HISTOGENESIS IN THREE-DIMENSIONAL SCAFFOLDS *IN VITRO*

JULIE GLOWACKI, PHD AND SHUICHI MIZUNO, PHD
SKELETAL BIOLOGY LABORATORY, BRIGHAM AND WOMEN'S HOSPITAL

INTRODUCTION

Methods for repairing skeletal deficiencies now include cell-based technologies. Current research efforts aim to develop new approaches for generating tissues *in vitro* that would integrate *in vivo* focus on cell carriers or scaffolds and methods to maintain the phenotype of the cells and to promote 3D histogenesis. Towards these ends, we developed porous 3D collagen sponge scaffoldings to deliver different cells and to support histogenesis. In addition, we used a simple system of medium perfusion and a more complex computer-driven system for application of fluid pressure for histogenesis *in vitro*.

THREE-DIMENSIONAL CULTURE OF CELLS

Monolayer, or two-dimensional, culture of disaggregated, anchorage-dependent cells *in vitro* has allowed for progress in understanding regulation of cell differentiation, growth, and function as well as cell-to-cell interactions that modulate these processes. Because monolayer culture does not model for the architecture of tissues and organs, analysis of integrated function or dysfunction of tissues or organs requires a different mode of experimentation. One approach is to retain cellular distributions within a block of tissue and to maintain it intact in what is called "organ culture." The major challenge in organ culture is to assure sufficient gas and nutrient exchange in order to maintain viability of cells throughout the tissue mass. A second approach is to grow disaggregated cells to high density with a three-dimensional (3D) geometry. Organoid structures will grow if kept supplied with fresh medium either by gyration or perfusion. Use of scaffolds can enhance histogenesis. Various materials have been tested as scaffolds to support the growth of musculoskeletal cells, including collagen, hydrogels, resorbable synthetic polymers, and calcium phosphates. Our laboratory uses collagen fibers prepared as a porous lattice; these have superior biocompatibility, reproducibility, and low cost. We reported that some preparations of collagen should be avoided because of their incompatibility *in vivo*.[1]

Julie Glowacki, PhD is an Associate Professor in Orthopaedic Surgery at Harvard Medical School.

Shuichi Mizuno, PhD, Research Staff, Brigham and Women's Hospital Orthopaedics Research Laboratories, Boston.

Address correspondence to:

Julie Glowacki, PhD
Orthopaedic Research Laboratory
Brigham and Women's Hospital
75 Francis Street
Boston, MA 02115

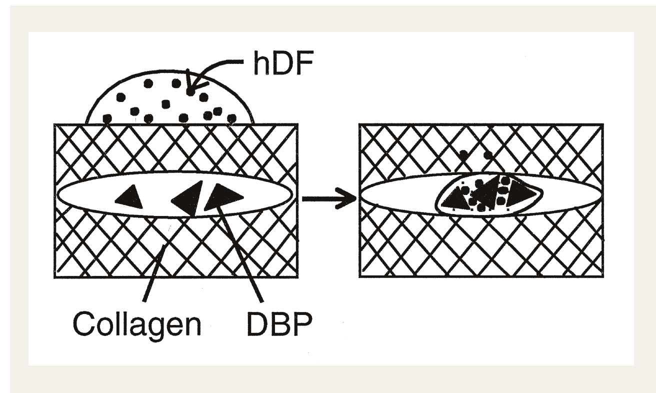

Figure 1. Representation of *in vitro* chondroinduction. Human dermal fibroblasts (hDF) are seeded on top of the collagen sponge which contains a filling of demineralized bone powder (DBP, 75-250 μm). The hDFs migrate through the collagen layer and those that invade the DBP are induced to produce cartilage matrix.

3D CULTURE OF CHONDROCYTES

Cartilage has been a model for research of *in vitro* engineered tissues on scaffolds because of its cellular homogeneity and avascularity.[2] On the other hand, chondrocytes require certain conditions to ensure that they maintain the features of differentiated, matrix producing cells when cultured *in vitro*.[3] Histochemical, immunohistochemical, and immunochemical analyses showed that cartilage matrix can accumulate in porous collagen sponges seeded with bovine articular chondrocytes.[4] Analysis of the cartilage-specific genes, aggrecan and collagen type II, have demonstrated preservation of the chondrocyte molecular signature compared to dedifferentiation in monolayer culture.[5] Preliminary investigations indicated that articular and endochondral chondrocytes retain their differences in these collagen sponges.[6]

The collagen sponge has been used to determine the effects of insoluble, soluble, and mechanical factors on chondrogenesis. For example, hyaluronan, another important constituent of cartilage matrix, was incorporated into the porous collagen sponges.[7] A small amount of hyaluronan (2% w/w) in 3D collagen scaffolds enhanced chondrogenesis, but a greater amount was found to be inhibitory. This finding lends support to the idea that biomimetic, or "smart" scaffolds can be developed with optimal signals for specific histogenesis.

