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Culture of canine synoviocytes on porcine intestinal submucosa scaffolds as a strategy for meniscal tissue engineering for treatment of meniscal injury in dogs

Jennifer J. Warnock [a,*], Jason Spina [a], Gerd Bobe [b,c], Katja F. Duesterdieck-Zellmer [a], Jesse Ott [a], Wendy I. Baltzer [a], Brian K. Bay [d]

[a] Department of Clinical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, USA
[b] Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR 97331, USA
[c] Linus Pauling Institute, Oregon State University, Corvallis, OR 97331, USA
[d] School of Mechanical, Industrial, and Manufacturing Engineering, Oregon State University, Corvallis, OR 97331, USA

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ABSTRACT

Meniscal injury is a common cause of canine lameness. Tissue engineered bioscaffolds may be a treatment option for dogs suffering from meniscal damage. The aim of this study was to compare in vitro meniscal-like matrix formation and biomechanical properties of porcine intestinal submucosa sheets (SIS), used in canine meniscal regenerative medicine, to synoviocyte-seeded SIS bioscaffold (SSB), cultured with fetal bovine serum (SSBfbs) or chondrogenic growth factors (SSBgf). Synoviocytes from nine dogs were seeded on SIS and cultured for 30 days with 17.7% fetal bovine serum or recombinant chondrogenic growth factors (IGF-1, TGFβ1 and bFGF). The effect on fibrochondrogenesis was determined by comparing mRNA expression of collagen types Iα and IIα, aggrecan, and Sry-type homeobox protein-9 (SOX9) as well as protein expression of collagens I and II, glycosaminoglycan (GAG), and hydroxyproline.

The effect of synoviocyte seeding and culture conditions on biochemical properties was determined by measuring peak load, tensile stiffness, resilience, and toughness of bioscaffolds. Pre-culture SIS contained 13.6% collagen and 2.9% double-stranded DNA. Chondrogenic growth factor treatment significantly increased SOX9, collagens I and IIα, aggrecan gene expression ($P < 0.05$), and histological deposition of fibrocartilage extracellular matrix (GAG and collagen II). Culture with synoviocytes increased SIS tensile peak load at failure, resilience, and toughness of bioscaffolds ($P < 0.05$). In conclusion, culturing SIS with synoviocytes prior to implantation might provide biomechanical benefits, and chondrogenic growth factor treatment of cultured synoviocytes improves in vitro axial meniscal matrix formation.

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Introduction

Avascular zone meniscal injuries are a common cause of painful stifle arthritis and joint dysfunction in dogs (Cox et al., 1975; Ralphs and Whitney, 2002). Therapeutic interventions to treat meniscal injuries in this region are particularly challenging, primarily because the avascular meniscus has a limited capacity to regenerate and heal (Arnoczky and Warren, 1983). The use of cell scaffolds has been investigated for the purposes of guiding meniscal regeneration in vivo, and in vitro, scaffolds provide cell support to guide fibrocartilage neotissue formation. The 'ideal' meniscal tissue engineering scaffold would possess meniscus-like extracellular matrix (ECM), lubricity, and biomechanical properties, specifically great tensile strength, resilience and toughness (Fithian et al., 1990; Villegas et al., 2007).

When implanted into large meniscal defects extending to the abaxial, vascular region of the meniscus, porcine submucosal intestinal scaffolds (SIS) promote regeneration of the canine avascular meniscus, thereby slowing secondary articular cartilage damage (Cook et al., 1999, 2006a, 2006b). However, regeneration of meniscal tissue does not occur in lesions located solely in the avascular region, despite SIS implantation (Welch et al., 2002). Forming axial meniscal tissue on the SIS prior to implantation might provide a solution to this problem. The frequently injured, avascular portion of the meniscus contains ECM and cell phenotypes similar to articular cartilage, including rounded cells in lacunae, glycosaminoglycan (GAG) and type II collagen (O'Connor, 1976; Stephan et al., 1998; Kambic and McDevitt, 2005; Valiyaveettil et al., 2005; Thomopoulos et al., 2011). Thus, culture conditions previously described for in vitro hyaline cartilage tissue

* Corresponding author. Tel.: +1 541 737 4812.
E-mail address: jennifer.warnock@oregonstate.edu (J.J. Warnock).

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engineering might also be applied to synthesis of axial meniscal ECM components.

