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Bone tissue engineering in a critical size defect compared to ectopic implantations in the goat

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Abstract

Since the application of the autologous bone graft, the need for an alternative has been recognized. Tissue engineering (TE) of bone by combining bone marrow stromal cells (BMSCs) with a porous scaffold, is considered a promising technique. In this study we investigated the potential of tissue engineered bone to heal a critical sized defect in the goat. Orthotopic bone formation was compared to ectopic bone formation in comparable constructs.

TE constructs were prepared from goat BMSCs and porous biphasic calcium phosphate ceramic scaffolds. These constructs and scaffolds without cells were implanted paired in critical sized iliac wing defects. Comparable samples were implanted intramuscularly. After 9 ($n = 7$) and 12 ($n = 8$) weeks implantation, the samples were analyzed histomorphometrically.

After 9-weeks implantation in the iliac wing defect, significantly more bone apposition was found in the TE condition. After 12 weeks, the defects were almost completely filled with bone, but no significant advantage of TE was determined anymore. This contrasted with the intramuscular samples where TE implants showed significantly more bone at both time points.

In conclusion, bone TE is feasible in critical sized defects. However, when appropriate osteoconductive/inductive materials are applied the effect of cell seeding may be temporary.

Keywords: Bone; Tissue engineering; Cells; Goat; Ectopic; Orthotopic

Introduction

Since the application of the autologous bone graft in orthopedic surgery, the need for an alternative has been recognized. Besides the well-known complications of the harvesting procedure [4,30], other important disadvantages of the autologous bone graft include the extended surgical procedure and limited availability. Therefore, many substitutes have been developed, all of which have specific disadvantages and generally do not perform as well as the autologous graft. In order to be more successful, it is conceivable that substitutes will need one or more of the features that result in the superior functioning of the autologous bone graft. Although little is known about the exact mechanisms involved in the use of autologous bone grafts [29], the osteoconductive and osteoinductive properties, in addition to a pool of viable osteoprogenitor cells, are likely to be such features [8,20]. Therefore, tissue engineering (TE) of bone by combining osteoprogenitor cells (usually bone marrow derived stromal cells, BMSCs) with an appropriate synthetic scaffold, to create a so-called hybrid construct, is a potentially interesting technique. The proof of the concept has been shown with genetically labeled cells in rodent studies both ectopically [1,13], and orthotopically [7,10,18]. Despite these successes in rodents, in larger animals only few studies have shown the feasibility of the technique orthotopically [6,24], and even less studies address the question whether clinically sized constructs are osteogenic ectopically [3,19,21]. A comparison between ectopic and orthotopic functioning in larger animals has never been reported. It is conceivable that functioning of the technique in clinically sized grafts with a delayed vascularization will be more challenging

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due to the difficulties that can be anticipated for cell survival. Therefore, insight regarding the influence of the surrounding tissue will improve the knowledge on how and when bone TE will function. In the present study, we investigated tissue-engineered grafts in an established critical sized defect model that allows paired comparisons [2]. In the same animals, comparable constructs were evaluated ectopically in a model previously shown to be successful for bone TE [19]. To investigate the growth dynamics, sequential fluorochrome labels were administered. The samples were analyzed after 9- and 12-weeks implantation with different parameters focussing on the apposition and the amount of newly formed bone.

Materials and methods

Scaffolds

Scaffolds were made of 50–60% macroporous, biphasic calcium phosphate (BCP, OsSatura™, IsoTis, The Netherlands). The ceramic consisted of $80 \pm 5\%$ hydroxyapatite (HA) and $20 \pm 5\%$ tricalcium phosphate (TCP) as confirmed by X-ray diffraction (XRD) and Fourier transformed infrared spectroscopy (FTIR), no additional phases or impurities where detected. The material was sintered at 1200 °C resulting in $15 \pm 5\%$ microporosity (pores < 10 μm). Previously, this ceramic has been shown to be osteoinductive ectopically in goats [31]. Disks of $\varnothing 17 \times 6$ mm (orthotopic implantation) and $7 \times 7 \times 7$ mm cubes (ectopic implantation) were cut, cleaned in ultrasonic baths and steam sterilized (Fig. 1(a)).

