A NOVEL PARADIGM FOR ENGINEERING BONE: THE IN VIVO BIOREACTOR

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INTRODUCTION

Bone is a highly vascularized organ with an intricate cellular architecture that continues to remodel throughout the lifetime of an individual. In spite of the regenerative capacity of bone, induction of new-bone at a site of injury can be extremely difficult. As a result reconstructive surgery of large-size bone defects pose an enormous challenge, which is further, compounded due to the limited availability of autologous bone. Currently, such defects are treated with allogenic bone obtained from human cadaver, with a potentially enormous risk of disease transmission. Bone regeneration therefore, represents a unique opportunity and challenge for Tissue Engineering (TE). To date attempts at engineering bone-like tissue *ex vivo* or *in vivo* using the principles of TE have largely been unsuccessful due to the complex nature of bone and the limitations of synthetic and biological grafts, in that, they lack the osteoinductive properties of autologous living bone (autografts). Furthermore, the requirement for cell expansions [1] *in vitro* in the presence of exogenous growth factors to aid in cell proliferation and differentiation is both costly and time consuming.

Here we propose a novel concept that maximizes the role of the body in the regeneration of neo-tissue by using the patient's own body as the scaffold and the bioreactor and obviates the need for *ex vivo* manipulation of cells. The crux of this novel paradigm, termed "*In Vivo Bioreactor*"; is the manipulation of an artificial space **(bioreactor)** created between the tissue of interest and a mesenchymal layer rich in pluripotent cells. In the context of bone TE, the "bioreactor", is a pocket between the bone (tissue of interest) and periosteum (fibrous mesenchymal tissue rich in progenitor cells), namely the "periosteal pocket". The composition within the periosteal pocket, which now serves as both the "bioreactor" and "scaffold", can be manipulated to elicit the desired outcome.

In this study we have demonstrated that large quantities of mature autologous compact lamellar bone suitable for transplantation can be generated utilizing this approach without the need for the *in vitro* culture of cells or exogenous growth factors. Specifically, the formation and remodeling of neo-tissue within sub-periosteal pockets filled with resorbable alginate, in the tibia of New Zealand rabbit models was followed over a 12 week in presence and absence of a combination of transforming growth factor-β1 (TGF-β1, morphogen) and basic-fibroblast growth factor (b-FGF, mitogen with angiogenic properties).

MATERIALS AND METHODS

Preparation of alginate gel

Alginate gel was prepared by ionically cross-linking a 2% (w/v) high G-content alginate (FMC Corp.) solution with 75 mM $CaCl_2$ in a 1:1 volume ratio [2]. Under these conditions the solidification of the gel occurred within 30 seconds.

Creation of periosteal pocket

Oval-shaped periosteal pockets (n = 16) of 1 cm in length and 0.75 cm in diameter with an elevation of 2 mm above the normal tibial plane were created bilaterally in the tibia of the hind legs of skeletally mature New Zealand white rabbits. The periosteal pocket was created utilizing a novel minimally invasive surgical technique that minimized the exposure of the periosteum to trauma [3]. In brief, the tip of a 22 gauge needle bent at a 45° angle was introduced between the cambium layer of the periosteum and the surface of the tibia and the periosteum was elevated by the injection of saline solution to create a pocket (Figure 1). The size of the pocket was manipulated by increasing the penetration distance of the needle and the volume of fluid. Since a needle and fluid is used to create and manipulate the pocket as opposed to a periosteum elevator, the damage to the periosteum is minimal. This ensures the preservation of blood flow to the periosteum and viability of the cambium layer. Furthermore, the use of a needle ensures the adaptability of the procedure to minimally invasive techniques.

Bone regeneration studies

The periosteum pocket was filled with alginate gel via the same needle-sized opening that was used to create the pocket. This pinhole opening was subsequently sealed with fibrin glue to prevent infiltration of cells from surrounding tissue and the leakage of the gel. 10 of the pockets were filled with alginate gel containing TGF-β1 and

b-FGF (10 ng/ml each) while the remaining was filled with alginate alone. The rabbits were sacrificed at 4, 6, 8 and 12 week time-points and the entire tibia was harvested. The tibiae were fixed in 10% formalin and then decalcified in EDTA prior to paraffin embedding. Histological analysis (H & E, safranin-O and thionin staining) of the artificial pocket and the surrounding area was performed.

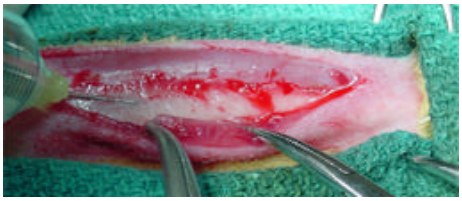

Figure 1. Creation of a periosteal pocket in the rabbit tibia.

RESULTS

The creation of the periosteal pocket using the aforementioned technique yielded reproducible pockets with a viable cambium. The presence of a viable cambium was confirmed by histology and organ culture studies using periosteal explants obtained by this procedure [4].

