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UNIVERSITY OF MIAMI

FIBRIN GELS: A POTENTIAL BIOMATERIAL FOR THE CHONDROGENESIS OF BONE MARROW MESENCHYMAL STEM CELLS

By

Melissa Anne Deitzer

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Master of Science

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the requirements for degree of
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FIBRIN GELS: A POTENTIAL BIOMATERIAL FOR THE CHONDROGENESIS OF BONE MARROW MESENCHYMAL STEM CELLS

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The purpose of this study was to develop a fibrin gel system capable of serving as a three dimensional scaffold for the chondrogenesis of rabbit bone marrow mesenchymal stem cells (BM-MSCs) and to examine the effect of two fibrinolytic inhibitors, aprotinin and aminohexanoic acid, on this system. Rabbit BM-MSCs were obtained from the tibias and femurs of New Zealand white rabbits. After chondrogenic potential of BM-MSCs was verified by pellet culture, 2 x 10^6 cells were pelleted and suspended in fibrinogen (80mg/ml) and then mixed with equal parts of thrombin (5 IU/ml). The specimen were then divided into four groups: aprotinin control (with aprotinin); aprotinin + transforming growth factor (TGF-β) (with aprotinin and TGF-β1); amino control (with aminohexanoic acid); and amino+TGF-β (with aminohexanoic acid and TGF- β1). Each of these groups was further divided into three groups depending on the concentration of the inhibitor. Both of the aprotinin groups received 0.0875, 0.175, or 0.35 TIU/ml of aprotinin and both of the aminohexanoic acid groups were supplemented with 2, 4, or 8 mg/ml of aminohexanoic acid. The gels were harvested and analyzed at 7, 14, and 21 days. All of the aprotinin+TGF-β groups exhibited a significantly higher aggrecan gene expression than control groups whereas only the amino+TGF-β group treated with

8mg/ml was significantly higher than those of the control groups. In addition, the 0.0875 and 0.175 TIU/ml aprotinin+TGF-β groups exhibited significantly higher levels of expression than the 2 and 4 mg/ml amino+TGF-β groups. There were no significant differences among the different concentrations of aprotinin or aminohexanoic acid with or without the treatment of TGF-β. Similar trends were also seen when the glycosaminoglycan (GAG) content was measured and analyzed. These findings suggest that fibrin gels are a suitable environment for the chondrogenesis of BM-MSCs and that aprotinin in combination with TGF-β1 is the optimal condition for stimulating BM-MSCs to differentiate into chondrocytes.

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TABLE OF CONTENTS

LIST OF FIGURES.. vi

LIST OF TABLES…... vii

I INTRODUCTION.. 1

II RATIONALE………………………………………………………… 4

II BACKROUND…... 6

1 ARTICULAR CARTILAGE.. 6

1.1 Chondrocytes.. 6

1.2 Proteoglycans... 7

1.3 Collagen... 8

1.4 Structure... 9

1.5 Injury.. 10

1.6 Repair... 12

2 TISSUE ENGINEERING.. 15

2.1 Cells.. 15

2.1.1 Stem Cells.. 16

2.2 Biomaterial Scaffolds.. 17

2.2.1 Fibrin Gels.. 18

2.3 Growth Factor.. 19

3 FIBRONLYTIC INHIBITORS 20

III MATERIALS AND METHODS…………………………………………… 21

1 STEM CELL HARVEST... 21

1.1 Bone Marrow Mesenchymal Stem Cell Isolation................. 21

1.2 Cell Culture... 21

2 FIBRIN GEL CONSTRUCTS.. 22

2.1 Preparation.. 22

2.3 Extraction.. 22

3 EXPERIMENTAL SETUP... 23

3.1 Evaluation of Potential of Fibrin Gels as a Scaffold for the Chondrogenesis of BM-MSCs.................................. 23

3.2 Effects of the Concentration of Fibrinolytic Inhibitors on Fibrin Gels.. 24

3.3 Effects of Cell Density on the Chondrogenesis of BM-MSCs in Fibrin Gels.. 24

3.4 Effects of Fibrinolytic Inhibitors on the Chondrogenesis of BM-MSCs in Fibrin Gels....................... 25

4 RNA ANALYSIS... 26

4.1 RNA Extraction.. 26

4.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis..…. 27
4.3 Gel Electrophoresis.. 28
5 GAG AND DNA ANALYSIS... 28
5.1 Extraction.. 28
5.2 GAG Content Measurement.. 29
5.3 DNA Content Measurement.. 29
6 HISTOLOGY AND IMMUNOHISTOCHEMISTRY..................... 30
6.1 Preparation of Slides... 30
6.2 Histology.. 30
6.3 Immunohistochemistry... 31
7 STATISTICAL ANALYSIS... 31

IV RESULTS.. 33
1 EVALUATION OF POTENTIAL OF FIBIN GELS AS A SCAFFOLD FOR THE CHONDROGENSIS OF BM-MSCS.. 33
2 EFFECTS OF THE CONCENTRATION OF FIBRINOLYTIC INHIBITORS ON FIBRIN GELS... 33
3 EFFECTS OF CELL DENSITY ON THE CHONDROGENSIS OF BM-MSCS IN FIBRIN GELS.. 34
4 EFFECTS OF FIBRINOLYTIC INHIBITORS ON THE CHONDROGENSIS OF BM-MSCS IN FIBRIN GELS…............... 34
4.1 Gene Expression.. 34
4.2 GAG Content.. 35
4.3 Histology and Immunohistochemistry................................. 36
4.4 DNA Content.. 36

V DISCUSSION
1 EVALUATION OF POTENTIAL OF FIBIN GELS AS A SCAFFOLD FOR THE CHONDROGENSIS OF BM-MSCS.. 37
2 EFFECTS OF THE CONCENTRATION OF FIBRINOLYTIC INHIBITORS ON FIBRIN GELS... 37
3 EFFECTS OF CELL DENSITY ON THE CHONDROGENSIS OF BM-MSCS IN FIBRIN GELS.. 38
4 EFFECTS OF FIBRINOLYTIC INHIBITORS ON THE CHONDROGENSIS OF BM-MSCS IN FIBRIN GELS…............... 39

VI CONCLUSION.. 41

VII APPENDIX A.. 46

VIII APPENDIX B... 49

IX REFERENCES.. 56

LIST OF FIGURES

Figure 1	Articular Cartilage……………………………………………………….	46
Figure 2	Proteoglycans…………………………………………………………	47
Figure 3	A collagen II:IX:XI heterofibril…………………………………….	47
Figure 4	Zones of Articular Cartilage………………………………………..	48
Figure 5	Typical chondrogenic gene expression of fibrin gels of the four groups at 14 days…………………………………………………...	49
Figure 6	Toluidine blue staining at 21 days………………………………….	49
Figure 7	The chondrogenic gene expression of different concentrations of aprotinin and aminohexanoic acid at 19 days………………………	50
Figure 8	Normalized aggrecan and collagen II gene expression of different concentrations of aprotinin and aminohexanoic acid at day 19…….	50
Figure 9	The chondrogenic gene expression of different cell densities at 7 and 14 days……………………………………………………....	51
Figure 10	Normalized aggrecan and collagen II gene expression of different cell densities at day 14…………………………………………...	51
Figure 11	Typical chondrogenic gene expressions of experimental groups after 14 days of culture…………………………………………	52
Figure 12	Statistical analysis of aggrecan gene expression after 14 days of culture…………………………………………………………	53
Figure 13	Statistical analysis of GAG content after 14 days of culture……….	54
Figure 14	Histology and Immunohistochemistry at 21 days…………………..	55

LIST OF TABLES

Table 1 Sequences of PCR primers………………………………………… 48

INTRODUCTION

Injured articular cartilage without penetration of subchondral bone has a limited capacity of self-repair due to inaccessibility of reparative cells and low cellularity. However, when defect disrupts subchondral bone, it stimulates formation of fibrin clot containing mesenchymal stem cells that migrate from the bone marrow. After differentiation of mesenchymal stem cells into cartilage-like cells under influence of various biologic and mechanical intra-articular factors, the fibrin clot remodels and results in a fibrocartilaginous repair tissue (Cheung et al., 1978; Johnson, 1986; Shapiro et al., 1993). Therefore, the fibrin clot is a potential natural biomaterial for supporting cartilage regeneration of mesenchymal stem cells derived from bone marrow (BM-MSCs).

Fibrin clots can be mimicked in vitro by combining fibrinogen and thrombin, both of which can be autologously harvested, thus limiting immunocompatibility issues. In addition, fibrin gels can be produced into an injectable, moldable form minimizing invasive implantation (Passaretti et al., 2001; Silverman et al., 1999; Westreich et al., 2004; Xu et al., 2004). Previous studies have also shown that fibrin gels are capable of supporting cartilage formation of chondrocytes (Fortier et al., 1997, 1998, 2002; Fussenegger et al., 2003; Nixon et al., 1999; Meinhart et al., 1999, Passaretti et al., 2001, Silverman et al., 1999; Sims et al., 1998; Westreich et al., 2004, Xu et al., 2004). Hendrickson et al. showed that allograft horse chondrocytes embedded within fibrin gels improved cartilage surface in comparison with the control defects, and showed a significantly greater aggrecan level, and a significantly higher proportion of type-II

collagen (Hendrickson et al., 1994). In addition, Fortier et al. found that autogenous fibrinogen derived from horses stimulated greater chondrocyte proliferation and supported higher proteoglycan accumulation when compared to commercially manufactured fractionated fibrinogen. This shows that autogenous fibrin components are capable of supporting chondrocytes and are preferential to their commercial analogs (Fortier et al., 1998). However, fibrin is intrinsically unstable due to fibrinolysis. Stabilization of fibrin clot can be achieved with two different kinds of fibrinolytic inhibitors, aprotinin and aminohexanoic acid, that have been tested for in-vivo safety and efficacy (Barrons and Jahr, 1996). Aprotinin is a small protein inhibitor of serine proteases belonging to the Kunitz family. It has a broad specificity for target serine proteases such as trypsin, plasmin, chymostrypsin, kallikrein, elastase, urokinase and thrombin. Aminohexanoic acid is an antifibrinolytic agent belonging to the lysine analogue class of inhibitors (Longstaff, 1994). Both antifibrinolytics inhibit plasmin, the main enzyme responsible for the breakdown of fibrin clots.

Recent *in-vitro* studies demonstrated that chondrogenesis of BM-MSCs could be induced with the treatment of cytokines such as transforming growth factor-β (TGF-β) and bone morphogenetic protein (Johnstone et al., 1998; Mackay et al., 1998; Pittenger et al., 2004; Solchaga et al., 1999; Yoo et al., 1998). Combined with their other advantages such as simple acquisition without permanently damaging tissues and efficient expansion in monolayers, BM-MSCs have been considered as candidate cells for cell transplantation and the development of autologous cartilaginous implants for the potential treatment of injured articular cartilage (Caterson et al., 2001, Wakitani et al., 2002). Therefore, fibrin gel is a potential biomaterial that can serve as a delivery vehicle in cell transplantation or

an initial 3-dimensional structure for BM-MSCs. The first goal of this study was to demonstrate that fibrin gels are capable of supporting the chondrogenesis of BM-MSCs. However, since fibrin gels have to be stabilized by fibrinolytic inhibitors, the second objective of this study was to examine the effects of two types of inhibitors, aprotinin and aminohexanoic acid, in various concentrations on the chondrogenesis of rabbit BM-MSCs.

RATIONALE

Many biomaterials have been used as a three dimensional scaffold for the chondrogenesis of BM-MSCs including agarose (Huang et al., 2004b), poly-lactide/alginate (Caterson et al., 2001), poly-lactic-glycolic acid (PLGA) (Uematsu et al., 2005), and gelatin (Ponticiello et al., 2000). Although fibrin gels have been shown to be successful in supporting chondrocytes (Fortier et al., 1997, 1998, 2002; Fussenegger et al., 2003; Nixon et al., 1999; Meinhart et al., 1999, Passaretti et al., 2001, Silverman et al., 1999; Sims et al., 1998; Westreich et al., 2004, Xu et al., 2004), they have not been investigated for the chondrogenesis of BM-MSCs. Fibrin gels have many positive characteristics that qualify them as good scaffolds for tissue engineering. They are capable of degradation and their degradation products are non-toxic. Their components can be autologously harvested, limiting immunocompatibility issues and they can be produced into an injectable, moldable form minimizing invasive implantation (Passaretti et al., 2001; Silverman et al., 1999; Westreich et al., 2004; Xu et al., 2004). Fibrin clots are also nature's solution to full thickness defects of the articular cartilage (Buckwalter, 2002).

The overall goal of this thesis is to examine the feasibility of using fibrin gels as a biomaterial for the chondrogenesis of BM-MSCs. To achieve this goal, we have 2 specific aims.

Specific Aim 1. To examine the roles of cell density and TGF-β in induction of chondrogenesis in BM-MSC grown in fibrin gels.

Specific Aim 2a. To test the efficacy of 2 inhibitors, aprotinin and aminohexanoic acid, in controlling the degradation of the fibrin gels

Specific Aim 2 b. To test whether the inhibitors affect the differentiation of BM-MSC in fibrin gels.

