*Key Engineering Materials* *Online: 2003-12-15*
*ISSN: 1662-9795, Vols. 254-256, pp 817-820*
*doi:10.4028/www.scientific.net/KEM.254-256.817*


# Chondrocyte Maturation on Biphasic Calcium Phosphate Scaffold: A Preliminary Study

Cristina C. Teixeira[1], Racquel Z. LeGeros[2], Claudia Karkia[2], and Yelena Nemelivsky[1]

[1]Department of Basic Science & Craniofacial Biology, New York University College of Dentistry, NY, USA, ct40@nyu.edu

[2]Department of Biomaterials, New York University College of Dentistry, NY, USA, rzl1@nyu.edu

**Keywords:** chondrocytes, biphasic calcium phosphate, maturation, collagen, alkaline phosphatase

**Abstract.** In recent years artificial bone replacement materials have been developed that can be coated with growth factors and loaded with osteoblasts to induce osteogenesis. All these attempts from the bone tissue engineering field disregard the fact that skeletal development can proceed by two pathways: intramembranous or endochondral bone formation. Here we propose to mimic nature by creating an *in vitro* cartilage template that could after implantation *in vivo*, remodel into bone, as is normally carried out during the process of endochondral bone formation. The specific aims of this study are: (1) to establish a method of growing chondrocytes in a well characterized biphasic calcium phosphate (BCP) scaffold, and (2) to induce chondrocyte hypertrophy and cartilage matrix deposition in cells growing in the BCP scaffold. Materials and Methods: Chondrocytes isolated from 18-day chick embryo tibial growth plates were grown on particles of macroporous biphasic calcium phosphate (MBCP®, *Biomatlante, France*). Chondrocytes were grown in culture continuously for 3 weeks, and fed every day with Dulbecco's modified Eagle medium (GIBCO) containing 10% Nu serum (Fisher), 2mM L-glutamine, 100 U/ml penicillin/streptomycin, and 50 μg/ml ascorbic acid. After one week, cultures were treated daily with 100 nM all trans-retinoic acid (RA), to induce chondrocyte maturation, and extracellular matrix synthesis. Chondrocyte proliferation was observed using scanning electron microscopy (SEM) on appropriately prepared specimens. Alkaline phosphatase (AP) activity was measured spectrophotometrically. Levels of proteoglycans and levels of type X collagen were determined using Alcian blue staining and Western blot analysis, respectively. Results: Chondrocytes attached, and proliferated on the BCP scaffold. Levels of AP, proteoglycans, and type X collagen increased in the presence of RA. An unexpected observation was made regarding changes in chondrocyte morphology in the presence of RA. Chondrocytes grown on BCP scaffold assumed an elongated morphology in an intricate net of collagenous proteins, while cells grown on tissue culture plate surfaces maintained a polygonal and flat morphology. Conclusion: Results from this study demonstrated for the first time the proliferation, maturation of chondrocytes, and cartilage matrix deposition on a macroporous calcium phosphate scaffold, and the potential of such a scaffold in tissue engineering through the endochondral bone formation mechanism.

## Introduction

Bone grafting has been used for many years to restore areas deficient in bone due to trauma, growth defects or pathology. Human derived bone grafts were historically the only osteoinductive replacement materials, providing cellular elements and growth factors necessary for osteogenesis. In recent years, a number of artificial bone replacement materials have been developed that can be coated with growth factors and loaded with osteoblasts to induce bone formation after implantation *in vivo*. Until now, the focus has been to engineer bone grafting materials from direct differentiation of osteoprogenitor cells, all attempts geared toward the creation of intramembranous bone, disregarding the fact that there are two distinct mechanisms of skeletal development: intramembranous and endochondral bone formation [1]. Intramembranous bone forms by differentiation of mesenchymal cells directly into osteoblasts, while during endochondral ossification there is the gradual and partial replacement of a cartilage model by bone. The residual cartilage acts as a growth plate or an articular surface. The long bones, pelvis, vertebral column, base of the skull and mandible are formed through the endochondral mechanism [1].

Here, we propose to mimic nature, by creating a cartilage template of the correct size and shape that, after implantation *in vivo*, will remodel into the required bone, as is normally carried out in the body during the process of endochondral bone formation. Our approach to the field of bone tissue engineering, presents significant advantages including the resistance of chondrocytes to low oxygen [2, 3] and the fact that these cells provide the signaling system for vascular invasion and osteogenesis [4, 5]. These characteristics will allow the generation of an improved osteoconductive and osteoinductive scaffold. By successful inducing endochondral bone formation from an artificially created cartilage model, we will be able to address situations where current bone grafting techniques have failed to correct severe bony defects. In the future, a large cleft palate, a severely resorbed dental alveolar ridge, or a large bone defect caused by cancer, may be repaired by placing a cartilaginous scaffold (artificially created) in the area, to provide the template and induce new bone formation. This new bone will be indistinguishable from natural bone, will present the same properties, respond to loads and have the potential to grow, just as the patient own bone.

