



Human adipose derived stem cells are superior to human osteoblasts (HOB) in bone tissue engineering on a collagen-fibroin-ELR blend



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ABSTRACT

The ultrastructure of the bone provides a unique mechanical strength against compressive, torsional and tensional stresses. An elastin-like recombinamer (ELR) with a nucleation sequence for hydroxyapatite was incorporated into films prepared from a collagen – silk fibroin blend carrying microchannel patterns to stimulate anisotropic osteogenesis. SEM and fluorescence microscopy showed the alignment of adipose-derived stem cells (ADSCs) and the human osteoblasts (HOBs) on the ridges and in the grooves of microchannel patterned collagen-fibroin-ELR blend films. The Young's modulus and the ultimate tensile strength (UTS) of untreated films were 0.58 ± 0.13 MPa and 0.18 ± 0.05 MPa, respectively. After 28 days of cell culture, ADSC seeded film had a Young's modulus of 1.21 ± 0.42 MPa and UTS of 0.32 ± 0.15 MPa which were about 3 fold higher than HOB seeded films. The difference in Young's modulus was statistically significant ($p: 0.02$). ADSCs attached, proliferated and mineralized better than the HOBs. In the light of these results, ADSCs served as a better cell source than HOBs for bone tissue engineering of collagen-fibroin-ELR based constructs used in this study. We have thus shown the enhancement in the tensile mechanical properties of the bone tissue engineered scaffolds by using ADSCs.

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1. Introduction

Musculoskeletal disorders are the second biggest reason for long term disability of patients in the world after the mental and behavioral disorders [1]. Fractures or diseases such as osteoporosis lead to a decrease in bone quality and mechanical integrity. Autografts and allografts are used in the conventional therapy of bone defects. Bone autografts are highly preferred over the allografts in order to eliminate infection and immune rejection risks. Availability of vasculature and mechanical properties adequate for cortical bone [2] are important properties of autografts, but their

scarcity, site morbidity, infection and bleeding at the extraction site are serious problems. In order to have mechanically strong grafts, ceramics such as hydroxyapatite and tricalcium phosphate [3], which can directly bond to the bone surface, were tested but not preferred because they are brittle and have a high density. Other synthetic options such as bioactive glass [4] were also unsuitable due to their brittleness and tendency to fracture upon cyclic loading or when drilling during implant surgery. On the other hand, bone tissue engineering using synthetic or biological polymers can overcome most of these drawbacks (except mechanical weakness). Besides, degradation products of polyesters such as poly(lactic acid) (PLA) [5], poly(glycolic acid) (PGA) [6] and their copolymers were shown to create an acidic environment. In addition, the synthetic polymers do not possess cell signaling sequences that are naturally present in the structure of biological polymers such as fibronectin, collagen, vitronectin and fibrinogen.

Tissue engineering (TE) can still be an appropriate alternative

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for small and medium sized bone defects because TE can introduce to the scaffolds properties like osteoconduction, osteoinduction and osteointegration. The main constituents of bone tissue engineering are biocompatible and biodegradable porous scaffolds and autologous cells to be seeded into the scaffolds to obtain biologically and mechanically adequate tissue substitutes [7]. Recently, protein based scaffolds gained importance in load bearing sites in the body such as bone, cartilage, tendon, meniscus, vessels, skin, bladder and cornea [8], but since they lacked the required mechanical strength, additional materials such as hydroxyapatite for bone needed to be added to improve their mechanical strength [9].

Collagen type I has a special place among the natural polymers because it is secreted by a variety of cells including osteoblasts and constitutes the main organic phase of extracellular matrix (ECM) [10] and contributes significantly to the viscoelasticity of bone [11]. In bone, collagen fibrils are reinforced by plate-shaped HAP nanocrystals 10–20 nm in length and 2–3 nm width [12]. However, reconstituted collagen has a much lower mechanical strength than the bone to be substituted [13]. The reason for this is partly the lack of fibrillar arrangement due to hydrolysis during reconstitution. Additionally, collagen denatures during sterilization and this decreases its resistance to enzymes and mechanical strength [14]. As an alternative to collagen, silk fibroin has been proposed as protein biomaterial for load bearing TE applications due to its unique mechanical properties. Self assembled *Bombyx mori* fibroin molecules have a crystalline β -sheet structure which gives its high tensile strength and toughness, and the remaining amorphous region provides the elasticity needed [15]. However, handling of pure silk fibroin scaffolds is an issue and therefore, it is advantageous to use it as a blend with more flexible materials such as collagen, to achieve optimum mechanical strength and ease of use.