BACKROUND

1. ARTICULAR CARTILAGE

The function of articular cartilage is to enable smooth, pain-free gliding of joints during skeletal motion (O'Connor et al., 2000). Articular cartilage is a unique tissue, but especially with regard to its structural, metabolic, and functional interactions (Akeson, 2003). It endures extreme levels of mechanical stress over many decades and is in a constant flux of skeletal morphogenesis, development and growth such that the anabolic and catabolic activities of the cartilage are balanced (O'Connor et al., 2000). Articular cartilage is composed of 75% to 80% water, a dense extracellular matrix consisting of 50% to 73% collagen II and 15% to 30% proteoglycan macromolecules, and chondrocytes that occupy less than 10% of the tissue (Figure 1). Water and proteoglycans are dispersed through out the collagen framework as a soluble gel, making the matrix biphasic. The collagen fibrils, which can withstand tension but not compression, provide the matrix with high tensile strength, whereas proteoglycans assist in resistance to compression (Athanasiou et al., 2001). The chondrocytes are responsible for the normal homeostasis of the tissue (Ulrich-Vinther et al., 2003).

1.1 Chondrocytes

Chondrocytes are the cells of articular cartilage. They reside in lacunae and interact with extracellular matrix by means of cell surface receptors called integrins. They are responsible for orchestrating a balance between matrix synthesis and breakdown that facilitates normal tissue metabolism (Ulrich-Vinther et al., 2003). Because articular

cartilage is avascular, chondrocytes derive both oxygen and nutrition from the synovial fluid by simple diffusion. As a result, intermittent mechanical force is essential for normal chondrocyte nutrition. Under compression, interstitial fluid, which contains cell and tissue metabolites, flows out of the permeable collagen-proteoglycan matrix. When the load is removed, fluid, containing nutrients, flows back into the matrix. The energy requirements of chondrocytes are met primarily through glycolysis, whereby glucose is metabolized under anaerobic conditions into lactate (Ulrich-Vinther et al., 2003).

1.2 Proteoglycans

Proteoglycans are a family of glycoconjugates with a central core protein to which one or more glycosaminoglycan (GAG) side chains are covalently linked post-translationally (Kiani et al., 2002). Figure 2 shows the disaccharide-repeating unit for the glycosaminoglycans of articular cartilage: chondroitin-4-sulfate, chondroitin-6-sulfate, hyaluronan, and keratan sulfate (Akeson, 2003). Among the cartilage proteoglycans, the most crucial to the proper functioning of articular cartilage is aggrecan. Almost 90% of aggrecan mass is comprised of substituted GAG chains that are mostly chondroitin sulfate chains, but also includes keratan sulfate chains with N- and *O*-linked oligosaccharides (Kiani et al., 2002). Aggrecan further aggregates by combining with hyaluronan through a noncovalent linkage, which is facilitated and strengthened by low molecular weight proteins called link proteins (Figure 2). The ability of the proteoglycan molecules to form aggregates of even greater molecular size amplifies its physiological functional properties (Akeson, 2003).

At high concentrations, proteoglycans create a large osmotic swelling pressure and draw water into the tissue. This occurs because all of the negatively charged anionic groups on the GAG chains of aggrecan carry with them mobile counter ions such as Na^+. This creates a large difference in ion concentration between the cartilage and surrounding tissue and an imbalance amongst the freely diffusible anions and cations. Water is drawn into cartilage because of this osmotic imbalance and because aggrecan is too large and immobile to redistribute itself. The addition of water causes aggrecan-rich matrix network to swell and expand. This water-swollen matrix is critical to the biomechanical properties of cartilage. Not only is aggrecan greatly restricted in its ability to move within the matrix, but also the collagen/aggrecan network is stiff and resistant to deformation. Aggrecan also offers great resistance to any fluid flow and redistribution of water. Thus, cartilage is referred to as a visco-elastic tissue in that it behaves like the stiff elastic polymer resistant to sudden impact loading, yet shows some slow inelastic deformation with sustained loads (Kiani et al., 2002).

1.3 Collagen

Collagen accounts for approximately two thirds of the total dry weight of adult articular cartilage. It is a protein with significant tensile force-resisting properties and is the key protein in musculoskeletal stability, providing the mechanical properties and imparting the “connect” to connective tissue. The tensile force-resisting properties of cartilage derive from the precise molecular configuration of the collagen macromolecule. This molecule is one of the largest in the body, forming a rigid, rod like structure that is made up of three subunits, α chains, folded into a right-handed collagen triple helix. The

collagen in cartilage differs in composition from that of most fibrous connective tissues. This collagen contains a different type of α chain, which is called α_2, type II. The collagen in most cartilages consists of three such identical chains and the abbreviated nomenclature is $(\alpha_1\ [II])_3$, or type II collagen (Akeson, 2003). In addition, articular cartilage also contains small amounts of type IX collagen and type XI collagen that make critical contributions to the organization and mechanical stability of the type II collagen fibrillar network. More specifically, type IX is a short fibrillar collagen that contains a proteoglycan moiety. It forms cross-links with type II along the surface of collagen fibrils and integrates with proteoglycan aggregates in the extracellular matrix. Although type XI collagen forms fibrils, its main function seems to be as a regulator of the fibril diameter of type II, with which it forms copolymers (Figure 3) (Akeson, 2003; Eyre, 2002; Ulrich-Vinther et al., 2003).

1.4 Structure

Architecturally, articular cartilage has four zones of depth from the articular surface to the subchondral bone (Figure 4). Zone 1, also called the superficial layer, makes up approximately 10% of the cartilage, determines its load-bearing ability, and serves as the gliding surface for the joint. The top portion of the superficial layer, also called the lamina splendens, is a clear film consisting of a sheet of small fibrils with little polysaccharide and no cells. Deeper in this layer, flat chondrocytes and collagen fibers are compacted tangentially to the articular surface. This tangential orientation of collagen fibers imparts higher tensile strength and stiffness to zone 1. It also affects significantly the tissue's compressive behavior. Its removal increases permeability and,

indeed, disruption of the superficial layer is an early sign of experimentally induced osteoarthritis. Zone 2 is the intermediate or transitional layer and is composed of spherical chondrocytes and randomly oriented collagen fibers. Compared to the superficial zone, the transitional zone has a higher concentration of proteoglycans, lower concentration of collagen, and lower concentration of water. In the deep layer (zone 3), the collagen fibers and clusters of chondrocytes surrounded by the matrix are perpendicular to the subchondral plate (Athanasiou et al., 2001). In addition, the collagen perforates the calcified basal layers of cartilage through the tidemark regions and eventually enters the subchondral bone layer, where they are attached firmly. This feature is crucial for cartilage to be able to resist shearing forces that would otherwise tend to peel cartilage away from the subchondral surface (Akeson, 2003). The calcified layer, zone 4, joins the deep zone of uncalcified cartilage to the subchondral bone. There are few cells and an abundance of calcium salts, making it a place for growth of underlying bone tissue. The largest and smallest water contents are found in zones 1 and 4, respectively. The highest and lowest proteoglycan contents are in zones 4 and 1, respectively (Athanasiou et al., 2001).

1.5 Injury

Cartilage damage occurs through injury to the joint or progressive degeneration of the cartilage, resulting in defects, intra-articular bleeding, osteoarthritis, and other ailments. A full thickness defect or subchondral defect is a defect that can perforate into underlying bone. This type of defect will lead to exposure of a blood supply, formation of a fibrin clot, and recruitment of bone marrow mesenchymal stem cells. Soon after

injury, blood escaping from the damaged bone blood vessels forms a hematoma that temporarily fills the injury site. Fibrin forms within the hematoma and platelets bind to fibrillar collagen. A continuous fibrin clot fills the bone defect and extends for a variable distance into the cartilage defect. Platelets within the clot release vasoactive mediators and growth factors or cytokines, which stimulate the invasion and migration of undifferentiated cells into the clot and influence the proliferative and synthetic activities of the cells (Buckwalter, 2002). The marrow cells produce an outgrowth of granulation type tissue, consisting of mostly immature vascular cells, fibroblast, and macrophages. Upon maturation, the repair tissue transforms into fibrous type of cartilage provided that the surface is protected from compressive and shear forces in the early repair stages. Although repair is achieved, the fibrocartilage that is formed is remarkably different form hyaline articular cartilage. The fibrocartilage surface is deficient in the precise morphology and composition of normal articular cartilage. The fibrous matrix lacks the precise morphology of arcades in the deeper layers and packing of thin, parallel fiber organization at the surface, and it is not anchored securely into the subchondral plate. The proteoglycan content is also only a fraction of that of normal articular cartilage. In addition, fibrocartilage is lacking from a biomechanical standpoint. In fact, mechanical tests performed on experimental arthroplasty surfaces show a resistance to compression of only one third of that of normal articular cartilage. As a result, the durability of regenerated arthroplasty surfaces of this type is limited, and the fibrocartilage surface is gradually worn away (Akeson, 2003; Hunziker, 2001).

Chondral or partial thickness defects are defects of the articular cartilage that do not penetrate the subchondral bone. They can be divided into two groups: (1) cartilage

matrix and cell injuries; that is, damage to the joint surface that does not cause visible mechanical disruption of the articular surface; and (2) chondral fissures, flap tears, or chondral defects; that is, visible mechanical disruption of just the articular cartilage.

Acute or repetitive blunt trauma can cause alterations of the articular cartilage matrix including a decrease in proteoglycan concentration and a disruption of the collagenous fibril framework. The ability of chondrocytes to sense changes in matrix composition and synthesize new molecules makes it possible for them to repair damage to the macromolecular framework. However, it is presumed that once the loss of proteoglycans exceeds the capabilities of chondrocytes to repair, the injury becomes irreversible and leads to the progressive loss of articular cartilage (Buckwalter, 2002; Hunzinker, 2001).

Acute and repetitive trauma can also cause focal mechanical disruption of articular cartilage including fissures, chondral flaps or tears, and loss of a segment of articular cartilage. The lack of blood vessels and lack of cells that can repair significant tissue defects limit the response of cartilage to injury. Chondrocytes respond to tissue injury by proliferating and increasing the synthesis of matrix macromolecules near the injury; however, the newly synthesized matrix and proliferating cells do not fill the tissue defect, and soon after injury the increased proliferative and synthetic activity ceases (Buckwalter, 2002).

1.6 Repair

As mentioned above, the spontaneous and inadequate natural repair response to injuries of the articular cartilage forces the need for alternative repair and regeneration

options. A number of therapeutic interventions have been developed without the use of active biologics including lavage, arthroscopy, shaving, debridement, laser abrasion, abrasion chondroplasty, pridie drilling, and microfracture technique. The first four are aimed at reducing pain to the patient. The lavage of a joint with solutions of sodium chloride, Ringer or Ringer and lactate, using the closed-needle-hole procedure, and arthroscopic surgery have been deemed beneficial in osteoarthritic or trauma patients with painful joints (Chang et al., 1993; Hunziker, 2001). Shaving, debridement, and laser abrasion are all aimed at the mechanical removal of diseased chondral tissue. These treatments are met with various results and lack prospective clinical trials that would provide the necessary information to justify the performance of any one of these methodologies or substantiate, claimed positive effects (Hunziker, 2001).

Abrasion chondroplasty, as well as pridie drilling and the microfracture technique, involve surgical access to the bone-marrow spaces, which, together with other vicinal compartments (such as the vascular and perivascular spaces, the bone tissue itself and adipose tissue), are consequently stimulated. These three interventions essentially lead to a spontaneous repair response, which is based upon therapeutically-induced bleeding from the subchondral bone spaces and subsequent blood-clot formation. The provocation of such a spontaneous repair response is well known from animal experiments to yield highly variable and non-reproducible healing results, the tissue formed being fibrous in nature and not durable (Hunziker, 2001).

Another approach to the repair of articular cartilage is autologous tissue transplantations. Perichondrial or periosteal tissue has been investigated as an autotransplantation material for repair induction in articular cartilage defects for

approximately thirty years (Engkvist et al., 1975). The biological rationale behind this transplantation lies in the observation that the cambial layer of perichondrial tissue manifests continuous, life-long, chondrogenic activity. A pool of adult-type stem cells must therefore reside in this germinative layer and be capable of reactivation for tissue neoformation. The assumptions made in adopting this principle is that when the floor of a full-thickness articular cartilage defect, its proliferative and tissue-differentiation activities will be resumed and result in the formation of repair cartilage within the lesion void (Hunziker, 2001). A number of experiments, mostly with rabbits, have been conducted to study the potential of this procedure and have been met with positive results (Engkvist et al., 1975; Homminga et al., 1991). Most recently, perichondrial transplants have been used in combination with a variety of biological factors such as human amniotic fluid (Ozgenel et al., 2004), collagen (Ozbek et al., 2003), and transforming growth factor-beta 1 (Miura et al., 1994) to enhance the effects of the transplants.