CHONDROINDUCTION *IN VITRO*

Demineralized bone has been used clinically and in experimental animals to induce endochondral osteogenesis.[8] The first steps appear to be migration of nearby connective

tissue cells to the demineralized bone and their transdifferentiation into chondrocytes. In an attempt to identify the mechanisms of chondroinduction, we cultured human dermal fibroblasts in collagen sponges that contained particles of demineralized bone (Figure 1). One of the most critical parameters for chondroinduction is a high packing density of the demineralized bone.[1,9] Histochemical, immunohistochemical, and immunochemical evidence documented that after culture with demineralized bone, human dermal fibroblasts produced cartilage extracellular matrix (ECM)(4) and expressed cartilage-specific matrix genes.[8] Analysis of gene shifts prior to full expression of the cartilage matrix genes should reveal controlling genes that are specifically up-regulated by exposure to demineralized bone. Recently, a new method of comparing cellular expression of genes, Representational Difference Analysis (RDA), was used to distinguish a pool of genes that were up-regulated during the initial steps of chondroinduction of human fibroblasts.[10] The identified genes represented several functional classes, including cytoskeletal elements, protein synthesis and trafficking molecules, and transcriptional regulators. Some of the identified genes are novel and others are known sequences with unknown functions. Thus, the culture system has the power to reveal important changes that forerun chondroblastogenesis.

3D culture of human marrow cells

Bone marrow contains adherent cells that give rise to osteoblasts and non-adherent cells that give rise to osteoclasts. Marrow was obtained during the course of total hip arthroplasty from 39 men aged 37-86 years.[11] Cells were cultured in porous collagen sponges and were assessed for alkaline phosphatase activity, as a marker of osteoblast differentiation. A quantitative, competitive reverse transcription-polymerase chain reaction (RT-PCR) assay showed that patients ≤ 50 years of age had 3-fold more mRNA for alkaline phosphatase than those ≥ 60 years of age ($p = 0.021$). Pearson correlation indicated a significant decrease in mRNA for alkaline phosphatase with age ($r = -0.78$, $p = 0.028$). These molecular and histoenzymatic data suggest that the osteogenic potential of human bone marrow cells decreases with age.

Different culture conditions are needed for the differentiation of bone-resorbing osteoclasts. Recently, it was shown that osteoclastogenesis is inhibited by a product of osteoblasts or marrow stromal cells, called osteoprotegerin (OPG). We tested whether age influenced OPG expression using bone marrow cells from 18 subjects, aged 38-84 years.[12] Expression of OPG in the younger group was 500% greater than in the older group ($p = 0.034$). Decline in the expression of OPG with age may increase the capacity of stromal/osteoblast cells to support osteoclastogenesis.

Human marrow stromal cells also have the capacity to differentiate into chondrocytes. Molecular analysis of chondrocyte and adipocyte genes expressed in marrow cells cultured in 3-D collagen sponges showed that TGF-β1 promoted chondrogenesis and inhibited adipogenesis.[13]

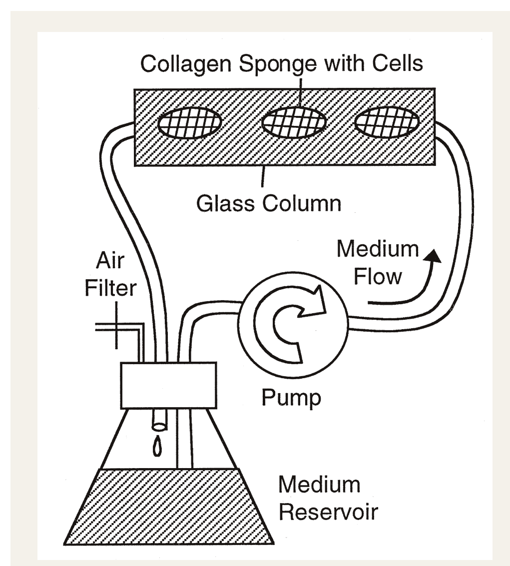

Figure 2. Schematic of the system to perfuse medium through the porous collagen sponges. Sponges are suspended in medium in a glass chromatography column. A peristaltic pump perfuses medium through the system. All components except the pump are held in a 37°C incubator.

