A cultured living bone equivalent enhances bone formation when compared to a cell seeding approach

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The development of cell therapy methods to confer osteogenic potential to synthetic bone replacement materials has become common during the last years. At present, in the bone tissue engineering field, two different approaches use patient own cultured osteogenic cells in combination with a scaffold material to engineer autologous osteogenic grafts. One of the approaches consists of seeding cells on a suitable biomaterial, after which the construct is ready for implantation. In the other approach, the seeded cells are further cultured on the scaffold to obtain *in vitro* formed bone (extracellular matrix and cells), prior to implantation. In the present study, we investigated the *in vivo* osteogenic potential of both methods through the implantation of porous hydroxyapatite (HA) scaffolds coated with a layer of *in vitro* formed bone and porous HA scaffolds seeded with osteogenic cells. Results showed that as early as 2 days after implantation, *de novo* bone tissue was formed on scaffolds in which an *in vitro* bone-like tissue was cultured, while it was only detected on the cell seeded implants from 4 days onwards. In addition, after 4 days of implantation statistical analysis revealed a significantly higher amount of bone in the bone-like tissue containing scaffolds as compared to cell seeded ones.

Introduction

The regeneration of large bone defects caused by injury, cancer, infection, congenital malformations and fracture non-union, remains a great challenge in orthopedic surgery. Autologous bone grafting is considered the golden standard in the treatment of such defects. It provides osteoprogenitor cells present in bone marrow and an extracellular matrix containing collagen, hydroxyapatite and a range of osteoinductive growth factors. However, the supply of bone to be harvested is quite limited with this therapy, while its collection is painful and associated with infections and donor site morbidity [1]. Allogenic bone grafting is also a sub-optimal treatment since it can elicit immunological responses and its success in bone regeneration is lower as compared to autologous bone due to the low or absent cellular function of allogeneic bone [2]. To overcome these problems, researchers are testing new ways to replace bone. Although a wide range of biomaterials is currently available to fill bone defects, the success of these materials is limited due to their general lack of osteogenic and/or osteoinductive properties.

The process of *in vivo* bone formation comprises a sequence of events that involve the recruitment and proliferation of osteoblastic precursors, followed by cell differentiation, matrix formation and, ultimately, mineralization [3–4]. Growth factors and proteins contained in the bone matrix are involved on the regulation of cell growth, differentiation and mineralization [3–7].

In recent years, the possibility of *in vitro* engineering an autologous graft with osteogenic properties has been investigated. The goal is to develop an alternative to the traditional autologous bone graft that achieves similar success in bone regeneration. In this approach, a small biopsy of the relevant cells is taken from the patient, cells are then expanded in culture and, finally, combined with a biomaterial. The biomaterial functions as a scaffold for the formation of new bone tissue, as a carrier for the transplanted cells and it also provides volume to better fill the bone defect. Several investigators [8–17] have reported the ability of culture expanded bone marrow stromal cells to form bone in ectopic sites when seeded on a biomaterial shortly before implantation. However, such an approach lacks the existence of an extracellular matrix on the implants, which can be essential to rapid healing since it contains a variety of bone related proteins and growth factors. A second approach, therefore, utilizes the culture of a bone-like tissue layer on the scaffolds prior to implantation. In fact, it is known that *in vitro* bone formation by osteogenic cultures is similar to the initial process of bone formation *in vivo* [18, 19], which indicates that by culturing osteogenic cells on a

suitable biomaterial scaffold an autologous bone equivalent can be obtained [20–22]. Several investigators have widely reported ectopic *in vivo* bone formation induced by such hybrid constructs of cultured bone and biomaterial [23–29]. However, to our knowledge no study has compared the osteogenic potential of the two above-mentioned techniques. In summary, two cell therapy approaches are currently investigated in the bone tissue engineering field. One is to seed cultured osteogenic cells on a biomaterial scaffold after which the construct is implanted. The other approach aims at culturing a layer of autologous bone equivalent on the scaffold before implantation. The objective of the current study is to evaluate both methods by investigating whether porous hydroxyapatite scaffolds coated with a layer of *in vitro* formed bone would induce faster bone formation in a ectopic implantation site, as compared to cell seeded hydroxyapatite.

Materials and methods

Isolation and culture of bone marrow cells

Bone marrow cells were obtained from the femora of young adult male F344 rats (150–180 g). The marrow cell preparation procedure was described in a previous report [25]. Briefly, femora were removed and washed in an antibiotic solution with a concentration 10 times higher than on culture medium. After the removal of the epiphyses, the bone marrow cells were flushed out with culture medium (see below). The bone marrow obtained from all the rats was pooled and plated in 75 cm^2 flasks at a density equivalent to a femur per flask. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and the culture medium consisted of alpha-minimum essential medium (α-MEM, Life Technologies, The Netherlands), 15% fetal bovine serum (FBS, Life Technologies, The Netherlands), antibiotics, 0.2 mM L-ascorbic acid 2-phosphate (AsAP, Life Technologies, The Netherlands), 0.01 M β-glycerophosphate (βGP, Sigma, The Netherlands) and 10 nM dexamethasone (dex, Sigma, The Netherlands). The culture medium was refreshed after 24 h and thereafter three times a week. At near confluence, the adherent cells were washed with phosphate buffered saline solution and enzymatically released by means of a 0.25% trypsin–EDTA solution (Sigma, The Netherlands).