BMSCs culture and seeding conditions

Autologous serum (AS) was derived from 100 ml venous blood that was taken at the time of BM aspiration [19]. The BMSCs were derived from 30 ml iliac wing aspirates that were counted before plating in tissue culture flasks. The adherent cells were culture expanded according to previously described methods [19]. When sufficient numbers were achieved, the cells were cryopreserved in medium containing 30% fetal bovine serum (FBS, Gibco, Paisly, Scotland, lot# 3030960S) and 10% dimethylsulfoxide (DMSO, Sigma, The Netherlands). Ten days before surgery, the cells were thawed in pure FBS, centrifuged for 10 min at 100G and after washing with culture medium, replated in medium containing 30% FBS. After 3 days, cells were washed with PBS, then detached and centrifuged before resuspending at a concentration of 1×10^7 cells/ml in medium containing 15% AS. The disks were statically seeded by dropping 500 μl of cell suspension on each side of the disks ($= 8 \times 10^6$ cells per cm^3 scaffold). The cubes were similarly seeded with 275 μl of cell suspension per cube ($= 8 \times 10^6$ cells per cm^3). The constructs were incubated at 37 °C for two hours to allow cell attachment. Constructs and control scaffolds without cells were cultured for seven days in AS medium with 10 nM dexamethasone and 10 mM β-glycerophosphate (DEX and BGP, Sigma) that has been shown to stimulate osteogenic differentiation of rat and human BMSCs [22,23]. Seeding efficiency and cell proliferation during culture were determined on the $7 \times 7 \times 7$ mm cubes ($n = 6$) by a DNA quantification assay (CyQUANT® kit Molecular Probes, Eugene, US) as described before [19]. A methylene blue staining was done on both the TE cubes and disks after seeding and after the 7-day culture period to evaluate cell attachment and the distribution of cells throughout the constructs. Also this allowed the assessment of the extracellular matrix that formed after 7 days culture.

Animals and implantation

After approval of the local animal care committee, 15 adult female Dutch milk goats (24–36 months) were obtained at least 4 weeks prior to surgery. The surgical procedures were performed under standard conditions [19]. After shaving and disinfection of the dorsal thoracolumbar area, a central skin incision T8-L5 was made to expose the muscle fascia. Both iliac crests were identified and cleared of muscle tissue. Under constant saline cooling, central guide holes were drilled before ∅17 mm trephine holes were made [2]. The implants were press fit placed into the bilateral defects according to a randomized scheme. The muscles were then sutured tight to the remaining fascia on the crests.

Bilateral intramuscular pockets were created by blunt dissection after separate fascia incisions in the paraspinal muscles (L1-3). After inserting the implants according a randomized scheme, the fascia was closed with a non-resorbable suture. The skin was closed in two layers. Postoperatively, pain relief was given by buprenorphine (Shering–Plough, The Netherlands). The goats received sequential fluorochrome labels at 3 weeks (Calcein green, 10 mg/kg intravenously, Sigma), 5 weeks (Oxyteracycline, 32 mg/kg intramuscular, Engemycine, Mycofarm, The Netherlands) and 7 weeks (Xylenol orange, 80 mg/kg i.v. Sigma) [19,25]. Animals were killed by an overdose of pentobarbital (Organon, The Netherlands) after 9 weeks ($n = 7$) and after 12 weeks ($n = 8$).

Post-mortem sample acquisition, histology and histomorphometry

Explanted samples were fixated in 4% glutaraldehyde/5% paraformaldehyde, dehydrated by graded ethanol series, and embedded in polymethylmethacrylate. Semi-thin sections (10 μm) were made with a sawing microtome (Leica, Nussloch, Germany) [28]. Only the mid-section through the samples was used for histomorphometry. To obtain the mid-section, the explanted iliac wing was ground in the plane parallel to the cortex, until the outer (circular) margin of the implant appeared. A central section, 3 mm below the ground surface, was then cut from the 6 mm thick disks. After cutting 3–4 central slides,