Effects of Medium Perfusion on Histogenesis

Once cells proliferate within 3D scaffolds, dense aggregates of cells and the accumulation of ECM may impede transfer of nutrients and wastes. Recently, we reported that medium perfusion enhanced the viability and function of murine bone marrow cells[14] with a perfusion culture system made from standard laboratory equipment (Figure 2). One advantage of 3D collagen sponge scaffolding is the ease of combining multiple cell types. Murine marrow stromal cell and IL-3-dependent hematopoietic cells were co-cultivated within collagen sponges. Viability of these cells was poor under standard conditions. Perfusion of medium, however, stimulated both the proliferation of the stromal cells and their ability to support the growth of the factor-dependent hematopoietic cells.

The perfusion system enhanced histogenesis by bone-forming cells, murine K8 osteosarcoma cells, maintained in 3D collagen sponges.[15] With perfusion, there was greater viability, more alkaline phosphatase-positive cells, and more mineralized tissue after 21 days than in non-perfused control sponges. Quantitative measures of cell proliferation, DNA content, calcium accumulation, and expression of the bone-specific genes, collagen type I and osteocalcin, were dramatically increased with perfusion. Obstacles to clinical application of engineered bone include optimization of starting cells, tissue vascularization, and both acute and long-term compatibility of scaffolds or their degradation products.[16]

Recently, we demonstrated that perfusion profoundly increased the viability and growth of human oral mucosal cells on porous scaffolds.[17] Clinically, thin layers of engineered epithelial tissue are technically difficult to transplant. With perfusion for one week, the keratinocytes formed multiple layers almost twice as thick as without perfusion.

Efficient medium exchange is not beneficial to all cell types. Because articular cartilage is avascular and exposed to relatively poor nutrient and low oxygen conditions, we wondered whether chondrocytes in 3D scaffolds would benefit by medium perfusion. Bovine articular chondrocytes (bACs) were grown in 3D collagen sponges with or without medium

perfusion (0.33 ml/min) for up to 15 days.18 The influence of medium perfusion was evaluated using markers of cartilage matrix accumulation, synthesis, and gene expression. These measures showed significantly better chondrogenesis without perfusion. Thus, the perfused conditions that are beneficial for other cell types inhibit chondrogenesis.

Because increased oxygen exchange may have been deleterious to the chondrocytes, we tested low oxygen concentration (tension) in this system.[19] In the growth plate, oxygen tension is low in the reserve zone (20 mm Hg; 2.6%) and highest in the proliferative zone (57 mm Hg; 7.6%). Even during fracture repair, oxygen tension varies considerably (~20-30 mm Hg; 2.6 - 4%) during callus formation. Collagen sponges were exposed to medium perfusion at either 2% or 19% (atmospheric) oxygen concentrations. Matrix synthesis was greater without perfusion in standard conditions of 19% oxygen. Reduction to 2% resulted in 130% increase in matrix synthesis. Thus, chondrogenesis was restored by reduction of oxygen concentration in the perfused medium to 2%.

With this system, the shear stress caused by medium perfusion at 1.3 ml/min was 0.00157 dynes/cm^2, which is 0.1-1% of the magnitude of shear stress achieved in veins. In the experiments with flow rate at 0.3 ml/min, the shear stress was 0.00037 dynes/cm^2. Even at these low shear stresses, cell surface receptors such as integrin could theoretically be stimulated, with subsequent signal transduction to regulate gene expression. The mechanisms of such mechanotranduction are the subject of intense research. Our homemade culture system is capable of defining these dynamic mechanisms.

Effects of Fluid Pressure on Chondrogenesis in Porous Collagen Sponges

All skeletal tissues are under compressive loading and stretching tension. We designed a novel pressure/perfusion culture system to apply fluid pressure (FP) to the medium perfusing the sponges.[20] The magnitude of pressure was 2.8 MPa, which was within the physiological range of 0 - 3.5 MPa that is achieved at the knee during normal walking. After 15 days, there was 300% more accumulated matrix with FP, applied either continuously or intermittently (0.015 Hz). Matrix synthesis was 40% greater with FP than control. With this novel fluid pressure culture system, 2.8 MPa fluid pressure stimulated synthesis of cartilage specific proteoglycans in chondrocytes cultured in 3D collagen sponges.

