. In dynamic procedures use is made of specially developed devices, such as a cell chamber,[7] spinner flask,[8–10] or bioreactor.[11–13]

Consequently, the purpose of the present study was to learn more about the effect of seeding and loading techniques on the osteogenic differentiation *in vitro* of rat bone marrow cells in titanium fiber mesh. The procedures that were used included a so-called droplet, cell suspension (high and low cell density), and rotating plate method. DNA and osteocalcin analysis, scanning electron microscopy (SEM) analysis, and calcium measurements were used to monitor cellular proliferation and differentiation during cell culture.

MATERIALS AND METHODS

Substrates

Sintered titanium fiber mesh (Bekaert, Zwevegem, Belgium) with a volumetric porosity of 86%, a density of 600 g/m^2, and a fiber diameter of 50 μm was used in these studies. The average fiber spacing distance of the mesh was about 250 μm. The prepared meshes were disc shaped with a diameter of 6 mm, a thickness of 0.8 mm, and a weight of about 15 mg. In total, 180 titanium discs were made for each experimental run. All discs were sonicated for 10 min with isopropanol and then sterilized by autoclaving for 15 min at 121°C. Before use in the experiments, half of the total number of discs received a radiofrequency (RF) glow discharge treatment (PDC-3XG [Harrick Scientific, Ossining, NY], argon, 0.15 torr, for 5 min) to increase the wettability of the titanium fiber mesh.

Cell culture

Rat bone marrow (RBM) cells were isolated and cultured according to the method described by Maniatopoulos *et al.*[14] Briefly, for each experimental run RBM cells were obtained from the femora of eight male Wistar rats. Femora were washed four times in α-MEM (minimal essential medium; GIBCO-BRL, Life Technologies, Breda, The Netherlands) culture medium with gentamicin (0.5 mg/mL; Sigma, St. Louis, MO) and Fungizone (3 μg/mL; Sigma). Epiphyses were cut off and diaphyses were flushed out with 15 mL of culture medium α-MEM, supplemented with 10% fetal calf serum (FCS; GIBCO), ascorbic acid (50 μg/ml; Sigma), gentamicin (50 μg/mL), 10 mM sodium β-glycerophosphate (Sigma), and 10^{-8} M dexamethasone (Sigma). The pooled RBM cells were incubated in a humidified atmosphere of 95% air, 5% $CO)_2$ at 37°C.

After 7 days of primary culture, cells were detached with trypsin–EDTA (0.25% [w/v] trypsin–0.02% EDTA; Sigma). The cells were concentrated by centrifugation at 1500 rpm ($400 \times g$) for 5 min and resuspended in a known amount of medium (5 mL). Cells were counted with a Coulter counter (Coulter Electronics, Luton, UK) and then added to the substrates, using various seeding techniques. After seeding, the substrates were incubated for predetermined times. During incubation, the medium was changed every 2 or 3 days. Further, two separate runs of experiments were always performed and in each run all materials were present in triplicate.

Seeding techniques

Titanium fiber mesh discs were placed at the bottom of 24-well tissue culture plates; one disc per well. Subsequently, two techniques were used to seed the RBM cells in the titanium discs, that is, a droplet technique and a cell suspension technique.

In the droplet technique, 8×10^4 cells, resuspended in 30 μL of culture medium, were placed as a droplet on top of each mesh and left for 3 h to allow cell attachment.

In the cell suspension technique, 8×10^4 cells (low cell density) or 8×10^5 cells (high cell density), resuspended in 1 mL of culture medium, were added to each well and left for 3 h to allow cell attachment. During this time, the well plates were manually shaken every half-hour. The cell suspension present in the wells was homogenized by gentle shaking in the horizontal direction (5 cm) for 10 s.

Further, another suspension group was formed: 25 mesh discs were placed in 10-mL tubes, to each of which 10 mL of culture medium was added containing 8×10^6 cells (8×10^5 cells/mL). The tubes were placed on a cell-rotating plate that rotated at 2 or 12 rpm.

In all the above-mentioned procedures, discs were used

as received or treated by glow discharge. In this way, in total 10 experimental groups were created (Table 1).

Finally, all specimens were placed in an incubator with a humidified atmosphere of 95% air, 5% CO_2 at 37°C.

DNA analysis

DNA was measured on days 4 and 8. Medium was removed from each mesh and the cell layer was washed twice with phosphate-buffered saline (PBS). The subsequent addition of 0.5 mL of 1 M NaOH to each mesh lysed the cells. Thereafter, the cells were harvested in 1.5-mL Eppendorf tubes (Merck, Darmstadt, Germany), and the cell suspensions were sonicated for 10 min. DNA was collected and stored at −20°C until use. Before analysis, each sample was neutralized with an equal volume of 1 M HCl-PO_4. For the assay, a DNA standard curve was made with salmon testis DNA (0–4 mM). A 125-μL volume of each sample or standard was added to a 96-well plate. Subsequently, after adding 125 μL of Hoechst dye (Sigma) working solution to each well, the well plate was incubated in the dark for 3 h. After incubation, the plate was scanned on a fluorescence plate reader (excitation, 365 nm; emission, 450 nm). All samples and standards were assayed in duplicate.

Calcium content

Calcium content in the samples was measured by the *ortho*-cresolphthalein complexone (OCPC) method (Sigma). Calcium content was examined on day 16. Substrates were washed twice in PBS and placed at the bottom of 24-well plates. Acetic acid (0.5 N, 500 μL) was added to the wells and the substrates were incubated overnight. Samples were frozen at −20°C until use.

OCPC solution was prepared as follows: 80 mg of OCPC was added to 75 mL of demineralized H_2O with 0.5 mL of KOH (1 M) and 0.5 mL of 0.5 N acetic acid. To prepare sample solution, 5 mL of OCPC solution was added to 5 mL with 14.8 M ethanolamine–boric acid buffer (pH 11), 2 mL of 8-hydroxyquinoline (5 g in 100 mL of 95% ethanol), and 88 mL of demineralized water. Finally, 300 μL of sample solution was added to 10 μL of sample. To generate a standard curve, serial dilutions of CaCl2 were made (1–200 μg/mL). The plate was incubated at room temperature for 10 min and subsequently read at 575 nm. All samples and standards were assayed in duplicate.

