Chapter 4

THE OSTEOGENICITY OF AUTOLOGOUS BONE TRANSPLANTS IN THE GOAT

Summary

Introduction: Little is known about the specific mechanisms that make autologous graft bone (AG) superior to the current alternatives. A potential mechanism is the active bone formation by the osteoprogenitor cells within the AG. However, whether these cells survive the transplantation is questionable, especially in non-vascularized clinically sized grafts. In the present study we investigated the role of viability in AG, implanted ectopically and orthotopically in the goat.

Methods: Eight goats were operated on twice. At the first operation, pieces of vital or devitalized autologous cortical bone were implanted in the paraspinal muscles. Eight weeks later, corticocancellous plugs were taken from the femoral condyles, morselized and re-implanted as either vital or devitalized orthotopic grafts. The goats received fluorochrome labels at 5, 7 and 9 weeks after the first operation. At twelve weeks the goats were killed and the samples were examined histologically.

Results: Ectopically, new bone had formed in both the vital and devitalized grafts. In the vital grafts, all three fluorochrome labels were present, indicating an early osteogenic mechanism. Within the devitalized grafts, only the 9 weeks label was observed. Histomorphometry indicated significantly more new bone in the vital grafts, 10.3 vs. 1.7% in the devitalized grafts. Orthotopically, both vital and devitalized grafts showed new bone. Again, graft viability was advantageous in terms of new bone formation (14.5 vs. 9.3%).

Conclusion: The cells inside the autologous bone transplants most likely survived transplantation and were capable of initiating and sustaining new bone formation.
Introduction

Currently, bone is one of the most frequently transplanted tissues and applied in many orthopedic, neurosurgical and maxillofacial interventions. The non-vascularized autologous bone graft (AG), as a structural graft or morselized, is the gold standard for most applications. However, many disadvantages, such as donor site pain and limited availability, are inherent to this graft. A fundamental difference with alternative graft materials, such as allograft bone, is the presence of a viable osteoprogenitor cell pool in the autograft at implantation. Besides many other important factors, subsequent bone formation by these cells may be responsible for the superiority of the AG. However, contrary to vascularized bone grafts, cell survival and participation in bone formation in non-vascularized grafts is largely unknown and highly controversial. After transplantation, the cells are exposed to the harsh environment of a haematoma and may be deprived of vascular supply for weeks. Some authors report an almost total loss of vital cells in the AG, others report substantial survival, although limited to a diffusion depth of 300µm. Elves and Gray reported the osteogenicity of ectopically implanted AG in rats. The implants were not osteogenic if devitalized by freezing before transplantation. As direct proof of osteogenicity, Boynton et al. used immunohistology to identify human cells within new bone appositions on human bone chips implanted ectopically in immune-compromised mice. These studies indicated that the cells inside small (cubic mm's) grafts have the potential to survive and form new bone ectopically. To our knowledge, this has never been shown in larger mammals with transplants of clinically relevant size (cubic cm's), where cell survival is expected to be more difficult because of the 1000-fold increased volume and subsequently delayed re-vascularization.

Considering orthotopic bone transplants, only a few experiments are reported that compared vital and devitalized autografts in large mammals. In these experiments, the contribution of viable cells in the autografts was considered insignificant, however, these grafts were analyzed after incorporation in the host bone. We did not find studies that analyzed grafts early after orthotopic transplantation, before incorporation, when bone formation as a function of transplanted progenitor cells is more likely to be determined.

At present, much research is focussed on cell-based tissue engineered bone as an alternative for autologous bone. The concept of this technique is to form a hybrid construct from an osteoconductive scaffold and cells that have bone forming capacity. In rodent studies, bone that formed in these hybrid constructs contained the implanted cells. Therefore, the success of tissue engineering is likely to rely on survival and subsequent functioning of these cells. As for the
autologous bone graft, this is questionable when clinically sized constructs are applied. Therefore, as a first step to obtain a better understanding regarding the importance of cell survival for bone grafting, we performed a study on autologous bone grafts in a large animal model. The aim of this study was to investigate whether the viability of autologous bone grafts influenced new bone formation, ectopically and orthotopically in the goat, which would be an indication for cell survival and functioning in these grafts.