Mosaicplasty and allogeneic osteochondral grafts are also options in the repair of cartilage. The first takes multiple plugs from osteochondral patellar autografts to fill an articular cartilage defect (Smith et al., 2005) and the latter aims to replace lost or failed tissue with healthy articular cartilage derived from cadavers (Hunziker, 2001). Advantages of mosaicplasty are that defects can be filled immediately with mature, hyaline articular cartilage. However, donor site morbidity and a mismatch between donor and treated cartilage sites are a big concern with this procedure (Hunziker, 2001; Smith et al., 2005). In the case of allogeneic osteochondral grafts, the goal is not to induce a cartilage repair response, but to represent a means of substituting failed tissue. This treatment has experienced positive results in clinical studies with success rates between

65% and 85% (McDermott et al., 1985). Although, the main drawbacks include the scarcity of fresh donor material, problems connected with handling and storage of frozen tissue, and the small but ever-present risk of disease transmission (Hunziker, 2001).

2 TISSUE ENGINEERING

Another biological based therapy that has potential for articular cartilage repair is tissue engineering. The first definition of tissue engineering was proposed in 1988 as follows:

> The application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissue and the development of biological substitutes to restore, maintain, or improve tissue function (Palson and Bhatia, 2004).

Most recently, the term tissue engineering is used quite loosely in conjunction with any component that, either by itself or in combination with others, mediates the formation of repair tissue. Three constituents form the basis of a tissue engineering approach, a matrix scaffold, cells, and signaling molecules. One strategy is acellular matrices, where matrices are used alone and which depend on the body's natural ability to regenerate for proper orientation and direction of new tissue growth. The other strategy transplants cell into the matrix and implants the cell matrix into the body (Koh and Atala, 2004).

2.1 Cells

When cells are used for tissue engineering, a small piece of donor tissue is dissociated into individual cells. These cells are either implanted directly into the host or expanded in culture, attached to a support matrix and then reimplanted in the host after

expansion. Cells can either be autologous, allogeneic, or xenogenic. The use of autologous cells is ideal because it avoids rejection and the use of immunosuppressive medications (Koh and Atala, 2004). The types of cells used are either specific to the tissue being regenerated, i.e. chondrocytes for cartilage regeneration or various types of stem cells.

2.1.1 Stem Cells

Stem cells are cells that can self-renew and have the potential to differentiate along on or two lineages. Stem cells are categorized by the kinds of progeny they can produce: unipotent (can produce one cell type), multipotent (can produce many cell types) or totipotent (can produce all cell types). There are only a few stem cell types that can be cultured and qualify for true totipotent status including embryonal carcinoma, embryonic stem, and embryonic germ cells. Multipotent stem cells can be cultured from a number of fetal and adult sources. Perhaps the best known source is bone marrow, which contains both hematopoietic stem cells and mesenchymal stem cells (Shamblott et al., 2000). Mesenchymal stem cells (MSCs) contribute to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma (Pittenger et al. 1999). A subpopulation of MSCs called multilineage adult progenitor cells has also been shown to differentiate into cardiomyocytes (Fukuda 2003) and hepatocytes (Schwartz et al., 2002).

In addition to their multipotency, MSCs can be acquired by bone marrow aspiration without permanently damaging tissues and efficiently expanded in monolayers by serial passages without altering their differentiation potential. Differentiation of BM-

MSCs can be induced by culturing the cells at a high cell density in a three dimensional matrix with a defined serum free, high glucose medium (Mackay et al., 1998) that includes TGF-β and dexamethasone (Johnstone et al., 1998; Yoo et al., 1998). For these reasons, BM-MSCs have been considered as candidate cells for cell transplantation and the development of autologous cartilaginous implants for the potential treatment of injured articular cartilage (Caterson et al., 2001; Wakitani et al., 2002).

2.2 Biomaterial Scaffolds

A scaffold is designed to provide a structural template for cell attachment and tissue development. A scaffold should be porous so as to permit the migration of cell through its interstices, the infiltration of native ones from the implant surroundings, and the transport of nutrients, metabolites and regulatory molecules to and from the cells within the matrix. A scaffold should also be made of a biodegradable and a biocompatible material. The material should be biodegradable to achieve isomorphous tissue replacement that is the scaffold should biodegrade at a rate matching the rate of extracellular matrix deposition. The material and its degradation products must also be biocompatible, causing a minimal adverse reaction from the cells transplanted within material or from the cells and tissue surrounding the implant. Finally, the material must meet the mechanical requirements of the tissue it is aiming to replace. For example, materials used in repair of articular cartilage must be resistant to compression once implanted inside the body (Hunziker, 2001; Vunjak-Novakovic, 2003). A number of different materials, both synthetic and natural, have been investigated for use in articular cartilage repair including collagen (Wakitani et al., 1998; Willers et al., 2005), fibrin

(Silverman et al., 1999; Hendrickson et al., 1994), chitosan (Kim et al., 2003), polylactic/polyglycolic acids (Banu et al., 2005), agarose (Huang et al., 2003; Buschmann et al., 1992), and hydrogels (Passaretti et al., 2001).

2.2.1 Fibrin Gels

Fibrin is a natural biomaterial that mimics the last step of the coagulation mechanism. It consists of two major components, thrombin and fibrinogen. Thrombin splits off fibrinopeptide A and B from the respective A α and B β chains of fibrinogen to form fibrin monomers that polymerize by hydrogen bonding to a fibrin clot. Factor XIII further catalyzes the crosslinking between the fibrin molecules, increasing the mechanical strength (Martinowitz and Saltz, 1996). Fibrin gels have other advantages such as its components can be autologously harvested, its degradation products are physiological and therefore nontoxic, and it can be formed into an injectable moldable form (Passaretti et al., 2001; Silverman et al., 1999; Westreich et al., 2004; Xu et al., 2004). For these reasons, fibrin gels have been studied in collaboration with articular cartilage repair. Hendrickson et al. showed that injecting articular cartilage defects with fibrin gels loaded with chondrocytes resulted in an improved cartilage surface when compared with the control defects, a significantly greater aggrecan level, and a significantly higher proportion of type-II collagen (Hendrickson et al., 1994). In addition, Passaretti et al. showed that cultured porcine chondrocytes suspended in a fibrin matrix will form cartilage when injected in the subcutaneous spaces of nude mice (Passaretti et al., 2001). The use of fibrin gels as a delivery system for MSCs has also been studied. Bensaid et al. showed that human MSCs were able to adhere, spread and proliferate when loaded into

fibrin scaffolds and Worster et al showed that fibrin gels were capable of supporting the chondrogenesis of MSCs when supplemented with IGF-I (Bensaid et al., 2003; Worster et al., 2001). The main downfall to fibrin gel is that it is unstable and is quickly disintegrated by cells. To control fibrinolysis, a fibrinolytic inhibitor can be added to the system (Meinhart et al., 1999; Fussenegger et al., 2003) or the concentration of fibrinogen can be increased (Silvermann et al., 1999).

2.3 Growth Factors

Growth factors influence various cell activities including proliferation, migration, matrix synthesis, and differentiation. Many of these factors, including insulin-like growth factors (IGF) (Fortier et al., 2002), bone morphogenetic protein (BMP) (Gooch et al., 2002), basic fibroblastic growth factor (bFGF) (Mastrogiacomo et al., 2001) and transforming growth factor-beta (TGF-β) (Yoo et al., 1998), have been shown to affect chondrocyte metabolism and chondrogenesis.

TGF-β is a ubiquitous protein cell regulator that has been identified in bone matrix, cartilage, platelets, activated lymphocytes, and other tissues. Multiple subtypes of TGF-β exist, each with a different profile of activity. It is produced by articular chondrocytes and remains in the cartilage matrix in a latent form (Pedrozo et al., 1999), and it has been reported to influence the proliferation of human articular chondrocytes, tibial periosteum, pericranial periosteum, and osteoblasts (O'Connor et al., 2000). In addition, TGF-β has been shown to assist in the differentiation of MSCs (Yoo et al., 1998) and the re-expression of the chondrocytic phenotype in chondrocytes (Goldberg et al., 2005).

3 FIBRINOLYTIC INHIBITORS

Fibrinolytic inhibitors necessary to stabilize fibrin gels include aprotinin, aminohexanoic acid, and transexamic acid. They are used clinically to limit perioperative bleeding and reduce the need for donated blood transfusions during cardiac surgery. In addition, each has been proven to be safe and effective and both aprotinin and aminohexanoic acid are FDA approved for prophylactic use to reduce perioperative blood loss and the need for blood transfusion in patients undergoing cardiopulmonary bypass in the course of coronary artery bypass graft surgery and when fibrinolysis contributes to excessive bleeding, respectively (Barrons and Jahr, 1996).

Aprotinin, also know as Trayslol, is a small protein inhibitor of serine proteases belonging to the Kunitz family. In common with many other Kunitz inhibitors, aprotinin has a broad specificity for target serin proteases and will inhibit trypsin, plasmin chymotrypsin, kallikrein, elastase, urokinase and thrombin in decreasing order of effectiveness (Longstaff, 1994). Aminohexanoic acid is an antifibrinolytic agent belonging to the lysine analogue class of inhibitors. This class of agents has been shown to interact with the active site of plasmin, even though their primary mode of action as antifibrinolytic agents is usually ascribed to their ability to block lysine binding sites and prevent association of plasminogen and tissue plasminogen activator with fibrin (Longstaff, 1994).

MATERIALS AND METHODS

1 STEM CELL HARVEST

1.1 Bone Marrow Mesenchymal Stem Cell Isolation

Rabbit BM-MSCs were isolated and the chondrogenic potential of BM-MSCs was examined using pellet cultures in the previous study (Huang et al., 2004b) according to previous reported protocol (Solchaga et al., 1999). Briefly, bone marrow was obtained from the tibias and femurs of five New Zealand White rabbits by either aspirating or flushing out with a 16-gauge needle and a 10-ml syringe containing 1 ml of heparin (3000 U/ml). After placing in a 50-ml tube containing 5 ml of low-glucose Dulbecco's modified eagle's medium (DMEM) (GibcoBRL, Grand Island, NY), the bone marrow was centrifuged at 600 g for 10 min to obtain cell pellet. Following removal of supernatant, cells were resuspended in 10 ml of low-glucose DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotics and then 10^5 cells were plated and cultured in 10-cm dishes at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

1.2 Cell Culture

Nonadherent cells were removed by changing the culture medium after 5 days of culture. The culture medium was subsequently changed every 2-3 days. After two weeks of primary culture, cells were stored in liquid nitrogen. When needed, the cells were thawed in a water bath of 37°C and plated in a 10-cm culture dishes. To expand the cells, medium was removed and the cells were washed with 5ml of phosphate buffer solution

(PBS) (Gibco). The PBS was vacuumed and 1.5-2 ml of trypsin was added to each plate. Most of the trypsin was vacuumed and the plates were incubated at 37ºC for about 3-4 minutes until the cells looked round and detached. The cells were washed and removed from the plate with 2ml of DMEM, added to a 50ml plastic tube, and counted with a hemocytometer. The passaged cells were seeded on 10-cm culture dishes at a density of 10^5 cells per dish and incubated at 37ºC. Fresh medium was replaced every 3-4 until cells in plates were confluent.

2 FIBRIN GELL CONSTRUCTS

2.1 Preparation

The rabbit BM-MSCs of the 4th passage were suspended in a fibrinogen solution, which consisted of equal parts of 80 mg/ml bovine fibrinogen (Sigma, St. Louis, MO) and 2x high-glucose Dulbecco's modified eagle's medium (DMEM) (Invitrogen, Carlsbad, CA), and then mixed with an equal volume of 5 IU/ml bovine thrombin solution (Sigma, St. Louis, MO) to produce a mixture of 10^7 cells/ml. A cell-fibrin construct was formed by added 200 μl of mixture to each well of a 48 well plate and incubating at 37ºC for 3 hours.

2.2 Extraction

After specified culture time, the cell-fibrin constructs were removed first by scraping the circumference of the construct with a small spatula and then by using the spatula to slowly scrape the bottom and lift the construct onto a clean 10-cm culture dish.

The construct was then cut in half with a razor blade. Half of the construct was used for RNA analysis and the other half was used for DNA and glycosaminoglycan (GAG) analysis.

3 EXPERIMENTAL SETUP

3.1 Evaluation of the Potential of Fibrin Gels as a Scaffold for the Chondrogenesis of BM-MSCs

As a preliminary experiment, fibrin gels were examined for their ability to act as a three dimensional matrix for the chondrogenesis of BM-MSCs. The constructs from the same donor were divided into four groups: aprotinin control, aprotinin+TGF-β, aminohexanoic acid control, aminohexanoic acid+TGF-β. The control groups were cultured in a defined serum-free medium consisting of high-glucose DMEM, 1% ITS+ Premix (final concentration: insulin, 6.25 g/ml, transferrin, 6.25 g/ml, selenous acid, 6.25 ng/ml, bovine serum albumin, 1.25 mg/ml, linoleic acid, 5.35 g/ml) (BD Biosciences, Bedford MA), 50μg/ml ascorbic acid, 0.01M HEPES buffer and 10^{-7} M dexamethasone (Sigma, St. Louis, MO) while the TGF-β groups were cultured in the defined serum-free medium supplemented with 10 ng/ml TGF-β1. In addition, the medium of the aprotinin groups was supplemented with 0.35TIU/ml of aprotinin and the medium of the aminohexanoic acid groups were supplemented with 4mg/ml of aminohexanoic acid. Samples were harvested at 7, 14, and 21. Since fibrinolytic inhibitors are necessary to stabilize the fibrin gels, we were unable to compare the

experimental groups to a control group that lacked inhibitor, thus prohibiting us from examining the effects of the inhibitors themselves.