Osteocalcin

Cell layers were collected on days 16 and 24. Cell layers were washed twice in PBS. The subsequent addition of 0.5 mL of sample buffer to each mesh lysed the cells. After that, cell suspensions were homogenized by sonication for 10 min. The suspensions were stored at −20°C until the assay was performed. Osteocalcin was measured with an enzyme immunoassay kit (Biomedical Technologies, Stoughton, MA). Briefly, 100 μL of sample was added to each well and the plate was incubated at 4°C for 24 h. Thereafter, wells were washed three times with 0.3 mL of wash buffer. Subsequently, 100 μL of osteocalcin anti-serum was added to each well and the plate was incubated for 1 h at 37°C. The plate was then washed three times and 100 μL of donkey anti-goat IgG–peroxidase was added and incubated for 1 h at room temperature. One volume of TMB solution was mixed with 1 volume of hydrogen peroxidase solution. The plate was rinsed three times with wash buffer and 100 μL of substrate solution was added to each well. The plate was then incubated for 30 min at room temperature (in the dark). Finally, 100 μL of stop solution was put in each well and absorbance was read at 450 nm. Osteocalcin stock was

Table 1. Cell-Seeding Techniques Used in Current Study

	Seeding methods
Dr+GD/Dr-GD	Droplet technique; meshes with or without glow discharge treatment
LDM+GD/ LDM-GD	Cell suspension technique with a low cell density and the meshes with or without glow discharge treatment; 24-well plate was shaken manually every half-hour during 3 h of attachment time
HDM+GD/HDM-GD	Cell suspension technique with a high cell density and the meshes with or without glow discharge treatment; 24-well plate was shaken manually every half-hour during 3 h of attachment time
HDR12+GD/ HDR12-GD	Cell suspension technique with a high cell density; the meshes with or without glow discharge treatment; 25 meshes were put in 10-mL tubes and were rotated on a rotating plate for 3 h during treatment; rotating speed was 12 rpm
HDR2+GD/ HDR2-GD	Cell suspension technique with a high cell density; the meshes with or without glow discharge treatment; 25 meshes were put in 10-mL tubes and were rotated on a rotating plate for 3 h during attachment, rotating speed was 2 rpm

diluted to generate standards from 0.25 to 20 ng/mL. All samples and standards were assayed in duplicate.

Scanning electron microscopy

After 8 and 16 days of incubation, meshes were washed twice with PBS. Fixation was carried out for 30 min in 2% glutaraldehyde (Sigma). Substrates were then washed twice with 0.1 M sodium cacodylate buffer (pH 7.4) (Sigma), dehydrated in a graded series of ethanol, and dried with tetramethylsilane. The specimens were sputter coated with gold and examined and photographed, using a JEOL (Tokyo, Japan) 6310 SEM at an acceleration voltage of 15 kV.

Statistical analysis

This study was done by performing two separate runs of experiments. In each run all materials were present in triplicate. Data from both runs are presented separately. Statistical analysis of each run was done, first, by a two-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls test.