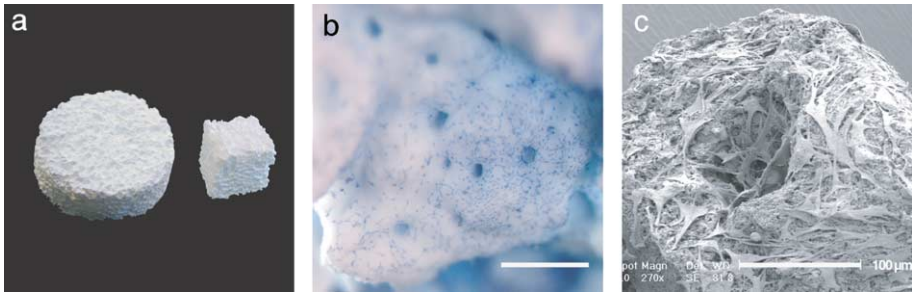

Fig. 1. In vitro imaging of scaffolds and cultured constructs. (a) Macroscopic image of the $\varnothing 17 \times 6$ mm disk that was used for orthotopic implantation and the $7 \times 7 \times 7$ mm cube that was used for ectopic implantation. (b) Stereomicroscopy of central part of a 7-days cultured disc as used for iliac defect implantation. Methylene blue stained cells are spread and cover the concavity of the pores. The small orifices are the interconnections between the pores (bar = 500 μm). (c) SEM image of an exterior part of a 7-days cultured cube. Flattened cells with extra cellular matrix positioned around an interconnection pore orifice (bar = 100 μm).

additional sections were made perpendicular to the plane of the central section, to evaluate bone formation on the axial faces of the disk that had been exposed to the soft tissue. A similar method was used to obtain central sections from the ectopically implanted samples. Slides were cut 3.5 mm below the outside of the $7\times7\times7$ mm cubes. Sections were either stained with methylene blue and basic fuchsin for routine histology and histomorphometry or left unstained for epifluorescence microscopy with a light microscope (E600 Nikon, Japan) equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558 and 640 nm, Omega filters, The Netherlands).

For histomorphometry, high resolution (300 dpi), low magnification ($10\times$) digital micrographs were made of blinded sections. Using Adobe Photoshop 5.5, bone and scaffold were pseudocolored red and green respectively. The area of interest was defined by adjusting the radius of a circle to exactly fit the perimeter of the iliac implant. The area of interest of the cubic ectopic samples was outlined with straight lines bridging the pores that interrupted the exterior contour. Image analysis was performed using a PC-based system equipped with the KS400 version 3.0 software (Zeiss, Oberkochen, Germany). A special program was developed to measure the following parameters of bone: (1) the percentage bone in the entire region of interest (fill%); (2) the percentage bone in the available (pore) space (B/Parea% = bone area/pore area $\times$ 100%); (3) the area of bone related to the area of scaffold (B/Sarea% = bone area/scaffold area $\times$ 100%) and (4) the percentage of available scaffold perimeter in contact to bone (contact% = scaffold perimeter length/bone-scaffold contact length $\times$ 100%). Fill% was measured to allow comparison with previous studies. This also accounts for the frequently used B/Parea% that is more informative on the filling of the available space [19]. In addition, the B/Sarea% was measured based on previous work indicating that tissue engineered bone formation is strongly associated with the scaffold surface [19]. It therefore seems more appropriate to relate the quantity of TE bone formation to available scaffold rather than available pore space. Similarly, contact% is more sensitive for early bone apposition which always occurs on the ceramic surface and has relatively little volume. Developmental differences between the above parameters indicate the growth dynamics of newly formed bone.

Statistics

Data are shown as mean ± standard deviation. Two-sided, student *t*-tests were performed to analyze differences between tissue engineered and control implants at each time point (paired) and between the time points (unpaired). The level of significance was set at $p = 0.05$.

Results

In vitro

The BM aspirates contained $9.7\pm2.6\times10^6$ (mean ± SD) nucleated cells/ml. Colonies formed after 6–8 days culture and the BMSCs were harvested when confluent, after 10–14 days. After the second passage, $0.8–1.5\times10^8$ BMSCs were cryopreserved. Thawed cells showed minimal dead cells (<5% according trypan blue exclusion) and proliferated well before seeding onto the scaffolds. The seeding efficiency of 8×10^6 cells/cm^3 cube, determined by DNA quantification, was 49.1 ± 14.7% and, after 1 week of culture, cells doubled to $8.7\pm3.7\times10^6$ cells/cm^3. Stereomicroscopy after seeding of both the cubes and the disks showed a comparable homogeneous cell attachment throughout the entire scaffolds. The 1 week cultured constructs showed the cells were spread on the ceramic surface (Fig. 1(b)) with dense multilayers and extracellular matrix on the outside of the constructs. This was confirmed by SEM imaging that showed the scaffolds were covered with cells and extracellular matrix (Fig. 1(c)).

In vivo results of orthotopic implants

There were no surgical complications and no macroscopic or microscopic signs of infection. The iliac wing implants were overgrown by tissue and could not be detected visually or manually (no motion).