Materials and Methods

Experimental design and groups
A total of 8 adult Dutch milk goats were operated on twice during the experiment, for which the local animal care committee gave approval. At the first operation, autologous cortical bone grafts, obtained from a femoral diaphyseal segment, were implanted in the paraspinal muscles after one of the following treatments:

1. Vital cortex – no specific treatment;
2. Devitalized cortex – by freezing in liquid nitrogen;
3. Morselized vital cortex and

This resulted in a sample-size of eight for each treatment group. Fluorochrome labels were administered at 5, 7, and 9 weeks.

Eight weeks after the first operation the goats were operated on again. A corticocancellous plug was taken from both the medial and lateral aspects of the left femoral condyle. Both plugs were morselized and implanted in the trephine shaft opposite the location of origin after one of the following treatments:

1. Vital corticocancellous chips – no specific treatment;
2. Devitalized corticocancellous chips – by freezing in liquid nitrogen.

The animals were killed 4 weeks later (12 weeks after the first operation). Ectopic and orthotopic bone formation was investigated by normal histology, fluorescence microscopy, and histomorphometry of nondecalcified sections.
General procedures

Goats (19-26 months) were obtained from a professional stockbreeder, at least four weeks prior to surgery. Surgery was performed under general inhalation anesthesia, preceded by i.v. detomidine sedation (Domosedan®, Pfizer, The Netherlands). Thiopental 10mg/kg (Rhone-Merieux, The Netherlands) was introduced i.v. and anesthesia was maintained by a halothane gas mixture (Sanofi, The Netherlands). Postoperatively, pain relief was provided by buprenorfine hydrochloride (Shering-Plough, The Netherlands). Three fluorochrome labels (Sigma-Aldrich, The Netherlands) were administered intravenously: Alizarin Red (30mg/kg) at 5 weeks, Calceine green (10mg/kg) at 7 weeks and Xylenol orange (100mg/kg) at 9 weeks.[131] After twelve weeks the animals were killed by an overdose of pentobarbital (Euthesate®, Organon, The Netherlands).

Ectopic grafts

Autologous cortical bone was derived from a femoral segment that was excised as part of a segmental defect model studied in the same goats. The 2.3cm, diaphyseal segment was excised by sawing under constant saline cooling and cleaned of periosteum and medullary contents. It was then sectioned in the sagittal and frontal planes to provide four grafts with a volume of approximately 1cm³ each. Two grafts were morselized with a mortar and pestle into bone chips (approximately 2x2x2mm). One intact and one morselized grafts was then devitalized by freezing twice for 5 minutes in liquid nitrogen.[282] The grafts were implanted according to a randomized scheme into separate pockets, created by blunt dissection, in the paraspinal muscles.[131] The intact grafts were cut into two 1.1cm pieces before implantation. The fascia was closed with a non-resorbable suture to facilitate implant localization at explantation and the skin was closed in two layers.

Orthotopic grafts

At the second operation, eight weeks after the first operation, both condyles of the left femur were exposed. Corticocancellous bone plugs of 10mm length were taken[283] with a Ø9.5mm hand trephine and extractor (Mathys, Bettlach, Switzerland) and stored in warm saline. A 12mm Ø1.2mm Kirschner wire was placed centrally in the trephine shaft to serve as a reference during explantation and histology. Per goat, one of the plugs was devitalized in liquid nitrogen. Both plugs were then morselized (approximately 2x2x2mm) and implanted press-fit in the opposing location. The origin of the devitalized plugs and the operative order of the
condyles were randomized. The periosteaum was closed carefully before closing the skin in two layers.

Validation of osteogenicity
To determine the osteogenicity of the applied bone grafts in an established model of osteogenicity,\cite{49,121,183} vital and devitalized pieces (1-5mm$^3$) of both the cortical and corticocancellous grafts of each goat were implanted subcutaneously in nude mice for four weeks.

Validation of devitalization
Cortical ($n=8$) and corticocancellous ($n=6$) grafts, as used for ectopic and orthotopic transplantation, were morselized and cultured under standard conditions for goat bone marrow stromal cells (BMSC’s)\cite{131} in 10cm$^2$ culture dishes (Nalge Nunc, Denmark) for 30 days to detect for surviving adherent cells. Another eight vital and devitalized corticocancellous grafts were cultured for one week to expand the number of any surviving cells. A 48h alamarBlue™ (AB. Biosource, Camarillo, US) assay was then done to detect metabolic activity.\cite{284}