3.2 Effects of the Concentration of Fibrinolytic Inhibitors on Fibrin Gels

Once it was determined that the fibrin gels were a potential biomaterial for the chondrogenesis of BM-MSCs, the minimum amount of inhibitor to prohibit degradation was examined. The constructs were divided into the same groups as above, however, for this experiment, each control group was further broken down into three inhibitor concentrations. The culture medium of aprotinin control groups were supplemented with either 0.0875, 0.175, or 0.35 TIU/ml of aprotinin while the culture medium of aminohexanoic acid control groups were supplemented with either 1, 2, or 4, mg/ml of aminohexanoic acid. The samples supplemented with TGF-β1 were not divided into different concentrations in order to conserve the amount of TGF-β1 available. Samples were harvested at 19 days and visually examined for structural integrity.

3.3 Effects of Cell Density on the Chondrogenesis of BM-MSCs in Fibrin Gels

To assess the effects of cell density on the chondrogenesis of BM-MSCs in fibrin gels, the constructs were broken down into four groups: high cell density (HCD) control, HCD+ TGF-β, low cell density (LCD) control, and LCD+ TGF-β. The HCD groups were seeded with $5x10^7$ cells/ml and the LCD groups were seeded with 10^7 cells/ml. Only 100 μl of the fibrinogen/thrombin/cell solution was added to the each well of “special plates”. These plates also enabled for each construct to receive 200 μl of medium instead of the normal 100 μl. Each of the groups received the same culture

medium discussed above, however, all groups were supplemented with 0.175 TIU/ml of aprotinin. The medium was changed everyday for the first 3 days and every other day after. The samples were harvested at 7 and 14 days.

3.4 Effects of Fibrinolytic Inhibitors on the Chondrogenesis of BM-MSCs

Based on the results of the previous experiments, a more thorough experiment was conducted to examine the effects of various concentrations of fibrinolytic inhibitors on the chondrogenesis of BM-MSCs. Again, the constructs from the same donor were divided into four groups: aprotinin control, aprotinin+TGF-β, aminohexanoic acid control, aminohexanoic acid+TGF-β. The control groups were cultured in a defined serum-free medium discussed above while the TGF-β groups were cultured in the defined serum-free medium supplemented with 10 ng/ml TGF-β1. In addition, each of the four groups was further divided to test various concentrations of the two inhibitors. The earlier experiment showed that the minimum concentration of inhibitors for preventing fibrinolysis of fibrin gels was 0.0875 TIU/ ml for aprotinin and 2 mg/ml for aminohexanoic acid. Therefore, the culture medium of aprotinin groups was supplemented with either 0.0875, 0.175, or 0.35 TIU/ml of aprotinin while the culture medium of aminohexanoic acid groups was supplemented with either 2, 4, or 8 mg/ml of aminohexanoic acid. All cell-fibrin constructs were cultured in a humidified incubator maintained at 37ºC in 5% CO_2. The culture medium was changed every everyday.

4 RNA ANALYSIS

4.1 RNA Extraction

According to the manufacturer's instructions, the half cell-fibrin construct was transferred to a grinder and 1ml of Trizol was added. The constructs were homogenized for approximately 2 minutes or until the construct was no longer visible. The mixture was transferred to a 1.5 ml tube and incubated at room temperature for 5 minutes. 200 µl of chloroform was then added to the tube and vigorously shaken for approximately 30 seconds. After incubating the solution for 15 minutes at room temperature, it was centrifuged at 12000 rpm and 4ºC for 15 minutes. The upper aqueous phase containing the RNA was then transferred to a sterile 1.5 ml tube and the bottom phase was stored at -20ºC.

The RNA from the aqueous phase was precipitated by adding 0.50 ml of isopropanol. The solution was inverted to mix, incubated at room temperature for 30 min, and then centrifuged at 12000 rpm and 4ºC for 15 minutes. The supernatant was removed and the RNA was washed with 1 ml of 75% ethanol and again the solution was centrifuged at 12000 rpm and 4ºC for 20 minutes.

A majority of the ethanol was removed from the RNA pellet by using a pipette and the rest was removed by air drying for about 20 minutes. The RNA pellet was then solubulized by adding 10µl of diethylpyrocarbonate- treated (DEPC) water. Once the RNA was completely dissolved, 1µl of the solution was transferred to a separated tube and diluted up to 100µl with DEPC water for RNA measurement. The RNA content was measured using a 640 spectrophotometer at 260 nm, assuming 40µg/ml of RNA equals

one absorbance unit. The remaining 9µl of dissolved RNA was used for RT-PCR analysis.

4.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

To examine gene expression, RT-PCR was performed using the GeneAmp PCR system (Applied Biosystems, Foster City, CA) and the Thermo Script RT-PCR system (Invitrogen, Carlsbad, CA). According to the manufacturers instructions, cDNA was synthesized by combining 1µl of 50µM Oligo $(dT)_{20}$ primer, 2µl of 10mM dNTP mix, solubilized RNA, and enough DEPC water to adjust the total volume to 12µl. The RNA and primer are denatured by incubating the solution at 65ºC for 5 minutes and then the solution was placed on ice. Meanwhile, a master reaction mix was prepared by mixing 4µl of 5x DNA Synthesis Buffer, 1µl of 0.1M DTT, 1µl of RNase Out (40 U/µl), 1 µl of DEPC water, and 1 µl of Thermoscript RT. Once completed, 8µl of the master reaction mix was pipetted into the 12 µl of denatured RNA solution and incubated in the GeneAmp PCR system at 60ºC for 50 minutes and terminated at 85ºC for 5 minutes. The cDNA was placed on ice and 10% of the solution was used for PCR.

According to the manufacturer's instructions, 2 µl of the cDNA was primed with 0.5µl of 10 µM sense and .05 µl of 10 µM antisense primer. The primers used were collagen II, aggrecan, and glyceraldehydes-3-phosphate dehydrogenase (GAPD) gene. The GAPD gene is a house keeping gene and was used to normalize the gene expression. The PCR primers are shown in Table 1. A 22 µl of the following mixture was then added to the primed cDNA: 2.5µl of 10x High Fidelity PCR Buffer, 1 µl of 50mM $MgSO_4$, 0.5 µl of 10mM dNTP mix, 0.1 µl of Platinum Taq High Fidelity, and 17.9 µl of DEPC

treated water. The solution had a final volume of 25 µl and was amplified using the GeneAmp PCR system. The PCR profile was 1 cycle at 95ºC for 2 minutes, 25 cycles for GADPH and 40 cycles for collagen II and aggrecan of 45ºC for 30 seconds, 55º for 30 seconds, and 72º for 45 seconds, and 1 cycle of 72ºC for 10 minutes. After the cycles were complete, samples were placed on ice and used for gel electrophoresis.

4.3 Gel Electrophoresis

The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide and photographed with a low light image system (ChemiImager 4000, Alpha Innotech Corporation, San Leandro, CA). A DNA mass ladder (100bp) was used to identify PCR fragments. Integrated density value (IDV) of each PCR product from the electrophoresis image was measured by the AlphaEase software (Alpha Innotech Corporation, San Leandro, CA).

5 GAG AND DNA ANALYSIS

5.1 Extraction

Remaining half of the cell-fibrin construct (n=5 per group) was weighed, lyophilized, and digested for 24 hours at 60°C in 1 ml buffer of 100 mM sodium phosphate, 10 mM EDTA, and 10 mM cysteine HCl (pH 7.0) 250 µg/ml papain (Sigma, St. Louis, MO) (1 ml papain solution per 4-40 mg dry weight of sample).

5.2 GAG Content Measurement

Total sulfated GAG content of fibrin samples was determined spectrophotometry using dimethylmethylene blue dye. One hundred microliters of the papain-digested sample was reacted with 2 ml of dimethylmethylene blue dye solution (Farndale et al., 1986) in a disposal cuvette and the GAG content was measured spectrophotometrically at 525 nm. A standard curve was constructed using bovine chondroitin sulfate.

5.3 DNA Content Measurement

The DNA content of constructs was measured using the Hoechst 33258 fluorometric assay described in the previous study (Kim et al., 1988). The stock solution (1mg/ml) of Hoechst 33258 dye was made by dissolving Hoechst 33258 dye in distilled water. A working buffer was prepared by diluting the stock solution to 0.15 μg/ml in 100mM Tris, 1mM EDTA, 0.2 M NaCl, pH=7.4, immediately before use. One hundred microliters of papain-digested sample was mixed with 2 ml working buffer in a disposal cuvette. The fluorescence measurement of Hoechst 33258 dye was performed using a spectrofluorometer (RF-1501, Shimadzu Scientific Instruments INC., Norcross, GA) with an excitation wavelength of 365 nm and an emission wavelength of 458 nm. A standard curve was generated using 50 to 400 ng of calf thymus DNA.

6 HISTOLOGY AND IMMUNOHISOCHEMISTRY

6.1 Preparation of Slides

In order to allow sufficient deposition of proteoglycans and type II collagen protein for the histological and immunohistochemical analysis, some constructs were cultured for 21 days. The samples were fixed in 10% buffered formalin for one hour, washed with PBS three times and left in PBS overnight at 4ºC. For dehydration, the samples were placed inside a cassette, which was subsequently placed in a beaker filled with 80% ethanol for up to 1 hour. A graded series of increasing concentrations of ethanol were used to complete the dehydration. The samples were placed in two different solutions of 80%, 95%, and 100% ethanol solution for 10 minutes each. Each sample was then cleaned with two separate solutions of xylene for 10 and 15 minutes respectively. Finally, the samples were embedded in paraffin, cut into 5 µm sections and placed on slides.

6.2 Histology

The slides were used to detect proteoglycans by staining sections with safranin-O or toluidine blue. Slides were put into a carriage and dipped into the respective stain for approximately 3 minutes. The slides were taken out of the staining and wiped down. The slides were then laid out to dry for about 1 hour before examination under a microscope.

6.3 Immunohistochemistry

The slides were also used to detect the deposition of type II collagen protein by immunohistochemical analysis. First, the slides were deparrafinized by placing the slides in two separated stations of 100% xylene for 5 minutes each. The slides were then placed in 50% xylene 50% ethanol solution for 5 minutes, two stations of 100% ethanol for 10 minutes each, and finally in one station of 95%, 70%, and 30% ethanol for 10 minutes each. After being rinsed in distilled water, the sections were incubated in 0.3% hydrogen peroxide in distilled water for 30 minutes, rinsed, then incubated in the 10% normal horse serum for 1 hour to block nonspecific background, rinsed, and finally incubated with mouse monoclonal anti-human type II collagen antibody (II-4CII, 5 μg/ml; ICN Biomaterials, Aurora, OH) at 4°C overnight. Following extensive washing with phosphate buffer solution to remove the primary antibody, immunoactivity was detected by incubating the sections with biotinylated horse anti-mouse antibody, followed by incubation with avidin-biotin-peroxidase complex (ImmunoPure ABC peroxidase staining kits; Pierce, Rockford, IL). Peroxidase activity was visualized using 3-3'-diaminobenzidine (DAB) as the substrate. The sections were incubated with 0.06% DAB in 0.1M Tris-HCL, pH 7.5, containing 0.03% hydrogen dioxide, followed by counterstaining with hematoxyline. All incubations were performed in a humidified chamber.

7 STATISTICAL ANALYSIS

During data analysis, the average value of the aprotinin control group served as a reference point and was used to normalize the data. To examine the effects of the

inhibitors, differences in gene expression, GAG content, and DNA content among experimental groups were analyzed statistically using a two-way (+/-TGF-β and inhibitor) analysis of variance (ANOVA). A two-way (+/-TGF-β and inhibitor level) ANOVA was also used to examine the effects of the different concentrations for each inhibitor. Significance was assumed for $p<0.05$. Statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL).

RESULTS

1 EVALUATION OF THE POTENTIAL OF FIBRIN GELS AS A SCAFFOLD FOR THE CHONDROGENESIS OF BM-MSCS

The chondrogenic gene expressions of the four groups at two time periods are shown in Figure 5. After 14 days, the aprotinin and TGF-β1 group exhibited a stronger gene expressions of aggrecan than the aprotinin control group and both aminohexanoic acid groups. Also at this time period, the control groups for both inhibitors show a weaker expression for collagen II than their TGF-β1 treated groups. After 21 days, the TGF-β1 treated groups for both aprotinin and aminohexanoic acid show a stronger gene expression for both chondrogenic genes than their respective control groups. In addition, the specimens of the TGF-β1 groups exhibited stronger toluidine blue staining as compared with the specimens of the control groups for both inhibitors (Figure 6). The aprotinin and TGF-β1 group also exhibited a much stronger staining than the aminohexanoic and TGF-β1 group, suggesting that aprotinin is more conducive to deposition of extracellular matrix.

