Optimization of Bone-Tissue Engineering in Goats

Moyo C. Kruyt,[1] Wouter J. A. Dhert,[1] Cumhur Oner,[1] Clemens A. van Blitterswijk,[2,3] Abraham J. Verbout,[1] Joost D. de Bruijn[2]

[1] Department of Orthopedics, University Medical Center Utrecht, The Netherlands

[2] IsoTis NV Bilthoven, The Netherlands

[3] Twente University, The Netherlands

Received 9 December 2002; revised 4 April 2003; accepted 23 April 2003
Published online 27 February 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.b.10073

Abstract: Successful bone-tissue engineering (TE) has been reported for various strategies to combine cells with a porous scaffold. In particular, the period after seeding until implantation of the constructs may vary between hours and several weeks. Differences between these strategies can be reduced to (a) the presence of extracellular matrix, (b) the differentiation status of the cells, and (c) the presence of residual potentially immunogenic serum proteins. These parameters are investigated in two types of calcium phosphate scaffolds in a goat model of ectopic bone formation. Culture-expanded bone-marrow stromal cells from eight goats were seeded onto two types of hydroxyapatite granules: HA60/400 (60% porosity, 400-μm average pore size) and HA70/800. Scaffolds seeded with cells and control scaffolds were cultured for 6 days in medium containing autologous or semisynthetic serum, in the presence or absence of dexamethasone. Other scaffolds were seeded with cells just before implantation in medium with or without serum. All conditions were implanted autologously in the paraspinal muscles. After 12 weeks, bone had formed in 87% of all TE constructs, as demonstrated by histology. Histomorphometry indicated significantly more bone in the HA70/800 scaffolds. Furthermore, a significant advantage in bone formation was found when the constructs had been cultured for 6 days. In conclusion, both scaffold characteristics (porosity) and TE strategy (culturing of the constructs) were demonstrated to be important for bone TE. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 69B: 113–120, 2004

Keywords: tissue engineering, serum, extracellular matrix, bone, ectopic, goats, cells

INTRODUCTION

Tissue engineering of autologous bone is a promising alternative for the surgically derived autologous bone graft. Since the early nineties, many investigators have demonstrated the concept of combining osteoprogenitor cells with an appropriate scaffold to be osteogenic ectopically in rodents and larger mammals.[1–4] Recently, ectopic tissue-engineered bone formation in a goat model has been demonstrated.[5,6] Although less informative with respect to clinical application, the advantage of an ectopic model is that it allows fundamental research without the disturbing influence of host bone as present orthotopically. Furthermore, many experimental conditions can be evaluated in one animal for comparative studies.

The scaffolds most frequently selected for bone TE are porous structures of hydroxyapatite (HA), often as a composite with β-tricalcium phosphate (TCP).[2,7] The osteoconductivity and biocompatibility of these materials are advantageous for future orthotopic application. The cells mostly used are bone marrow stromal cells (BMSCs).[2,8,9] These BMSCs can be administered by soaking the scaffold in fresh bone marrow,[10,11] or after culture expansion,[8,12,13] which was shown to be superior.[1,4,6,14,15]

Although many scaffold characteristics and the selection and expansion of BMSCs is quite similar between various studies, the strategy for building the final construct is remarkably different. For instance, some investigators seed undifferentiated "stem" cells under serum-free conditions on the scaffold, followed by implantation of the constructs within several hours after seeding.[4,15,16] Others culture the cells in the constructs 1 or more weeks prior to implantation, to allow extracellular-matrix formation and to stimulate osteogenic differentiation by addition of specific differentiation factors such as dexamethasone.[3,5,14,17,18] Differences between these strategies may be reduced to the following parameters: (a) the

Correspondence to: Moyo C. Kruyt, Department of Orthopedics, G05.228, University Medical Center, Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands (e-mail: m.c.kruijt@chir.azu.nl)

Contract grant sponsor: The Netherlands Technology Foundation (STW), contract grant number UGN.4966)

© 2004 Wiley Periodicals, Inc.

