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Pericellular plasma clot negates the influence of scaffold stiffness on chondrogenic differentiation

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ABSTRACT

Matrix stiffness is known to play a pivotal role in cellular differentiation. Studies have shown that soft scaffolds (<2–3 kPa) promote cellular aggregation and chondrogenesis, whereas, stiffer ones (>10 kPa) show poor chondrogenesis *in vitro*. In this work we investigated if fibrin matrix from clotted blood can act as a soft surrogate which nullifies the influence of the underlying stiff scaffold, thus promoting chondrogenesis irrespective of bulk scale scaffold stiffness. For this we performed *in vitro* chondrogenesis on soft (~1.5 kPa) and stiff (~40 kPa) gelatin scaffolds in the presence and absence of pericellular plasma clot. Our results demonstrated that in absence of pericellular plasma clot, chondrocytes showed efficient condensation and cartilaginous matrix secretion only on soft scaffolds, whereas, in presence of pericellular plasma clot, cell rounding and cartilaginous matrix secretion was observed in both soft and stiff scaffolds. More specifically, significantly higher collagen II, chondroitin sulfate and aggrecan deposition was observed in soft scaffolds, and soft and stiff scaffolds with pericellular plasma clot as compared to stiff scaffolds without pericellular plasma clot. Moreover, collagen type I, a fibrocartilage/bone marker was significantly higher only in stiff scaffolds without plasma clot. Therefore, it can be concluded that chondrocytes surrounded by a soft fibrin network were unable to sense the stiffness of the underlying scaffold/substrate and hence facilitate chondrogenesis even on stiff scaffolds. This understanding can have significant implications in the design of scaffolds for cartilage tissue engineering.

Statement of Significance

Cell fate is influenced by the mechanical properties of cell culture substrates. Outside the body, cartilage progenitor cells express significant amounts of cartilage-specific markers on soft scaffolds but not on stiff scaffolds. However, when implanted in joints, stiff scaffolds show equivalent expression of markers as seen in soft scaffolds. This disparity in existing literature prompted our study. Our results suggest that encapsulation of cells in a soft plasma clot, present in any surgical intervention, prevents their perception of stiffness of the underlying scaffold, and hence the ability to distinguish between soft and stiff scaffolds vanishes. This finding would aid the design of new scaffolds that elicit cartilage-like biochemical properties while simultaneously being mechanically comparable to cartilage tissue.

1. Introduction

Biomaterial based scaffolds provide a transient framework for cells to assemble and organize into three dimensional neotissues. Though earlier attempts at scaffold development focused on providing a cell supporting material, recent insights on the influence of physical and chemical features of the extracellular milieu have led to the development of cell instructive scaffold systems. Amongst various physical features, matrix stiffness plays a crucial role in determining cell fate [1].

Like many other tissues [2–5], *in vitro* cartilage formation too has been shown to be significantly influenced by scaffold stiffness [6,7]. Most of these studies show that while soft matrices (films/hydrogels/sponges) (~<3 kPa) facilitate cell condensation, rounding and chondrogenic differentiation, stiffer ones (>10 kPa) favor cell proliferation and non-cartilaginous phenotype [6–8]. Surprisingly, such a trend is not observed when translated to the *in vivo* environment. In fact, many *in vivo* studies show good quality cartilage formation even in extremely stiff scaffolds

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(macroporous sponges) made up of materials such as silk (E′ ~0.4 to 1 MPa) and PLGA (E = 0.3 MPa) [9,10]. Therefore, it is very interesting to note that while *in vitro* studies suggest the use of soft materials for better chondrogenesis, previously reported *in vivo* experiments have shown success – in terms of cartilage formation – in a wide array of materials ranging from extremely soft (<2 kPa) to extremely stiff (⩾0.1 MPa) [6–10].

We speculated that there might exist certain factors that can either disguise or override the matrix stiffness mediated response. A closer look into the existing literature revealed that the most widely used animal model was the osteochondral defect (OCD) model [11]. During creation of the OCD, blood exudes out into the defect when the subchondral bone vasculature is damaged. As a result, blood also perfuses into the porous structure of an implanted scaffold and clots *in situ*, giving rise to a soft fibrin network. It is known that fibrin clot at physiological concentrations of fibrinogen has a storage modulus of around 1.5 kPa [12], which is well suited for chondrogenic differentiation [8]. Moreover, it has been reported that plasma and whole blood clots act as excellent matrices for chondrocyte survival and differentiation leading to formation of hyaline like neo-cartilage [13]. Therefore, we posed a question that can fibrin matrix from clotted blood act as a soft surrogate which nullifies the influence of the underlying stiff scaffold, thus promoting chondrogenesis irrespective of scaffold stiffness.

In the current study, the role of scaffold stiffness on cell behavior was investigated in the presence and absence of plasma clot. For this, *in vitro* expanded/dedifferentiated chondrocytes were seeded onto scaffolds of varying stiffness in the presence and absence of plasma and were characterized for changes in cell morphology and cartilaginous matrix deposition.