Scaffold material

Porous granules of hydroxyapatite (HA, IsoTis NV, The Netherlands) with a porosity of approximately 60% were used as scaffold material. The interconnected pores had a median diameter of 430 μm and the size of the implanted particles was approximately $3 \times 2 \times 2\,mm^3$.

Cell seeding and culture on the scaffolds

First passage cells were seeded on the HA particles placed on bacteriological grade plates. Aliquots of 50 μL of cell suspension were seeded into each scaffold (see cell densities below) and cells were allowed to attach on the HA samples for 4 h, after which time an additional 2 mL of culture medium was added. Four experimental groups were defined as stated in Table I: (1) cells seeded at a density of 100 000 cells per particle followed by an additional culture period of 5 days prior to implantation; (2) cells seeded at a density of 750 000 cells per particle for 16 h prior to implantation. This seeding density is at least equivalent to the cell number present on the scaffolds seeded with 100 000 cells after 5 days of culture (the number was obtained by extrapolating the results of cell growth rate on tissue culture polystyrene plates); (3) cells seeded at a density of 100 000 cells per particle for 16 h prior to implantation. This group was used to analyze the effect of 5 days of cell culture versus cell seeding and implantation for an additional period of 5 days; (4) control HA particles without cells.

TABLE I Experimental groups and design

Experimental group	Seeding density/ scaffold	Seeding/culture time	Implantation times (days)
I	100 000	5 days	2, 4, 7, 9 and 12 *
II	750 000	16 h	2, 4, 7, 9 and 12 †
III	100 000	16 h	2, 4, 7, 9 and 12 *
IV	0	—	2, 4, 7, 9 and 12 *

*$n = 8$ per implantation time.
†$n = 6$ per implantation time.

Light and scanning electron microscopy

Prior to implantation, samples were fixed, dehydrated and either embedded in methyl methacrylate, sectioned using a diamond saw (SP1600, Leica, Germany) stained with a 1% methylene blue solution and examined by light microscopy ($n = 3$) or critical point dried (Balzers model CPD 030 critical point drier), sputter coated with carbon (Balzers sputter coater model SCD 004) and examined in a Philips XL30 ESEM-FEG scanning electron microscope ($n = 3$), at an accelerating voltage of 10–15 kV.

In vivo implantation

Prior to implantation, tissue engineered samples from the four experimental groups were soaked in serum free medium and then washed in phosphate buffered solution pre-warmed to 37 °C. Fifteen male syngeneic F344 rats (300–350 g) were anesthetized, the surgical sites cleaned with ethanol and subcutaneous pockets were created, in which the samples were inserted randomly (two samples per pocket, three–four pockets per rat). After 2, 4, 7, 9 and 12 days of implantation, the samples ($n = 8$ per experimental group and per survival period, except for group II, in which $n = 6$ due to the large cell number required) were removed and fixed in 1.5% glutaraldehyde in 0.14 M cacodylic acid buffer, pH 7.3.

Histology of the implanted samples and extent of bone formation

The fixed samples were dehydrated and embedded in methyl methacrylate. The sections were processed on a histological diamond saw (Leica SP1600, Leica, Germany) and stained with a 1% methylene blue solution and a 0.3% basic fuchsin solution in order to visualize bone formation. Osteogenesis was blindly estimated by

three independent investigators (SCM, MS, AM). The following scale was used: (0) no bone formation; (1) first signs of bone formation in few sections of the sample; (2) bone tissue occupied less than 10% of the pore area; (3) bone occupied between 10% and 20% of the pore area; (4) bone tissue spread over 20–50% of the pore area; and (5) bone occupied more than half of the pore area. For each survival period, the average score for the extent of osteogenesis was calculated for each sample of the three experimental groups ($n = 6$ to 8). Statistical analysis was performed using both the Kruskal–Wallis and the Mann–Whitney U tests, which are appropriated to the non-parametric and ordinal nature of the bone formation score. Statistical significance was defined as $p < 0.05$.

Results

Light and scanning electron microscopy

Light and scanning electron microscopy examination revealed that HA scaffolds seeded with 100 000 cells which were further cultured for 5 days (group I) were entirely covered with multilayers of cells (Fig. 1(a) and (b)). In between cell layers numerous collagen-like fibers could be observed (Fig. 1(c)). On scaffolds seeded with 750 000 cells for 16 h (group II), numerous cells were present throughout the porous materials although cells did not cover the entire surface of the scaffold and the presence of extracellular matrix was not detected (Fig. 2). In the higher cell density areas rounded cells were still detected, indicating that cell spreading was still in process. On samples from group III (HA scaffolds seeded with 100 000 cells for 16 h), isolated cells were seen, uniformly distributed throughout the porous scaffolds. The degree of cell-to-cell contact was quite low (Fig. 3). On these scaffolds cell density was clearly lower as compared to the samples of groups I and II.