Recently, recombinant proteins have become protein materials of choice due to their tailor-made properties. Elastin-like recombinamers (ELRs) are such recombinant proteins coded in a synthetic DNA and expressed via the use of high yield vectors. VPGXG repeating sequences, where X is a natural or modified amino acid except L-proline originates from elastin which is an ECM protein found in many tissues including bone and is responsible for their elasticity. The amino acid sequences are combined in repeating fashion to form the backbone of ELRs [16]. Most ELRs are thermoresponsive materials and aggregate at temperatures higher than their inverse transition temperature (ITT). This feature aids purification of ELRs via solubilization-precipitation [17]. ELRs have earlier been used in bone tissue engineering and shown to be biocompatible [18]. ELRs with special sequences were reported for bone tissue engineering in order to increase cell adhesion [19], to form an antifouling coat on titanium implants [20] or to enhance nucleation of HAP on the implant surfaces [21].

In this paper, swelling and crystallinity of films of pure collagen, fibroin and their blends were studied. ELR was added to the blend composition to compare the suitability of the stem cells and primary osteoblasts for bone tissue engineering. In this study, an ELR with [(VPGIG)₂ (VPGKG) (VPGIG)₂]₂ DDDEEKFLRRIGRFG [(VPGIG)₂ (VPGKG) (VPGIG)₂]₂ repeats was used (Fig. 1a). The DDDEEKFLRRIGRFG region is responsible for nucleation of hydroxyapatite. This analog fragment was adapted from statherin, a salivary protein, that has high affinity for calcium and phosphate ions and helps maintain calcification dynamics of tooth enamel [22]. This fragment was shown to induce mineralization on ELR containing surfaces in a supersaturated solution of divalent ions such as simulated body fluid (SBF) [21]. We aimed anisotropic cell growth to mimic the osteon organization. Microchannel pattern decorated films were seeded with human osteoblasts (HOBs) and human adipose-derived stem cells (ADSCs) to align the cells and expected them

to secrete ECM in a parallel fashion as in cortical bone. It was hypothesized that alignment could lead to anisotropic mechanical strength to resist the compressive, tensional and torsional forces [23]. In this study the organization of bone lamellae was mimicked with a collagen-fibroin-ELR blend film carrying oriented collagen fibers and the influence of cell type on the mechanical properties of the resultant scaffolds were studied using HOBs and ADSCs (Fig. 1b). This is the first study in the literature that compares mature and stem cells with respect to their proliferation potential and influence on the mechanical strength of the scaffold for their potential use in bone tissue engineering.

2. Materials and methods

2.1. ELR expression and purification

ELR [(VPGIG)₂ (VPGKG) (VPGIG)₂]₂ DDDEEKFLRRIGRFG [(VPGIG)₂ (VPGKG) (VPGIG)₂]₂ carrying an HAP nucleating sequence was produced and characterized by Prof. José Carlos Rodríguez-Cabello (Universidad de Valladolid, Spain). The theoretical mass of the ELR was calculated according to recombinamer design and found as 31,877 Da [24]. Briefly, *E. coli* system was used for the oligopeptide synthesis. Cells were lysed by ultrasonication and protein was purified by applying a series of cold and warm centrifugation steps and dialysis. Purification was carried out by using aggregation of the recombinamer with a lower critical solution temperature (LCST) above its transition temperature. ITT was found to be 32 °C at pH = 7.36 as determined by size measurement between 20 °C and 40 °C by using Nano-ZS (Malvern, Worcestershire, UK). Molecular weight and purity of protein were confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) with a sharp peak at 31,857.21 Da and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a distinctive band around 30 kDa that was close to the theoretical mass.

2.2. Template preparation

The chemical etching method was applied for the production of patterned silicon wafer at Bilkent University (Ankara, Turkey). Microchannel dimensions were 5 μ m, 10 μ m, and 5 μ m, for ridge width, groove width and depth, respectively (Fig. 1c). Negative replicas were produced from poly(dimethylsiloxane) (PDMS) by mixing PDMS prepolymer and curing agent (Sylgard 184 Elastomer Kit, Dow Corning, Midland, Michigan, USA) in 10:1 ratio and heating at 70 °C for 3 h [25] (Fig. 1d).

2.3. Film preparation

Collagen type I was isolated from Sprague–Dawley rat tails and fibroin was purified from silk fibers of *Bombyx mori* according to previously published methods [26]. *Bombyx mori* silk threads were gift from Prof. Esra Karaca, Uludag University (Bursa, Turkey). In brief, for collagen type I isolation, tendons were removed and dissolved in cold acetic acid (0.5 M). Filtered solution was dialyzed against phosphate buffer (24 mM, pH 7.2) and centrifuged. Pellets were dissolved in acetic acid (0.15 M) and NaCl (5%) was added to solution. Next day, precipitated collagen was separated via centrifugation and dissolved in acetic acid (0.15 M). After a week of dialysis, solution was centrifuged and collagen was sterilized in ethanol (70%) for 2 days. Following the centrifugation step, collagen was lyophilized for long term storage. For silk fibroin isolation, silk threads (12.5 g) were washed in boiled Na₂CO₃ (0.02 M) for 30 min and dried at 37 °C. Fibroin was dissolved in 9.3 M LiBr (60 °C) and filtered. Solution was dialyzed against water and lyophilized.

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