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Chondrocyte Cell Culture and Tissue Engineering on BD BioCoat™ Osteologic™ Discs and BD™ 3D Calcium Phosphate Scaffolds

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Introduction

In addition to culturing bone cells, BD BioCoat™ Osteologic™ Discs and MultiTest Slides provide an excellent substrate for the growth of chondrocytes. Chondrocytes play a vital role in the development and healing of bone as well as other structural tissues in the body. A major structural function of cartilage is at the articulating surfaces of joints, especially the knees, where the unique mechanical load bearing properties of cartilage allow human mobility on a few millimeters of tissue.

Given the low population of cells in functional cartilage, it is difficult to stimulate the tissue to repair once damaged. This characteristic has made cartilage, and the chondrocytes which comprise this tissue, a focus of tissue engineering research for viable solutions. The *ex vivo* culture of chondrocytes for tissue repair is, therefore, a viable option for the treatment of this problem. However, chondrocytes can be difficult to culture and are prone to dedifferentiation following several passages. In the following examples, human and porcine chondrocytes were cultured on BD BioCoat Osteologic Discs and BD™ 3D Calcium Phosphate (BD™ 3D CaP) Scaffolds respectively, to demonstrate the biocompatibility and growth characteristics of this unique biomaterial for the study of chondrocytes.

Skelite™ Bone Biomaterial from Millenium Biologix Inc. is a 100% synthetic, multi-phase calcium phosphate-based material that exhibits excellent biocompatibility and is ideally suited for supporting the proliferation and differentiation of numerous cell types. The BD 3D CaP Scaffold is a

porous format of Skelite™ that has an open pore reticulated structure and possesses the ideal pore size and porosity for *in vitro* and *in vivo* studies. BD BioCoat Osteologic is a thin film format of the Skelite bone biomaterial.

2D Cell Proliferation on BD BioCoat™ Osteologic™ Discs and MultiTest Slides

Method

Human chondrocytes isolated from sequential enzymatic digestion of a knee biopsy were cultured in DMEM/F12 medium (Gibco Cat. No. 11320-033) supplemented with 10% FBS and Penicillin/Streptomycin, 100 U/100 µg/mL (Gibco Cat. No. 15140-122). Cells were passaged once in BD Falcon™ 75 cm² flasks, harvested and seeded onto BD BioCoat Osteologic Discs in 24-well plates at 1×10^4 cells per well at Passage 2 (P2). Control cells were seeded directly into plastic wells without discs on the same plate. Parallel plates were prepared for a time course study with cell counts taken at 0, 2, 4 and 7 days. Cells were trypsinized and counted by hemocytometer using the Trypan blue method. A second set of plates were plated with the same cells after culturing in flasks for an additional Passage 3 (P3). Plates were cultured for 7 days, fixed with 1% glutaraldehyde in PBS, and then stained using a combination of Periodic Acid Schiff (PAS) and Alcian blue for detection of proteoglycans (Figure 2).

Results

P2 Chondrocytes

P2 human chondrocytes cultured on BD BioCoat Osteologic Discs exhibited elevated levels of proliferation when compared to that cultured on standard TC plastic (Figure 1). The BD BioCoat Osteologic Discs supported rapid log phase growth between 4 and 7 days in culture. At day 7, the cell number associated with the BD BioCoat Osteologic Discs was 2.5 fold greater than that observed with the TC plastic. This increase was achieved in the absence of additional growth factors.

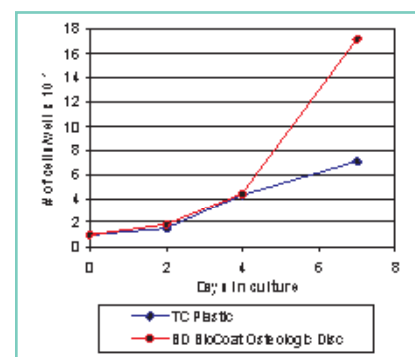


Figure 1: Growth curve of primary human articular chondrocytes (P2) cultured on BD BioCoat Osteologic Discs and standard TC plastic. Cell counts by Trypan blue exclusion method at day 0, 2, 4 and 7.



