Biomaterials 112 (2017) 313–323

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Repair of bone defects *in vivo* using tissue engineered hypertrophic cartilage grafts produced from nasal chondrocytes

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ARTICLE INFO

Article history:
Received 7 March 2016
Received in revised form
3 October 2016
Accepted 11 October 2016
Available online 11 October 2016

Keywords:
Bone tissue engineering
Nasal chondrocytes
Bone graft
Hypertrophic cartilage
Bone regeneration
Maxillofacial reconstruction

ABSTRACT

The regeneration of large bone defects remains clinically challenging. The aim of our study was to use a rat model to use nasal chondrocytes to engineer a hypertrophic cartilage tissue which could be remodelled into bone *in vivo* by endochondral ossification.

Primary adult rat nasal chondrocytes were isolated from the nasal septum, the cell numbers expanded in monolayer culture and the cells cultured *in vitro* on polyglycolic acid scaffolds in chondrogenic medium for culture periods of 5–10 weeks. Hypertrophic differentiation was assessed by determining the temporal expression of key marker genes and proteins involved in hypertrophic cartilage formation. The temporal changes in the genes measured reflected the temporal changes observed in the growth plate. Collagen II gene expression increased 6 fold by day 7 and was then significantly downregulated from day 14 onwards. Conversely, collagen X gene expression was detectable by day 14 and increased 100-fold by day 35. The temporal increase in collagen X expression was mirrored by increases in alkaline phosphatase gene expression which also was detectable by day 14 with a 30-fold increase in gene expression by day 35. Histological and immunohistochemical analysis of the engineered constructs showed increased chondrocyte cell volume (31–45 μm), deposition of collagen X in the extracellular matrix and expression of alkaline phosphatase activity. However, no cartilage mineralisation was observed in *in vitro* culture of up to 10 weeks. On subcutaneous implantation of the hypertrophic engineered constructs, the grafts became vascularised, cartilage mineralisation occurred and loss of the proteoglycan in the matrix was observed. Implantation of the hypertrophic engineered constructs into a rat cranial defect resulted in angiogenesis, mineralisation and remodelling of the cartilage tissue into bone. Micro-CT analysis indicated that defects which received the engineered hypertrophic constructs showed 38.48% in bone volume compared to 7.01% in the control defects.

Development of tissue engineered hypertrophic cartilage to use as a bone graft substitute is an exciting development in regenerative medicine. This is a proof of principal study demonstrating the potential of nasal chondrocytes to engineer hypertrophic cartilage which will remodel into bone on *in vivo* transplantation. This approach to making engineered hypertrophic cartilage grafts could form the basis of a new potential future clinical treatment for maxillofacial reconstruction.

1. Introduction

Bone is the second most transplanted tissue after blood. Despite this, the repair of large bone defects, whether congenital in nature or caused by trauma or disease remains clinically challenging [1]. Crucially, survival of transplanted bone tissue into a bone defect is dependent on the tissue receiving adequate blood supply to supply essential oxygen and nutrients and removal of waste products. Currently, autologous bone is the clinical 'gold standard' for bone tissue transplantation. Autologous bone is a living tissue with osteoconductive and osteoinductive properties which will not be rejected by the patient's immune system. There are, however, drawbacks to using autologous bone tissue including lasting post-

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http://dx.doi.org/10.1016/j.biomaterials.2016.10.014
0142-9612/

operative pain from the donor site and donor site morbidity [2]. There are also limitations in the amount of healthy tissue that can be taken from a patient without causing instability in the remaining bone structures, an issue particularly pertinent in children [2]. Bone allografts and xenografts are used for bone repair, but these have to be decellularised to reduce their immunogenicity and as a consequence, are osteoconductive but have little osteoinductive capacity. Similarly, to date, current biomaterial bone graft substitutes have good osteoconductive properties but their osteoinductive properties are limited. Therefore, there is a strong clinical need to develop new bone graft materials for the repair and regeneration of bone defects. Tissue engineering and regenerative medicine technologies are promising 'tools' to generate bone graft substitutes to fill this demand.

Most tissue engineering approaches have focussed on producing bone tissue by the route of intramembranous ossification in which mesenchymal stem cells are differentiated directly into osteoblastic cells to produce a bone-like extracellular matrix directly. One of the main challenges of bone tissue engineering by this route is the provision of a sufficient supply of nutrients and oxygen essential for the survival of the osteoblastic cells. During the culture of large three-dimensional tissue constructs, limited diffusion can cause problems in cell proliferation, differentiation and cell survival. Approaches to overcome these limitations include use of bioreactors [3,4] to provide dynamic culture conditions and engineering prevascularised grafts by including endothelial cells with differentiation signals to stimulate formation of blood capillaries [5].