Histology of the implanted samples and extent of bone formation

In control HA samples without osteogenic cells (group IV), bone tissue formation did not occur at any of the survival periods studied. The histological findings in cell containing scaffolds are summarized in Table II. As early as 2 days after implantation, all bone-like matrix containing scaffolds (group I) presented the first signs of *in vivo* bone formation. Cells acquired a more cuboidal shape and, in few areas, osteoid was formed (Fig. 4(a)). Both on high (group II) and low (group III) cell density seeded scaffolds only fibrous tissue was present (Fig. 4(b)), indicating that the culture of cells on HA scaffolds prior to implantation induces faster bone formation as compared to cell seeding only.

In group I, all implants harvested after 4 days of implantation showed bone tissue, which on average occupied more than 10% and less than 20% of the implant pore area (average bone score 2.2, Table II). For the same survival period, 4 of the 6 implants seeded with 750 000 cells for 16 h (group II) had less than 10% of their pore area filled with bone tissue, while in the remaining two implants, osteogenesis had not started (average bone score 1.1, Table II). Also after 4 days of implantation, half of the low cell density seeded implants

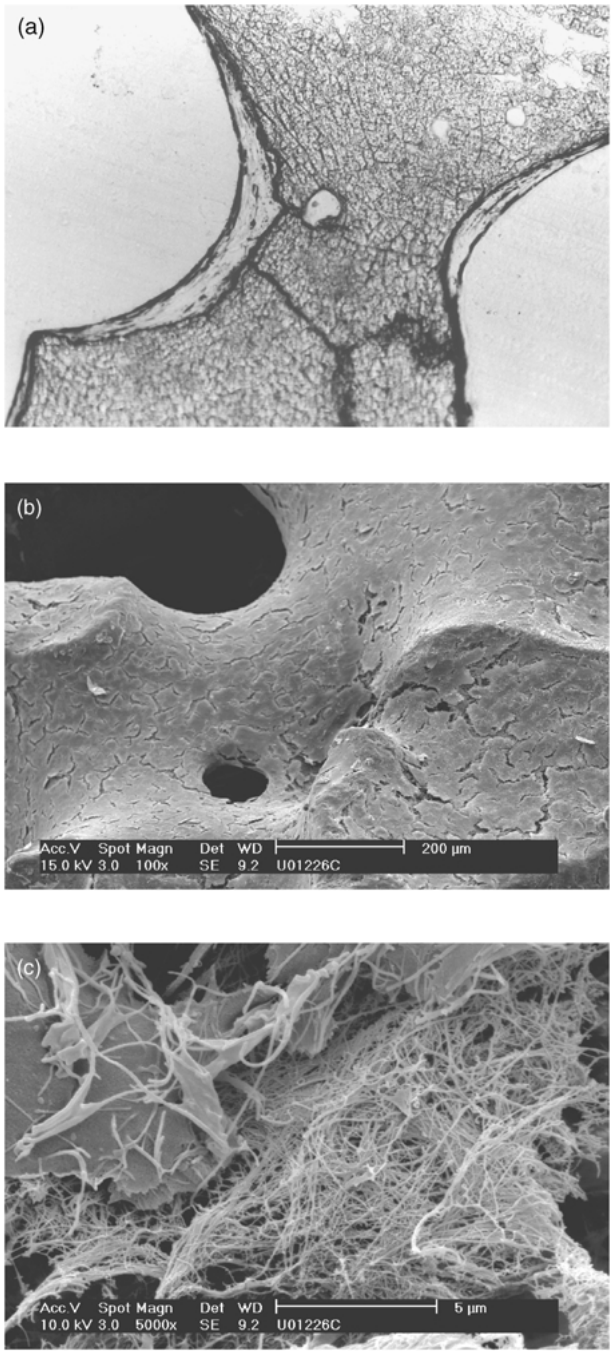

Figure 1 (a) Light micrograph (200 ×), (b) scanning electron micrograph (100 ×) and (c) scanning electron micrograph (5000 ×) of rat bone marrow cells grown for 5 days on porous HA particles. Cell seeding density: 100 000 cells/scaffold. Group I. Note the abundant presence of extracellular matrix and the numerous collagen-like fibers in between cell layers.

(group III) did not show signs of bone tissue, while on the other half the first signs of bone formation appeared (average bone score 0.1, Table II). At this survival period, statistical analysis revealed a significantly higher degree of osteogenesis in group I, as compared to groups II and III ($p = 0.032$ and $p = 0.019$, respectively), indicating a positive effect of bone-like matrix containing scaffolds with regard to *in vivo* bone formation. With respect to the cell seeding density, the extent of bone tissue at day 4 in the high cell density seeded scaffolds (group II) was not statistically different from the degree of bone formation on the low cell density seeded scaffolds (group III) ($p = 0.271$).