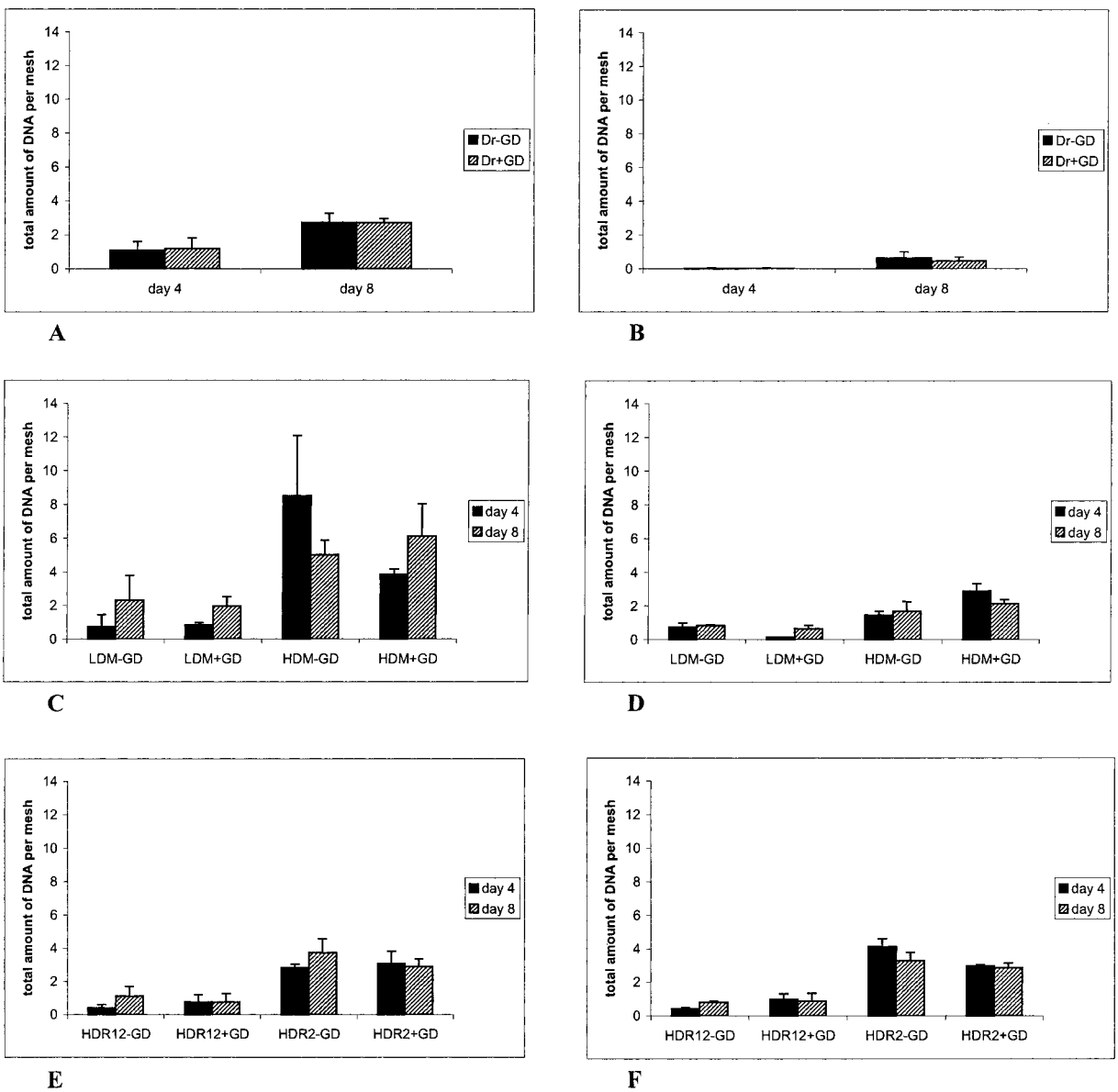

FIG. 1. DNA content of osteoblast-like cells after 4 and 8 days of culture on titanium fiber mesh that was seeded by various cell-seeding techniques. (**A** and **B**) First and second run Dr; (**C** and **D**) first and second run LDM and HDM; (**E** and **F**) first and second run HDR12 and HDR2. Values represent means ± SD. Dr, Droplet method; LDM, low cell density, manually; HDM, high cell density, manually; HDR12, high cell density, rotation 12 rpm; HDR2, high cell density, rotation 2 rpm.

RESULTS

DNA

All obtained results are graphically depicted in Fig. 1A–F.

Analysis of these data showed that proliferation proceeded significantly ($p < 0.05$) faster in the first versus the second run for Dr, LDM, and HDM specimens (see Table 1 for seeding method abbreviations). No significant differences were found between the different runs for HDR2 and HDR12 samples.

Subsequent evaluation of the effect of glow discharge treatment on DNA amount showed that no effect of glow discharge treatment could be observed in either run for the Dr substrates. On the other hand, some differences were found in the cell suspension and rotation groups. Nevertheless, the effect of glow discharge was not clear and distinct. For example, the LDM samples showed a significant effect ($p < 0.05$) only for glow discharge treatment on days 4 and 8 in the second run, while the HDM samples showed a significant effect ($p < 0.05$) on day 4 in both runs and on day 8 in the second run only. Further, for HDR2 substrates there existed a significant effect ($p < 0.05$) on day 4 in the second run and on day 8 in both runs. HDR12 specimens showed a significant effect ($p < 0.05$) only on day 4 for both runs.

Finally, we tested the effect of cell-seeding density as well as rotational movement during the inoculation period. Comparison of the LDM and HDM samples revealed that during all incubation periods and in both runs a high cell density resulted in significantly ($p < 0.05$) more DNA compared with a low cell density. Statistical analysis of the rotational speed data showed that samples subjected to a low rotational speed (HDR2) always had a significantly ($p < 0.05$) higher DNA content than when a higher rotational speed (HDR12) was used.

Calcium

As can be seen in Fig. 2A and B, the results of the calcium measurements revealed again the existence of differences in calcium deposition between the two experimental runs. Calcium deposition differed ($p < 0.05$)

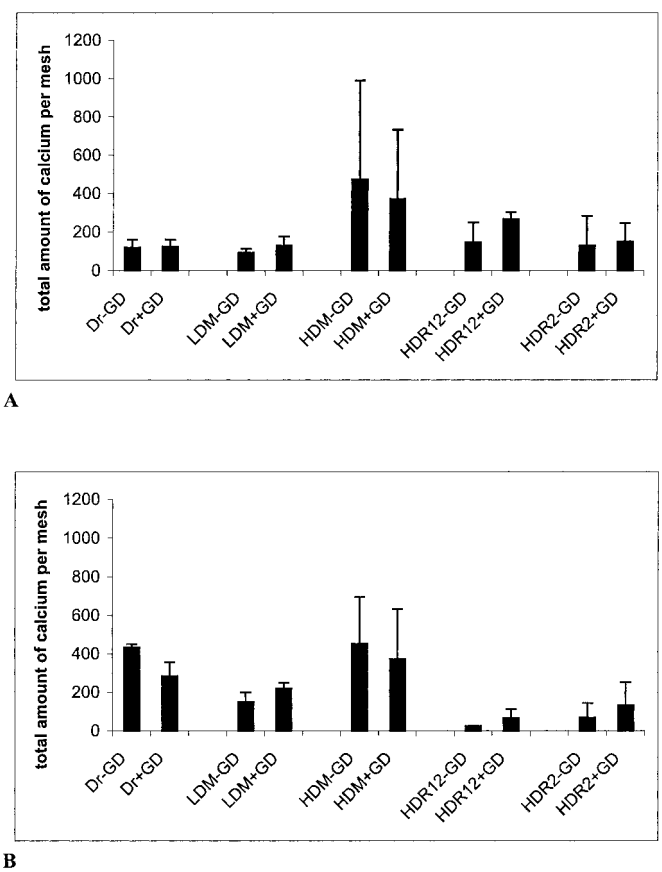

FIG. 2. Calcium content of osteoblast-like cells after 16 days in culture. The osteoblast-like cells were seeded by various cell-seeding techniques on titanium fiber mesh. (**A**) First run calcium content on day 16; (**B**) second run calcium content on day 16. Values represent means ± SD. Dr, Droplet method; LDM, low cell density, manually; HDM, high cell density, manually; HDR12, high cell density, rotation 12 rpm; HDR2, high cell density, rotation 2 rpm.

between the first and second runs for all Dr, LDM, and HDR12 samples. HDM and HDR2 specimens showed similar amounts of calcium in both runs.