There are two specific aims in the current investigation: 1) to establish a method of growing chondrocytes in a well characterized BCP scaffold, 2) to induce chondrocyte maturation and cartilage matrix deposition in cells growing in the BCP scaffold.

## Materials and Methods

The material we chose to use in this project is macroporous biphasic calcium phosphate (Biomatlante France) consisting of a mixture of 60% Hydroxyapatite and 40% ß-tricalcium phosphate. This material has been extensively characterized and shown to be biocompatible and an adequate bone replacement scaffold [6-8]. Chondrocytes were isolated from 18 day chick embryo tibia, using a method described by Rajpurohit et al [2] and plated in flat bottom polystyrene 100mm tissue culture dishes. After one week, chondrocytes were sub cultured (0.125 million cells/well) into 24-well tissue culture plates containing the BCP particles, no attempt being made to seed the cells into the scaffold. Chondrocytes were grown continuously without further subculturing for 3 weeks at a temperature of $37^{o}$ C and at a 5% carbon dioxide atmosphere. Cultures were fed every day with Dulbecco's modified Eagle medium (GIBCO) containing 10% Nu serum (Fisher), 2mM L-glutamine, 50 µg/ml ascorbic acid, and 100 U/ml penicillin/ streptomycin. After one week, cultures were treated daily with 100 nM all-trans retinoic acid to induce chondrocyte maturation, and matrix synthesis. To access chondrocyte attachment and proliferation in the BCP scaffold, the samples were examined by scanning electron microscopy at the end of each week, and photographed. To evaluate chondrocyte maturation, we investigated the expression of two important markers of chondrocyte hypertrophy: alkaline phosphatase activity and type X collagen protein levels. Alkaline phosphatase activity was measured using a method described by Leboy et al [9], and western blot analysis was performed using an antibody against chick type X collagen. Protein content in each sample was analyzed using the DC Protein Assay (BioRad, CA) according to manufacturers instructions. In addition, AP staining and Alcian blue staining were performed to visualize levels of this enzyme activity and proteoglycans deposited on the BCP scaffold.

**Results**

Results show that chondrocytes attach and divide on the BCP scaffold. Scanning electron microscopy demonstrates that at the end of the first week in culture chondrocytes became confluent or almost confluent on the scaffold. After 3 weeks in culture, a continuous layer of polygonal chondrocytes covered the BCP particle surface and pores (data not shown). Once it was established that chondrocytes could attach and proliferate in the BCP surface, cell maturation was induced by adding retinoic acid (RA) to the cultures. Chondrocytes were allowed to proliferate for one week before treatment with 100 nM RA was initiated. After two weeks of daily RA treatment, BCP particles were collected and used for different analysis. Macroscopic analysis was performed, by staining the beads to visualize both proteoglycan production and AP activity. RA treatment caused a marked increase in AP activity evidenced by deep red coloration. An increase in proteoglycans deposition on the extracellular matrix was characterized by the presence of an intense blue staining (data not shown). The level of AP activity was also measured by spectophotometry. A significant increase in the alkaline phosphatase activity was observed in the presence of RA in the culture media, and this increase in enzymatic activity was dependent on the duration of treatment (Fig. 1).

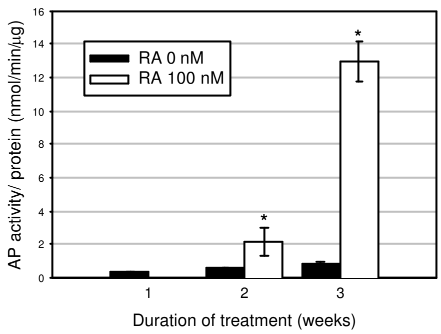


Fig. 1: Effect of retinoic acid on alkaline phosphatase activity. Tibial chondrocytes growing on BCP were treated with RA (100 nM) for 2 weeks, and chondrocytes collected for alkaline phosphatase analysis. Note that RA causes a time dependent increase in the enzymatic activity. * Significantly different from control. (RA 0nM)

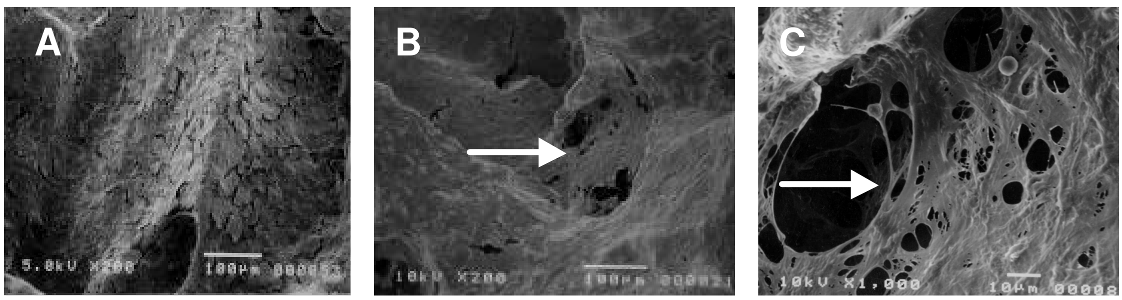


Fig. 2: Scanning electron microscopy of chondrocytes after 3 weeks in culture. Chondrocytes were grown to confluence for 1 week, and then treated with RA for 2 additional weeks. The samples were fixed and dehydrated, sputter coated with gold, viewed using SEM (Joel JSM 5400, Tokyo, Japan) and photographed. Note that the cells grown in the presence of 100nM of RA (B,C) secreted a matrix rich in collagen fibers (arrow in C) that even covered the pores (arrow in B). I n the absence of RA (A) the chondrocytes presented the characteristic polygonal shape and formed a thin cellular layer.