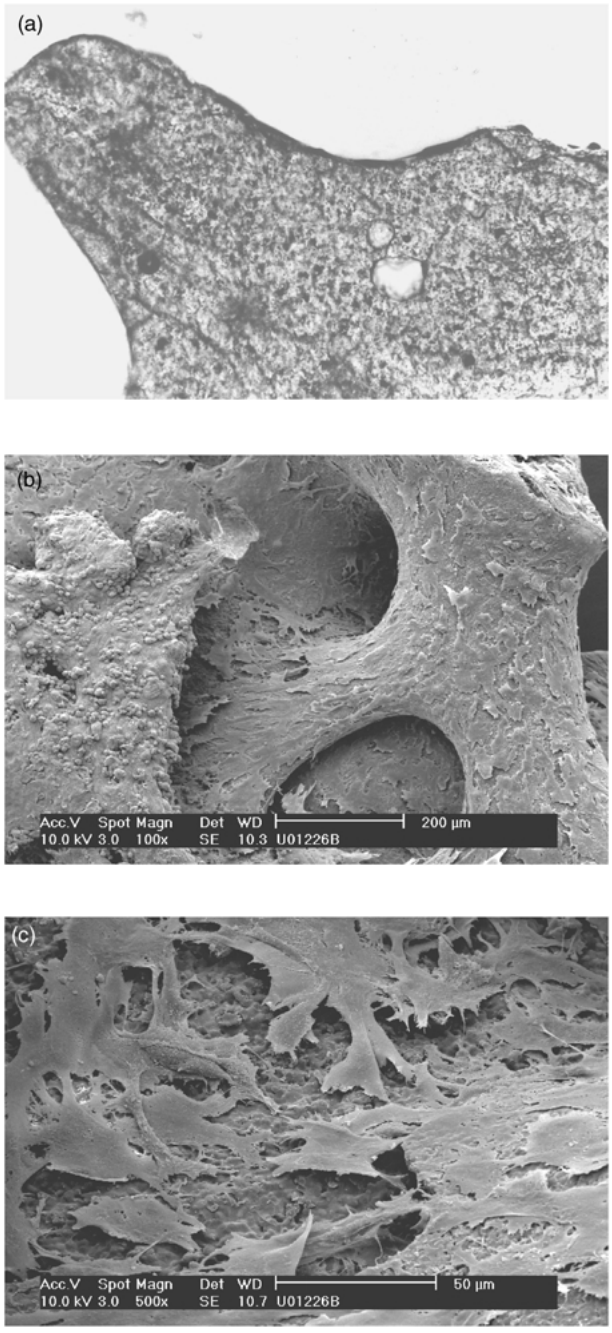

Figure 2 (a) Light micrograph (200 ×), (b) scanning electron micrograph (100 ×) and (c) scanning electron micrograph (500 ×) of rat bone marrow cells seeded for 16 h on porous HA particles. Cell seeding density: 750 000 cells/scaffold. Group II. Note the abundant cell number but the absence of extracellular matrix.