Concerning the glow discharge treatment, no effect was seen for HDM and HDR2 specimens in both runs. Further, a significant effect ($p < 0.05$) of glow discharge was observed for LDM and HDR12 substrates in both runs, while this effect for Dr specimens existed only in the second run.

Finally, we observed that the HDM samples showed the most consistent results, with a relatively high amount of calcium in both experimental runs.

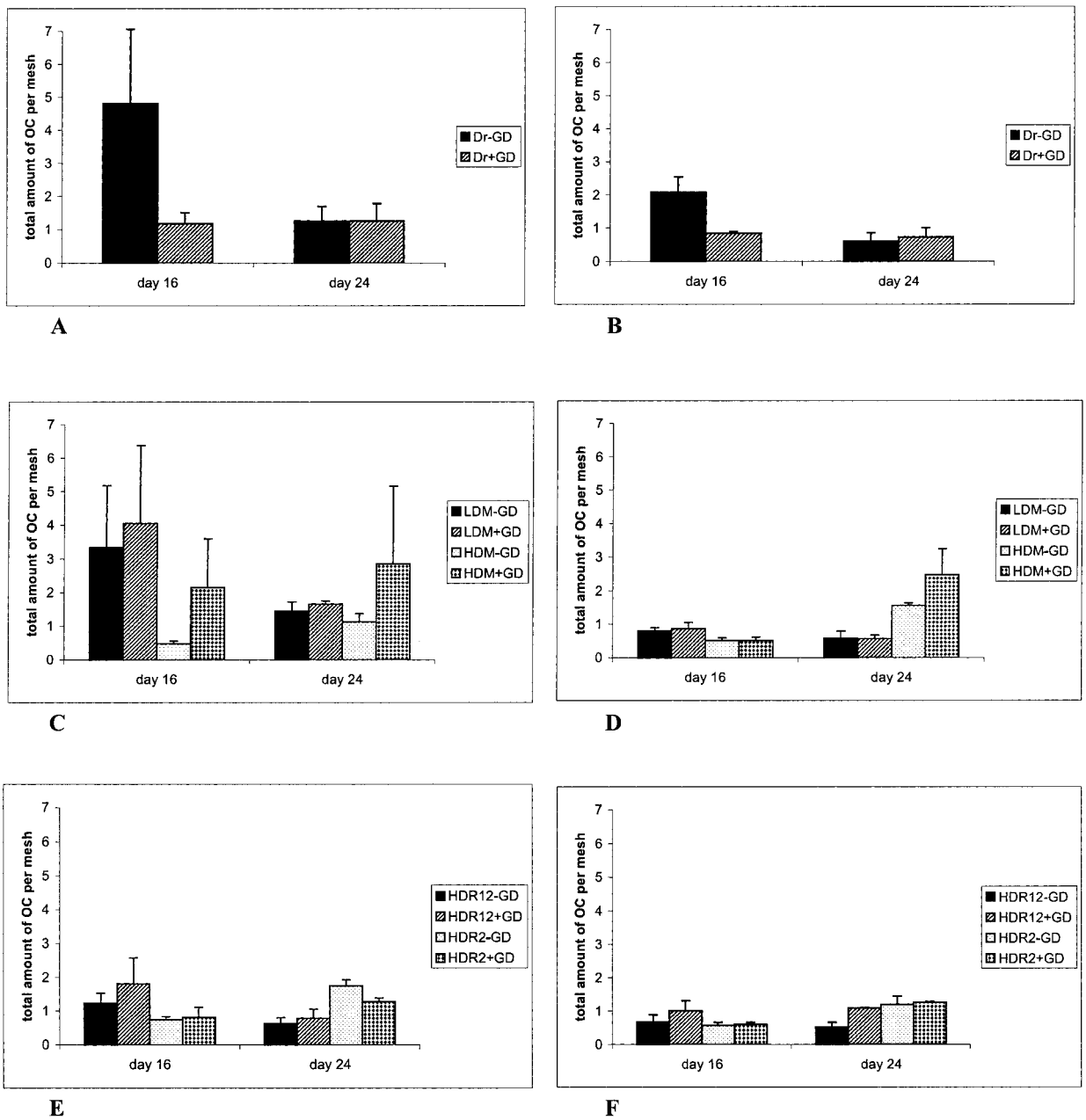

FIG. 3. Osteocalcin content of osteoblast-like cells after 16 and 24 days in culture. The osteoblast-like cells were seeded by various cell-seeding techniques on titanium fiber mesh. (**A** and **B**) first and second run Dr; (**C** and **D**) first and second run LDM and HDM; (**E** and **F**) first and second run HDR12 and HDR2. Values represent means ± SD. Dr, Droplet method; LDM, low cell density, manually; HDM, high cell density, manually; HDR12, high cell density, rotation 12 rpm; HDR2, high cell density, rotation 2 rpm.

Osteocalcin

Evaluation of the data, as presented in Fig. 3A–F, showed that the occurrence of differences between experimental runs could apparently be prevented by using high cell densities during inoculation of the scaffolds. No differences in osteocalcin content were observed between the two runs for HDM, HDR2, and HDR12 specimens. In contrast, the osteocalcin expression for Dr and LDM samples differed significantly ($p < 0.05$) between the first and second runs.

The effect of glow discharge treatment on osteocalcin expression was inconsistent and seemed to disappear with prolonged incubation. HDM and HDR12 specimens showed a significant effect ($p < 0.05$) of glow discharge only at 16 days of incubation, while no effect at all was seen for LDM and HDR2 samples.

Further, no differences in osteocalcin expression were observed between the different cell-seeding (inoculation) methods.

Scanning electron microsopy

SEM examination indicated that cells proliferated on all meshes in all experimental groups. By day 8, multilayers of cells could already be seen. Still, the various cell-seeding methods resulted in different cell coverage and mesh invasion patterns.