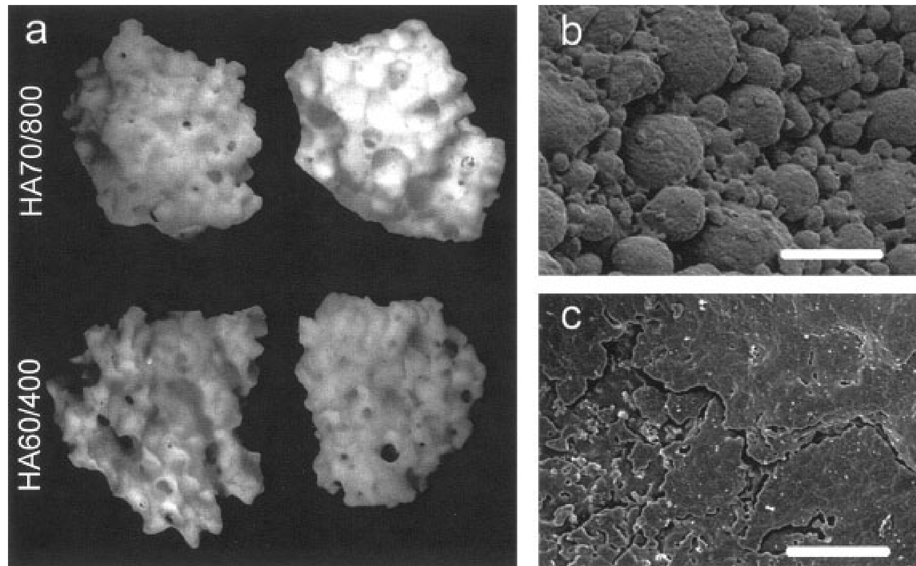

Figure 1. Scaffolds. (a) Two granules (Ø = 3–4 mm) of both scaffold types that were combined in the treatment units. Macroscopically little difference can be seen. (b) Scanning electron microscope image of the HA70/800 surface. The material is composed of small HA beads resulting in high microporosity between the individual beads (bar = 20 μm). (c) SEM image of the HA60/400 surface. The material is smoother, resulting in less microporosity (bar = 20 μm).

presence of extracellular matrix in the scaffolds at time of implantation, (b) the differentiation status of the cells at time of implantation, and (c) the presence of potentially immunogenic serum proteins after seeding and culturing the constructs in the presence of commercial sera.

In order to optimize the present bone tissue-engineering approach, these parameters were investigated in two different calcium–phosphate scaffolds in a goat model of ectopic bone formation.

MATERIALS AND METHODS

Experimental Design

The local animal care committee gave approval for the experiment, which utilized a total of eight adult goats. Forty-eight (8 × 6) units consisting of granules of each scaffold type (HA70/800 and HA60/400), were prepared and treated as single samples for all further treatment-group-related procedures (Table I). One week before implantation, four treatment groups were created by seeding cultured BMSCs on the units under conditions mentioned in the paragraph on culturing and seeding conditions. These constructs were cultured for another 6 days in specific media. The night before implantation, another two treatment groups were created. Because these constructs were not subsequently cultured, a higher cell load was seeded to compensate for the cell increase on the cultured constructs. The units were implanted randomly in the paraspinal muscles of the goat from which the BMSCs were derived from (autologous implantation). This resulted in a sample size of eight for each treatment group. To monitor bone formation in time, fluorochrome labels were administered after 5, 7, and 9 weeks and the animals were killed after 12 weeks. Bone formation in the scaffolds was investigated by histology and histomorphometry of non-decalcified sections.

Scaffolds

Two different scaffolds were used in the current experiment:

1. HA70/800, a 70% porous hydroxyapatite with an average pore size of 800 μm (CAM Implants, The Netherlands). This scaffold was produced with spray-dried HA powder, and the interconnected pores were created with the use of an H_2O_2 foaming method. According to the manufacturer the ceramic was sintered at a temperature above 1100 °C. This resulted in a marked microporosity, as shown in Figure 1.
2. HA60/400, a 60% porous hydroxyapatite with an average pore size of 400μm (IsoTis NV, The Netherlands). This scaffold was produced with commercial HA powder (Merck, The Netherlands) with the use of a wax incorporation method to create an interconnected porous structure.[19] The sintering temperature was 1200 °C. Microporosity was less prominent in this scaffold (Figure 1).

The chemical composition of the materials was analyzed by X-ray diffraction (XRD) and Fourier-transform infrared (FTIR) spectroscopy and indicated HA without additional phases or impurities. Granules of 30–50 mm^3 of both scaffold types were selected by sieving, cleaned in an ultrasonic bath, and then autoclaved. Two granules of each scaffold type (total four granules) were combined in a 10-ml tube and

TABLE I. Treatment Groups: Cells were Loaded on Units of Four Granules (2*HA60/400 + 2*HA70/800). The Constructs Were Cultured 6 Days before Implantation in Media with Ultroser G (US) or Autologous Serum (AS) with or without DEX and BGP Administration. Other Constructs Were Seeded Short before Implantation with Ultroser Medium or Serum Free (USS, SF). Controls Were Maintained in the US+ Condition without Cells.

Condition	Seeding Load (cells/cm^3 Scaffold)	Cultured	DEX and BGP	Serum Type
1. US−	1E7	Yes	No	Ultroser G
2. US+	1E7	Yes	Yes	Ultroser G
3. AS+	1E7	Yes	Yes	Autologous
4. Control	0	Yes (no cells)	Yes	Ultroser G
5. USS−	5.5E7	No	No	Ultroser G
6. SF−	5.5E7	No	No	No

processed as one unit for all further treatments. Eight units were impregnated with 100 μg/ml fibronectin (Micronic, the Netherlands) to provide the units for the serum-free seeding condition.[7]

Culturing and Seeding Conditions (Table I)