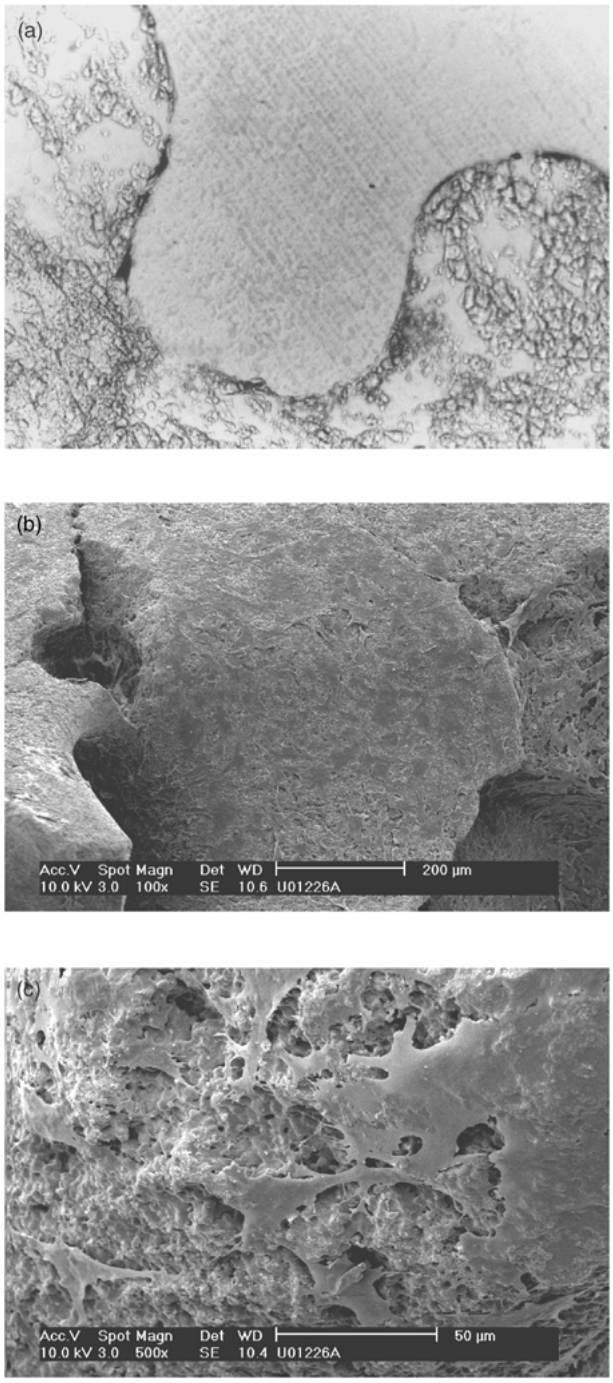

Figure 3 (a) Light micrograph (200 ×), (b) scanning electron micrograph (100 ×) and (c) scanning electron micrograph (500 ×) of rat bone marrow cells seeded for 16 h on porous HA particles. Cell seeding density: 100 000 cells/scaffold. Group III. Note the presence of isolated cells equally distributed throughout the scaffold surfaces.

At the end of one week survival, bone was detected in all samples from all experimental groups (except control group IV), (Fig. 5). The tissue was composed of a mineralized matrix, with embedded osteocytes and a layer of osteoblasts surrounding the outer surface of the newly formed bone. In groups I and II the average bone formation score was 3.1 and 2.3, respectively. The differences between the two groups failed to be statistically significant ($p = 0.724$). From day 7 on, bone formation in groups I and II increased with the implantation period (Table II). Although samples from group I exhibited a slightly higher extent of bone formation when compared to samples from group II, the differences were not statistically significant ($p = 0.564$, day 9 and $p = 0.372$, day 12).

With respect to the low cell density seeded scaffolds (group III), at day 7, the extent of bone formation varied from less than 10% (score 0.1–1, Table II) to between 10% and 20% (score 1.1–2, Table II), with an average bone formation score of 1.0 (Table II). At this implantation period, a significant difference was found between this group and the high cell density seeded one ($p = 0.034$). This difference was maintained both at 9 and 12 days post implantation, indicating that the extent

TABLE II Bone formation in HA scaffolds containing rat bone marrow stromal cells. Effect of cell seeding versus cell seeding and culture

Implantation period (days)	Experimental group	Total number of implants (#)	Bone formation score						Average bone formation score (± SD)
			0	0.1–1	1.1–2	2.1–3	3.1–4	4.1–5	
2	I	8		6	2				0.8 ± 0.2
	II	6	6						0.0 ± 0.0
	III	8	8						0.0 ± 0.0
4	I	8				8			2.2 ± 0.1
	II	6	2		4				1.1 ± 1.0
	III	8	4	4					0.1 ± 0.1
7	I	8				4	4		3.1 ± 0.7
	II	6				2	4		2.3 ± 0.6
	III	8		6	2				1.0 ± 0.2
9	I	8					8		3.4 ± 0.1
	II	6				4	2		3.1 ± 0.7
	III	8			6	2			1.9 ± 0.2
12	I	8					6	2	3.7 ± 0.4
	II	6				2	4		3.4 ± 0.5
	III	8	2	2	2	2			1.2 ± 1.1

Experimental group I: HA scaffolds seeded with 100 000 cells, which were cultured for 5 days prior to implantation.
Experimental group II: HA scaffolds seeded with 750 000 cells for 16 h prior to implantation.
Experimental group III: HA scaffolds seeded with 100 000 cells for 16 h prior to implantation.
The following scale was used to estimate bone formation: (0) no bone formation; (1) first signs of bone formation in few sections of the sample; (2) bone tissue occupied less than 10% of the pore area; (3) bone occupied between 10 and 20% of the pore area; (4) bone tissue spread over 20 to 50% of the pore area; and (5) bone occupied more than half of the pore area.