Developmental tissue engineering, mimicking the natural pathways of tissue formation in the body is a potential route to producing graft tissues for transplantation. Endochondral ossification is responsible for the formation of the axial skeleton and long bones [6] during embryogenesis and, is a potential and as of yet largely unexplored pathway of producing a suitable, alternative graft material for bone repair. Endochondral ossification involves the formation of a hypertrophic cartilage template which undergoes mineralisation, invasion by blood vessels and subsequent remodelling into bone. Hypertrophic cartilage formation and its remodelling into bone by endochondral ossification can be seen in the growth plate of young children and adolescents during periods of active growth and in the adult during healing of bone fractures where the 'soft' cartilage callus undergoes ossification into woven bone. In the growth plate, proliferative columnar chondrocytes produce a complex hyaline-cartilage extracellular matrix (ECM), in which the predominant protein is collagen type II. These chondrocytes then undergo terminal differentiation to the hypertrophic chondrocyte phenotype and produce collagen type X in the ECM which is essential for mineralisation [7,8].

Cartilage constructs have several advantages over the more extensively researched bone tissue constructs formed by intramembranous ossification. Cartilage can withstand lower oxygen concentrations than bone tissue containing osteoblasts and mesenchymal stem cells [9]. This advantage of cartilage allows for both the culture of larger grafts, as well as enabling better survival of the cartilage graft once implanted at the site of injury. Hypertrophic cartilage also contains the required biological factors to promote matrix remodelling [10], angiogenesis [11,12], and bone formation [13], within the tissue. Therefore, once implanted *in vivo,* hypertrophic cartilage should recruit blood vessels and be remodelled into bone tissue via the normal physiological process of endochondral ossification. Hence, the potential of utilising tissue engineered hypertrophic cartilage warrants further research.

There has been widespread interest in the use of mesenchymal stem cells in regenerative medicine and tissue engineering for repair/regeneration of hyaline cartilage defects in the body; for example regeneration of articular cartilage in joints [14,15] and cartilage defects in the head and neck area [16] for example, the nasal septum [17,18]. In comparison, there has been little research focussed on engineering hypertrophic cartilage. Scotti et al. demonstrated that bone marrow mesenchymal stem cells (BM-MSCs) in pellet culture were able to differentiate into hypertrophic-like chondrocytes which expressed both collagen type X and alkaline phosphatase and mineralised on subcutaneous implantation [7]. Scotti et al. also reported that seeding BM-MSCs on a shaped collagen scaffold followed by chondrogenic priming, formed a mineralised trabecular-like bone on subcutaneous implantation [19]. Sheehy et al. [20] have also recently reported that BM-MSCs encapsulated in a shaped hydrogel structure formed layer of mineralised tissue in the periphery of the constructs after *in vitro* chondrogenic priming followed by 8weeks of subcutaneous implantation. While mesenchymal stem cells have been approved for clinical use [21], other adult cells may offer an advantageous source for the production of a hypertrophic cartilage. Mesenchymal stem cells have been shown to produce cartilage with lower levels of ECM and reduced mechanical strengths when compared to adult chondrocytes [22,23]. Primary adult hyaline chondrocytes, however, have been shown to produce high levels of GAG in the ECM when cultured *in vitro*, for the production of hyaline cartilage [22,24].

In terms of using chondrocytes to form hypertrophic cartilage, previous research has shown that immortalised adult articular chondrocytes become hypertrophic and mineralised in pellet culture [25]. Also, immortalised chondrocytes derived human embryonic femurs were reported to produce osteoinductive signals in the culture medium [26]. There are, however, significant regulatory and safety concerns associated with the use of genetically modified cells for clinical applications. Two sources of adult chondrocytes, nasal septum and rib cartilage, have potential as sources of adult hyaline chondrocytes which can undergo terminal differentiation to the hypertrophic phenotype. Both tissues have capacity *in vivo* for the cartilage to undergo endochondral ossification; for example, where the nasal cartilage is attached to the facial bone. Also, both tissues can be harvested clinically by biopsy. The nasal septum is potentially a simpler biopsy procedure with a lower risk of donor site morbidity. In addition, chondrocytes extracted from the nasal septum have been broadly studied in tissue engineering for their capacity to produce a hyaline cartilage matrix [24,27–29] and have been used clinically for repair of septal defects [17]. Only one detailed *in vivo* study to date has considered the use of nasal chondrocytes for tissue engineering a hypertrophic cartilage template for bone regeneration [30]. Here engineered constructs did not mineralise following sub-cutaneous implantation, but were capable of some mineralisation following osteogenic pre-culture and implantation within ceramic scaffolds. More recently, a population of nasoseptal chondrocytes with progenitor features were described by Rotter and co-workers [31].

The overall aim of this study was to investigate whether chondrocytes isolated from the nasal septum, a potentially clinically relevant cell source, could produce a hypertrophic cartilage tissue which would remodel into bone on transplantation into a bone defect *in vivo*. Our first objective was to use a rat model in which to isolate and culture nasal chondrocytes and investigate whether the chondrocytes would produce a hypertrophic cartilage matrix in 3D culture using a poly-L-glycolic acid (PGA) scaffold support. Chondrocytes were also isolated from the rib and cultured in the same way as a comparator. The second objective was to implant the engineered hypertrophic cartilage subcutaneously *in vivo* to investigate biocompatibility, and angiogenesis in the implant. The engineered hypertrophic cartilage was also implanted in an *in vivo* bone defect in the calvaria of the rat to determine whether the cartilage would undergo remodelling into bone.