Plain histology of the iliac wing implants revealed two cases (one for each condition in the 9-week group) of partial resorption of the inferior iliac wing border, resulting in only 80–90% of these implants being surrounded by bone. Besides these two implants, the scaffolds were totally integrated by the surrounding cancellous bone without interposition of fibrous tissue. No signs of scaffold resorption in terms of material degradation or osteoclast/giant cell activity were observed at both implantation periods. In the 9-week implants, bone formation appeared advanced in the TE group, although neither of the conditions showed bone formation in the center of the implanted disks. In the absence of bone, well vascularized loose fibrous tissue and abundant fat tissue was observed in both conditions (Fig. 3(a)). At the disk surfaces that were exposed to soft tissue, a similar distribution of new bone in close association with the surrounding bone was seen, while it was absent in the center. In the 12-week implants, considerable bone had formed and some defects were almost completely filled with bone in both conditions (Figs. 2 and 3(a)).

Fluorescent microscopy indicated bone was growing away from the scaffold surface and confirmed that no bone had formed in the middle of the defect in the first 9 weeks. No obvious differences in the presence or pattern of the labels was observed between TE and control samples (Fig. 3(b) and (c)).

Histomorphometry of the explanted samples indicated a porosity of 53.7 ± 10.9% for the 9-week implants and 65.0 ± 5.2% for the 12-week implants (mean ± SD). After 9 weeks, the bone fill%, the B/Parea% (in available space) and the B/Sarea% (bone area related to scaffold area) were not significantly different between the TE and control samples (Table 1). However, at this follow-up, the contact% was significantly higher in the TE group as compared to the controls (24.7 ± 9.3 vs. 17.9 ± 8.4, $p < 0.01$) (Fig. 4(a)). Because of the observed differences in porosity between the 9- and 12-weeks implanted samples, comparisons for the fill% and B/Parea% in time were considered not applicable and only the parameters that related bone directly to the scaffold were addressed. After 12 weeks, the B/Sarea% had increased significantly for both the TE and control condition when compared to the 9-week group. The contact% had increased less and did not show significantly more bone in the TE

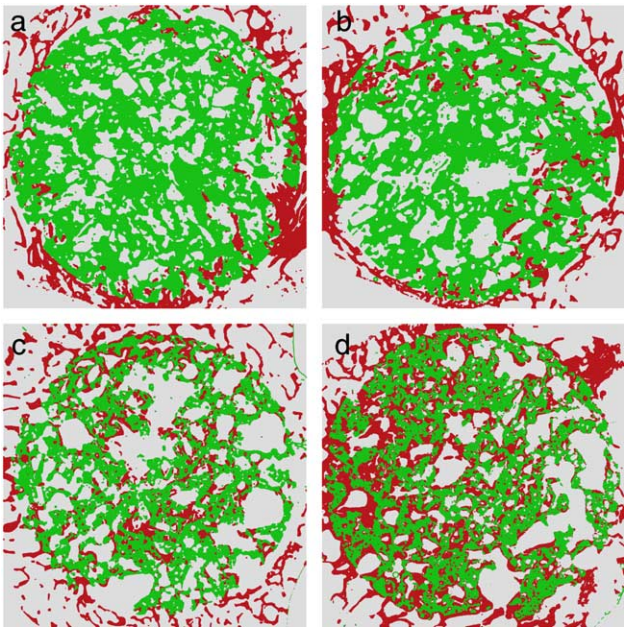

Fig. 2. Pseudocolored images of the ∅17 mm iliac wing implants. (a) Control scaffold implanted for 9 weeks. Surrounding bone (red) covers the periphery of the entire scaffold (green). (b) TE sample implanted for 9 weeks in the same goat as (a). No bone formation was observed in the middle of the 9-weeks implants. (c) Control scaffold implanted for 12 weeks. The entire scaffold is covered with bone. (d) TE sample implanted for 12 weeks contralateral to sample (c). The scaffold is completely integrated in the surrounding cancellous bone.

condition. Comparing the TE condition to the controls after 12 weeks did not reveal significant differences.

In vivo results of ectopic implants

All samples were retrieved without macroscopic or microscopic signs of infection.

Plain histology showed small foci of bone, lined by osteoblasts, in 5/7 controls (= 71%) after 9 weeks, and in 6/8 (= 75%) after 12 weeks. In all tissue engineered samples, ample bone was present. In these samples, bone lined by osteoblasts was closely related to the scaffold surface after 9 weeks and had a more trabecular appearance after 12 weeks. Bone was never found on the exterior of the scaffolds and seemed more abundant centrally in the scaffolds (Fig. 3(d)–(f)). No signs of material resorption were found.