2 EFFECTS OF THE CONCENTRATION OF FIBRINOLYTIC INHIBITORS ON FIBRIN GELS

Upon visual examination, the 0.35 TIU/ml aprotinin gel seemed to have the best consistency and was the most firm of all the gels harvested. In addition, the 1mg/ml aminohexanoic acid gel disintegrated within one week of initial preparation. The typical chondrogenic gene expression is shown in Figure 7. The aprotinin and TGF-β1 group showed a slightly stronger staining than aminohexanoic and TGF-β1 group for aggrecan

and almost double the intensity of staining for collagen II. The gene expression also changed with the amount of inhibitor. The aprotinin group supplemented with .0175 TIU/ml had similar gene expression as the aprotinin and TGF-β1 group for both aggrecan and collagen II. Also, there was a huge decrease in the aggrecan gene expression from groups supplemented with 4mg/ml to groups supplemented with 2mg/ml of aminohexanoic acid.

3 EFFECTS OF CELL DENSITY ON THE CHONDROGENESIS OF BM-MSCS IN FIBRIN GELS

The typical gene expression for the different groups is shown in Figures 9. The aggrecan gene expression for both high and low cell densities supplemented with TGF-β1 are approximately the same, whereas, the HCD control is twice that of the LCD control. The collagen II gene expression for the high cell density is twice that of the low cell density for both the control and TGF-β1 supplemented groups.

4 EFFECTS OF FIBRINOLYTIC INHIBITORS ON THE CHONDROGENESIS OF BM-MSCS

4.1 Gene Expression

The typical gene expressions of the four groups are shown in Figure 1. The aprotinin+TGF-β groups exhibited significantly higher aggrecan gene expression than the control groups whereas only aggrecan gene expression of the TGF-β group with the treatment of 8mg/ml aminohexanoic acid was significantly higher than those of the

control groups (Figure 12). Significant differences were found on aggrecan gene expression between the aprotinin+TGF-β and aminohexanoic acid+TGF-β groups with the groups supplemented with 0.0875 and 0.175 TIU/ml of aprotinin exhibiting higher level of expression than those supplemented with 2 and 4 mg/ml of aminohexanoic acid. In addition, no significant effects of different inhibitor concentrations were found on aggrecan gene expression among the aprotinin control groups, the aminohexanoic acid control groups, the aprotinin+TGF-β groups, or the aminohexanoic acid+TGF-β groups. However, the TGF-β group supplemented with 0.35 TIU/ml of aprotinin tended to exhibit a lower level of aggrecan gene expression than the other two aprotinin+TGF-β groups while aggrecan gene expression of the TGF-β group supplemented with 8 mg/ml of aminohexanoic acid tended to be higher that those of the other two aminohexanoic acid+TGF-β groups. Furthermore, no significant differences were found on expression of type II collagen gene between the treatments of two inhibitors in either TGF- β or control groups or among the different inhibitor concentrations

4.2 GAG content

Three aprotinin+TGF-β groups exhibited significantly higher GAG content than the control groups whereas only the GAG content of the TGF-β group supplemented with 2mg/ml aminohexanoic acid was significantly higher than those of the control groups (Figure 13). No significant effects of different inhibitor concentrations were found on GAG content among the aprotinin control groups, aminohexanoic acid control groups, aprotinin+TGF-β groups, or aminohexanoic acid+TGF-β groups. Although there is no significant difference on GAG content between the aprotinin+TGF-β and aminohexanoic

acid+TGF-β groups after a 14-day culture, the aprotinin+TGF-β groups tended to exhibit more GAG content than the aminohexanoic acid+TGF-β group.

4.3 Histology and Immunohistochemistry

After a 21-day culture, the TGF-β groups exhibited a stronger safranin-O staining than those of the control group while the aprotinin+TGF-β group exhibited stronger staining when compared to the aminohexanoic acid+TGF-β group (Figure 4). Generally, safranin-O staining was more evident in the surface zone of TGF-β1 treated samples and more intense around cells. Immunohistochemical analysis of type II collagen protein deposition showed positive reaction only on the surface zone of the TGF- groups (Figure 14) but no differences between the aprotinin+TGF-β and aminohexanoic acid+TGF-β groups.

4.4 DNA content

There is no significant difference in DNA content between TGF-β and control groups and among the groups treated with aprotinin or aminohexanoic acid of three different concentrations.

DISCUSSION

1. EVALUATION OF THE POTENTIAL OF FIBRIN GELS AS A SCAFFOLD FOR THE CHONDROGENESIS OF BM-MSCS

This initial study was to assess the feasibility of using fibrin gels as a biomaterial scaffold for the chondrogenesis of BM-MSCs. Two clinically used fibrinolytic inhibitors, aprotinin and aminohexanoic acid, were chosen to see which was better to prevent fibrinolysis. At the concentrations used, both inhibitors were capable of preventing the degradation of the fibrin gel. In addition, the analysis of the chondrogenic gene expression verified our hypothesis that fibrin gels are capable of supporting the chondrogenesis of rabbit BM-MSCs. Unexpectedly, the fibrinolytic inhibitors needed to stabilize the fibrin gels seemed to have an affect on the chondrogenesis of the BM-MSCs. The aprotinin samples had a stronger chondrogenic gene expression than the aminohexanoic acid samples and the toluidine staining showed that the aprotinin promotes extracellular matrix deposition when compared to aminohexanoic acid.

2. EFFECTS OF THE CONCENTRATION OF FIBRINOLYTIC INHIBITORS ON FIBRIN GELS

This study was conducted to assess the minimum amount of inhibitor necessary to prevent degradation of the fibrin gel. The concentration used in the last experiment was the starting point and was divided in half for the second concentration and divided in half again for the third concentration. Only the control samples were divided into three concentrations because the amount of TGF-β1 available was limited. However, a TGF-β

sample was put into each inhibitor using the same concentration from the last experiment to repeat the study. The 1mg/ml sample of aminohexanoic acid quickly disintegrated and was removed as a viable option. The remaining samples were all capable of preventing degradation for up to 19 days. Upon visual examination, the 0.35 TIU/ml aprotinin sample had the best physical integrity. In addition, similar to the previous experiment, the aprotinin and TGF-β group exhibited the best results. Interestingly, the concentration of the aprotinin seemed to affect the chondrogenesis. The difference between the TGF-β group and the control of the same concentration was two fold, however, the next lower concentration brought the relative gene expression for both chondrogenic genes back to the level exhibited when treated with TGF-β1. This observation was extremely significant since it showed that it may be possible to promote chondrogenesis without the addition of TGF-β1 or that by varying the concentration of the inhibitor, the chondrogenesis of the BM-MSCs may be positively affected.

3. EFFECTS OF CELL DENSITY ON THE CHONDROGENESIS OF BM-MSCS IN FIBRIN GELS

To further optimize the fibrin gel system, this experiment studied the effects of cell density of the chondrogenesis of BM-MSCs. Cell density will affect the diffusion of nutrients and metabolic waste, the rate of degradation of the fibrin gel, and the proliferation and differentiation of cells. In atelocollagen constructs, a low cell density (10^5 cells/ml) of chondrocytes proliferated best, whereas a high cell density (10^6 and 10^7 cells/ml) showed an increased capability of producing chondroitin 6-sulfate (Iwasa et al., 2003). Also, Silverman et al. showed that high cell density implants (40 million cells/cc)

formed a more solid homogeneous cartilage, similar in histologic appearance to normal swine articular cartilage (Silverman et al., 1999). The results showed that at 14 days the gene expression for aggrecan was similar for both cell densities when supplemented with TGF-β1. However, the collagen II gene expression for the HCD and TGF-β group was twice that of the LCD and TGF-β group. Unexpectedly, the control group showed a stronger collagen II gene expression than the TGF-β groups for both cell densities and similar aggrecan gene expression in the HCD group. The reason for this observation is unknown and may be explained through ratio of fresh medium to the amount of cells in the HCD samples. Although special plates were used to circumvent this issue, the medium still required that it be changed more often, suggesting that the amount of medium was still not sufficient. Also, the amount of TGF-β1 was not increased in the HCD samples.

4. EFFECTS OF FIBRINOLYTIC INHIBITORS ON THE CHONDROGENESIS OF BM-MSCS

This study examined the effects of aprotinin and aminohexanoic acid on chondrogenesis of BM-MSCs. Aprotinin and aminohexanoic acid belong to different classes of fibrinolytic inhibitors and have demonstrated clinical effectiveness in decreasing blood loss during cardiac surgery (Barrons and Jahr, 1996). By using these inhibitors, degradation rate of fibrin clot can be slowed down to allow sufficient deposition of new extracellular matrix before breakdown. This study demonstrates for the first time that fibrinolytic inhibitors affected TGF-β induced chondrogenesis of BM-MSC in fibrin gels. The RT-PCR analysis showed significant differences on aggrecan gene

expression of TGF-β1 treated constructs between the treatments of two inhibitors but no effects of both inhibitors on gene expressions of the control groups. It suggests that both inhibitors can influence BM-MSCs with TGF-β1 treatment, not by itself. In addition, the TGF-β1 treated constructs exhibited higher GAG content and stronger safranin-O staining, suggesting that fibrin gel can support BM-MSC chondrogenesis. The measurement of GAG content and safranin-O staining also indicated that the constructs treated with aprotinin and TGF-β1 exhibited more proteoglycan deposition than those treated with aminohexanoic acid and TGF-β1. It suggests that aprotinin is a better fibrinolytic inhibitor to control degradation of fibrin clot when used for chondrogenesis of BM-MSCs. Furthermore, although the inhibitors may influence BM-MSC chondrogenesis, no significant effects were found on DNA content among either the aminohexanoic acid or aprotinin groups, suggesting that both fibrinolytic inhibitors may not affect proliferation of BM-MSCs.

CONCLUSION

Partial thickness defects of articular cartilage are usually not repaired because reparative cells are not able to access to the defect site due to avascularity of articular cartilage and neighboring chondrocytes are not effective in repairing adjacent defects. When articular cartilage defects that penetrate the subchondral bone tend to initiate a homeostatic response due to disruption of blood supply, a fibrin clot is formed and recruits mesenchymal stem cells from the bone marrow to begin repair on the defect. Therefore, the fibrin clot is a natural three-dimensional scaffold for chondrogenesis of BM-MSCs. Fibrin gels have already been proven successful in the transplantation of chondrocytes (Fortier et al., 2002; Fussenegger et al., 2003; Hendrickson et al., 1994; Nixon et al., 1999; Westreich et al., 2004) and in supporting the osteogenic differentiation of mesenchymal stem cells (Bensaid et al., 2003). Although the fibrin gel is a natural and successful scaffold with many advantages, its main drawback is fibrinolysis. Fibrinolysis is a natural process that leads to the degradation of fibrin clots. In order to prevent fibrinolysis, either a fibrinolytic inhibitor or a higher concentration of fibrinogen can be used (Passaretti et al., 2001; Silverman et al., 1999).

This project set out to assess the feasibility of using fibrin gels as a biomaterial scaffold for the chondrogenesis of bone marrow mesenchymal stem cells. The results showed that BM-MSCs will exhibit classic chondrogenic markers, increased aggrecan and collagen II expression, in the fibrin gel system proposed. In addition, an unexpected observation came from the studies, the fibrinolytic inhibitor needed to control the degradation of the system, seemed to affect the chondrogenesis of the BM-MSCs. In an attempt to better understand the system and reproduce our initial results, three

concentrations of two fibrinolytic inhibitors were tested for their effects on chondrogenesis. It was confirmed that aprotinin was a more effective inhibitor to control degradation when used for the chondrogenesis of BM-MSCs than aminohexanoic acid. The RT-PCR analysis showed significant differences on aggrecan gene expression of TGF-β1 treated specimens between the treatments of two fibrinolytic inhibitors while histological analysis demonstrated more proteoglycans deposited in the TGF-β groups supplemented with aprotinin than those supplemented with aminohexanoic acid. However, no significant effect of different concentrations of individual inhibitor was found on either aggrecan gene expression or proteoglycan deposition of rabbit BM-MSCs. Furthermore, both inhibitors did not influence gene expression of type II collagen and proliferation (i.e. DNA content) of rabbit BM-MSCs. An exact explanation of these result is unknown, however, it may be caused by the way the two inhibitors interact with plasmin.