Concerning the Dr and LDM specimens, cells were present only on one side of the mesh (Fig. 4B and C). Which side of the mesh the cell sheet grew on depended on the additional use of glow discharge treatment. If the materials were glow discharge treated, the cells penetrated through the mesh and formed a cell sheet at the bottom side.

HDM specimens looked like Dr and LDM samples, except that the cell sheet seemed denser, with almost complete filling of the mesh voids.

The appearance of the samples that were subjected to rotation during cell seeding was completely different. Here, no cell sheet formation was observed. All cells had

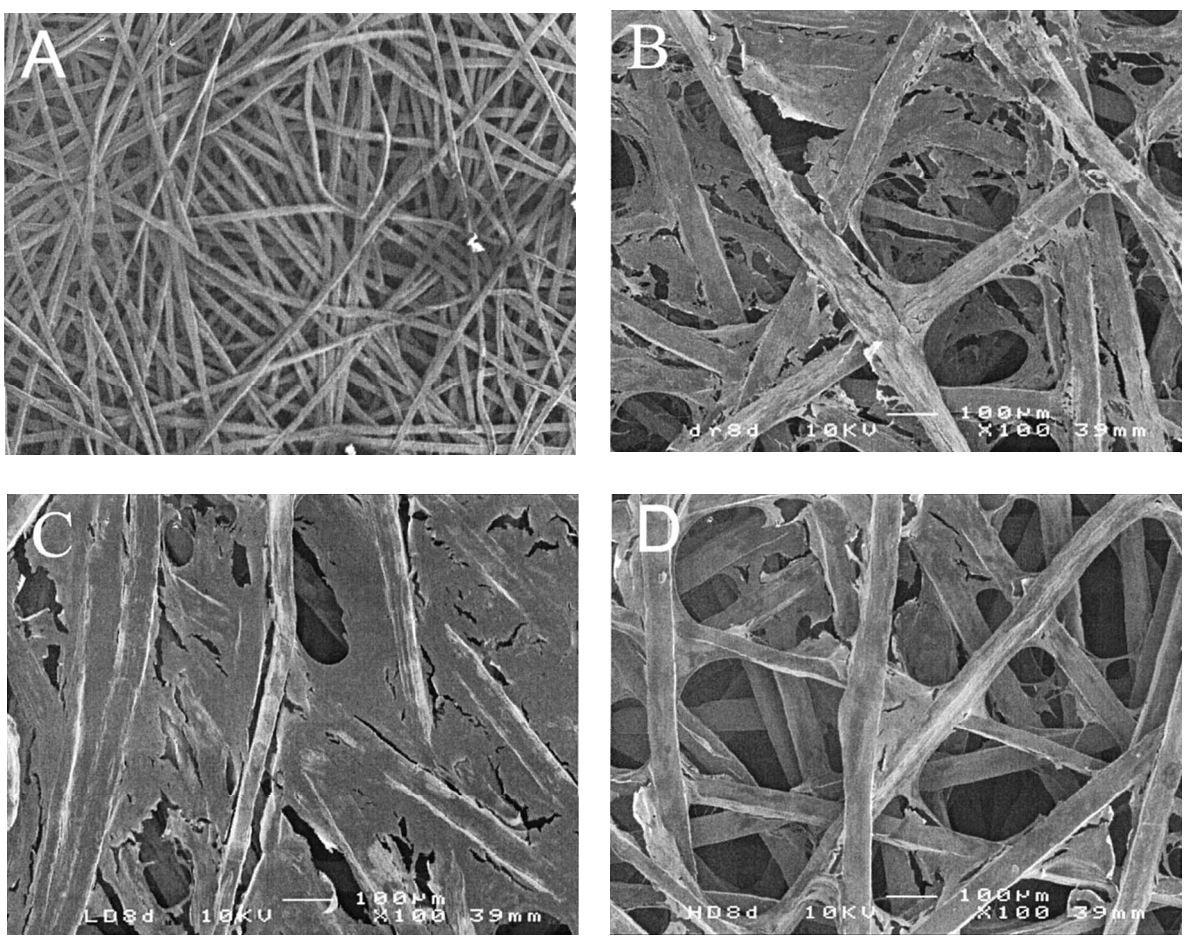

FIG. 4. SEM pictures of titanium fiber meshes after 8 days in culture. The osteoblast-like cells were seeded by various cell-seeding techniques on titanium fiber mesh. Cells have started to form a multilayer of cells on the titanium fibers. (**A**) Untreated starting material; (**B**) Dr; (**C**) LDM; (**D**) HDR12. Original magnification: ×100. Dr, Droplet method; LDM, low cell density, manually; HDR12, high cell density, rotation 12 rpm.

invaded the mesh and were growing around the titanium fibers (Fig. 4D). No effect of the glow discharge procedure could be observed.

After 16 days of incubation, we observed that a layer of calcified globular accretions associated with collagen bundles was deposited on and into the mesh material (Fig. 5). No clear differences in appearance between the various cell-seeding techniques were visible.

DISCUSSION

The results of our current study confirmed our earlier studies showing that titanium fiber mesh supports the attachment, growth, and differentiation of rat bone marrow stromal cells. After 16 days of incubation, this even resulted in the appearance of calcified globular accretions and collagen bundles. In general, our findings corroborate other studies in which different scaffold materials have been used. Despite this agreement, we still must note that a direct comparison of assays and data between the various studies is difficult and frequently even impossible. For example, the scaffold materials used differ not only in chemical properties but also in three-dimensional geometry. The titanium fiber mesh is fabricated by interengaging and intertwining a multiplicity of thin titanium fibers. The fibers are bound at their points of contact by a sinter process. This results in an open, interconnected porosity. In contrast, polymer scaffolds in which the porosity is created by sodium chloride crystals and a solution leaching process present a less interconnected porosity. Further, most studies differ in cell source as well as cell culture conditions. Some researchers use calvarial cells[4,15,16] to examine the osteogenic capacity of the scaffold material, while others use bone marrow cells.[4,17,18] Besides, different types of rats are used for cell retrieval, that is, Wistar[18,19] or Sprague-Dawley.[4,20] Also, the rats used varied in age. All these discrepancies in primary conditions can have severe implications for the final results. The same is true for the cell culture conditions. In most experiments, dexamethasone is added as a supplement in order to enhance or induce the osteogenic differentiation of the cells. However, this supplement is added at different stages during cell culturing. When dexamethasone is provided for the first time after the first passage of rat bone marrow cells, the osteogenic expression of the cells is reduced.[21]