Since harvesting of meniscal cells themselves would most likely further injure the animal, synoviocytes might be an ideal cell source for this purpose. We have previously demonstrated that canine synoviocytes are readily harvested through minimally invasive procedures during routine stifle surgery (Warnock et al., 2012) and canine synoviocytes are also readily cultured on SIS (Fox et al., 2006). While canine synoviocytes harvested from osteoarthritic joints might have decreased synthetic potential in monolayer culture (Warnock et al., 2011), exposure to a more bioactive environment might mitigate against these intrinsic limitations. Additionally, violation of a normal joint to obtain cellular material is clinically unattractive.

In humans, synoviocytes from osteoarthritic joints can form axial meniscus-like matrix in vitro (Pei et al., 2008). Culture of human osteoarthritic synoviocyte pellets with basic fibroblast growth factor (bFGF) increases GAG and actin content, as well as type II collagen gene expression (Lee et al., 2009). A combination of transforming growth factor beta 1 (TGFβ1) and insulin-like growth factor 1 (IGF-1) increases type II collagen formation and promotes a chondrocytic cell phenotype in human osteoarthritic synoviocytes (Sakimura et al., 2006), while culture with bFGF, TGFβ1 and IGF-1 maximizes hyaline chondrogenesis in human osteoarthritic synoviocytes (Pei et al., 2005; Pei et al., 2008). We have previously reported that in canine synoviocyte monolayer culture, this protocol produced ECM components of fibrocartilage, but failed to produce neotissue with histological characteristics of axial meniscus (Warnock et al., 2011). Therefore, in the present study we tested the efficacy of chondrogenic growth factor treatments (bFGF, IGF-1 and TGFβ-1) on canine synoviocytes cultured on SIS, rather than simple monolayer culture, with a view to formation of axial meniscal-like ECM.

The aim of this study was to evaluate the effect of canine synoviocyte culture on SIS bioscaffold (SSB), using either fetal bovine serum (SSBfbs) or chondrogenic growth factors (SSBgf) on their matrix and biomechanical properties. The hypothesis was that culturing synoviocytes on SIS would promote synoviocyte fibrochondrogenesis and improve SIS biomechanical properties, and that culture with chondrogenic growth factors would enhance fibrochondrogenesis.

Materials and methods

Tissue samples

Synovial villi were obtained as previously described (Warnock et al., 2012) from nine dogs undergoing arthroscopy for investigation/treatment of cranial cruciate ligament rupture and meniscal tear, with informed owner consent for use of tissue in research and with approval from the institutional Animal Care and Use Committee. Harvested synovial villi were immediately placed in 40 mL of Dulbecco's modified Eagle medium (DMEM, Invitrogen) with 10% fetal bovine serum (FBS, Invitrogen), warmed to 37 °C.

Cell culture

Villi were centrifuged at 313 *g*, and tissue transferred to a digestion solution containing 10 mg/mL type 1A clostridial collagenase in RPMI 1640 medium (Invitrogen) and incubated at 37 °C for 2–4 h. Cells were cultured in monolayer to isolate type B and C synoviocytes (Vasanjee et al., 2008; He et al., 2009) at 37.8 °C, 5% CO_2, 95% humidity with daily medium changes consisting of DMEM containing 17.7% FBS and other supplements (0.021 mg/mL glycine, 0.025 mg/mL L-alanine, 0.037 mg/mL L-asparagine, 0.038 mg/mL L-aspartic acid, 0.042 mg/mL L-glutamic acid, 0.033 mg/mL L-proline, 0.03 mg/mL L-serine, 0.23 mg/mL pyruvate, 0.52 mg/mL L-glutamine, 6.75 mg/mL HEPES buffer, 177 units/mL penicillin, 177 μg/mL streptomycin, 0.44 μg/mL amphotericin; all from Invitrogen).

To create SSB, single-ply, 4 cm × 2.3 cm, non cross-linked SIS (Vet BioSIST, Smiths Medical), were folded around 2 cm diameter cerclage wire hoops and placed in 6-well plates containing 8 mL of supplemented DMEM medium per well. The SSB (10 per dog) were statically seeded (Fox et al., 2006) by slowly pipetting 1×10^6 fourth-passage synoviocytes, suspended in 0.5 mL supplemented DMEM, directly onto the SSB. After 7 days of culture SSB were randomized into two groups: five SSB continued treatment with supplemented DMEM containing 17.7% FBS (SSBfbs), and five SSB were cultured with serum-free supplemented DMEM containing the three chondrogenic growth factors (SSBgf). Growth factors (all from Invitrogen) consisted of recombinant human bFGF (100 ng/mL, for the first 3 days only), TGFβ1 (20 ng/mL) and IGF-1 (500 ng/mL) (Pei et al., 2008; Warnock et al., 2011).