BMSCs were derived from both iliac wings and culture expanded as described in detail previously.[17] In brief, 30 ml aspirates were plated in culture flasks (5.10^5 nucleated cells per cm^2) and cultured in a standard culture medium containing 15 vol% fetal bovine serum (FBS, Gibco, Scotland, Lot No. 3030960S). When colonies of adherent cells had formed, these were replated at 5000 cells/cm^2. At the third passage, 1 week before surgery, the cells were partially replated for culturing another week, or seeded on scaffold units. For seeding dynamically (on a roller bank) the cells were resuspended in different culture media at 5E5 cells/ml and 3.2 ml of this cell suspension was added to a tube containing one unit (= 1E7 cells per cm^3 scaffold). This procedure constituted the first four conditions:

1. US −: The BMSCs were resuspended in medium containing 2 vol% Ultroser G (US Invitrogen, The Netherlands) as a replacement of the 15 vol% FBS in the standard culture medium. US was expected to be less immunogenic, as it is mainly synthetic. The exact constituents would not be given by the manufacturer, but they did not exclude the presence of human- and animal-derived components.
2. US +: as (1), with the addition of 10-nM dexamethasone and 10-mM β-glycerophosphate (DEX and BGP, Sigma, the Netherlands) to stimulate osteogenic differentiation.[20] This was the standard condition published before,[6] from which the other groups were deduced.
3. AS +: the medium contained 15 vol% autologous serum (AS) instead of FBS plus DEX and BGP. AS was obtained from 200 ml venous blood that was allowed to clot in a sterile glass jar overnight. Before application, the serum was heat inactivated for 30 min at 56–58 °C and filtered through a 0.2-μm filter.
4. Control: Units were maintained in US + medium without cells seeded.

After seeding the units were taken to bacteriological 25-well plates and the medium was refreshed with the same medium type as used for seeding. Then the units were cultured statically for another 6 days before implantation. In a previous study, scanning electron microscopy (SEM) showed the 6-day culture period resulted in cell expansion and abundant ECM formation covering all of the scaffold surface.[6]

The cells that were not seeded were replated and maintained in the US- culture medium for another 6 days. Then the cells were resuspended at 5E5 cells/ml, and 17.6 ml of this cell suspension was added to the units (55E6 cells per cm^3 scaffold) 14–16 hours before implantation according to two protocols:

5. USS −: Cells were resuspended and seeded in US − medium.
6. SF −: Cells were resuspended in serum-free medium and seeded on fibronectin-coated scaffolds.

Determination of Cell Seeding Load for Noncultured Constructs

To standardize the cell number for all groups at implantation, we studied the required seeding load to normalize the cell number on the noncultured constructs (USS − and SF −) in a separate study, prior to the *in-vivo* constructs. The first cell numbers on 6-day cultured US − constructs (HA60/400, $n = 6$) were quantified by a tetrazolium salt assay, which measures mitochondrial metabolic activity (MTT, sigma).[21] The constructs were incubated in 200-μl MTT solution for 2 h and then lysed with 200 μl DMSO. The light absorbance of the formazan product in the lysate was read at 570 nm. Subsequently, constructs seeded with similar, 4-, 5-, and 7-times higher cell loads than the 6-day cultured constructs were analyzed ($n = 6$) 14 h after seeding in US − medium or serum-free medium. The seeding load for an intensity equal to the cultured US − constructs was calculated from the function of the curve as shown in Figure 2(a).

Cell Quantification

To determine the seeding efficiency and subsequent cell increase for the different scaffold materials and different

medium conditions, including the FBS medium, another cell quantification method that was described in detail before was applied in a separate *in vivo* study (CyQUANT® kit Molecular Probes, Eugene, OR).[6,22] It was thought that absolute numbers (for seeding efficiency) could be calculated more accurately with this method as compared to the MTT assay. The constructs ($n = 5$) were digested in a collagenase solution overnight on a shaking platform to retrieve all DNA. The cell number was calculated from a standard curve of identical cells.

Alkaline Phosphatase Measurement

The alkaline phosphatase activity (ALP) in relation to the cell number (ALP/DNA) was measured to analyze cell response to DEX and BGP and to identify differences between the media. The standard culture medium with FBS, the US medium, the AS medium, and medium containing 15% allogeneic goat serum were studied. Cells of one batch were plated in 6-well plates (5E4 cells per well) and cultured for 6 days to allow formation of confluent sheets.[20] The cell sheet was resuspended in 1 ml 0.05% Triton solution and homogenized by sonification. 500 μl was used for the CyQUANT assay. Another 100 μl was transferred to 100 μl p-nitrohenyl phosphate (PNP, 104[R] Sigma) in alkaline phosphatase buffer and incubated for 15 min to determine the rate of conversion into p-nitrophenol. The reaction was stopped with 500 μl 0.1-N NaOH. Absorbance was read at 405 nm.