Fluorescence microscopy indicated that bone formation was always directed away from the scaffold surface and had started before 3-weeks implantation in the TE group (Fig. 3(e)) and only after 5-weeks implantation in the controls, which occasionally showed the 7-week label.

Histomorphometry of all slides indicated a scaffold porosity of 48.9 ± 4.1 for the 9-week samples and 61.8 ± 4.4 for the 12-week samples. After 9 weeks, all parameters indicated an about 50 times higher yield of bone inside the TE scaffolds ($p < 0.01$) (Table 1 and Fig. 4(b)). To analyze an effect of time, again the fill% and B/Parea% where not considered. After 12 weeks, the B/Sarea% had significantly increased for the TE condition, while the contact% had slightly decreased from 25.8% to 21.4%, which was significant ($p = 0.019$). The substantial difference between TE and control groups remained for all parameters.

Discussion

In the current study in goats, we investigated bone formation in tissue engineered constructs both ectopically and orthotopically. The purpose of this study was to demonstrate differences between implant locations. The study was not designed to elucidate why differences may exist. Therefore, we chose our standard $7 \times 7 \times 7$ mm implants for the ectopic implantations because cell survival limitations were expected to be an important issue here as well as in the $\varnothing 17 \times 6$ mm orthotopic implants, due to the diffusion depth up to 3.5 mm.

Ectopically, the scaffolds without cells were shown to be osteoinductive in the majority of the goats for both implantation periods, as was expected based upon previous observations [31]. It is conceivable that this inductive capacity stimulated (part of) the seeded BMSCs, which population contains both determined and inducible osteoprogenitor cells (DOPCs and IOPCs) [9,12]. Because of this inductive stimulus, the 1-week predifferentiation of the cells as was done in this study, may be irrelevant. Furthermore, as was also shown in a previous study [21], at the moment of implantation only slightly more cells were present than the number of cells that was originally seeded (8.7 cells/cm^3 present, compared to 8×10^6 cells that were seeded with about 50% efficiency). This also supports the frequently used alternative of combining the cells and scaffold just prior to surgery [6,10,17].

While the effect of TE was obvious ectopically, orthotopically the effect was less pronounced. Only at the shortest period of 9 weeks, did the tissue engineered samples demonstrate significantly more bone apposition on the available scaffold surface. This effect could not be detected after 12-weeks implantation, at which time the TE group showed only about 10% higher values for all parameters. It should be mentioned that due to the large standard deviation, despite paired comparisons, the effect needed to be in the order of 40–50% to be detectable with a power of 80%.

Compared to other reports on orthotopic implantation for up to 5 months in large animal models [6,24,26,27], the impact of TE in our study was modest. The most rational explanation for this lies in the use of different hybrid constructs. Both the osteogenicity of the TE constructs and the scaffold material itself should be