Post-mortem sample acquisition, histology and histomorphometry
At explantation, the ectopic implants were localized and excised together with surrounding muscle. The orthotopic implants were sawed en-bloc from the condyles. Explants were fixated in a 4%paroformaldehyde-5%glutaraldehyde mixture, then dehydrated by alcohol series and embedded in polymethylmethacrylate. Semi-thin sections (10µm) were obtained with a sawing microtome (Leica, Nussloch, Germany) and analyzed with fluorescent microscopy using a fluorescence/light microscope (E600 Nikon, Japan) with double filter block (dichroic mirror 505nm and 590nm). Other sections were stained with methylene blue and basic fuchsin for routine histology and histomorphometry. The bright red, highly cellular, newly formed bone was identified and distinguished from the pale, mainly acellular grafted bone. The mid-section through the implants was used for histomorphometry. For the ectopic implants, this was the section providing the largest sample area. For the orthotopic implants, the explanted condyle blocks were ground in the sagittal plane until the outer (circular) margin of the grafted defect appeared. Then the block was cut 5mm below and parallel to the ground surface through the middle of the 10mm defect. Image analysis was done by a blinded
observer using a VIDAS system (KS400, Zeiss, Munich, Germany) coupled to a light microscope. For the ectopic implants, the area of interest was defined by outlining the graft. Within this area the percentage of newly formed bone was measured. In the orthotopic implants, the area of newly formed bone was measured within four quadrants of the Ø9.5mm outlined defect at 25x magnification. The area percentage newly formed bone was then calculated.

Statistics
Results are given as mean±standard deviation (SD). Two-tailed paired student t-tests were performed after the data were analyzed for normal distribution with SPSS10 software (p<0.05 was considered significant).

Results

Efficiency of devitalization and osteogenicity of the cells
The devitalized grafts did not show remaining viable cells during one month of culture. The vital grafts, however, showed outgrowing fibroblastic cells within one week. When these cells were detached, seeded onto 70% porous hydroxyapatite scaffolds (Cam Implants, The Netherlands) and implanted in nude mice, these constructs were shown to be osteogenic.[121] The 48 hour alamarBlue™ assay also did not indicate any vitality in the devitalized grafts, whereas, in the viable control grafts, metabolic activity could be measured within 1 hour of incubation. The subcutaneous implants of cortical and corticocancellous grafts in mice showed new bone formation only in the vital implants, indicating a cell-dependent osteogenicity of these small grafts (Table 1).

General
Two of the 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 ectopic implants of these two goats were used for qualitative measurements and for paired comparisons within the animal. The orthotopic grafts were excluded from quantitative analysis because these had been implanted for only half the planned evaluation time.
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Table 1  Bone formation in the study groups
The fraction of samples showing new bone and the area percentage new bone (Mean±SD) are shown. The time points at which the observed fluorochrome labels were administered to the goats are shown in the right column.

<table>
<thead>
<tr>
<th>Group</th>
<th>New bone ectopically in mice</th>
<th>New bone ectopically in goats (area%)</th>
<th>Fluorochrome labels (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vital morselized cortex</td>
<td>N.A.</td>
<td>8/8 (18.9±8.7%)</td>
<td>5,7,9</td>
</tr>
<tr>
<td>Vital intact cortex</td>
<td>8/8</td>
<td>8/8 (10.3±5.0%)</td>
<td>5,7,9</td>
</tr>
<tr>
<td>Devitalized intact cortex</td>
<td>0/8</td>
<td>8/8 (1.7±1.2%)</td>
<td>9</td>
</tr>
<tr>
<td>Devitalized morselized cortex</td>
<td>N.A.</td>
<td>Resorbed</td>
<td>Resorbed</td>
</tr>
<tr>
<td>Vital corticocancellous chip</td>
<td>8/8</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Devitalized corticocan. chip</td>
<td>0/8</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Ectopic grafts (Table 1)
At retrieval, all devitalized morselized cortex chips had been resorbed. All other implants were surrounded by well-vascularized muscle tissue without signs of inflammation. Histology showed new bone formation in all retrieved implants ranging from minute spots on the devitalized implants to abundant trabecular bone formation in the morselized vital implants (Fig. 1). The vital morselized cortex typically formed rigid ossicles (Fig. 1) as a result of extensive new bone bridging between the implanted chips. The interior of these ossicles was filled with fat cells (Fig. 2a).
Within the vital intact cortex, new bone had formed between the two implanted pieces, however, bone apposition on the walls of resorbed haversian channels was responsible for most of the newly formed bone (Fig. 2c). The devitalized intact cortex had decreased in size substantially when compared to the vital intact implants. Inside these implants, minimal resorption was found. On the periphery, however, extensive resorption was present and small amounts of bone had formed on the bottom of resorbed cavities in all samples (Fig. 2e). Fluorochrome analysis of the vital morselized cortex indicated that mineralization was present within the newly formed bone at 5, 7 and 9 weeks after transplantation (Fig. 2b). In the vital intact cortex, the 7 and 9 weeks labels were typically present in the remodeled bone within the osteons (Fig. 2d). Within the bone that had formed on the devitalized implants only the 9 weeks label was detectable (Fig. 2f).
Figure 1 Micrographs of ectopic autologous implants in goats. (☞ p. 180)