Animals and Implantation

Adult Dutch milk goats (19–26 months) were obtained at least 4 weeks prior to surgery. The surgical procedures were performed under general inhalation anesthesia, preceded by i.v. detomidine sedation (Pfizer, the Netherlands) and maintained by a halothane gas mixture (Sanofi, The Netherlands). After the lumbar area was disinfected and shaved, six separate skin incisions were made. By using blunt dissection, intramuscular pockets were created and filled with one unit according to a randomized scheme. Subsequently, the fascia was closed with a nonresorbable suture and the skin was closed in two layers. Postoperative pain relief was given by buprenorphine (Shering-Plough, The Netherlands). In addition to the intramuscular implantations, the goats were subjected to a femur-defect operation that will not be discussed in this article.

Fluorochromes (all Sigma) were administered intravenously. At 5 weeks, Alizarin red (30 mg/kg), at 7 weeks Calceine green (10 mg/kg), and at 9 weeks Xylenol orange (100 mg/kg).[23] After 12 weeks, the animals were killed by an overdose of pentobarbital (Organon, The Netherlands). As a control for osteogenicity, per goat, two HA70/800 granules of each condition and two granules of HA60/400 of the US + condition were implanted subcutaneously for 4 weeks in nude mice (two mice per goat).[17] These implants were scored as bone or no bone in the 16 (8×2) granules that were implanted of each condition.

Postmortem Sample Acquisition, Histology and Histomorphometry

After explantation, the units were fixated in 1.5% glutaraldehyde, dehydrated by graded ethanol series and embedded in polymethylmethacrylate. Semithin sections (10 μm) were made with a Leica sawing microtome, stained with methylene blue and basic fuchsin for routine histology,[24] and histomorphometry, or left unstained for epifluorescence microscopy. Per unit 4–10 sections were made to provide sections through the center of each individual granule. The sections were evaluated for general tissue response, bone formation, and the fluorochrome labels with a fluorescence/light microscope (E600 Nikon, Japan) with a double filter block (dichroic mirror 505 and 590 nm). The midsection through the single granules was chosen for histomorphometry. The percentage of bone occupying available space within the granules was measured with the use of a VIDAS image analysis system (KS400, Zeiss, Munich, Germany), coupled to a light microscope. First, the area of interest was defined by outlining the specific granule. Pores that interrupted the exterior scaffold contour were crossed with straight lines. Then, after blinding the samples, the pore area and the bone area were identified and measured. Bone formation was expressed as percent bone in available space (bone area/pore area $\times$ 100%).

Statistics

To analyze quantitative DNA and ALP/DNA data, ANOVAs with Bonferroni corrected post hoc tests were performed. Histomorphometric results were analyzed by Friedman paired-rank test and post hoc Wilcoxon signed-rank tests. All tests were performed with SPSS10 for Macintosh (significance level: $p < 0.05$).

RESULTS

In Vitro Results (Figure 2)

To normalize the cell numbers in the constructs that had not been cultured after seeding to cultured constructs, a 5.5-times higher seeding load was calculated for the USS − condition. The SF − condition could not be normalized as can be seen in Figure 2(a). The average seeding efficiency according the CyQUANT® assay was about 60%, without significant difference between the sera or scaffold materials. After the 6-day culture period, the total DNA reflecting cell numbers had doubled once on average, with about 35% less DNA in AS +condition as compared to the US + condition ($p = 0.018$). This indicated a relatively low proliferation of the cells that, when cultured in flasks, have a doubling time of only one day.[6] Other comparisons did not show significant differences [Figure 2(b)]. The ALP/DNA analysis as a reflection of cell differentiation demonstrated a comparable alkaline phosphatase activity for all study conditions. The effect of dexamethasone and BGP admission was ambiguous and not significant as can be seen in Figure 2(c).

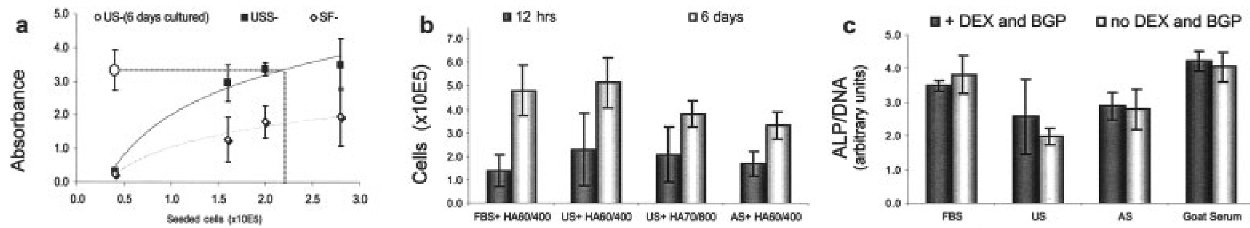

Figure 2. *In vitro* measurements (error bars indicate the SD). (a) Relative cell quantities (by MTT assay) in HA60/40 constructs 14 h after seeding with increasing cell loads compared to constructs seeded with 4.10E5 cells and cultured for another 6 days. The open circle represents the cultured construct. Higher cell loads seeded in Ultroser medium (USS −) or in serum-free medium (SF −) resulted in higher cell numbers in the noncultured constructs. A seeding cell load of 2E6 cells per granule was calculated from the USS − curve to normalize the cell number in the noncultured constructs to the cultured construct. SF − could not be normalized. (b) Estimated cell numbers in different combinations of scaffold and culture conditions by DNA quantification. The 6-day culture resulted in an average doubling of the DNA quantity. Culture of HA60/400 constructs in autologous serum (AS +) resulted in 35% less DNA as compared to identical constructs cultured in Ultroser medium (US +) ($p < 0.02$). (c) Relative alkaline phosphatase activity of goat cells cultured for 6 days in media containing FBS, Ultroser (US), autologous serum (AS), or allogenic goat serum. The response to DEX and BGP admission was ambiguous. No conditions were significantly different when all conditions were compared.