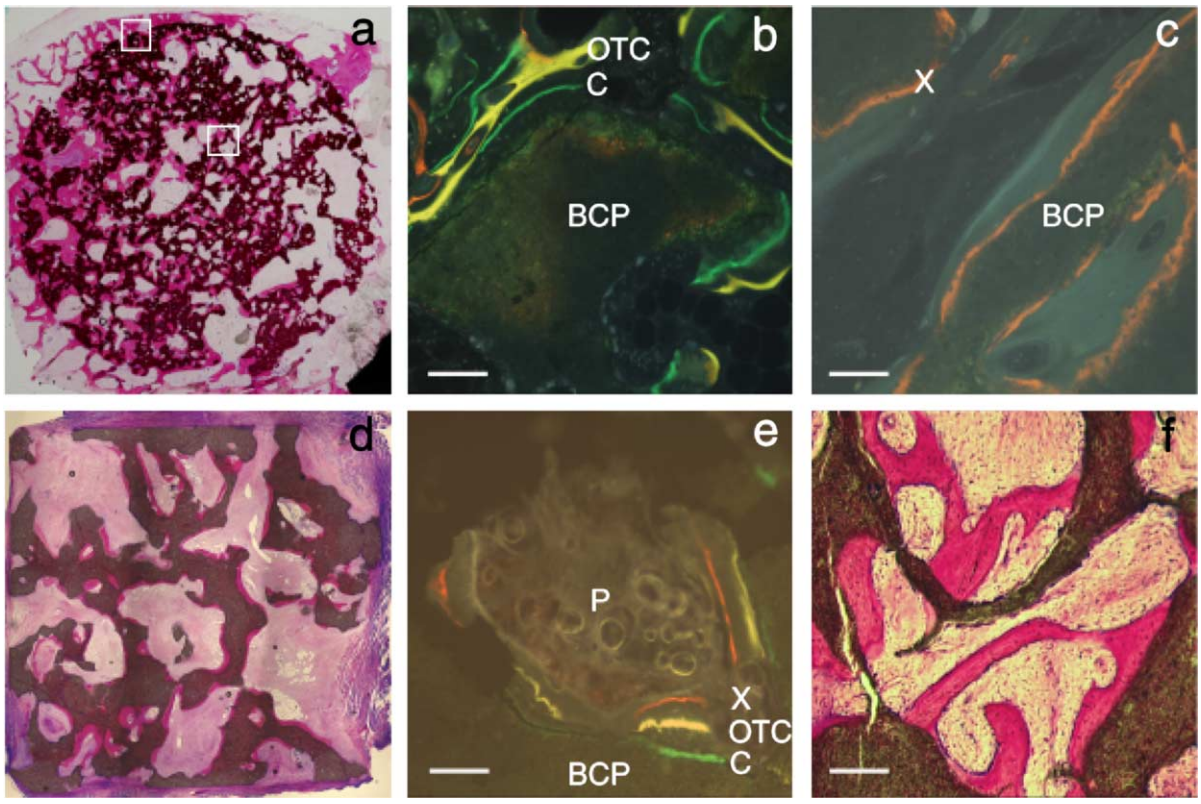

Fig. 3. Histology. (a) Low magnification micrograph of the ∅17 mm iliac implant as shown in Fig. 2(d) (12-weeks TE). Squares indicate the source for the fluorescence microscopy images (b) and (c). (b) High magnification fluorescence microscopy of a peripheral area of the implant in (a). Bone formation originated before 3 weeks on the outer side and inner side of the ceramic, as indicated by the Calcein green (C, 3 week) label. Fusion between surrounding bone and the scaffold was accomplished around 5 weeks as indicated by the yellow OTC label (bar = 50 μm). (c) High magnification fluorescence microscopy of a more central part. Only the Xylenol orange (X, 7 weeks) was present close to the BCP scaffold (bar = 50 μm). (d) Low magnification micrograph of 7×7×7 mm TE construct implanted ectopically for 9 weeks. Bone formation was in close association with the scaffold surface, but never occurred on the exterior. (e) Fluorescence microscopy of bone formed in the middle of 9-weeks implanted TE scaffold. All three fluorochrome labels were present. Growth dynamics can be deduced from the line pattern showing growth started from the BCP surface towards the pore (P) center (bar = 100 μm). (f) High magnification micrograph of TE construct implanted ectopically for 12 weeks. Typical trabecular bone formation lined with osteoblast zones (bar = 50 μm).

Table 1
Histomorphometry of orthotopic and ectopic implants

Location	Parameter	9 weeks		12 weeks		Level of significance	
		NC	TE	NC	TE	NC vs. TE	9 vs. 12 week
Orthotopic (mean ± SD)	Total fill%	10.1 ± 4.3	11.6 ± 3.9	16.7 ± 5.4	19.5 ± 5.5	NS	NA
	B/Parea%	18.9 ± 9.3	22.1 ± 8.3	25.1 ± 7.1	29.0 ± 10.0	NS	NA
	B/Sarea%	23.3 ± 12.1	26.5 ± 11.8	51.1 ± 20.5	54.4 ± 16.2	NS	<0.01
	Contact%	17.9 ± 8.4[*1,2]	24.7 ± 9.4[*1]	30.3 ± 8.8[*2]	34.5 ± 16.9	<0.01[*1]	0.016[*2]
Ectopic (mean ± SD)	Total fill%	0.1 ± 0.1	5.6 ± 0.9	0.3 ± 0.5	6.1 ± 1.6	<0.01	NA
	B/Parea%	0.2 ± 0.2	11.7 ± 2.5	0.6 ± 0.8	10.2 ± 2.9	<0. 01	NA
	B/Sarea%	0.2 ± 0.2	10.7 ± 1.8[*3]	0.9 ± 1.4	15.3 ± 4.6[*3]	<0.01	0.025[*3]
	Contact%	0.4 ± 0.5	25.8 ± 2.4[*4]	2.1 ± 3.0	21.4 ± 3.6[*4]	<0.01	0.019[*4]

For both the orthotopic and ectopic locations, the area% bone in the whole sample (total fill%); the area% bone in available pore space (B/Parea%); the area% bone related to scaffold area (B/Sarea%); and the percentage of the scaffold perimeter in contact to bone (contact%) are shown. The effect of TE vs. the control with no cells (NC) and the effect of implantation time was analyzed. NA = not applicable (see text) and NS = not significant.