The fibrinolytic system includes plasminogen, which is ubiquitously distributed throughout the human body. Plasminogen can be activated by tissue-type or urokinase-type plasminogen activator and form plasmin that is mainly responsible for the fibrinolysis of fibrin in vivo. Plasmin can also directly degrade some components of extracellular matrix (such as laminin and fibronectin) and activate zymogens of the matrix metalloproteinases (MMPs) that can degrade major components of extracellular matrix. As a result, molecular interactions between the matrix metalloproteinase and the plasminogen/plasmin system may affect the deposition of extracellular matrix (Lijnen, 2002). In addition, plasmin has been shown to transform latent TFG-β1 to active TGF-β1 (Pedrozo et al., 1999) while its activation of latent TGF-β1 is dose-dependently inhibited

by fibrinolytic inhibitor (Herbert and Carmeliet, 1997). Aprotinin and aminohexanoic acid inhibit the fibrinolytic activity of plasmin in different mechanisms. Aprotinin inhibits plasmin by forming reversible enzyme-inhibitor complexes between the active serine residue of plasmin and the lysine-15 residue of aprotinin. Aminohexanoic acid mimics the side chain of lysine that can interact with the lysine-binding sites of plasmin to disrupt the binding of plasmin to fibrin and potently inhibit fibrinolysis. Because of different mechanisms of plasmin inhibition, aprotinin and aminohexanoic acid may differently affect the plasmin activation of latent TGF-β1 and MMP zymogens, which may consequently influence chondrogenic differentiation and extracellular matrix deposition of BM-MSCs.

Aprotinin and aminohexanoic acid have been also shown to exhibit different functions other than fibrinolytic inhibition. Aprotinin can reduce inflammatory response (Donahue and Price, 2002) and promote platelet adhesion (Bradfield and Bode, 2003), and decrease thrombin antithrombin III complexes, fibrin-split products, fibrinopeptide 1+2, prothrombin fragments, and all markers of thrombin formation (Swartz et al., 2004). Aminohexanoic acid can stimulate the production of alpha2-antiplasmin during and after cardiopulmonary bypass surgery (Ray et al., 2001). When compared with aminohexanoic acid, aprotinin can reduce cytokine-induced production of nitric oxide by inhibiting expression of nitric oxide synthase (Hill et al., 1997) and attenuate the increase in the proinflammatory and anti-inflammatory cytokines IL-6 and IL-10 after cardiac surgery (Greilich et al., 2001). Therefore, different functions between aprotinin and aminohexanoic acid may result in different effects on the TGF-β induced chondrogenesis of BM-MSCs.

Although the results of this project were positive, a limitation of each study was the plates in which the gels were formed and cultivated. The plates were not large enough to hold an excess of culture medium. The amount of culture medium used was enough nutrients for the first day but by the second day, the medium was very orange indicating that the pH was high and a lot of metabolic waste was present. In addition, the gels settled on the bottom of the plates allowing for the culture medium to access the cells only through diffusion from the top. As a result, the bottom and middle layers did not receive as much nutrients and supplements as the top. This is evident in the histology and immunohistochemistry results where the staining is more prevalent near the surface of the gel. It may also be the reason that the DNA content was the same for all time periods. It is possible that there was proliferation in the cells near the top of the gel but the cells on the bottom were dying off. Since the analysis included the top and bottom of the gel, the DNA content seemed to be staying constant. In response to these issues, a better bioreactor should be designed for future studies. A bioreactor that allowed for the constant flow of culture medium through the system or a bigger well that allowed for the culture medium to access all sides of the gel could potentially increase the success of the fibrin gel system.

The future of this project is extremely promising. The result reported by this paper show how to control the degradation of the gel and what effect the inhibitors have on the chondrogenesis of the BM-MSCs. A next logical step is to further optimize the fibrin gel system. Once fibrinogen is cleaved, a self assembly step occurs in which the fibrinogen monomers come together and form a noncovalently cross-linked polymer gel via the proteolytic exposure of binding sites. This phenomena is the basis for the current

fibrin gels. However as the fibrin clot gels in vivo, intermolecular cross-linking between fibrin molecules and between soluble plasma fibronectin (pFN) found in the clot and fibrin proceeds via activated factor XIII. While the factor XIII crosslinking of fibrin should increase the gels structural stability and possibly prolong its degradation, the pFN will be important for cell and protein adhesion and cFN will provide a framework for subsequent collagen deposition (Corbett et al., 1997 and Schense and Hubbell, 1999).

In summary, this project demonstrated that fibrin gels are capable of supporting the chondrogenesis of BM-MSCs and that two fibrinolytic inhibitors, aprotinin, and aminohexanoic acid, exhibited different effects on TGF-β1 induced chondrogenesis of BM-MSCs in fibrin gels. A fibrin clot is the natural way to heal an injury to the articular cartilage assuming it penetrates the subchondral bone. The results reported and the studies proposed in this paper build on that basic concept. With further experimentation and a better understanding of the mechanisms of cartilage formation, the fibrin gel can potentially be the future of cartilage repair.

APPENDIX A

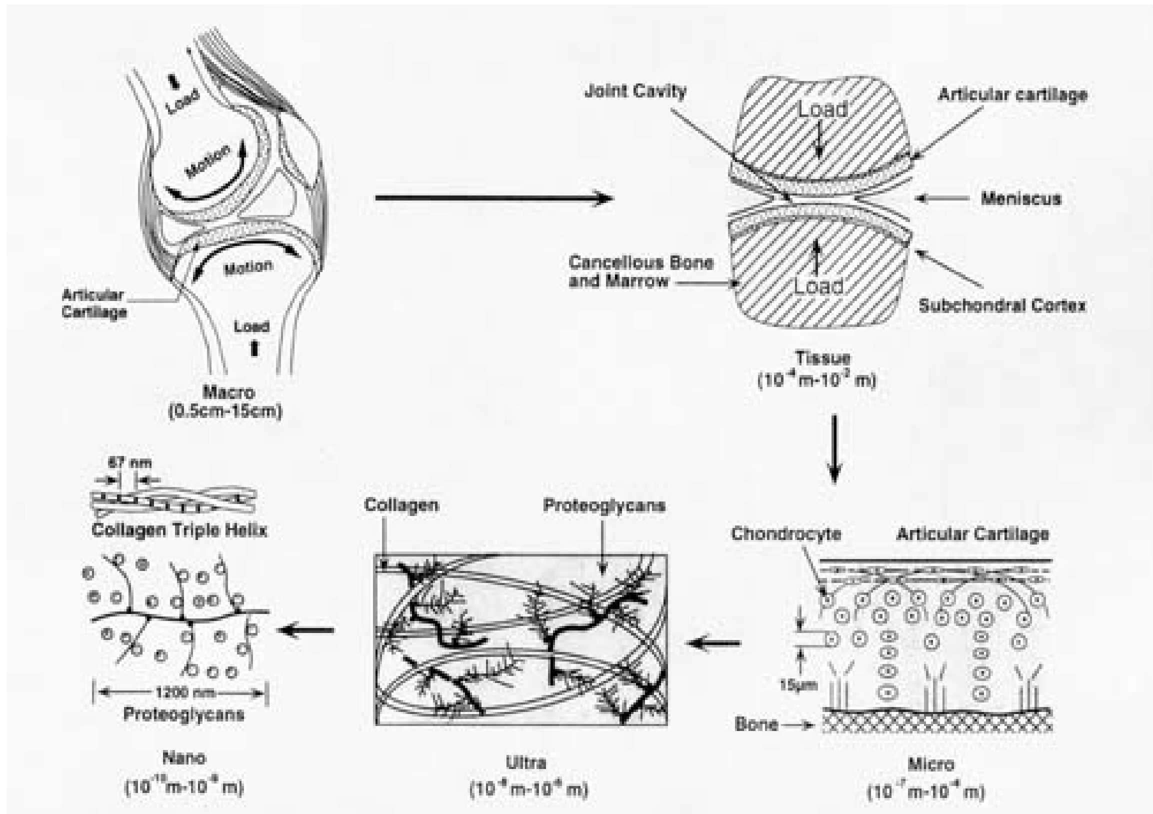

Figure 1. Articular Cartilage
(http://www.engin.umich.edu/class/bme456/cartilage/cart.htm)

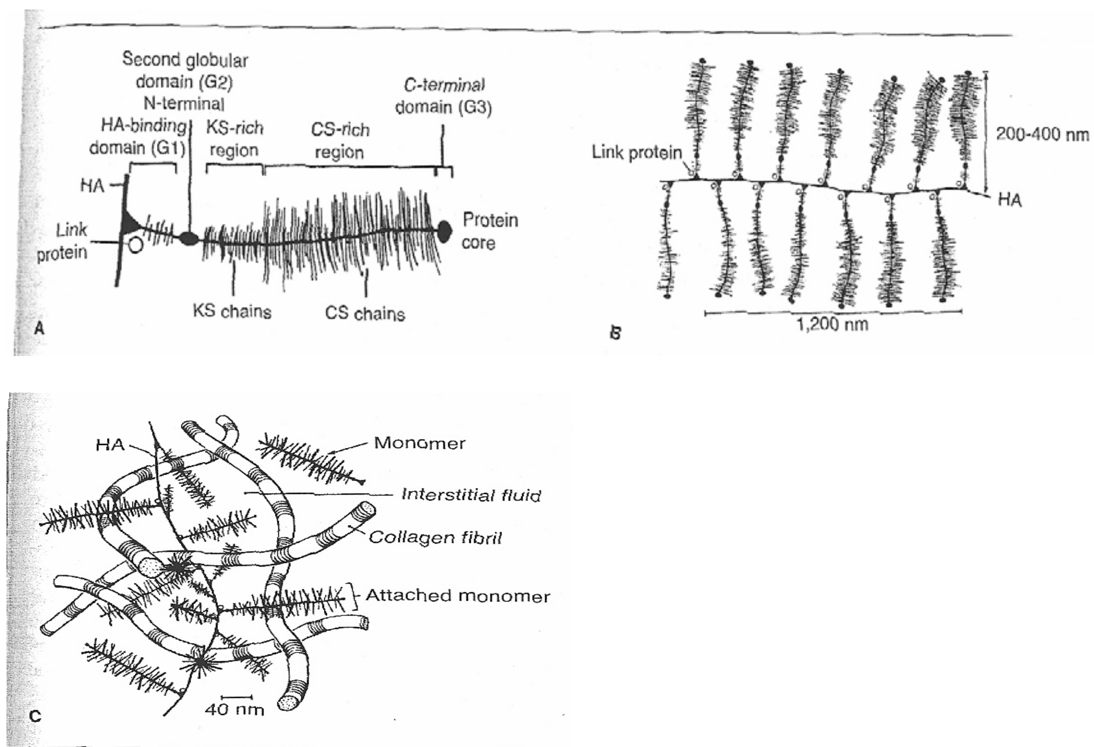

Figure 2. Proteoglycans (Ulrich-Vinther et al., 2003)

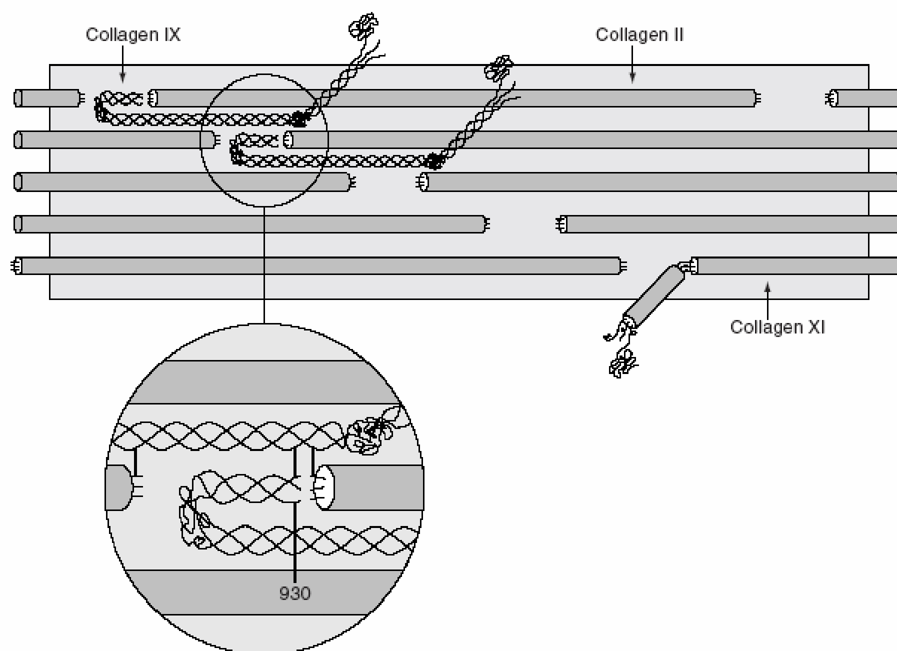

Figure 3. A collagen II:IX:XI heterofibril (Eyre, 2002)