In Vivo Results

Although there were no surgical complications, two goats developed a painful hoof disease for which they were terminated after 10 weeks instead of 12 weeks. Autopsy and cultures of blood and tissue did not indicate a cause related to the procedure. The data of these two goats were applied for qualitative analysis and paired comparisons within the animal. Histology showed that all granules were embedded in well-vascularized soft tissue without signs of inflammation. Bone formation was found in all but the control conditions in amounts varying in and between the goats (Figure 3). The bone was typically present on the HA surface of the smaller pores with an obvious preference for the HA70/800. In 35 of all 40 tissue engineered units (87%), bone had formed, and in 33 cases the HA70/800 implants yielded more bone than the HA60/400 implants ($p < 0.01$). Figure 4 shows the result of histomorphometry on the 12-week implants. Although the standard deviation (between goats) was high, paired comparisons (within goats) revealed that the condition/material combination significantly influenced the percentage of bone area ($p < 0.01$), also when considering only HA70/800 ($p = 0.01$). The three conditions that were cultured for 6 days (US −, US +, AS +) had comparable bone percentages (9–13%) that were higher than the two conditions that were implanted directly after seeding (3–5%). Post hoc analysis of the US − condition (no DEX and BGP admission) versus the USS − condition demonstrated a significant difference ($p = 0.02$) in bone formation. Fluorochrome analysis showed the fluorochrome labels as distinct lines within the newly formed bone. The occasional presence of the 5-week label and the line pattern were comparable for all conditions. The fluorochrome line order indicated bone formation had started on the HA surface and was directed toward the pore center. The line shape often indicated bone was budding from the surface toward the opposite surface to bridge the pore (Figure 3). Because the distances between the different labels was very variable, no attempt was made to calculate the bone-formation rate.

In the control granules implanted in mice, bone was also present in all cell-containing groups ranging from 62.5% (10/16) in the US − constructs to 100% (16/16) in the SF −constructs. Remarkably, HA60/400 and HA70/800 (both US +) showed similar bone formation, both 90%.

DISCUSSION AND CONCLUSIONS

In the present study, the effect of five different TE strategies was investigated ectopically in a goat model. Bone was found in all cell-based TE conditions, with an obvious preference for the HA70/800 scaffolds and for constructs that were cultured before implantation. Neither the serum type nor dexamethasone admission influenced bone formation. An explanation for these findings could be that only the scaffold type and the presence of extracellular matrix is relevant for bone formation without any role for the cells *in vivo*. This hypothesis can be supported by literature that reports a potential bone inductive capacity of porous ceramics and extracellular matrix.[25–28] However, the scaffolds without cells failed to show bone induction, and a previous study showed that identically cultured TE constructs that contained extracellular matrix and were devitalized before implantation also failed to show any bone.[5,6] Therefore, the hypothesis of an osteoinductive mechanism without a contribution of the cells is not plausible. Different parameters for this cell-dependent bone TE were investigated: The presence of extracellular matrix; the differentiation status of the cells; and the presence of potential immunogenic serum proteins.

The effect of extracellular matrix was investigated by comparing constructs cultured for 6 days to constructs that