A major observation in our study was the variance in results between different experimental runs. For example, large deviations were found when the droplet and manual shaking methods were used. For the manual shaking method, this large deviation can be explained by the difficulty in reproducing the shaking maneuver. The problem with the droplet-seeding technique is that the cell type can vary in each droplet. In view of this, we proved that the bone marrow population is heterogeneous in nature, which implies that different cultures can show different sensitivity. This will result in a differential response that cannot be prevented even by cell pooling or multiple experiments.[22,23] We must emphasize that this problem will always occur when primary cells are used and the experiments are repeated. This can be prevented

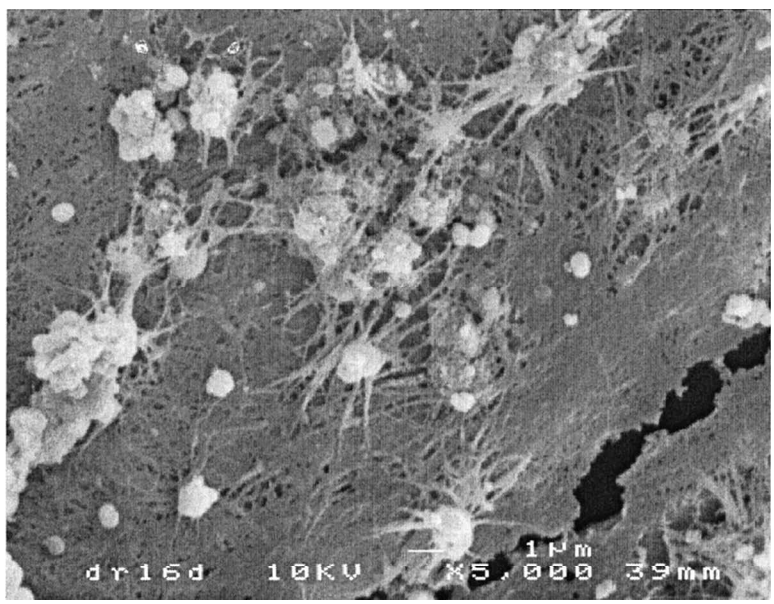

FIG. 5. SEM picture of titanium fiber mesh after 16 days in culture. Shown is the formation of multilayer with globular accretions and collagen fibers after 16 days in culture. Original magnification: ×5000. Dr, Droplet method.

only by using homogeneous cell populations, that is, cell lines. On the other hand, this simply emphasizes the problems encountered in producing reliable and standardized tissue-engineered bone constructs when bone marrow is used as the cell source. Therefore, we can only stress that a solution must be found for the proper selection of osteoprogenitor cells from multicellular bone marrow aspirate.

Besides the problem with the cell source, another experimental problem exists. In our 16- and 24-day calcium and osteocalcin assay, we did not normalize our data to DNA. We observed that extensive mineralization occurred within the scaffold porosity after 16 and 24 days of incubation. This prevents reliable extraction of DNA, as confirmed in previous experiments in which we measured low DNA values after 16 days of culture. Cell proliferation is supposed to decline when osteogenic differentiation starts. Nevertheless, this must result in similar cell numbers after 16 days compared with 8 days of culturing, as we know from rat bone marrow cultures on flat substrate surfaces. We suppose now that the apparent decline in DNA at 16 days is caused by difficulties in extracting DNA from the scaffolds, which showed extensive matrix mineralization. Because of this experimental flaw, comparison of the calcium and osteocalcin data between the experimental runs is difficult. Still, we must stress that this problem occurs in all experiments with porous three-dimensional scaffolds and mineralizing cells. Consequently, we suggest that other parameters or methods be found to interpret the osteogenic differentiation of these kinds of cells in porous materials.

As part of our experiment, we investigated the effect of glow discharge treatment on seeding efficiency. Glow discharge improves the wettability of a material.[24,25] We hypothesized that in this way the penetration of cells into the scaffold porosity could be improved. Unfortunately, we did not observe such an effect.[26,27] In the droplet-loaded specimens, the glow discharge procedure caused only a complete invasion and penetration of the RBM cells through the mesh pores to the bottom side. This can be concluded from the SEM results, which showed the presence of a cell sheet on the top side of nontreated titanium mesh and on the bottom side of glow discharge-treated mesh. Apparently, because of the increased wettability, the medium–cell droplet just "fell" through the fiber mesh material (notice that the fiber mesh is only 0.8 mm thick). Of course, in suspension culture this is impossible. Nevertheless, also under these conditions only a minor effect was seen in the DNA assessment. Titanium already possesses a hydrophilic surface. Therefore, we suppose that the increase in wettability, as achieved by glow discharge, does not further sustain cell behavior and appears to be superfluous. This suggestion is supported by previous findings of our group, in which we also could not observe a beneficial effect of titanium glow discharge treatment on the growth of fibroblasts and epithelial cells.[28,29]

Further, we compared the influence of seeding technique on the final osteogenic expression of the cells in the titanium mesh. On the basis of the results, we cannot claim that any one of the methods used is superior. On the other hand, we observed that the results became more consistent when high cell densities were used during dynamic seeding. Before discussing this observation, it appears appropriate to discuss the reason for the use of a high cell density and why this was done only for the dynamic seeding method. High cellularity is known to enhance cell-to-cell contact and communication between cells. This is essential for phenotypic expression of cells *in vitro*.[30] The droplet, as used in the droplet technique, consists of a small volume with a high cell density suspension, that is, 8×10^4 cells, resuspended in 30 μL of culture medium. We assumed that cell–cell contact and communication are already optimal in this droplet. This is in contrast to the dynamic method, in which cells were resuspended in a large medium volume and significantly less cell–cell contacts will occur.