These 3 platform technologies - porous collagen sponges, the perfusion system, and fluid pressure apparatus - are being used to optimize 3D histogenesis of different tissues. Rational manipulation of soluble factors, insoluble factors, and physical factors are expected to result in greater understanding of the cellular and tissue mechanisms of growth and in practical applications for repair of skeletal tissues.

References

1. **Mizuno S, Glowacki J.** A three-dimensional composite of demineralized bone powder and collagen for *in vitro* analysis of chondroinduction of human dermal fibroblasts. Biomaterials 1996;17:1819-25.
2. **Glowacki J.** Engineered cartilage, bone, joints, and menisci. Cells Tissues Organs: In Press.
3. **Glowacki J.** In vitro engineering of cartilage. J Rehabil Res Dev 2000;37:171-7.
4. **Mizuno S, Glowacki J.** Chondroinduction of human dermal fibroblasts by demineralized bone in three-dimensional culture. Exp Cell Res 1996;227:89-97.
5. **Allemann F, Yates KE, and Glowacki J.** Preservation of chondroblastic gene expression signature in 3D culture devices. J Bone Miner Res 2000;15:S468.
6. **Eid K, Mizuno S, Glowacki J.** Effect of PTH on matrix production by articular and endochondral chondrocytes in 3D collagen sponges. Bone 1998;23:S349.
7. **Allemann F, Mizuno S, Eid K, Yates KE, Zaleske D, Glowacki J.** Effects of hyaluronan on engineered articular cartilage ECM gene expression in 3-dimensional collagen scaffolds. J Biomed Mater Res:In Press.
8. **Glowacki J, Yates K, Little G, Mizuno S.** Induced chondroblastic differentiation of human fibroblasts by three-dimensional culture with demineralized bone matrix. Mater Sci Eng C 1998;6:199-203.
9. **Mizuno S, Lycette C, Quinto C, Glowacki J.** A collagen/DBP sponge system designed for *in vitro* analysis of chondroinduction. Mat Res Soc Symp Proc 1992;252:133-40.
10. **Yates KE, Mizuno S, Glowacki J.** Early shifts in gene expression during chondroinduction of human dermal fibroblasts. Exp Cell Res: In Press.
11. **Mueller SM, Glowacki J.** Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. J Cellular Biochem: In Press.
12. **Makhluf HA, Mueller SM, Mizuno S, Glowacki J.** Age-related decline in osteoprotegerin expression by human bone marrow cells cultured in three-dimensional collagen sponges. Biochem Biophys Res Comm 2000;268:669-72.
13. **Eid K, Glowacki J.** In vitro chondrogenesis of human marrow stromal cells in novel 3-D collagen devices. J Bone Miner Res 1999;14:S533a.
14. **Glowacki J, Mizuno S, Greenberger JS.** Perfusion enhances functions of bone marrow stromal cells in three-dimensional culture. Cell Trans 1998;7:319-26.
15. **Mueller SM, Mizuno S, Gerstenfeld LC, Glowacki J.** Medium perfusion enhances osteogenesis by murine osteosarcoma cells in three-dimensional collagen sponges. J Bone Miner Res 1999; 14:2118-26.
16. **Mueller SM and Glowacki J.** Construction and regulation of three-dimensional bone tissue *in vitro*. In: Bone Engineering, JE Davies, ed., University of Toronto Press, Toronto, 2000.
17. **Navarro FA, Mizuno S, Huertas JC, Glowacki J, Orgill DP.** Medium perfusion enhances viability and growth of human oral mucosal tissue constructs. Plastic Surg Res Council, Milwaukee, WI, June 9-12, 2001.
18. **Mizuno S, Allemann F, Glowacki J.** Effects of medium perfusion on matrix production by bovine chondrocytes in three-dimensional collagen sponges. Submitted.
19. **Mizuno S and Glowacki J.** Effects of 2% and 19% oxygen on matrix production by bovine articular chondrocytes in 3-D culture. J Bone Miner Res 2000;15:S467.
20. **Mizuno S, Tateishi T, Ushida T, Ohshima N, Sledge CB, Glowacki J.** Effects of soluble, insoluble, and mechanical factors on bovine chondrocytes in 3-dimensional culture. Orthopedic Research Society, 1997.