2. Materials and methods

2.1. Fabrication of gelatin scaffolds

Gelatin scaffolds were fabricated using a modified freeze drying method [14]. For this, gelatin type B (~225 Bloom viscosity) from Sigma-Aldrich, St Louis, MO, USA was dissolved in deionized water containing 2% (w/v) salt at a final polymer concentration of 3% (w/v). The solution was poured in disposable syringe molds (5/8 mm diameter) and that were placed at −135 °C in liquid nitrogen vapor phase for 12 h. The solution was allowed to freeze overnight followed by freeze-drying at −45 °C and 0.2 mbar (Christ-Alpha 1–2 LD) for 36 h to obtain porous scaffolds. The scaffolds were then cross-linked using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC)/N-hydroxysuccinimide (NHS) (Sigma-Aldrich, St Louis) at a concentration of 1 mg/ml/0.24 mg/ml for 2 h and 3 mg/ml/0.72 mg/ml for 24 h in 90% ethanol to obtain soft and stiff scaffolds respectively. The cross-linked soft and stiff scaffolds were then washed in ethanol to remove unreacted EDC and NHS and stored in absolute ethanol until further use.

2.2. Physicochemical characterization of scaffolds

Freeze dried scaffolds were mounted on copper stubs and sputter coated with gold–palladium alloy (Quorum Technologies SC7620). Morphology was then visualized by scanning electron microscopy (Zeiss EVO18). Pore size was quantified from SEM images using Image J software [15]. Pore size was quantified from 3 images each for 4 scaffolds and a total of at least 250 pores were analyzed for both soft and stiff scaffolds.

Degree of cross-linking of soft and stiff scaffolds was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay as reported previously [14]. Degree of cross-linking was calculated as the percentage of primary amine groups cross-linked.

2.3. Mechanical characterization

Cross-linked scaffold samples were swollen overnight at 37 °C in 1X PBS prior to mechanical testing (Bose® ElectroForce® 3200 equipped with 20 N load cell).

Compressive testing: Soft and stiff cylindrical scaffolds of dimensions 8 mm × 5 mm (diameter × height) were compressed uniaxially between flat platens at a strain rate of 0.01 mm/s and the stress–strain curves were recorded. For pure plasma clot samples, citrated plasma was clotted in presence of 0.1% $CaCl_2$ in disposable syringe molds of dimensions 8 mm × 5 mm (diameter × height). Compressive modulus was calculated from the slope of the linear fit line between 5% and 15% strain values using Origin software (OriginLab Corporation).

Tensile testing: Soft and stiff cuboidal scaffolds of dimensions 40 mm × 10 mm × 2 mm (length × breadth × thickness) with a functional length of 10 mm between the grips were tensile loaded at a strain rate of 0.01 mm/s and the stress–strain curves were recorded. Tensile modulus was calculated from the slope of the linear fit line between 5% and 15% strain values using Origin software (OriginLab Corporation). For pure plasma clot samples, citrated plasma was clotted in presence of 0.1% $CaCl_2$ on custom made molds of glass slides of dimensions 40 mm × 10 mm × 2 mm (length × breadth × thickness).

2.4. Cell culture

Goat chondrocytes were isolated from stifle joints of goats (collected from local slaughterhouse) and cultured in Dulbecco's modified Eagle's medium–high glucose (Sigma-Aldrich, St Louis) supplemented with 10% fetal bovine serum (Gibco, Carlsbad), 100 mM sodium pyruvate, 2.5 μg/ml amphotericin B and 100 U/ml penicillin–streptomycin (Himedia Labs, India) as reported previously [14]. Cells between passage numbers P2 to P4 were used for all experiments. For cell seeding, 2×10^6 cells were suspended in 25 μl of medium or citrated plasma and this suspension was added equally on both sides of a sterile scaffold (5 mm diameter × ~1 mm thickness) pre-equilibrated in cell culture medium. Prior to cell seeding, the scaffolds were dried by blotting them on sterile paper towels. The cell-seeded scaffolds were then incubated at 37 °C and 5% CO_2 and $CaCl_2$ was added to constructs with plasma at a final concentration of 0.1% after 30 min of incubation. Post one hour of cell seeding, all the constructs were transferred to well plates containing chondrogenic induction medium [DMEM high glucose supplemented with 100 mM sodium pyruvate, 2.5 μg/ml amphotericin B and 100 U/ml penicillin–streptomycin, 10 mM HEPES, 1.25 mg/ml BSA, 1 mM proline, 50 μg/ml ascorbic acid, 100 nM dexamethasone, 1X ITS+1 and 10 ng/ml TGF β1 (Sino Biologicals, China)]. The scaffolds seeded with cells in media have been referred to as 'soft' or 'stiff' and those with cells in plasma as 'soft + plasma' or 'stiff + plasma'. The cell-seeded constructs were harvested for morphological study, histology, biochemical analysis and immunohistochemistry at variable time points.

2.5. Morphological analysis

For visualizing the morphology of cells in cell-seeded constructs, cell-seeded scaffolds cultured for 2 days were stained with fluorescein diacetate (0.1 mg/ml for 10 min) and were imaged using a fluorescence microscope (Leica DM2500). Cryo-sections of the cell seeded constructs cultured for 14 days were stained for F-actin using phalloidin-FITC/DAPI and imaged on a laser scanning confocal microscope (Carl Zeiss LSM780NLO) as described previously [16]. The DAPI channel of these images were analyzed and used to quantify nuclear aspect ratio (NAR) of cells