Orthotopically an effect of TE existed after 9 weeks for the contact% (*1). In time, the B/Sarea% increased in both TE and NC condition, the contact% increased only for the NC condition (*2).

Ectopically the effect of TE was obvious for all parameters. In time, the B/Sarea% increased (*3) whereas the contact% slightly decreased in the TE condition (*4).

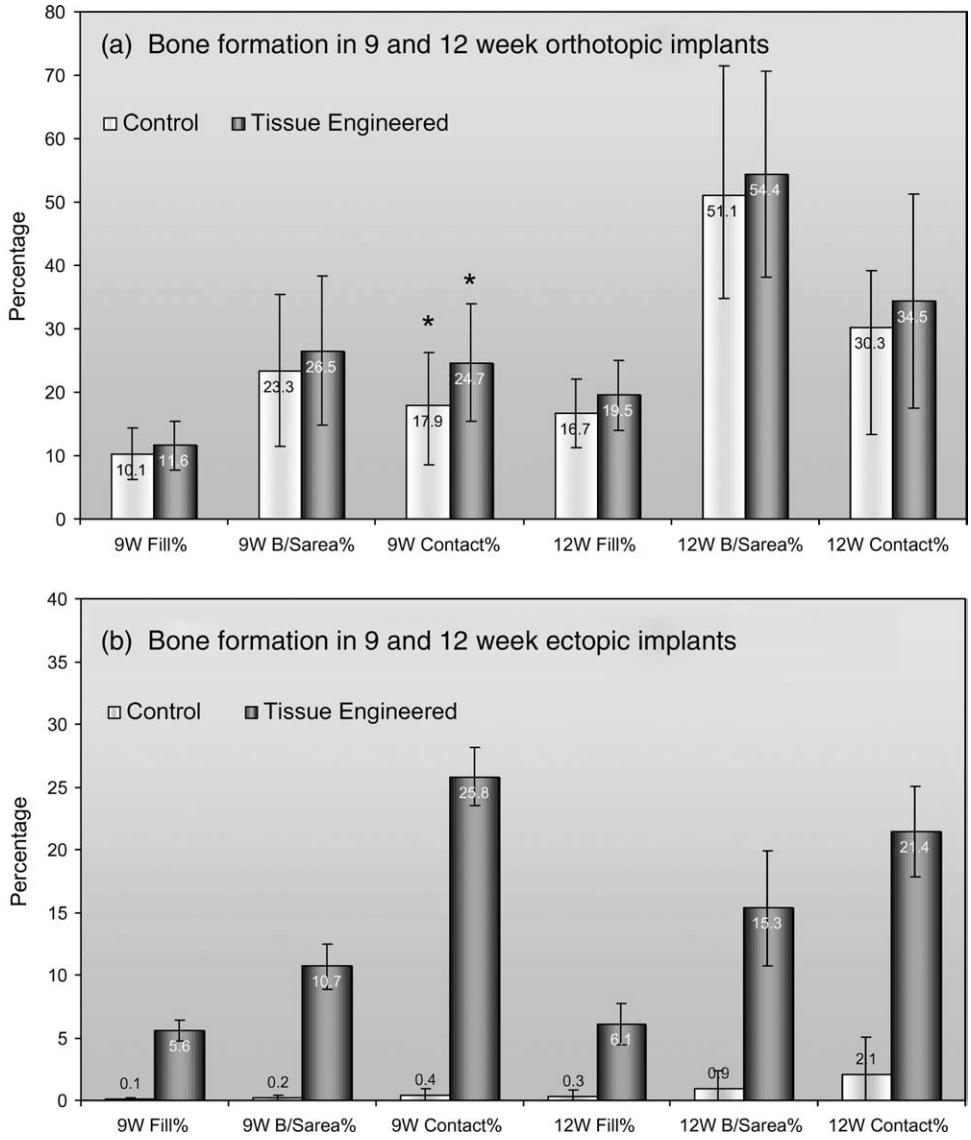

Fig. 4. Orthotopic and ectopic bone formation after 9 weeks ($n = 7$) and 12 weeks ($n = 8$). (a) Orthotopic implants in the iliac wing defect. The percentage of the total defect filled with bone (fill%); the area of bone related to the scaffold area (B/Sarea%) and the percent bone apposition on available scaffold outline (contact%) were measured. White bars reflect the controls; black bars the tissue engineered constructs. Error bars indicate the standard deviation. At 9 weeks, the contact% was significantly higher in the TE samples (^{*1} $p < 0.01$). (b) In the ectopic samples the same parameters were measured. Always significantly more bone was found in the tissue engineered samples.

