Evaluation of Chondrogenesis in Collagen / Chitosan / Glycosaminoglycan Scaffolds for Cartilage Tissue Engineering

Jong Eun Lee1, Mee Hyun Jeong1, Hyun Jeong Ahn1, Jung Kyu Kim2, Kuiwon Choi2, Chong Bum Chang1, Hee Joong Kim1, Sang Chul Seong1, and Myung Chul Lee1,*

1Department of Orthopedic Surgery, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-744, Korea
2Biomedical Research Center, Korea Institute of Science and Technology, 131 Cheongryang, Seoul 130-650, Korea.

(Received: Feb. 01, 2005; Accepted: Feb. 18, 2005)

Abstract: We investigated the behavior of rabbit chondrocytes seeded in vitro into collagen/chitosan/glycosaminoglycan (GAG) scaffolds with different chitosan contents (i.e., at collagen:chitosan ratios of 20 : 1, 5 : 1, and 1.25 : 1, w/w, respectively). The porous scaffolds containing collagen and chitosan were fabricated by using a freeze drying technique and crosslinked using 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) in the presence of chondroitin sulfate (CS). Scanning electron microscope (SEM) views of the scaffolds showed that all three had interconnected pores of mean diameter 164, 353, and 567 µm at collagen:chitosan ratios of 20 : 1, 5 : 1, and 1 : 1, w/w, respectively. GAG was covalently bound onto these scaffolds at 6.4% (w/w) in all three cases, i.e., regardless of chitosan content. However, increased chitosan content resulted in enhanced mechanical properties and increased pore size. Rabbit chondrocytes seeded onto these scaffolds were cultured for 1, 3, 7, and 14 days. Biochemical analysis of these scaffolds showed that GAG synthesis and proliferation rate increased with time, and this became significant for the collagen : chitosan (20 : 1)-CS scaffold on day 14. The histology of the cell-seeded constructs showed a significantly higher percentage of cells with spherical morphology, consistent with chondrocytic morphology, especially in the collagen : chitosan (20 : 1)-CS scaffold at each time point. This finding was supported by the observation that the pericellular matrix was stained positively for proteoglycans and type II collagen after 14 days. We conclude that the collagen : chitosan (20 : 1)-CS scaffold seems to be a useful carrier material for tissue engineered cartilage.

Keywords: tissue engineering, collagen, chitosan, glycosaminoglycan, scaffold, chondrogenesis

1. Introduction

Damaged articular cartilage (AC) has very little capacity for spontaneous healing. Although many repair techniques have been proposed over the past three decades, all fail to produce a long-lasting repair tissue.1 Recently, tissue engineering concepts have been applied to the development of a cell-based repair material for AC.2 The tissue engineering of AC involves the isolation of precursor cells that may be expanded in vitro, and the combining of these with a biocompatible matrix for cultivation and subsequent implantation into the joint. Not surprisingly, the choice of biomaterial is critical to the success of such tissue-engineered constructs.

Various scaffold materials have been tested, including both naturally occurring materials and synthetic polymers. These natural materials include, demineralized bone matrix, fibrin glue, collagen, hyaluronan, and chitosan, and the synthetic biodegradable polymers include, polyesters, such as polyglycolic acid (PGA), poly(L-lactic acid) (PLLA), and poly (latide-co-glycolide) (PLGA).3-10 Many studies using chondrocytes or cells with chondrogenic potential have shown that these quite different biomaterials can support the formation of collagen type II and proteoglycan-containing tissue. However, the results obtained have been inconsistent, with cartilage tissue morphologies ranging from hyaline-like to fibrous in quality. One reason for this variable quality may be related to the chemical compositions of the biomaterial used.11 Moreover, the use of a biomaterial simi-
lar to the target tissue’s extracellular matrix may be a key factor to the successful tissue engineering of AC.