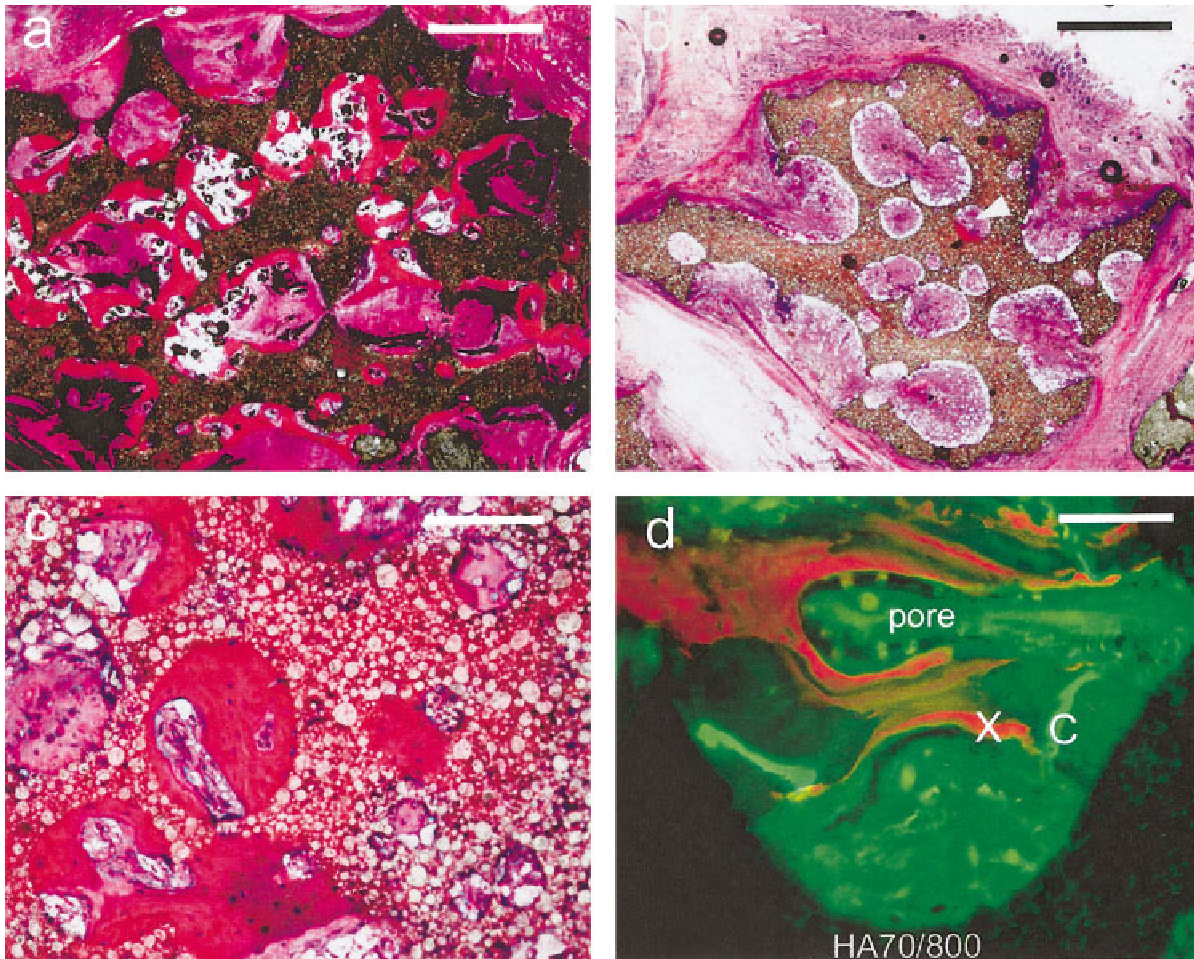

Figure 3. Bone formation shown with undecalcified histology (bone is red) and fluorescence microscopy after 12 weeks implantation in goats. (a) HA70/800 scaffold of AS + condition. Bone is present in all pores in close contact to the scaffold surface (bar = 500 μm). (b) HA70/800 granule of USS − condition. Only little bone had formed (triangle) (bar = 800 μm). (c) HA70/800 of SF − condition, detail of bone. Note scaffold composition of small beads (bar = 100 μm). (d) HA70/800 of US − condition. Fluorescent microscopy shows the budding of bone from the scaffold surface to the pore center where two buds united. C = Calceine label (7 weeks); X = Xylenol orange label (9 weeks) (bar = 50 μm).

were not cultured after seeding that do not contain ECM.[6] In agreement with earlier observations in rats,[14,18] significantly more bone was found in the cultured constructs, indicating an advantage for this situation. A remark that should be made is that the high cell load that was seeded on the noncultured constructs resulted in a cell distribution that was less homogeneous (more clotting) as on the cultured constructs.

To analyze the effect of cell differentiation, there was an attempt to stimulate differentiation into the osteogenic lineage by adding DEX and BGP to the culture medium.[20] The *in vitro* results of the ALP/DNA assay, however, showed a very moderate alkaline phosphatase activity in goat cells, with an ambiguous response to DEX and BGP admission. This is contrary to human or rodent cells frequently analyzed by the same method in which ALP/DNA is 10–20 times higher. This phenomenon was also observed by others with sheep BMSCs.[29] The absence of an ALP/DNA increase *in vitro* might explain why there was no significant effect *in vivo*.

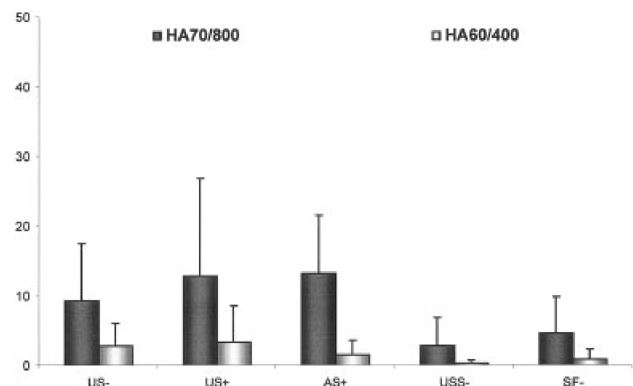

Figure 4. Area % bone in all treatment groups after implantation for 12 weeks in goats (error bars indicate SD). HA70/800 yielded more bone than the HA60/400 ($P < 0.01$) Post-hoc paired comparisons indicated US − (cultured) had more bone than USS − (noncultured) ($p = 0.02$).