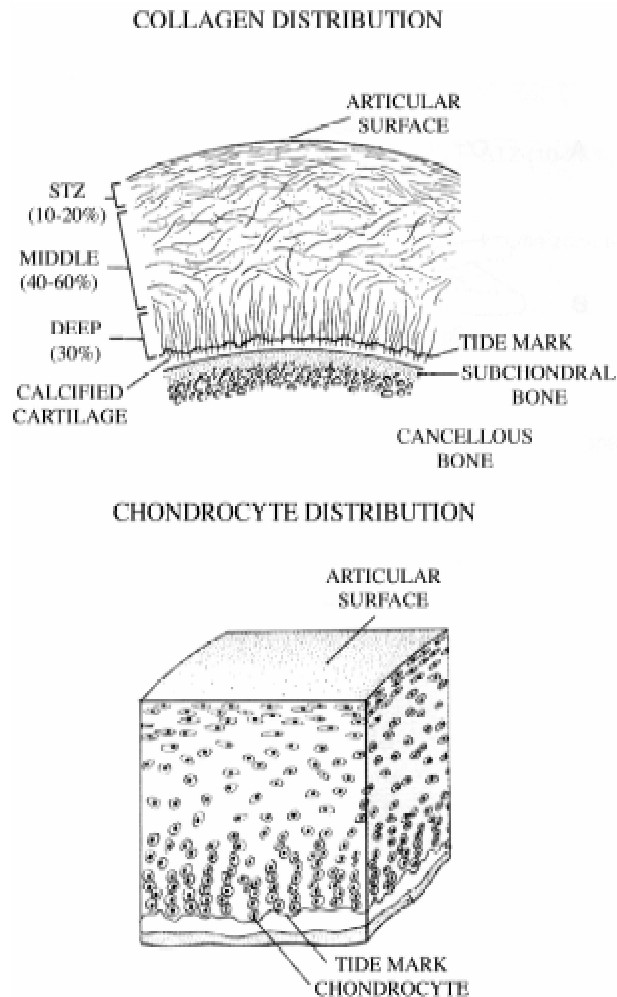

Figure 4. Zones of Articular Cartilage
(http://www.sfu.ca/~stever/kin402/lecture_outlines/lecture_9_cartilage.pdf#search='superficial%2C%20tangential%2C%20and%20deep%20layer%20of%20articular%20cartilage')

Gene	Sequence	Size	Reference
Collagen II (Sense) (Antisense)	5'-GCACCCATGGACATTGGAGGG-3' 5'-GACACGGAGTAGCACCATCG-3'	366 bp	Genbank S83370
Aggrecan (Sense) (Antisense)	5'-ACATCCCAGAAAACTTCTTT-3' 5'-CGGCTTCGTCAGCAAAGCCA-3'	276 bp	Genbank L38480
GAPDH (Sense) (Antisense)	5'-TCACCATCTTCCAGGAGCGA -3' 5'-CACAATGCCGAAGTGGTCGT-3'	293 bp	GenBank L23961

Table 1. Sequences of PCR primers

APPENDIX B

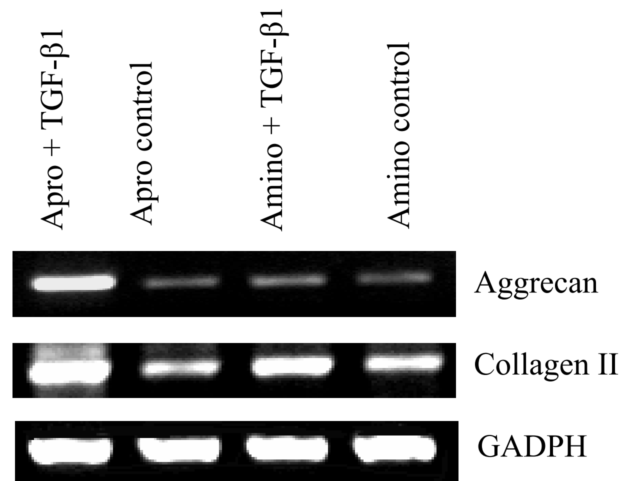

Figure 5. Typical chondrogenic gene expression of fibrin gels of the four groups at 14 days (Apro = Aprotinin and Amino = Aminohexanoic acid)

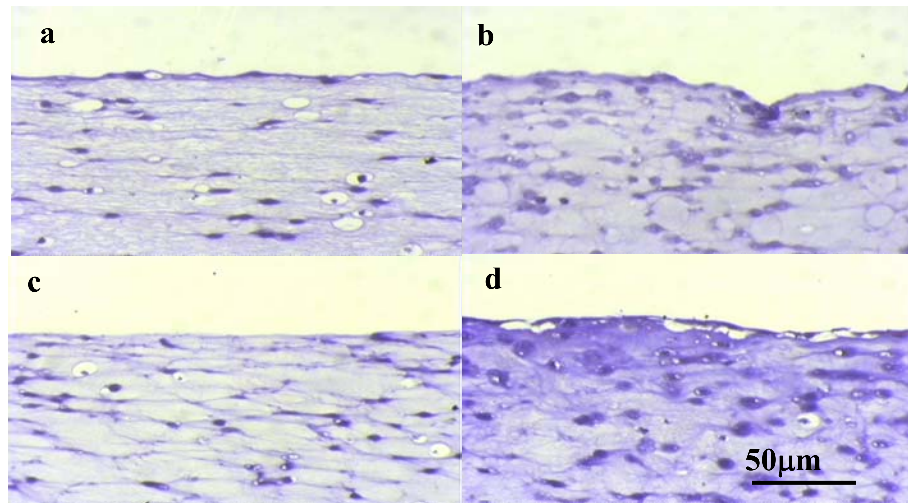

Figure 6. Toluidine blue staining of (a)(b) aminohexanoic acid and (c)(d) aprotinin treated specimens (a)(c) without and (b)(d) with the treatment of TGF-β1 at 21 days

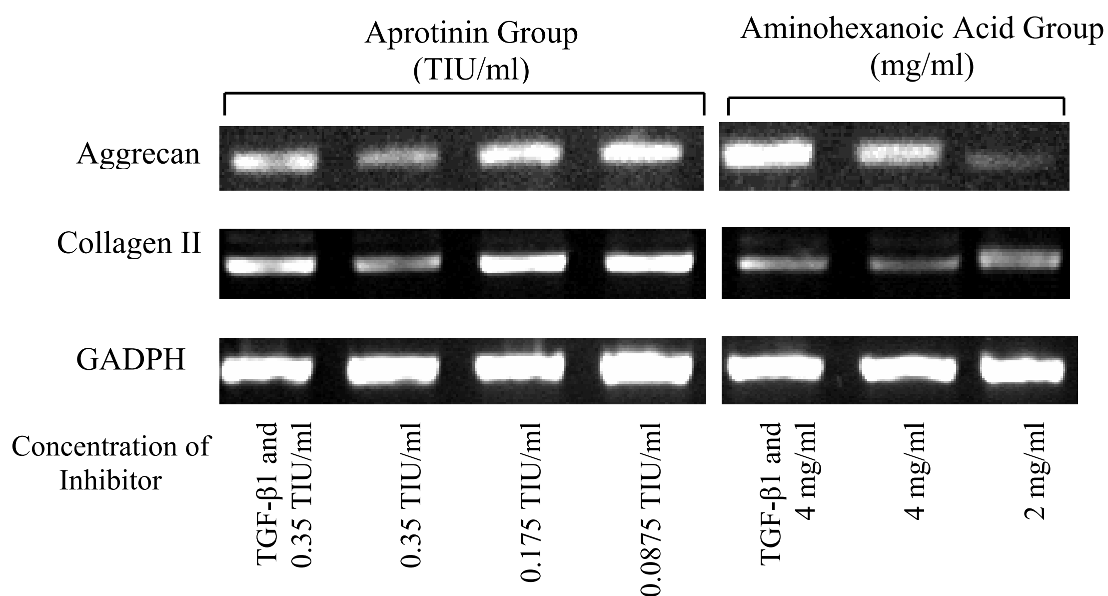

Figure 7. The chondrogenic gene expression of different concentrations of aprotinin and aminohexanoic acid at 19 days

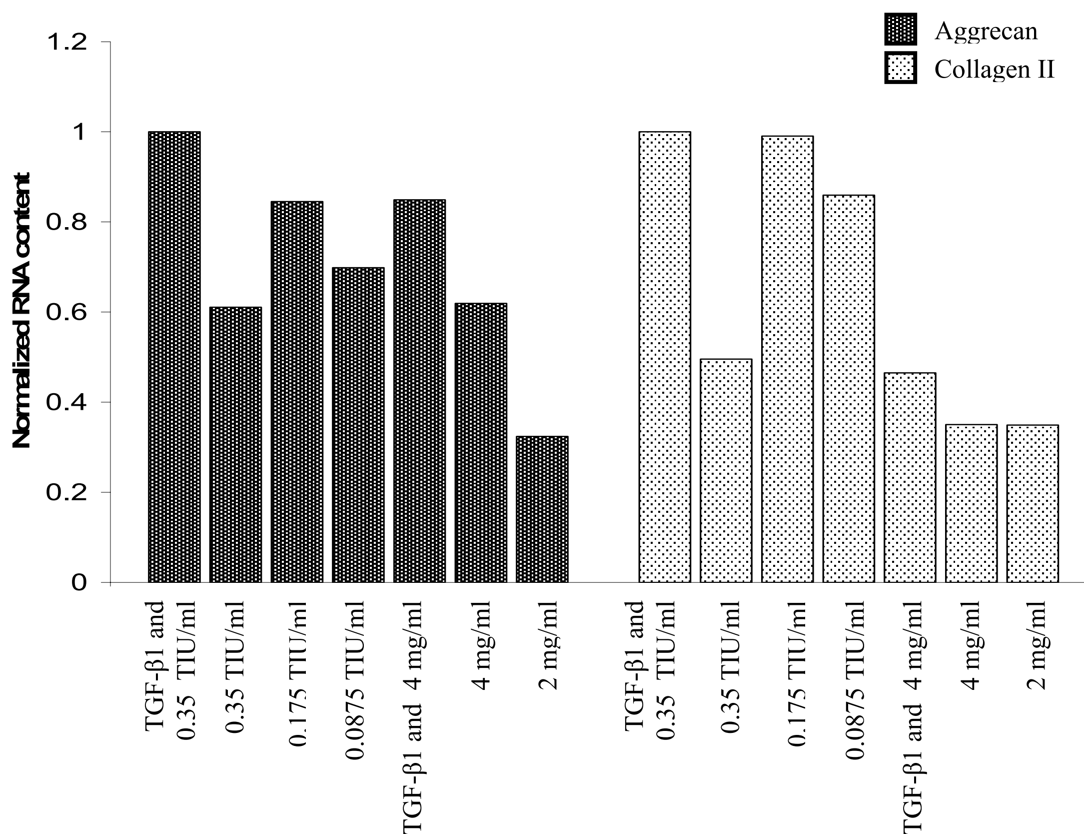

Figure 8. Normalized aggrecan and collagen II gene expression of different concentrations of aprotinin and aminohexanoic acid at day 19

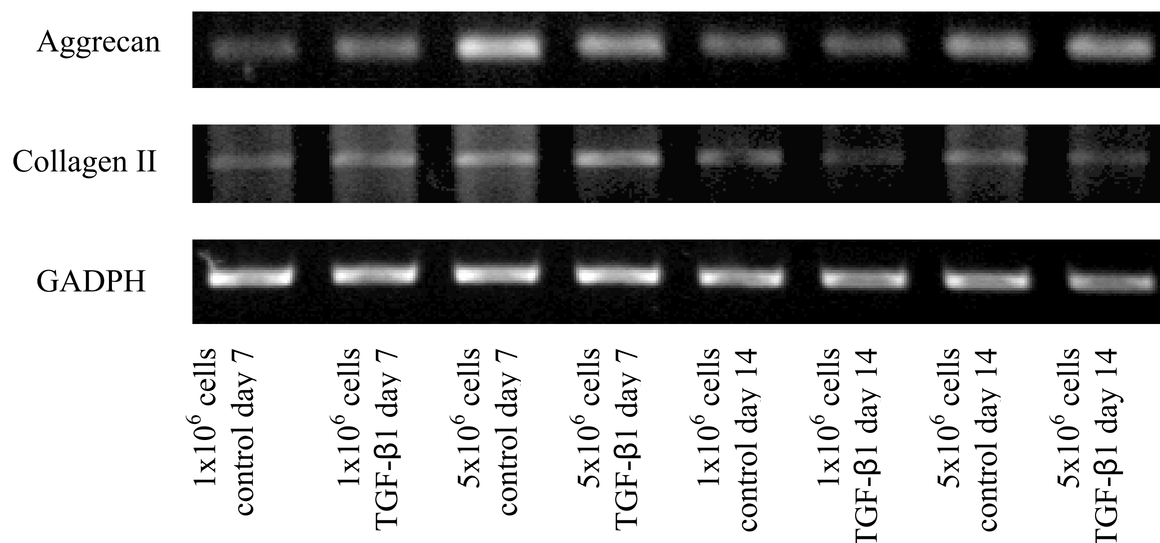

Figure 9. The chondrogenic gene expression of different cell densities at 7 and 14 days.