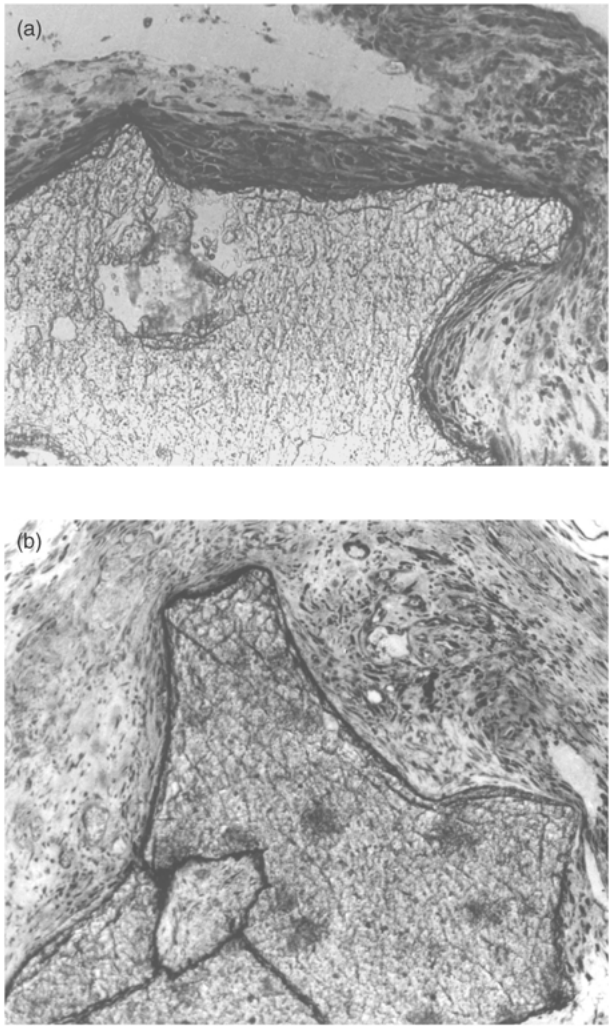

Figure 4 Light micrographs illustrating representative sections after 2 days of implantation. (a) First signs of *in vivo* bone formation on HA scaffolds in which rat bone marrow cells grown for 5 days, (group I, 200 ×); (b) Fibrous tissue is present on the cell seeded implants (group II, 100 ×).

of newly formed bone was directly proportional to the amount of seeded bone marrow cells.

An interesting analysis is to compare the *in vivo* osteogenic potential of bone-like matrix containing scaffolds (group I) at day 2, 4 and 7 to the lower cell density seeded scaffolds (group III) at day 7, 9 and 12, respectively. On both groups, HA particles were seeded with an equal cell amount, however, in group I cells were cultured for an additional period of 5 days prior to implantation. Therefore, when adding *in vitro* and *in vivo* testing periods samples of group I at day 2, 4 and 7 after implantation can be compared with samples from group III at day 7, 9 and 12 after implantation, respectively. Although no differences in bone formation could be detected between samples from group I at 2 days of implantation and samples from group III at day 7 ($p = 0.2381$), group I at day 4 and 7 exhibited significantly higher bone formation scores as compared to group III at day 9 ($p = 0.021$) and 12 ($p = 0.015$), respectively. This indicates that cell seeding and culture for 5 days prior to implantation seems more efficient than cell seeding followed by an extra implantation period of 5 days.

Discussion

Bone marrow has long been recognized to contain osteoprogenitor cells that are able to differentiate towards the osteogenic lineage when cultured in conditions permissive to osteobastic development [30]. In the present study, we used rat bone marrow cells to evaluate the potential of two cell therapy approaches used in the development of bone grafts with osteogenic properties. One approach aims at *in vitro* engineering an autologous bone graft through the use of porous scaffolds coated with a layer of bone-like tissue, while the second approach uses porous scaffolds in combination with

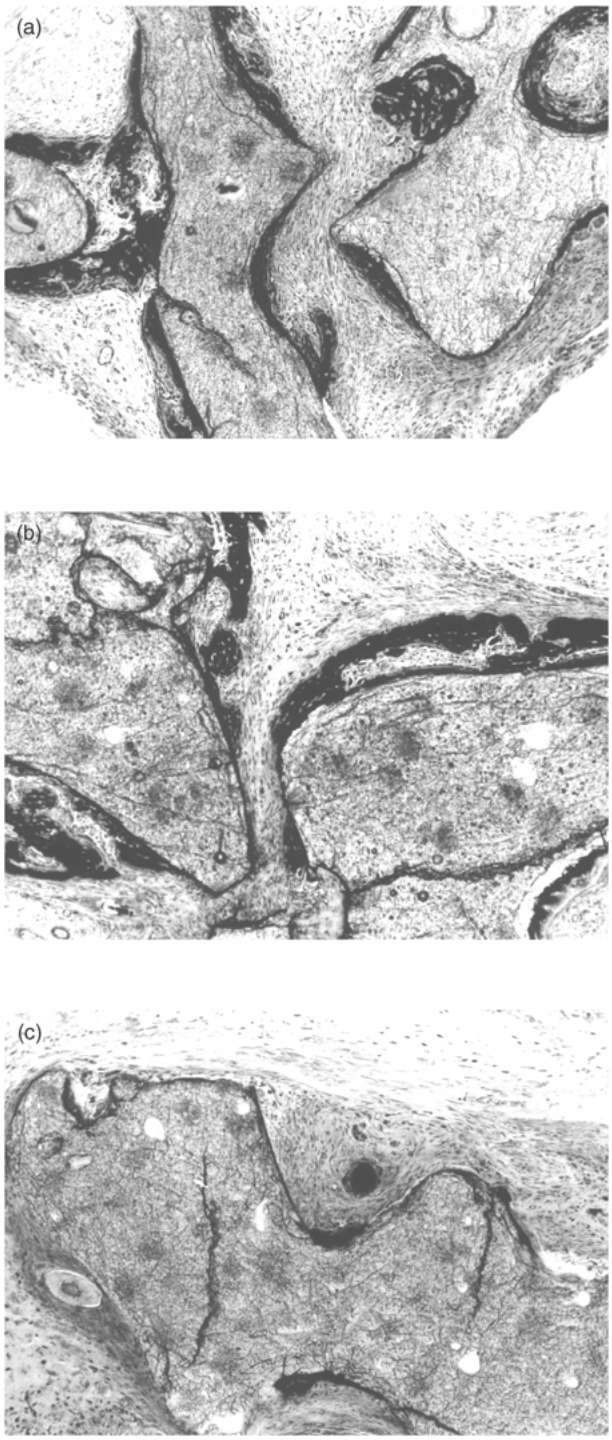

Figure 5 Light micrographs illustrating *de novo* formed bone after 7 days of implantation. (a) Rat bone marrow cells grown for 5 days on porous HA particles (group I, 100 ×); (b) rat bone marrow cells seeded for 16 h on porous HA particles (group II, 100 ×); (c) rat bone marrow cells seeded for 16 h on porous HA particles (group III, 100 ×).