As fibrochondrogenesis is time dependent, SSB were harvested for analysis after a total of 30 days in culture (Ando et al., 2008; Tan et al., 2010). Nine untreated, dry SIS scaffolds were removed from their sterile wrappings and immediately analyzed as controls for spectrophotometric assays and biomechanical testing, respectively.

Immunohistochemical analysis

Tissue analyses examined the presence of ECM responsible for meniscal form and function, including type I collagen (Kambic and McDevitt, 2005), type II collagen (Kambic and McDevitt, 2005), alpha smooth muscle actin (ASMA) (Kambic et al., 2000; Spector, 2001), and GAG (Adams and Ho, 1987; Stephan et al., 1998), including aggrecan (Valiyaveettil et al., 2005).

One SSB from each treatment group was fixed in 10% buffered formalin for 24–48 h, paraffin embedded, sectioned at 4–5 μm, and stained with hematoxylin and eosin, Masson's trichrome, or Toluidine blue. Immunohistochemistry was performed as previously described (Warnock et al., 2012) using antibodies against type I collagen (AB749P; 1:100 dilution; Millipore), type II collagen (AB746P; 1:100 dilution: Millipore), and ASMA (M0851; 1:30 dilution; Dako). The intensity and prevalence of immunoreactivity was scored as previously described (Wakshlag et al., 2011); immunoreactivity was localized to intracellular or extracellular staining and ECM immunoreactivity was described as mild, moderate, or strong staining. As determined by manual counting, intracellular immunoreactivity was categorized as positive in <10%, 10–50%, or >50% of cells.

Real-time quantitative PCR

Samples from each group were snap frozen in liquid nitrogen and stored at −80 °C. Quantitative real-time RT-PCR was performed as previously described (Chomczynski and Sacchi, 1986; Schmittgen and Livak, 2008) for Sry-type homeobox protein-9 (SOX9), an embryonic chondrogenic transcription factor, collagen type I α1, collagen type 2 α1, aggrecan, and GAPDH as a housekeeping gene control (Table 1) using proprietary, pre-designed primers and probes (Taq-Man Primers and Probes, Applied Biosystems). Fold changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008), whereby $\Delta\Delta CT = [(C_T \text{ gene of interest} - C_T \text{ GAPDH})_{SSBfbs} - (C_T \text{ gene of interest} - C_T \text{ GAPDH})_{SSBgf}]$ (Table 1).

Analysis of extracellular matrix composition

A construct from each group was lyophilized, dry weight obtained, digested, and used for double-stranded DNA, GAG, and collagen analysis (Warnock et al., 2012). The Quant-iT PicoGreen (Invitrogen) double stranded DNA quantification assay was performed according to the manufacturer's instructions; standard and sample fluorescence was read at 485 nm excitation, 528 nm emission using a fluorometer (Quibit Fluorometer, Invitrogen).

The concentration of GAG was determined by the di-methyl-methylene blue sulfated GAG assay (Farndale et al., 1986) using a spectrophotometer (Synergy HT – KC4, BioTec). Collagen content was determined by Erlich's hydroxyproline assay (Reddy and Enwemeka, 1996). The hydroxyproline content was converted to collagen content using the following equation: (μg hydroxyproline × dilution factor)/0.13 = μg collagen (Ignat'eva et al., 2007), based on hydroxyproline representing approximately 13% of the amino acid content of collagen in the human meniscus (Fithian et al., 1990).

The concentrations of GAG and collagen were standardized to tissue dry weight and expressed as per cent dry weight to allow comparison of the experimental neotissues to normal meniscal ECM content (Eyre and Wu 1983). The chondrogenic index was calculated using the following equation: μg GAG/μg dsDNA (Li and Pei, 2011). The collagen index was calculated using the following equation: μg collagen/μg dsDNA, to determine cellular GAG and collagen production, respectively. Total GAG and collagen content were also reported in μg/neotissue to allow comparison of total synthetic activity over the course of 30 days of in vitro culture between SSBfbs and SSBgf.

Biomechanical analysis

Scaffolds from each group were removed from their wire frames and tested using an electro-mechanical materials testing device (Insight, MTS Systems Corporation) with a 100 N load cell and analyzed with associated software (Testworks 4, MTS Systems Corporation). When insufficient SSB samples were available, a construct that was used to test cell viability was also biomechanically tested. For tensile testing, tissues were grasped using custom-modified Pennington forceps, so that tissues assumed a bar-bell shape. The Pennington forceps were affixed to aluminum arrow shafts, which were aligned in series with the actuator and load cell.

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