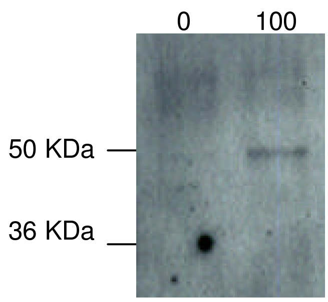


Fig. 3: Western Blot analysis of type X collagen in tibial chondrocytes grown on BCP. Tibial chondrocytes were treated with RA (0 and 100 nM) for 2 weeks. After extraction, proteins were transferred onto a nitrocellulose membrane and immunostained with antibody against type X chick collagen. Note that RA caused an increase in the protein level of type X collagen in chondrocytes.

Scanning electron microscopy performed at the end of the 3rd week in culture showed that in the absence of RA, chondrocytes form a confluent layer over the BCP surface (Fig. 2A) with a polygonal and flat cell morphology. Treatment with the retinoid induced formation of a thick extracellular matrix over the particle surface (Fig. 2B) and over the pores (arrow in Fig. 2B). Chondrocytes are embedded in this matrix composed of numerous fibers creating an intricate net of collagenous proteins (white arrow on Fig. 2C). Western blot analysis confirmed an increase in the protein level of type X collagen when chondrocytes were treated with the retinoid (Fig. 3).

## Discussion

While attempts to create an in vitro cartilage scaffold have been geared towards articular cartilage replacement therapies, here we propose the creation of an *in vitro* calcified cartilage scaffold as a bone replacement material. To the best of our knowledge, this is the first report of chondrocytes being grown on a mineralized scaffold, specifically BCP. This material has been used as a scaffold for bone grafting but always using osteoblasts as the cell precursor. Over the last 25 years calcium phosphate materials have been well tested clinically in craniofacial bone replacement therapies [10-12]. We reasoned that due to its characteristics, the material would be a suitable template for endochondral bone formation, by first allowing attachment and maturation of chondrocytes, and later supporting and inducing osteoblast ingrowth. Experiments described here reveal that indeed chondrocytes attach and proliferate on the BCP scaffold. After three weeks of continuous cell culture, the beads became completely covered by a continuous layer of cells and extracellular matrix. To test the hypothesis that chondrocytes grown in an already mineralized surface can undergo hypertrophy and maturation, we treated the cells with retinoic acid. Our results show an increase in 2 important markers of maturation, AP activity and type X collagen expression, in the presence of this agent.

In conclusion, we have successfully established a method of growing and inducing chondrocyte maturation on a mineralized template, BCP. Further studies will be conducted to test the suitability of this template as an intermediate in endochondral bone formation.

## References

[1] S.D. Howell, and D.D. Dean: Disorders of Bone and Mineral Metabolism (1992), p. 313-353, Raven Press.
[2] R. Rajpurohit, C.J. Koch, Z. Tao, C.C. Teixeira, and I.M. Shapiro: Journal of Cellular Physiology 168 (1996), 424-432.
[3] E. Schipani, H.E. Ryan, S. Didrickson, T. Kobayashi, M. Knight, and R.S. Johnson: Genes Dev 15 (2001), 2865-76.
[4] C. Maes, P. Carmeliet, K. Moermans, I. Stockmans, N. Smets, D. Collen, R. Bouillon, and G. Carmeliet: Mech Dev 111(2002), 61-73.
[5] W. Petersen, M. Tsokos, and T. Pufe: J Anat 201 (2002), 153-7.
[6] R.Z. LeGeros, J.R. Parsons, G. Daculsi, F. Driessens, D. Lee, S.T. Liu, S. Metsger, D. Peterson, and M. Walker: Ann N Y Acad Sci 523 (1988), 268-71.
[7] G. Daculsi, N. Passuti, S. Martin, C. Deudon, R.Z. Legeros, and S. Raher: J Biomed Mater Res 24 (1990), 379-96.
[8] R.Z. LeGeros: Clin Mater 14 (1993), 65-88.
[9] P.S. Leboy, L. Vaias, B. Uschmann, E. Golub, S.L. Adams, and M. Pacifici: Journal of Biological Chemistry 264 (1989), 17281-17286.
[10] T.E. Butts, L.J. Peterson, and C.M. Allen: J Oral Maxillofac Surg 47 (1989), 475-9.
[11] S.F. Hulbert, S.J. Morrison, and J.J. Klawitter: J Biomed Mater Res 6 (1972), 347-74.
[12] L.M. Wolford, R.W. Wardrop, and J.M. Hartog: J Oral Maxillofac Surg 45 (1987), 1034-42.