seeded osteogenic cells. For this purpose four experimental groups were developed (Table I) and studied. On HA scaffolds, in which cells were seeded and cultured for 5 days, light and scanning electron microscopy results revealed the presence of multilayers of cells embedded within extracellular matrix where collagen fibers were abundantly detected. Although, in this study, the identification of collagen was only based on microscopic observations, our group has previously reported the identification of collagen I on this type of constructs using immunological assays [26], demonstrating the presence of a bone-like tissue on such samples. On the contrary, scaffolds seeded with cells for 16 h were extracellular matrix free, consisting of cell/HA constructs. To determine whether porous HA scaffolds containing *in vitro* formed bone-like matrix would induce faster bone formation as compared to HA scaffolds with seeded osteogenic cells, the samples were subcutaneouslly implanted in syngeneic rats for periods of 2, 4, 7, 9 and 12 days. Our data indicated that the bone-like matrix containing scaffolds (group I), during the earlier implantation periods (day 2 and day 4), clearly induced faster bone formation as compared to the high cell density seeded scaffolds (group II). Such differences between the two groups may have resulted from several factors. It is likely that the cultured cells were in a further stage in the process of osteogenic differentiation, since they had been in the presence of the differentiation factor dexamethasone for an additional period of 5 days. In addition, and as suggested by Yoshikawa *et al*. [29], the immediate *in vivo* bone inducing ability of these constructs can be related to bone proteins and growth factors that are present in the formed extracellular matrix and contribute to enhanced osteogeneicity of the implants. In fact, previous research in our group [26] revealed that similar constructs, obtained from human bone marrow cells, expressed mRNA for alkaline phosphatase, osteopontin, osteocalcin and bone morphogenetic proteins 2 and 4.

For the implantation periods of 7, 9 and 12 days, the average degree of osteogenesis found in group I was slightly higher than in group II. This difference, however, was not statistically significant. Nevertheless, it should be noted that bone turn-over is very fast in rats. In a larger animal, the two types of implants would take longer than 7 days to achieve the same degree of bone formation. Therefore, it is likely that on a clinical relevant situation, such as a bone defect in a large animal, implant stability will be achieved earlier if bone-like tissue is present on the grafts at the time of implantation. These two tissue engineering approaches are currently being tested in a large animal model.

To compare the *in vivo* osteogenic potential of scaffolds in which cells were cultured for 5 days followed by implantation to scaffolds in which cells were seeded and implanted for an additional period of 5 days (so, identical total test periods), the extent of bone formation on samples from group I at days 2, 4 and 7 of implantation was compared to the extent of bone formation on samples from group III at days 7, 9 and 12. Results demonstrated that scaffolds from group I presented a significantly higher degree of bone tissue at day 4 and 7, as compared to scaffolds from group III at day 9 and 12, respectively. This data indicates that cell seeding and culture for 5 days prior to implantation is more efficient as cell compared to cell seeding followed by an extra implantation period of 5 days. As previously mentioned, these findings maybe related to the longer exposure of the cultured cells to dexamethasone, resulting in different degrees of cell differentiation in both experimental groups.

Conclusions

The results presented herein demonstrate that scaffolds which contain an *in vitro* formed matrix induce significantly faster bone formation as compared to scaffolds in which cells are only seeded. This suggests that a tissue engineered bone implant is more efficient when cells have already started to form a bone-like tissue *in vitro*. Moreover, the results indicate that longer implantation periods for the cell seeded implants do not achieve the degree of bone formation found in implants containing an *in vitro* cultured bone-like matrix.