Measurements of the cross-sectional area of the artificial pocket (pocket area (mm^2)), and the relative percentages of unresorbed alginate gel (Alginate), angiogenesis (Blood Vessels) and neo-osseous tissue (Osseous Tissue) occupying the artificial pocket are presented in Table 1. A striking observation is the formation of neo-bone even in the absence of any growth factors. We believe that this might be as a result of the calcium rich environment provided by injection of the calcium alginate gel. Calcium salts have been shown to promote the differentiation of mesenchymal cells into osteoblasts in both the embryo and during the fracture repair process [5].

Timepoint (weeks)	GF	Pocket Area (mm^2)		Alginate (%)		Blood Vessels (%)		Osseous Tissue (%)	
4 (n = 1)	T / F	3.175	0.01	5.5	0.21	8.98	0.2	85.51	0.41
6 (n = 2)	none	4.86	2.32	7.95	6.15	1.26	1.31	90.79	7.27
6 (n =1)	T / F	11.18	0.78	6.40	1.13	27.64	2.66	65.96	3.37
8 (n = 2)	none	1.90	0.59	0.74	1.27	0.95	0.85	70.71	8.00
8 (n = 4)	T / F	2.18	0.88	3.20	6.37	1.89	2.15	85.93	11.48
12 (n = 2)	none	2.95	1.12	5.00	8.62	2.42	2.12	85.61	14.21
12 (n = 4)	T / F	3.50	1.23	2.44	3.90	2.22	3.00	93.68	7.28

Table 1. Composition of cross-sections through the middle region of the periosteal pockets. T/F indicates the inclusion of TGF-β1 and b-FGF within the injected alginate gel formulation.

The introduction of exogenous growth factors TGF-β1 and b-FGF into the pocket appears to have no significant bearing on the final formation of neo-bone. However, the degree of angiogenesis, a process associated with the early stages of osteogenesis, is an order of magnitude greater at 6 weeks upon incorporation of these growth factors. The increased angiogenesis could prove beneficial in patients where the viability of the periosteum is diminished due to age or other disease related complications.

An H & E stain of a cross-section from the edge of the periosteal pocket to the bone marrow cavity after 6 weeks *in vivo* is shown in Figure 2. The demarcation between the original cortical bone of the tibia and the fully vascularized neo-tissue formed in the artificial pocket is clearly apparent. The lacunae, canaliculi and haversian systems of the neo-tissue, which is easily identified as osseous in

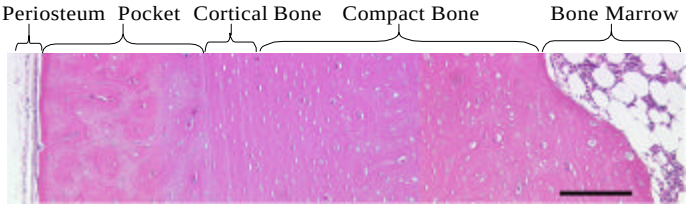

Figure 2. H & E stained cross-section from the edge of the artificial periosteal pocket to the central bone marrow cavity of the tibia after 6 weeks *in vivo.* Scale Bar: 300 μm

origin can be further visualized with a thionin stain. It can be seen from Figure 2, that there are some areas of less mature, woven bone within the neo-tissue as indicated by a lighter staining. However, after a period of 8 weeks, the neo-tissue stains both more intensely and uniformly indicating the development of a mature osseous tissue with a cellularity and blood vessel distribution analogous to compact bone.

DISCUSSION

We have demonstrated that an "*In Vivo* Bioreactor" concept can be applied in the regeneration of large quantities of fully vascularized compact bone in a skeletally mature rabbit model. We believe that this paradigm is not limited to neo-synthesis of bone but can be extended to the engineering of cartilage with proper manipulation of the pocket environment. In fact at early time points (10 days), we have observed the presence of hypertrophic chondrocytes in regions of the pocket (Figure 3).

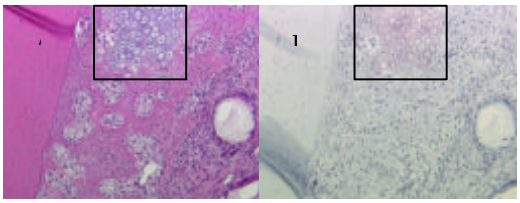

Figure 3. H&E (A) and safranin-O (B) stained section though the periosteal pocket after 10 days. The box indicates the area of neo-tissue occupied by hypertrophic chondrocytes.

The success of this novel approach maybe attributed to the fact that the patients' body is used as the scaffold and bioreactor, thus maximizing the role and impact of the healing process in defining the microenvironment. This paradigm could prove very powerful in engineering large quantities of human mature bone for autologous transplantation, which has proven to be elusive with conventional approaches. A major advantage of this paradigm is that the patient's own cells are utilized to engineer/regenerate a tissue mass, thus avoiding potential problems of immune rejection and eliminating the need for harvesting and *in vitro* culturing of cells.

ACKNOWLEDGEMENTS

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