Results (continued)

P3 Chondrocytes

P3 human chondrocytes cultured for 7 days on BD BioCoat™ Osteologic™ Discs exhibited elevated growth rates and higher culture density when compared to that cultured on standard TC plastic (Figure 2). Cells grown on TC plastic appear fibroblastic compared to the high density growth observed on BD BioCoat Osteologic Discs. Chondrocyte proteoglycan staining associated with the BD BioCoat Osteologic Discs was dramatically increased when compared to that observed on standard TC plastic. Cultures on TC plastic did not reach confluence and appeared to be growth limited. No additional growth factors were added to the media.

Conclusions

BD BioCoat Osteologic Discs and MultiTest Slides are an excellent substrate for the culture and study of human chondrocytes. Growth on these disks is superior to traditional TC plastic substrates. Proliferation of later passage cells is enhanced to the point of high-density confluence and extracellular matrix production of chondrocyte proteoglycans.

The convenient BD BioCoat Osteologic Discs (for 24-well plates) and MultiTest Slides (16-well format) allow for multiple sample study, culture optimization or drug development methodologies.

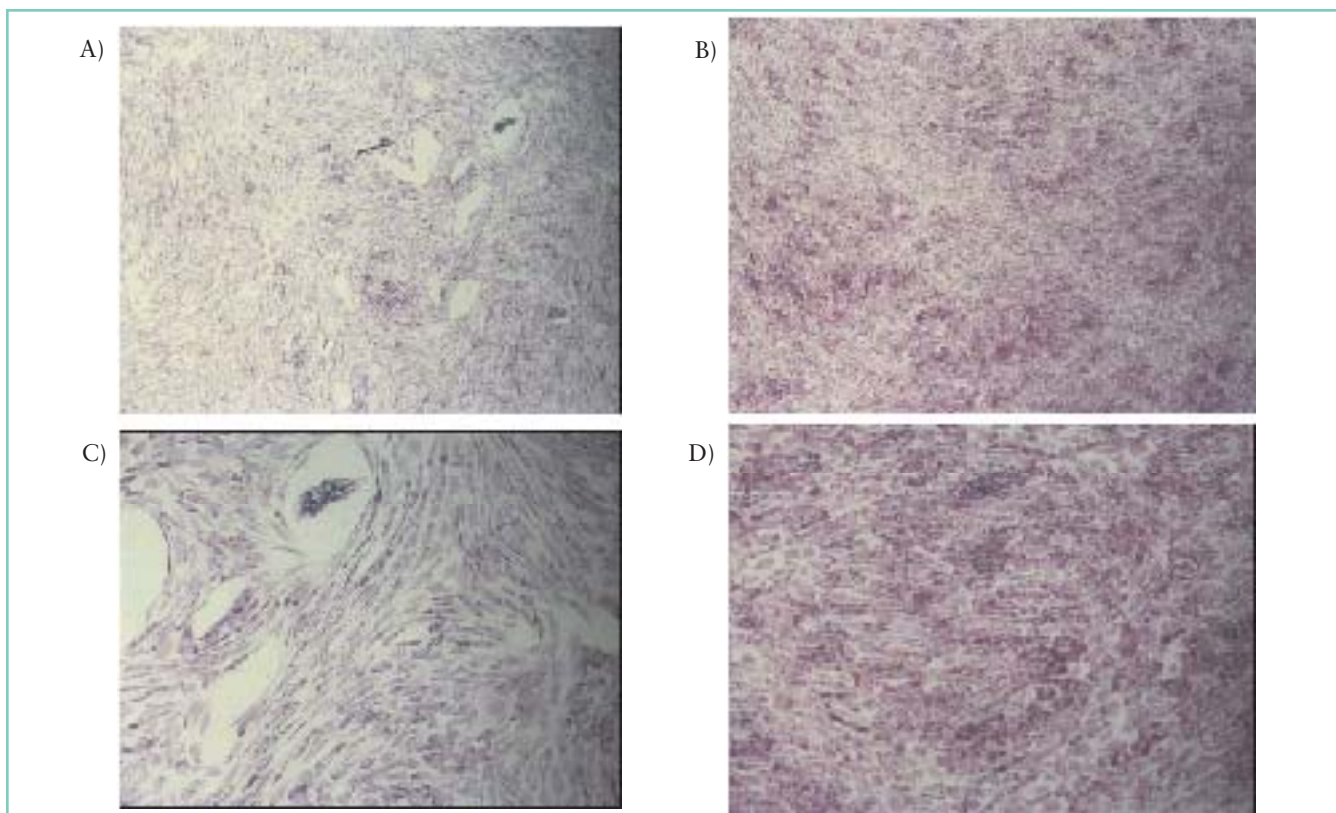


Figure 2: Growth of P3 human chondrocytes on TC plastic vs. BD BioCoat Osteologic Discs. Chondrocytes were stained with PAS and Alcian blue at day 7. (A) low power magnification of human chondrocytes on plastic, (B) same cells grown on BD BioCoat Osteologic Discs, (C) and (D) higher magnifications of human chondrocytes cultured on TC plastic and BD BioCoat Osteologic Discs, respectively.

3D Culture on BD™ 3D Calcium Phosphate Scaffold

Method