Similar experiments with the use of different cell densities have been done by Ishaug *et al.*[31] However, in contrast to our results, they observed that their highest cell density resulted in increased cell numbers after 7 days of incubation and increased mineralization at later evaluation periods. Unfortunately, we are not able to give a clear explanation for this discrepancy in observation. We can only hypothesize that this may be because the high cell density used was not the optimum density. Therefore, we recommend that in future studies additional cell densities be included to determine whether indeed such an optimum exists. Further, we must note also that in the dynamic method a large difference was present between the two experimental runs. Although interindividual differences in cell population can again explain this phenomenon, differences in cell population due to preferential adhesion of specific cell types during the rotation time can also influence the cell number and differentiation after dynamic seeding.

Our calcium measurements suggest that the additional use of dynamic movement during the initial cell-seeding process does not further increase the influence of cell density. Nevertheless, the SEM pictures show that the cells are more uniformly distributed in the specimens that are subjected to a low rotational speed. This corroborates other studies that also used dynamic seeding methods.[3,8,12,32] A uniform and well-established distribution of cells inside the pores of the scaffold materials can be relevant for the final *in vivo* bone-inducing capacity of the construct.

The three major seeding methods documented in the literature are dynamic (rotating wall vessels, spinner flasks), static, and perfusion bioreactor. Most of the re-

search concerning the effect of seeding techniques on the differentiation of osteoblast-like cells has been done with polymeric scaffolds. However, the choice of a seeding method depends on the spatial architecture as well as the chemical composition of the scaffold material used. In view of this, we know that metals have intrinsic surface properties other than polymers. Metals are hydrophilic while most polymers are hydrophobic. As already mentioned, the three-dimensional architecture of titanium fiber mesh differs completely from that of spongelike or foamlike scaffold structures. Because of its open structure, the exchange of medium and waste products is facilitated in titanium fiber mesh. Further, cells can adhere during seeding at points where contact exists between various fibers. Subsequently, they can also migrate along the fibers, in contrast to spongelike or foamlike materials, which to a great extent prevent the penetration of cells and exchange of waste products. Regrettably, a direct comparison between the various studies dealing with this topic is difficult, because of the cell culture problems pointed out earlier. Therefore, we can only suggest that for scaffolds with interconnective pores a perfusion bioreactor is probably a good system to optimize the osteogenic efficacy of a scaffold. This is supported by the findings of Burg *et al*,[32] who compared the effects of three seeding methods (perfusion bioreactor, stir flask, and static) on a three-dimensional scaffold, polyglycolide fibrous mesh. They found that seeding with a perfusion bioreactor achieved the best results as determined on the basis of metabolic activity, cellular attachment, and cellular proliferation. Other researchers[11,33] used rotating wall vessels and spinner flasks to increase the invasion of cells and cellular attachment inside their scaffolds.

In summary, we conclude that titanium fiber mesh is indeed suitable to support the osteogenic expression of bone marrow cells. Changing the initial cell density as well by making use of dynamic seeding methods can influence the osteogenic capacity of the scaffold. In further experiments, the consequences of these findings for final *in vivo* bone induction must be investigated. In addition, methods must be found to better select osteoprogenitor cells out of bone marrow aspirates in order to standardize and improve the reliability of bone tissue engineering.

ACKNOWLEDGMENTS

This research was supported by the Dutch Technology Foundation STW, the Applied Science Division of the NWO, and the Technology Program of the Ministry of Economic Affairs.

REFERENCES

1. Jansen, J.A., von Recum, A.F., van der Waerden, J.P., and de Groot, K. Soft tissue response to different types of sintered metal fibre-web materials. Biomaterials **13,** 959, 1992.
2. Vehof, J.W., Spauwen, P.H., and Jansen, J.A. Bone formation in calcium-phosphate-coated titanium mesh. Biomaterials **21,** 2003, 2000.
3. Vehof, J.W., de Ruijter, A.E., Spauwen, P.H., and Jansen, J.A. Influence of rhBMP-2 on rat bone marrow stromal cells cultured on titanium fiber mesh. Tissue Eng. **7,** 373, 2001.
4. Ishaug Riley, S.L., Crane, G.M., Gurlek, A., Miller, M.J., Yasko, A.W., Yaszemski, M.J., and Mikos, A.G. Ectopic bone formation by marrow stromal osteoblast transplantation using poly(DL-lactic-co-glycolicacid) foams implanted into the rat mesentery. J. Biomed. Mater. Res., **36,** 1, 1997.
5. Mendes, S.C., Van den Brink, I., De Bruijn, J.D., and Van Blitterswijk, C.A. In vivo bone formation by human bone marrow cells: Effect of osteogenic culture supplements and cell densities. J. Mater. Sci. Mater. Med., **9,** 855, 1998.
6. Jaiswal, N., Haynesworth, S.E., Caplan, A.I., and Bruder, S.P. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J. Cell. Biochem. **64,** 295, 1997.
7. Gao, J.M., Niklason, L., and Langer, R. Surface hydrolysis of poly(glycolic acid) meshes increases the seeding density of vascular smooth muscle cells. J. Biomed. Mater. Res. **42,** 417, 1998.
8. Kim, B.S., Putnam, A.J., Kulik, T.J., and Mooney, D.J. Optimizing seeding and culture methods to engineer smooth muscle tissue on biodegradable polymer matrices. Biotechnol. Bioeng. **57,** 46, 1998.
9. Vunjak-Novakovic, G., Obradovic, B., Martin, I., Bursac, P.M., Langer, R., and Freed, L.E. Dynamic cell seeding of polymer scaffolds for cartilage tissue engineering. Biotechnol. Prog. **14,** 193, 1998.
10. Xiao, Y.L., Riesle, J., van Blitterswijk, C.A. Static and dynamic fibroblast seeding and cultivation in porous PEO/PBT scaffolds. J. Mater. Sci. Mater. Med. **10,** 773, 1999.
11. Granet, C., Laroche, N., Vico, L., Alexandre, C., and Lafage-Proust, M.H. Rotating-wall vessels, promising bioreactors for osteoblastic cell culture: Comparison with other 3D conditions. Med. Biol. Eng. Comput. **36,** 513, 1998.
12. Carrier, R.L., Papadaki, M., Rupnick, M., Schoen, F.J., Bursac, N., Langer, R., Freed, L.E., and Vunjak-Novakovic, G. Cardiac tissue engineering: Cell seeding, cultivation parameters, and tissue construct characterization. Biotechnol. Bioeng. **64,** 580, 1999.
13. Kim, S.S., Sundback, C.A., Kaihara, S., Benvenuto, M.S., Kim, B.S., Mooney, D.J., and Vacanti, J.P. Dynamic seeding and in vitro culture of hepatocytes in a flow perfusion system. Tissue Eng. **6,** 39, 2000.
14. Maniatopoulos, C., Sodek, J., and Melcher, A.H. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res. **254,** 317, 1988.

