Archives of Biochemistry and Biophysics 489 (2009) 118–126

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Co-culture of mechanically injured cartilage with joint capsule tissue alters chondrocyte expression patterns and increases ADAMTS5 production

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ARTICLE INFO

Article history:
Received 3 April 2009
and in revised form 29 June 2009
Available online 14 July 2009

Keywords:
Cartilage injury
Joint capsule
Synovium
Aggrecan
Aggrecanase
Chondrocyte gene profiling
ADAMTS4
ADAMTS5
Matrix degradation
Cartilage

ABSTRACT

We studied changes in chondrocyte gene expression, aggrecan degradation, and aggrecanase production and activity in normal and mechanically injured cartilage co-cultured with joint capsule tissue. Chondrocyte expression of 21 genes was measured at 1, 2, 4, 6, 12, and 24 h after treatment; clustering analysis enabled identification of co-expression profiles. Aggrecan fragments retained in cartilage and released to medium and loss of cartilage sGAG were quantified. Increased expression of MMP-13 and ADAMTS4 clustered with effects of co-culture, while increased expression of ADAMTS5, MMP-3, TGF-β, c-fos, c-jun clustered with cartilage injury. ADAMTS5 protein within cartilage (immunohistochemistry) increased following injury and with co-culture. Cartilage sGAG decreased over 16-days, most severely following injury plus co-culture. Cartilage aggrecan was cleaved at aggrecanase sites in the interglobular and C-terminal domains, resulting in loss of the G3 domain, especially after injury plus co-culture. Together, these results support the hypothesis that interactions between injured cartilage and other joint tissues are important in matrix catabolism after joint injury.

Introduction

Joint injury in young adults leads to increased risk for development of osteoarthritis (OA)[2] [1–3] irrespective of surgical intervention to stabilize the joint [4,5]. Joint injury initiates a cascade of responses which can result in cartilage degeneration, pain, and compromised joint function. Following human knee joint injuries which involve damage to the anterior cruciate ligament (ACL) or meniscus, analysis of synovial fluid has revealed elevated levels of collagen II and aggrecan fragments [6–8], inflammatory cytokines (IL-1β, TNF-α, IL-6, and IL-8) [9], suppressors of inflammation (IL-1Ra and IL-10) [9], matrix degrading enzymes [6] and markers of matrix turnover and tissue repair (TIMP-1 and aggrecan synthesis) [6] compared to uninjured controls. These proteins and proteolytic fragments could originate from cartilage, joint capsule tissue, meniscus, ligament, tendon, or bone, and may participate in the initiation of cartilage degradation leading to OA.

Animal models of joint injury, such as transection of the ACL (ACLT), show changes in cartilage, bone, synovium, and joint capsule. These changes include cartilage fibrillation and full-thickness loss, chondrocyte cloning, osteophyte formation, hyperplasia of synovial lining, mononuclear cell infiltration into the synovium, and joint capsule fibrosis [10]. In rabbits, ACLT increased chondrocyte gene expression of collagen II, aggrecan, MMP-1,-3,-13, and decreased expression of decorin and fibromodulin [11,12], while increasing synovial cell expression of MMP-3 and IL-1β [12]. Together, human and animal studies highlight the complexity of events following joint injury and motivate the need for complementary *in vitro* studies to isolate particular factors and identify mechanisms of cartilage degradation.

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[2] *Abbreviations used:* OA, osteoarthritis; ACL, anterior cruciate ligament; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif; MMP, matrix metalloproteinase; RT, reverse transcription; DAB, 3,3′-diaminobenzidine; sGAG, sulfated glycosaminoglycan; DMMB, dimethylmethylene blue; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor α.

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doi:10.1016/j.abb.2009.07.006

In vitro models of mechanical injury to cartilage, alone, demonstrate damage to cartilage matrix resulting in increased water content [13–15], decreased stiffness [13,16], increased hydraulic permeability [17], sGAG loss to the culture medium [13,18–20], and damage to collagen [14,15,17]. Additionally, chondrocytes show decreased biosynthesis [13] and undergo apoptosis and necrosis [15,16,19,21–23]. Recently, we found that within 24 h following a single injurious compression of bovine cartilage explants, gene expression of MMP-1,-3,-9,-13, ADAMTS4,5, and TIMP-1 increased from 4- to 250-fold, while expression of matrix molecules such as aggrecan and type II collagen remained essentially unchanged compared to that in uninjured cartilage [24].