AC-specific extracellular matrix (ECM) components, such as collagen and glycosaminoglycan (GAG), are reported to play a critical role in regulating the expression of the chondrocytic phenotype and in supporting chondrogenesis both in vitro and in vivo. Among these, collagen has received the most attention as a material for cartilage tissue engineering. However, the use of a collagen scaffold for the repair of cartilage defects has limitations due to its inherent weak mechanical stability and rapid biodegradation. Various chemical crosslinking agents, such as glutaraldehyde, cyanamide, formaldehyde, and disiocyanate, have been introduced into collagen based scaffolds to prevent these defects; however, such chemical agents have associated cytotoxicities. Recently, in order to ensure the absence of toxic materials, the water soluble carboimide, 1-ethyl-3-(3-dimethyl aminopropyl)carboimide (EDC), has been examined as an alternative. EDC mediates ester/amide bond formation between the hydroxyl/amine and carboxyl groups. Therefore, it has been widely used for the crosslinking of various proteins and polysaccharides.

Chitosan is a natural polysaccharide, which is structurally similar to GAGs. Chitosan consists of (1 → 4) linked N-glucosamine residues, and has been reported to be nontoxic, bioabsorbable and to promote wound healing. Furthermore, the incorporation of chitosan into a collagen scaffold is known to increase its mechanical strength by forming an ionic complex between the positively charged chitosan and the negatively charged collagen.

In addition, studies upon the culturing of chondrocytes demonstrated that chitosan helps maintain cellular morphology and function, although it had no proliferate effects.

GAGs are negatively charged and unbranched polysaccharides, which are present in AC and are important for stimulating chondrogenesis. With the exception of hyaluronic acid, polysaccharides, such as heparin, and chondroitin sulfate, are covalently linked to a protein core, thereby forming proteoglycans. In particular, chondroitin sulfate (CS) is one of the major GAGs in ACs. Furthermore, CS has biocharacteristics, which include the binding and modulation of growth factors and cytokines, the inhibition of proteases, and an involvement in the adhesion, migration, proliferation and differentiation of cells. We hypothesized that the collagen/chitosan/GAG matrix would provide chondrocytes with a familiar and supportive environment due to the similarity between it and the GAG-rich ECM of the AC, and thus allow them to maintain many of the characteristics of differentiated chondrocytic phenotypes in vitro.

Although collagen, chitosan and GAG have been proposed separately as in vitro ECM materials, the influence of collagen/chitosan/GAG composite scaffolds on cell morphology, differentiation, and function has not been well studied, and no study of this type has been performed on chondrocytes. To this end, we characterized collagen/chitosan/GAG scaffolds containing collagen and chitosan in different proportions (20 : 1, 5 : 1, 1.25 : 1, w/w) physico-chemically and mechanically, and examined their abilities to regulate cellular activity.

2. Materials and Methods

**Scaffold Fabrication.** Blended collagen/chitosan (Col : Chi) scaffolds were made using the freeze-drying method described previously. A 0.5% (w/v) collagen (Type I, Sigma Chemical Co. St. Louis, MO, USA) solution and a 0.1% chitosan (Mw : 150,000, 81 mole, Fluka Chemie, Buchs, Switzerland) solution were both made in 0.1M acetate pH 4.5 buffer. The two solutions were mixed in the ratios 8 : 2, 5 : 5 and 2 : 8 (col : chi, v/v) and then the resultant ratios become 20 : 1, 5 : 1 and 1.25 : 1 w/w. These blends were then frozen at -70°C and lyophilized for 24 h in a freeze dryer (Labconco Co., Kansas, MO, USA).

**Crosslinking of Scaffolds in the Presence of Chondroitin Sulphate.** The scaffolds were crosslinked in the presence of GAGs by using 1-ethyl-3-(3-dimethyl aminopropyl)carboimide (EDC) (Fluka Chemie), as described previously with some modification. Briefly, scaffolds of uniform size (10 mm in diameter, 3 mm in thickness) were cross-linked by immersing them for 4 h in 2 ml of 40% (v/v) ethanol containing 50 mM 2-morpholinethane sulfonic acid (MES) (pH 5.0) (Fluka Chemie), 33 mM EDC, 8 mM N-hydroxsuccimide (Fluka Chemie) and 1% (w/v) chondroitin sulfate (CS, Sigma Chemical Co.). The acidities of the scaffolds were neutralized with 0.1M Na2HPO4 (pH 9.1) for 1 h. Excess base was then removed by repeated washing with distilled water until the matrix pH returned to a physiologic range (pH 7.0-7.4) and then freeze-dried.

**Determination of the Physico-Chemical/Mechanical Properties of Scaffolds.** Scanning electron microscopy