a): Vital morselized cortical implants had formed ossicles as a result of new bone (NB) bridging the grafted chips (bar=250µm).
b): Vital intact cortical implants showed new bone bridging the two implanted pieces and appositioned in the resorbed haversian channels (bar=250µm).
c): Devitalized intact cortical implants showed minute spots of new bone on the periphery (triangles). Note the absence of resorption around the haversian channels (bar=250µm).

Histomorphometry of the samples that were retrieved from the goat, indicated an area% of 18.9±8.7% (Mean±SD) new bone within the vital morselized cortex sample and 10.3±5.0% within the intact vital cortex. The devitalized intact cortex showed only 1.7±1.2% new bone, which was significantly less when compared to the vital intact cortex (p<0.01 Fig.3a). The histomorphometry data of the morselized cortex indicated that morselization resulted in a higher yield of new bone. However, statistical comparison with the intact cortex would be difficult, because in the intact cortical grafts the available area for bone formation was different and restricted to the framework that this structural graft provided. Meaningful histomorphometrical comparisons with respect to the influence of viability of morselized grafts could not be made, because the morselized devitalized cortex was completely resorbed.

Figure 2 Detailed histology of ectopic autologous implants in goats (☞ p. 180)

a): Vital morselized graft. Detail of new bone (NB) bridging the chips that were implanted vital. The interior of the ossicles was filled with fat cells (F) (bar=400µm).
b): Fluorescent image of morselized vital implant showing all labels A=Alizarine red (5 weeks) C= Calcein green (7 weeks) X= Xylenol orange (9 weeks) (bar=400µm).
c): Vital intact implant at high magnification. Bone resorption by osteoclasts (OC) and new bone apposition by osteoblasts (OB) is visible. The distinction between pale grafted (G) and red new bone (NB) is obvious (bar=100µm).
d): Fluorescent image of the vital intact implant showing the 7 and 9 weeks labels in the remodelled bone around haversian channels (bar=200µm).
e): Devitalized intact graft (G) with a thin layer of new bone present on the periphery (bar=400µm).
f): Fluorescent image of the devitalized implant showing only the 9 weeks label (bar=400µm).
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Figure 2

a) NB

b) F

c) OC

d) OB

e) G

f) NB

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Orthotopic grafts

Histology of the orthotopic implants showed good integration of both the vital and devitalized grafts in the surrounding bone without an apparent difference between the two groups at first observation (Fig. 4a-b). A distinct layer of new bone covered most surfaces of the grafted bone (Fig. 4c), occasionally reaching the center of the defect. The variance between goats was considerable, however, detailed microscopic and histomorphometric analyses of the paired samples indicated more new bone inside the defects grafted with vital bone, (14.5±3.5 vs. 9.3±4.9% \( p<0.02 \)). (Fig. 3b). The fluorochrome label given one week after implantation was occasionally present, both in the vital and devitalized grafts, without an obvious difference between the groups.

Figure 3  Area% new bone in vital and devitalized grafts implanted in goats
Statistical analysis with paired t-tests, error bars indicate the standard deviation.

- a): The area% newly formed bone within the vital and devitalized ectopic cortical implants (n=8, \( p<0.01 \)).
- b): The area% newly formed bone covering the vital and devitalized orthotopically grafted corticocancellous bone chips (n=6, \( p=0.02 \)).

Figure 4  Histology of orthotopic autologous grafts implanted for 4 weeks.