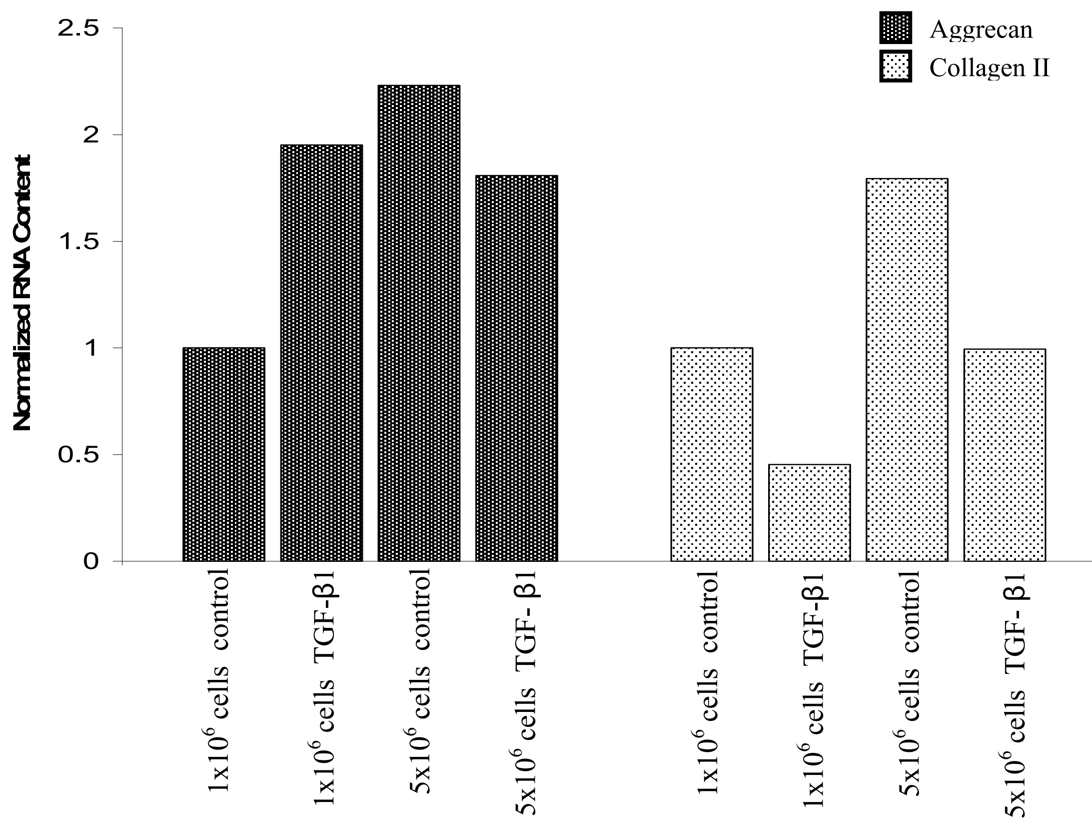

Figure 10. Normalized aggrecan and collagen II gene expression of different cell densities at day 14

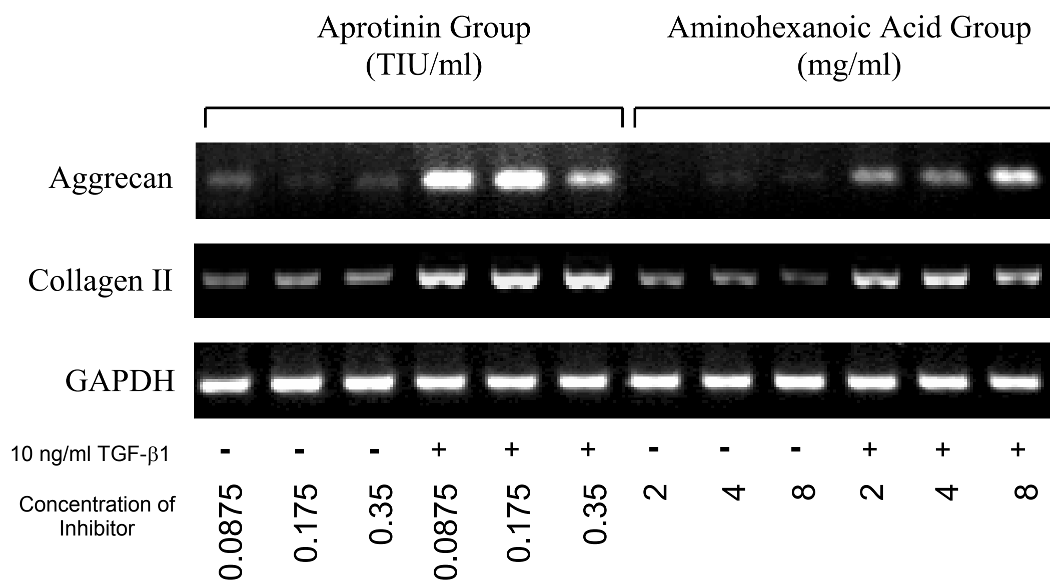

Figure 11. Typical chondrogenic gene expressions of experimental groups after 14 days of culture

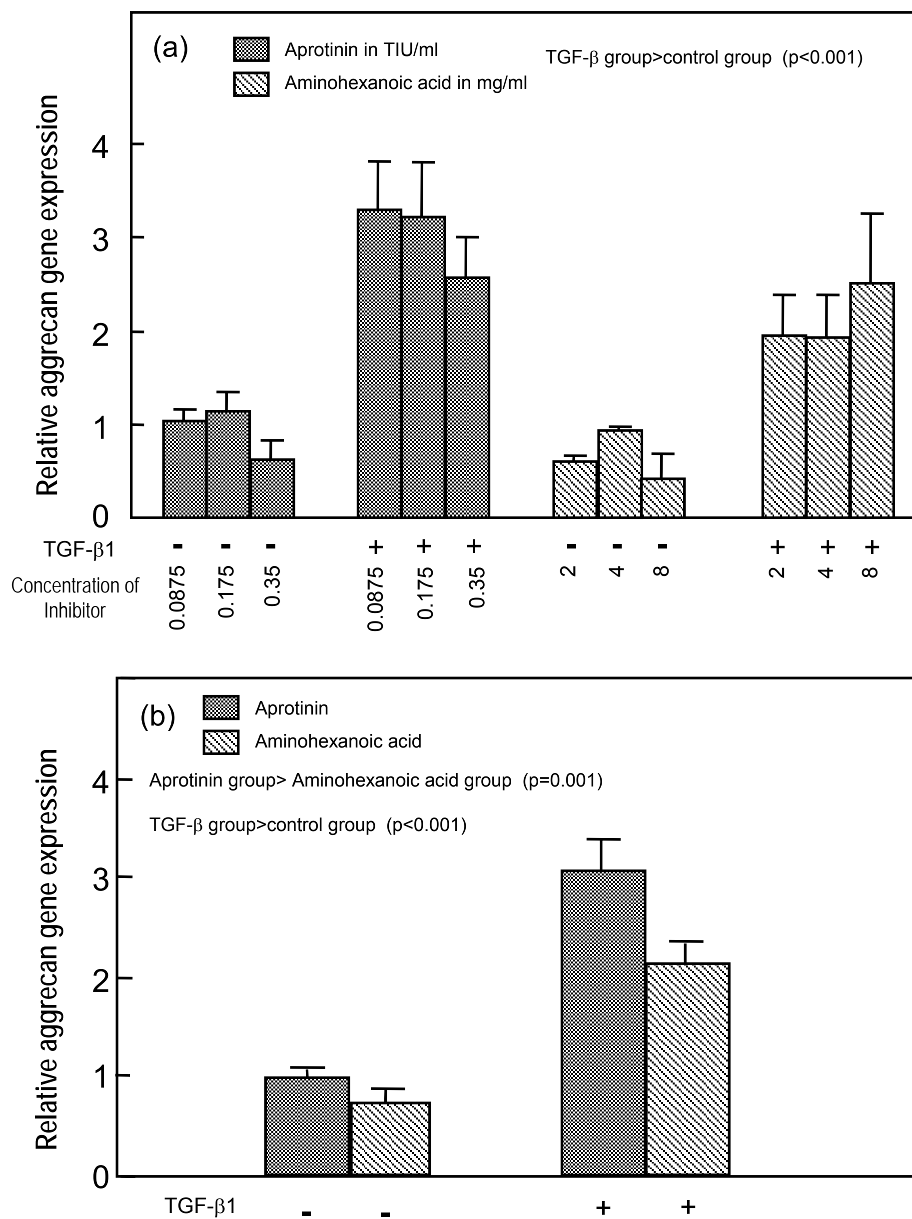

Figure 12. Statistical analysis of aggrecan gene expression after 14 days of culture. The relative aggrecan gene expressions of experimental groups were statistically analyzed using a two-way ANOVA. Factors are +/- TGF-β treatment and inhibitor level for each inhibitor in (a) (n=5 for each group) and +/- TGF-β treatment and inhibitor in (b) (n=15 for each group).

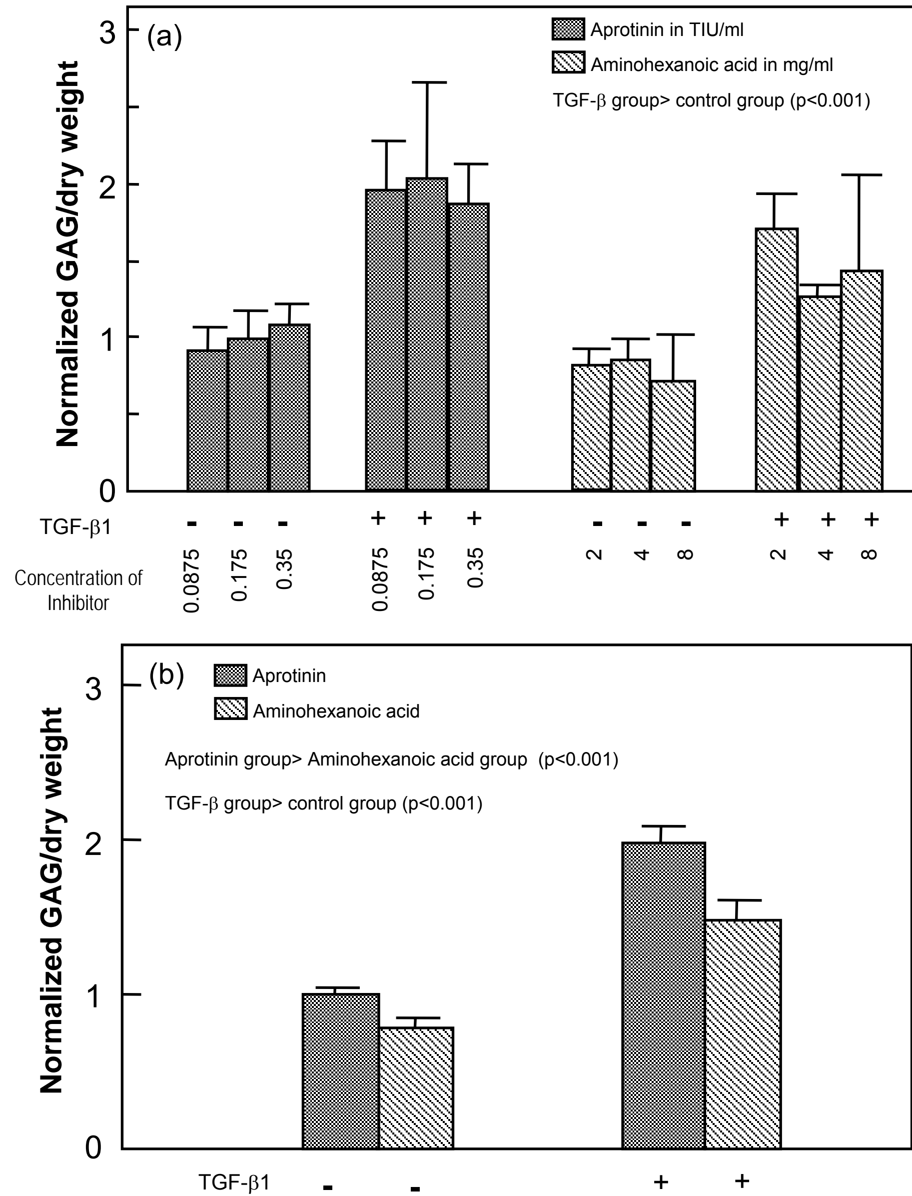

Figure 3. Statistical analysis of GAG content after 14 days of culture. The GAG contents of experimental groups were statistically analyzed using a two-way ANOVA. Factors are +/- TGF-β treatment and inhibitor level for each inhibitor in (a) (n=5 for each group) and +/- TGF-β treatment and inhibitor in (b) (n=15 for each group).

(A) (B) (C) (D) (E) (F)

Figure 14. Histology and Immunohistochemistry at 21 days. Typical safranin-O staining of proteoglycans (A-D) and immunohistochemical staining of type II collage protein (E,F) for (A,E) the aprotinin control group (0.35 TIU/ml aprotinin), (B,F) the aprotinin+TGF-β group (0.35 TIU/ml aprotinin), (C) the aminohexanoic acid control group (2 mg/ml aminohexanoic acid) and (D) the aminohexanoic acid+TGF-β group (2 mg/ml aminohexanoic acid) (Bar = 100μm for (A-D); 25μm for (E,F)).

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VITA

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