considered. Lack of osteogenicity of the constructs at the orthotopic location is unlikely because the osteogenicity was shown clearly in the ectopic model. In previous studies with this model, it was demonstrated that viable cells were crucial to accomplish bone formation, indicating the osteogenic role of the cells [19]. Unfortunately, the osteogenicity of the constructs (ectopically) used by other researchers was not reported in their animal model. Another explanation concerns the scaffold material itself, which was shown to be osteoinductive. It is possible, that bone conduction in combination with this inductive capacity resulted in so much bone apposition, that the TE-related osteogenesis was overruled. This hypothesis is supported by the finding that significant TE-related bone formation was only found at the early (9 weeks) evaluation. After 12 weeks, bone was present also throughout the control implant with bone filling 16.7% of the entire defect area. For comparison, in previous studies with this model after the same time period, empty controls contained 13.5% bone, a porous osteoconductive copolymer only 1.5%, allograft 13% and autograft 36% [2]. In that study also the grafted chips of the allo- and autograft were counted as bone, whereas in the current study only new bone was counted.

A more discouraging explanation for the modest effect of TE may be proposed by the absence of early osteogenesis in the center of the iliac defects. This is not

that surprising, considering the literature on cell survival in large unvascularized bone grafts, as cell survival is expected to be limited and not to exceed a distance of 300 μm from nutrient supply [11,14,15]. However, if this were true, bone formation ectopically should also have been limited, since the diffusion depth in these samples was also much larger (up to 3.5 mm). This appeared not to be the case, and on the contrary, bone seemed to be more abundant in the middle of these constructs. Although (micro)movement on the scaffold outside may account for absence of bone at the very exterior, the observation of extensive bone centrally does indicate that cells probably survived at relatively large diffusion depths.

Cell survival in the orthotopic location, however, has probably been more compromised than in the ectopic location, because of the greater trauma to reach the iliac wing, with subsequently delayed revascularization. The only way to find out whether this is indeed the limiting factor, is by techniques that can reliably identify the transplanted cells [1,18]. Another difference with the ectopic location is the fracture healing response, which may interfere with the implanted cells. Typically, fracture healing does not rely on cells present inside the defect, but recruits the cells from the surrounding mesenchymal tissue [5,16]. It is possible that cells inside the orthotopic defect were subject to phagocytosis during the immediate injury response.

The measured porosity of the 9- and 12-week implants was different (approximately 50% and 60%, respectively), resulting in different available spaces for bone formation. Although material degradation cannot be ruled out completely, according to the manufacturer this difference in porosity is likely to be the result of fluctuations in the normal production process. This complicated the investigation of growth dynamics by comparisons between the 9- and 12-week implants. Therefore, the fill% and the bone per available pore space (B/Parea%), which are directly related to the available pore space, were not statistically compared between this time points. By relating bone to the available scaffold material (B/Sarea%) and to the available scaffold perimeter (contact%), relevant comparisons could be made because bone apposition was always on the scaffold surface. Together with the fluorochrome markers, these parameters indicated bone formation was associated with the ceramic surface and grew centripetally after initiation. In the ectopic samples, the percent scaffold outline occupied by bone decreased between 9 and 12 weeks, suggesting no new bone formed on the scaffold surface after 9 weeks. However, based on the increasing B/Sarea% and the presence of osteoblast zones, bone formation itself continued (Fig. 3(f)).

In conclusion, we have shown the relative contribution of TE to bone formation ectopically and orthotopically. Although the constructs where obviously osteogenic ectopically, in the orthotopic location functioning was modest in comparison to the osteoconductive and osteoinductive capacity of the scaffold and the presence of cultured cells may have been irrelevant. In situations that may be considered to have an ectopic constituent, or where the inherent conductive and inductive nature of the scaffold is likely to be limited, e.g. posterior spinal fusions, bone TE may be helpful.

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