The presence of serum proteins in US and especially in FBS that could elicit an immune reaction was a major concern. Therefore, the FBS-cultured constructs were not implanted. However, a sensitization test (on guinea pigs) of Ultroser G that was performed in a separate experiment, indicated an immune response against US that was even stronger than the response to FBS. Despite this finding, bone formation in all cultured groups (US −, US + AS +) appeared similar. and no advantage of serum-free seeding was found in the noncultured groups (USS − and SF −). This implies the absence of a profound inhibitory immune reaction and also shows the feasibility of using autologous serum.

The type of scaffold appeared to affect the outcome more than any of the other parameters. This has been discussed earlier,[6] where it was emphasized that a prominent feature of the HA70/800 was the higher microporosity. The larger surface area might result in increased dissolution and subsequent precipitation with the possible integration of inducing factors in the scaffold surface.[14,30] This phenomenon might be relevant only after prolonged implantation periods, which would explain the absence of a material-related difference in the frequently applied nude mice model[31] (implanted for 4 weeks).

The MTT assay that we used for standardization of the cell numbers was not ideal because of conflicting data. The measured cell increase during the 6-days culture time was tenfold, whereas this quantity could be achieved with a 5.5 times higher seeding load (Figure 2). Others reported a selective adherence of the MTT to plastics,[32] which could have caused a relative underestimation of low cell numbers. The cell loads applied for normalization were more likely too high than too low as, according to the DNA quantification method, the 6-day cell increase was only twofold. Another reason to assume that the cell numbers on the noncultured constructs were sufficient is that the present seeding load is 55E6 cells/cm^3, whereas in the literature only 10E6 cells per cm^3 scaffold are seeded.[15,29,33]

Finally, it should be realized that the area% bone found in goats (on average 12% in the cultured constructs) is low when compared to rodents, where 25% was reported.[34,35] This could be the result of a delayed vascularization related to an increased implant volume and lower rate of metabolism.[36,37] Further research on this topic will be necessary to refine the technique for future clinical applications.

The authors thank Michiel van Tilborg, Mirella van den Doel, and Patrick Engelberts for assistance with histomorphometry, surgery, and *in vitro* analysis.

REFERENCES

1. Goshima J, Goldberg VM, Caplan AI. The osteogenic potential of culture-expanded rat marrow mesenchymal cells assayed *in vivo* in calcium phosphate ceramic blocks. Clin Orthop 1991; 2:298–311.
2. Krebsbach PH, Kuznetsov SA, Satomura K, Emmons RV, Rowe DW, Robey PG. Bone formation *in vivo*: Comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. Transplantation 1997;63:1059–1069.
3. Yoshikawa T, Ohgushi H, Nakajima H, Yamada E, Ichijima K, Tamai S, Ohta T. *In vivo* osteogenic durability of cultured bone in porous ceramics: A novel method for autogenous bone graft substitution. Transplantation 2000;69:128–134.
4. Anselme K, Noel B, Flautre B, Blary MC, Delecourt C, Descamps M, Hardouin P. Association of porous hydroxyapatite and bone marrow cells for bone regeneration. Bone 1999;25: 51S–54S.
5. Kruyt MC, De Bruijn JD, Oner FC, van Blitterswijk CA, Dhert WJ, Verbout AJ. Optimization of bone tissue engineering in goats. Trans. 49th ORS, New Orleans, LA. 2003.
6. Kruyt MC, de Bruijn JD, Wilson CE, Oner FC, van Blitterswijk CA, Verbout AJ, Dhert WJ. Viable osteogenic cells are obligatory for tissue-engineered ectopic bone formation in the goats. Tissue Eng 2003;9:327–336.
7. Dennis JE, Haynesworth SE, Young RG, Caplan AI. Osteogenesis in marrow-derived mesenchymal cell porous ceramic composites transplanted subcutaneously: Effect of fibronectin and laminin on cell retention and rate of osteogenic expression. Cell Transplant 1992;1:23–32.
8. Friedenstein AJ, Ivanov-Smolenski AA, Chajlakjan RK, Gorskaya UF, Kuralesova AI, Latzinik NW, Gerasimow UW. Origin of bone marrow stromal mechanocytes in radiochimeras and heterotopic transplants. Exp Hematol 1978;6:440–444.
9. Caplan AI. Mesenchymal stem cells. J Orthop Res 1991;9:641-650.
10. Ohgushi H, Okumura M. Osteogenic capacity of rat and human marrow cells in porous ceramics. Experiments in athymic (nude) mice. Acta Orthop Scand 1990;61:431–434.
11. Takigamay H, Muschler G, Matsukura Y, Boehm C, Valdevit A, Kambic H, Davros C, Powell K, Easley K. Spine fusion using allograft bone matrix enriched in bone marrow cells and connective tissue progenitors. In: Trans. 48th Ann. Meeting ORS, Dallas; 2002.
12. Friedenstein A, Kuralesova AI. Osteogenic precursor cells of bone marrow in radiation chimeras. Transplantation 1971;12: 99–108.
13. Goshima J, Goldberg VM, Caplan AI. Osteogenic potential of culture-expanded rat marrow cells as assayed *in vivo* with porous calcium phosphate ceramic. Biomaterials 1991;12:253–258.
14. Ohgushi H, Caplan AI. Stem cell technology and bioceramics: From cell to gene engineering. J Biomed Mater Res 1999;48: 913–927.
15. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue-engineered bone regeneration. Nat Biotechnol 2000;18:959–963.
16. Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S. Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. J Orthop Res 1998;16: 155–162.
17. de Bruijn JD, van den Brink I, Bovell YP, van Blitterswijk CA. Tissue engineering of goat bone: Osteogenic potential of goat bone marrow cells. Bioceramics 1998;11:497–500.
18. Mendes S, Sleijster M, Muysenberg Avd, De Bruijn JD, van Blitterswijk CA. A cultured living bone equivalent enhances bone formation when compared to a cell seeding approach. J Mater Sci (in press).
19. Li SH, De Wijn JR, Layrolle P, De Groot K. Synthesis of macroporous hydroxyapatite scaffolds for bone tissue engineering. J Biomed Mater Res 2002;61:109–120.
20. Maniatopoulos C, Sodek J, Melcher AH. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res 1988;254:317–330.
21. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric