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References

1. C. DAMIEN and R. PARSONS, *J. Appl. Biomater.* **2** (1991) 187.
2. H. H. DE BOER, *Clin. Orthop.* (1988) 226.
3. J. E. AUBIN and F. LIU, in ''Principles of Bone Biology'', edited by J. Bilizekian and G. Rodan (Academic Press, San Diego, USA, 1996) p. 39.
4. J. T. TRIFFITT and R. O. C. OREFFO, in ''Molecular and Cellular Biology of Bone: Advances in Organ Biology Series, London, 1998'', edited by M. Zaidi (JAI, London, 1998) p. 429.
5. A. SCUTT, H. MAYER and E. WINGENDER, *BioFactors* **4** (1992) 1.
6. M. J. YASZEMSKI, R. G. PAYNE, W. C. HAYES, R. LANGER and A. G. MIKOS, *Biomaterials* **17** (1996) 175.
7. M. LIND, *Acta Orthop. Scand.* **67** (1996) 407.
8. H. OHGUSHI and M. OKUMURA, *ibid.* **61** (1990) 431.
9. H. OHGUSHI, M. OKUMURA, S. TAMAI, E. SHORS and A. I. CAPLAN, *J. Biomed. Mater. Res.* **24** (1990) 1563.
10. S. E. HAYNESWORTH, J. GOSHIMA, V. M. GOLDBERG and A. I. CAPLAN, *Bone* **13** (1992) 81.
11. D. P. LENNON, S. E. HAYNESWORTH, S. P. BRUDER, N. JAISWAL and A. I. CAPLAN, *In Vitro Cellular Dev. Biol. Animal* **32** (1996) 602.
12. S. A. KUZNETSOV, P. H. KREBSBACH, K. SATOMURA, J. KERR, M. RIMINUCCI, D. BENAYAHU and P. G. ROBEY, *J. Bone Miner. Res.* **12** (1997) 1335.
13. P. H. KREBSBACH, S. A. KUZNETSOV, K. SATOMURA, R. V. B. EMMONS, D. W. ROWE and P. G. ROBEY, *Transplantation* **63** (1997) 1059.
14. J. E. DENNIS, E. K. KONSTANTAKOS, D. ARM and A. I. CAPLAN, *Biomaterials* **19** (1998) 1323.
15. S. P. BRUDER, A. A. KURTH, M. SHEA, W. C. HAYES, N. JAISWAL and S. KADIYALA, *J. Orthop. Res.* **16** (1998) 155.
16. Y. M. LEE, Y. SEOL, Y. T. LIM, S. KIM, S. B. HAN, I. C. RHYU, S. H. BAEK, S. J. HEO, J. Y. CHOI, P. R. KLOKKEVOLD and C. P. CHUNG, *J. Biomed. Mater. Res.* **54** (2001) 216.
17. P. BIANCO, M. RIMINUCCI, S. GRONTHOS and P. G. ROBEY, *Stem Cells* **19** (2001) 180.
18. J. E. DAVIES, B. CHERNECKY, B. LOWENBERG and A. SHIGA, *Cells Mater.* **1** (1991) 3.
19. J. D. DE BRUIJN, J. E. DAVIES, J. S. FLACH, K. DE GROOT and C. A. VAN BLITTERSWIJK, in ''Tissue-Inducing Biomaterials'', vol. 252, edited by L. Cima *et al.* (Res. Soc. Sp. Proc., Boston, USA, 1992) p. 63.
20. S. L. I. RILEY, G. M. C. KRUGER, M. J. YASZEMSKI and A. MIKOS, *Biomaterials* **19** (1998) 1405.
21. S. L. ISHAUG, M. CRANE, M. J. MILLER, A. W. YASKO, M. J. YASZEMSKI and A. G. MIKOS, *J. Biomed. Mater. Res.* **36** (1997) 17.
22. T. YOSHIKAWA , H. OHGUSHI, Y. DOHI and J. E. DAVIES, *Bio-Med. Mater. Eng.* **7** (1997) 49.
23. S. L. I. RILEY, M. G. CRANE, A. GURLEK, M. J. MILLER, A. W. YASKO, M. J. YASZEMSKI and A. G. MIKOS, *J. Biomed. Mater. Res.* **36** (1997) 1.
24. S. C. MENDES, I. VAN DEN BRINK I, J. D. DE BRUIJN and C. A. VAN BLITTERSWIJK, *J. Mater. Sci. Mater. Med.* **9** (1998) 855.
25. J. D. DE BRUIJN, I. VAN DEN BRINK, S. MENDES, R. DEKKER, Y. P. BOVELL and C. A. VAN BLITTERSWIJK, *Adv. Dental Res.* **13** (1999) 74.
26. S. C. MENDES, J. D. DE BRUIJN, K. BAKKER, A. A. APELDOORN, P. P. PLATENBURG, G. J. M. TIBBE and C. A. VAN BLITTERSWIJK, in ''Bone Engineering'', edited by J. E. Davies (em square incorporated, Canada, Toronto, 2000) p. 505.
27. S. C. MENDES, J. D. DE BRUIJN, J. M. TIBBE, M. VEENHOF, K. BAKKER, S. BOTH, P. P. PLATENBURG and C. A. VAN BLITTERSWIJK, *Tissue Eng.*, Accepted 2002.
28. T. YOSHIKAWA, H. OHGUSHI, T. UEMURA, H. NAKAJIMA, K. ICHIJIMA, S. TAMAI and T. TATEISI, *Bio-Med. Mater. Eng.* **8** (1998) 311.
29. T. YOSHIKAWA, H. OHGUSHI, T. UEMURA, H. NAKAJIMA, E. YAMADA, K. ICHIJIMA, S. TAMAI and T. OHTA, *Transplantation* **69** (2000) 128.
30. C. MANIATOPOULOS , J. SODEK and A. H. MELCHER, *Cell Tissue Res.* **254** (1988) 317.

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