15. Ishaug Riley, S.L., Crane Kruger, G.M., Yaszemski, M.J., and Mikos, A.G. Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. Biomaterials **19,** 1405, 1998.
16. Lynch, M.P., Stein, J.L., Stein, G.S., and Lian, J.B. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: Modification of expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. Exp. Cell Res. **216,** 35, 1995.
17. Holy, C.E., Shoichet, M.S., and Davies, J.E. Engineering three-dimensional bone tissue in vitro using biodegradable scaffolds: Investigating initial cell-seeding density and culture period. J. Biomed. Mater. Res. **51,** 376, 2000.
18. Bi, L.X., Simmons, D.J., and Mainous, E. Expression of BMP-2 by rat bone marrow stromal cells in culture. Calcif. Tissue Int. **64,** 63, 1999.
19. Aubin, J.E. Osteoprogenitor cell frequency in rat bone marrow stromal populations: Role for heterotypic cell–cell interactions in osteoblast differentiation. J. Cell. Biochem. **72,** 396, 1999.
20. Puleo, D.A. Dependence of mesenchymal cell responses on duration of exposure to bone morphogenetic protein-2 in vitro. J. Cell. Physiol. **173,** 93, 1997.
21. Ter Brugge, P.J. and Jansen, J.A. In vitro osteogenic differentiation of rat bone marrow cells cultured with and without dexamethasone. Tissue Eng, **8,** 321, 2002.
22. Dieudonne, S.C., van den Dolder, J., de Ruijter, J.E., Paldan, H., Peltola, T., van't Hof, M.A., Happonen, R.P., and Jansen, J.A. Osteoblast differentiation of bone marrow stromal cells cultured on silica gel and sol–gel-derived titania. Biomaterials **23,** 3041, 2002.
23. Ter Brugge, P.J., Torensma, R., de Ruijter, J.E., Figdor, C.G., and Jansen J.A. Modulation of integrin expression on rat bone marrow cells by substrates with different surfaces. Tissue Eng. **8,** 615, 2002.
24. Hesby, R.M., Haganman, C.R., and Stanford, C.M. Effects of radiofrequency glow discharge on impression material surface wettability. J. Prosthet. Dent. **77,** 414, 1997.
25. Ozden, N., Akaltan, F., Suzer, S., and Akovali, G. Time-related wettability characteristic of acrylic resin surfaces treated by glow discharge. J. Prosthet. Dent. **82,** 680, 1999.
26. Curtis, A.S., Forrester, J.V., McInnes, C., and Lawrie, F. Adhesion of cells to polystyrene surfaces. J. Cell Biol. **97,** 1500, 1983.
27. Ramsey, W.S., Hertl, W., Nowlan, E.D., and Binkowski, N.J. Surface treatments and cell attachment. In Vitro **20,** 802, 1984.
28. Jansen, J.A., van der Waerden, J.P., and de Groot, K. Effect of surface treatments on attachment and growth of epithelial cells. Biomaterials **10,** 604, 1989.
29. Jansen, J.A., van der Waerden, J.P., and de Groot, K. Fibroblast and epithelial cell interactions with surface-treated implant materials. Biomaterials **12,** 25, 1991.
30. Cheng, S.L., Lecanda, F., Davidson, M.K., Warlow, P.M., Zhang, S.F., Zhang, L., Suzuki, S., St John, T., and Civitelli, R. Human osteoblasts express a repertoire of cadherins, which are critical for BMP-2-induced osteogenic differentiation. J. Bone Miner. Res. **13,** 633, 1998.
31. Ishaug, S.L., Crane, G.M., Miller, M.J., Yasko, A.W., Yaszemski, M.J., and Mikos, A.G. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. J. Biomed. Mater. Res. **36,** 17, 1997.
32. Burg, K.J., Holder, W.D., Culberson, C.R., Beiler, R.J., Greene, K.G., Loebsack, A.B., Roland, W.D., Eiselt, P., Mooney, D.J., and Halberstadt, C.R. Comparative study of seeding methods for three-dimensional polymeric scaffolds. J. Biomed. Mater. Res. **51,** 642, 2000.
33. Temenoff, J.S., and Mikos, A.G. Review: Tissue engineering for regeneration of articular cartilage. Biomaterials **21,** 431, 2000.

Address reprint requests to:
John A. Jansen, D.D.S., Ph.D.
Department of Biomaterials, College of Dental Science
University Medical Center, University of Nijmegen
P.O. Box 9101
6500 HB Nijmegen, The Netherlands

E-mail: J.Jansen@dent.umcn.nl