- a): Overview of the outlined Ø9.5mm defect showing the grafted devitalized chips embedded in soft tissue and surrounded by host bone.
- b): Overview of the defect showing the vital graft.
- c): High magnification micrograph of the rectangle in b. At this magnification newly formed bone can be distinguished from the grafted bone. Triangles point to osteoblast linings (white bar=200\( \mu \)m)
Figure 4
Discussion and conclusions

In a study on transplants of human bone pieces (0.5mm$^3$) ectopically in nude mice, Urist et al. observed that graft cell survival was exceptional and limited to bone-tumor transplants. Whether cell death was due to insufficient oxygen and nutrition or the result of a residual humoral immunologic response was not clear. In the present study, we investigated new bone formation in ectopically and orthotopically transplanted autologous bone grafts. After implantation of these grafts, with a volume of about 1cm$^3$, poor oxygen and nutrition conditions can also be expected. To study the role of vital cells present in the graft, we devitalized the control grafts by freezing in liquid nitrogen. We chose this method as other methods, such as lysing, gamma irradiation or heating, might be insufficient or interfere with osteoinduction by devitalized bone matrix. Although freezing has minimal effect on bone morphogenetic protein (BMP) related osteoinductivity, we cannot rule out that freezing interfered with new bone formation in any other way. Oklund et al. postulated that freezing could result in calciolysis of bone grafts, thereby promoting ECM exposure and resorption. In our study, new bone formation in the frozen grafts was typically found on the periphery where mineralized bone had been partially resorbed, indicating a positive effect of organic matrix exposure. This is in agreement with the finding of Ripamonti that bone induction occurred on the resorbed surface of partially demineralized allografts in baboons. For these reasons, freezing probably did not negatively influence the osteoinductive capacity of the grafts in our study.

In pilot studies, we found that corticocancellous bone (as used for the orthotopic investigations) could not be used in the ectopic model. Although abundant new bone formed in viable corticocancellous grafts, all devitalized grafts were completely resorbed. We cannot exclude the possibility that the devitalized grafts resorb faster as a result of freezing. It is therefore possible that bone formation in the viable grafts may be the result of bone induction that initiated after the frozen samples had resorbed. Consequently, we chose cortical bone, which was more resistant to resorption, for ectopic investigations in the current study.

Increased bone formation was found in the viable ectopic implants, which showed the early fluorochrome label. This suggests an early, osteogenic mechanism, which indicates that cells do survive after transplantation and are involved in new bone formation. However, we cannot be sure how long cells survive or if they only initiated host derived bone formation by unknown or not fully understood mechanisms.

Another interesting finding was the absence of bone resorption around the haversian channels in the devitalized cortical grafts. In the vital cortical grafts,
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Bone resorption and subsequent new bone formation were abundant after 5 weeks of implantation, based on the presence of the fluorochrome labels (Figs. 1 and 2). Apparently, bone resorption for remodeling, according the principles of so-called “creeping substitution”,[166,287] requires an environment of vital bone, as was also postulated by others,[57] or the residence of vital osteoclasts within the transplant. The aim of studying the orthotopic model was not for comparison to the ectopic model but to investigate whether the findings on cell survival and function in the ectopic location were of any relevance orthotopically. Irrespective of cell osteogenicity, we expected progressive bone formation in the orthotopic grafts as a result of osteoconduction and induction. Therefore, we chose an evaluation time that would be most distinctive for cell related osteogenesis. In rat studies, consistent osteogenic bone formation was shown two weeks after subcutaneous implantation of an autologous bone graft.[179] In the goats, we expected new bone formation to occur later due to a longer re-vascularization period.[173] We therefore estimated that an implantation period of 4 weeks would allow us to easily detect bone formation. After this period, new bone was indeed present in both conditions, even centrally in the defects. Apparently, host initiated bone formation is the predominant mode of repair in this model. However, histomorphometry indicated a significantly higher percentage of new bone in the defects grafted with vital bone. We therefore conclude that at least a proportion of the grafted cells survived to promote bone formation, leading to an acceleration of this process. However, in agreement with observations by others,[23,181] no advantage of graft viability should be expected for such a model in the longer term.

In conclusion, the cells present in autografts in our ectopic and orthotopic models most likely survived transplantation and were capable of initiating new bone formation. With respect to tissue engineering of bone, these observations suggest that the combination of vital osteogenic cells with an appropriate scaffold may be advantageous to non-vital graft materials. This may be especially important in applications where host bone formation is limited, such as spinal fusion.

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