Porcine chondrocytes isolated from sequential enzymatic digestion of a knee biopsy were cultured in DMEM/F12 medium (Gibco Cat. No. 11320-033) supplemented with 10% FBS as prepared previously for 2D cultures on thin film. Cells were seeded onto a custom BD 3D CaP scaffold as a high-density culture. The construct was cultured for 4 weeks in medium supplemented with added insulin and ascorbic acid.

Histological Sectioning

Constructs were fixed in 10% neutral buffered formalin and stained with Trypan blue as a counter stain for photography before mineralized histological sectioning of the Scaffold. Trypan blue was washed away with several changes of PBS before embedding.

The specimen was dehydrated in ascending grades of ethanol and cleared with xylene. These steps were done in a Shandon automatic tissue processor. The specimen was then transferred into a solution of methylmethacrylate (MMA) and put in a glass desiccator at 4°C under constant vacuum for 3 days.

The specimen was embedded in a fresh solution of MMA in a glass vial and polymerized at 30°C in a dry incubator for approximately 3 days. Once poly-merized, the block was trimmed to remove excess plastic with an industrial vertical band saw and cut along its long axis with a diamond band saw (EXAKT standard saw). Ground polished sections of 20 µm thickness were made using the EXAKT micro grinder system from EXAKT Technologies, Inc., Oklahoma City, OK. Sections were stained with Toluidine blue for histological examination.

continued

Results

Porcine articular chondrocytes cultured on the BD™ 3D CaP scaffold resulted in a thick layer of cartilage-like tissue intimately and completely (without any splitting or non-attachment zones) covering the top of the implant (*Figure 3a*).

Histological ground sections (*Figure 3b*) indicate that the thick layer of cartilaginous-like tissue is composed of numerous round to ovoid cells, which is consistent with a chondrocytic phenotype.

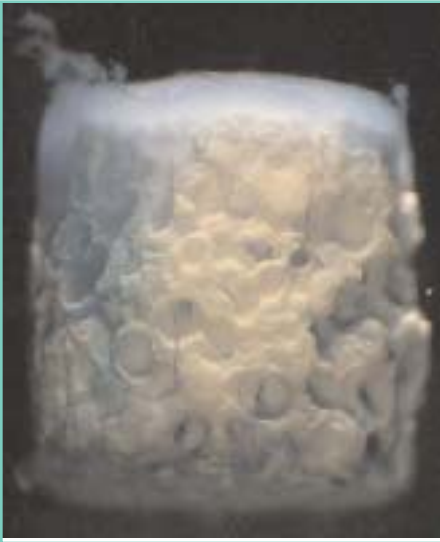


Figure 3a: Intact custom BD 3D CaP scaffold with 3D cartilage top layer.