assay: assessment of chemosensitivity testing. Cancer Res 1987; 47:936–942.
22. Jones LJ, Gray M, Yue ST, Haugland RP, Singer VL. Sensitive determination of cell number using the CyQUANT cell proliferation assay. J Immunol Methods 2001;254:85–98.
23. Rahn BA, Perren SM. Xylenol orange, a fluorochrome useful in polychrome sequential labeling of calcifying tissues. Stain Technol 1971;46:125–129.
24. vd Lubbe HBM, Klein CPAT, De Groot K. A simple method for preparing thin (10 μm) histological sections of undecalcified plastic embedded bone with implants. Stain Technol 1988;63: 171–176.
25. Ripamonti U. Osteoinduction in porous hydroxyapatite implanted in heterotopic sites of different animal models. Biomaterials 1996;17:31–35.
26. Yuan H, Yang Z, De Bruijn JD, De Groot K, Zhang X. Material-dependent bone induction by calcium phosphate ceramics: A 2.5-year study in dog. Biomaterials 2001;22:2617–2623.
27. Moehl T, Ripamonti U. Primate dentine extracellular matrix induces bone differentiation in heterotopic sites of the baboon (Papio ursinus). J Periodont Res 1992;27:92–96.
28. Naito T, Yokota M, Noguchi T. Presence of lactate dehydrogenase-containing vesicles in an intramembranous ossifying tissue: new-born mouse calvaria. Calcif Tissue Int 1993;53:65–68.
29. Kon E, Muraglia A, Corsi A, Bianco P, Marcacci M, Martin I, Boyde A, Ruspantini I, Chistolini P, Rocca M, Giardino R, Cancedda R, Quarto R. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. J Biomed Mater Res 2000;49:328–337.
30. Yuan H, Kurashina K, de Bruijn JD, Li Y, de Groot K, Zhang X. A preliminary study on osteoinduction of two kinds of calcium phosphate ceramics. Biomaterials 1999;20:1799–1806.
31. Kruyt MC, De Bruijn J, Veenhof M, Oner FC, Van Blitterswijk CA, Verbout AJ, Dhert WJ. Application and limitations of chloromethylbenzamidodialkylcarbocyanine for tracing cells used in bone tissue engineering. Tissue Eng 2003;9:105–115.
32. Nikolaychik VV, Samet MM, Lelkes PI. A new method for continual quantitation of viable cells on endothelialized polyurethanes. J Biomater Sci Polym Ed 1996;7:881–891.
33. Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am 1998;80:985–996.
34. Dennis JE, Konstantakos EK, Arm D, Caplan AI. In vivo osteogenesis assay: a rapid method for quantitative analysis. Biomaterials 1998;19:1323–1328.
35. Mendes S, Bezemer J, Claase MB, Grijpma DW, Belia G, Degli-Innocenti F, Reis RL, De Groot K, van Blitterswijk CA, De Bruijn JD. Evaluation of two biodegradable polymeric systems as substrates for bone tissue engineering. Tissue Eng 2002.
36. Deleu J, Trueta J. Vascularisation of bone grafts in the anterior chamber of the eye. J Bone Joint Surg Br 1965;47:319–329.
37. Coulson RA. Relationship between fluid flow and O_2 demand in tissues in vivo and in vitro. Perspect Biol Med 1983;27:121–126.