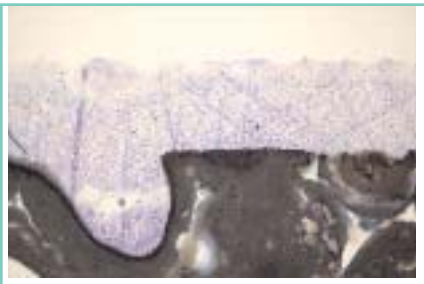


Figure 3b: 20 µm histology section of porcine chondrocytes on 3D Skelite scaffold, Toluidine blue stain.

BD 3D CaP Scaffold Physical and Biological Characteristics

Biocompatibility

Biocompatibility testing was performed in accordance with ISO Standard 10993. A summary of the conclusions from each test follows:

- 1. ISO MEM Elution using L-929 Mouse Fibroblast Cells (Cytotoxicity)**
The test article, Skelite™ Synthetic Bone Biomaterial, scored "0" at 24, 48 and 72 ± 4 hours and is considered non-toxic under the conditions of this test.
- 2. ISO Intracutaneous Reactivity Test (Irritation)**
Under the test conditions of this protocol, Skelite Synthetic Bone Biomaterial is considered a non-irritant.
- 3. ISO Acute Systemic Injection Test (Systemic Toxicity)**
These findings indicate that the requirements of the ISO Systemic Injection test have been met by Skelite Synthetic Bone Biomaterial.
- 4. In Vitro Genotoxicology Test (Bacterial Mutagenicity Test [Ames Assay])**
Based on the criteria of the assay the test article, Skelite Synthetic Bone Biomaterial, is considered non-mutagenic.
- 5. ISO Guinea Pig Maximization Sensitization Test**
Skelite Synthetic Bone Biomaterial is classified as Grade 1, weak sensitizer. In the Magnusson-Kligman model, weak sensitizers are not regarded as significant.
- 6. Materials Mediated Rabbit Pyrogen Test**
These results indicate that Skelite Synthetic Bone Biomaterial is non-pyrogenic.

Impurities

None of the trace elements identified in ASTM F1185-88 (i.e. As, Cd, Hg or Pb) could be detected within the Skelite Synthetic Bone Grafts using inductively coupled plasma spectroscopy (ICP).

Solubility and Degradation

Degradation of Skelite in a 0.1 mol tris(hydroxymethyl)aminomethane solution HCl buffered to a pH of 7.4 at 37°C is minimal with the solution concentration of Ca²⁺ and P⁵⁺ ions in the parts per million. With respect to the solubility of the hydroxyapatite component, the average equilibrium solubility product is 1.6×10^{-36} . Likewise, with respect to the solubility of the tricalcium phosphate component, the average equilibrium solubility product is 5.5×10^{-19} .

Preclinical Studies

The long term *in vivo* performance of Skelite Synthetic Bone Biomaterial was evaluated using critical size defects (2 cm) created in the ulnae of skeletally mature NZW rabbits. Autologous bone, harvested from the iliac crest, served as the contralateral control and the study was terminated at 12 weeks.

Both radiographic and histological results indicated an accelerated rate of healing associated with Skelite. Typically, defects treated with Skelite exhibited radiographic evidence of union at 6 weeks compared to 9 weeks for those defects treated with autologous bone. At termination, resorption of the Skelite implants was observed associated with active osteoclasts and active resorption pits. Additionally, all defects treated with Skelite consistently demonstrated substantial bone formation and an advanced state of healing.

Acknowledgements

Histology modified from **Method of Calcified Tissues Preparation**, G.R. Dickson (1984).

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