Directly relevant to the present study, models using normal or mechanically injured cartilage co-cultured with joint capsule have focused on multiple tissue involvement and tissue interactions that naturally occur during joint injury. Excision of tissues from the joint for these studies is considered a model of injury and has been shown to cause cell death at the cut surface [25,26]. For example, co-culture of normal cartilage with joint capsule tissue caused decreased chondrocyte biosynthesis [27,28] and loss of cartilage aggrecan and collagen [29]. Mechanical injury of cartilage followed by co-culture with joint capsule resulted in further reduction in chondrocyte biosynthesis than that occurring after injury or co-culture alone [28]. Capsular tissue released soluble factor(s) that caused aggrecanase cleavage of aggrecan in the interglobular domain [30], and conditioned medium from capsule tissue caused sGAG release from cartilage [31]. ADAMTS5 (aggrecanase-2) is expressed and active in normal bovine and osteoarthritic human synovium [32].

While previous co-culture studies showed degradative capacity of synovium, the combined effects of mechanical injury and co-culture with synovial tissue are not well characterized. The objectives of this study were (1) to determine if co-culture of normal or mechanically injured cartilage with excised joint capsule tissue (including synovial lining cell layers) affects gene co-expression profiles of matrix molecules, matrix degrading enzymes, inhibitors, growth factors, and inflammatory cytokines, and (2) to identify the kinetics of sGAG loss, the presence and tissue localization of ADAMTS4 and ADAMTS5 in the cartilage, and the specific cleavage of aggrecan observed in these injury models.

Materials and methods

Tissue harvest and culture

Cartilage disks (1 mm-thick × 3 mm-diameter) were harvested from the middle zone of femoropatellar grooves of 1- to 2-week-old bovine calves (48 disks per joint) as described [24]. Joint capsule tissue was cut from the medial side in the joint immediately proximal to the articular cartilage; full thickness samples consisted of fibrous tissue with a single layer of synovium (visualized in histological cross-section, not shown). Joint capsule tissue was cut into 36 pieces (~5 mm × 5 mm) using a razor. Cartilage and joint capsule explants were equilibrated separately in medium (low glucose DMEM supplemented with 10% fetal bovine serum, 10 mM Hepes buffer, 0.1 mM non-essential amino acids, 0.4 mM proline, 20 μg/ml ascorbic acid, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) for 2 days in a 37 °C, 5% CO_2 environment. Serum was replaced with 1% ITS for samples used in Western analyses.

Injurious compression, co-culture, and exogenous IL-1 treatment

Injurious compression of cartilage disks was performed using a custom-designed incubator-housed loading apparatus [33] as described previously [13,18,20,24]. A single unconfined compression displacement ramp was applied to a final strain of 50% at a velocity of 1 mm/s (strain rate 100%/s) in displacement control, followed by immediate release at the same rate. This strain waveform resulted in an average measured peak stress of ~20 MPa, shown previously to produce damage to the ECM, decreased cell viability, decreased biosynthesis by the remaining viable cells, increased sGAG loss to the medium, and altered chondrocyte gene expression when applied to similar bovine cartilage explants in the absence of co-culture with joint capsule tissue [13,16,18,20,24,34].

In co-culture studies, normal or injured cartilage disks were cultured in the same well with a single joint capsule explant after the time of cartilage injury (but not before). Normal cartilage maintained free swelling without joint capsule or exogenous cytokine was the negative control, and normal cartilage treated with 10 ng/ml rhIL-1α (R&D Systems, MN), added at the time of medium change every 2 days throughout the test, was the positive control. Cartilage disks in each treatment or control group were matched across depth and location along the joint surface to prevent bias based on anatomical location.

For analysis of time dependent changes in gene expression, groups of six cartilage disks from each condition were removed from culture at 1, 2, 4, 6, 12, and 24 h, flash frozen in liquid nitrogen, and stored at −80 °C. Groups of six non-injured control disks from the same animal were frozen at 4 and 24 h. This experiment was repeated to give three replicates in total, using one joint from each of three different animals (data are means ± SE, $n = 3$). For Western analyses, medium was collected at days 2, 4, 6, and 8 after initiation of treatment (injurious compression, co-culture, compression plus co-culture, or IL-1α) at the time of medium change and stored at −20 °C. Thus, these medium samples contained released molecules from day 0–2, 3–4, 5–6, and 7–8, respectively. Medium collected concurrently from free swelling cartilage served as controls.

RNA extraction

RNA was extracted from the six pooled cartilage disks by pulverizing the tissue and homogenizing in Trizol reagent (Invitrogen, CA) to lyse the cells. Extracts were transferred to Phase Gel Tubes (Eppendorf AG, Germany) with 10% v/v chloroform, spun at 13,000*g* for 10 min, and the clear liquid removed from above the phase gel. RNA was isolated using the RNeasy Mini Kit (Qiagen, CA). Genomic DNA was removed by DNase digestion (Qiagen, CA) during purification. Absorbance measurements at 260 and 280 nm gave the concentration of RNA extracted from the tissue and the purity of the extract. The average 260/280 absorbance ratio was 1.91 ± 0.10. Reverse transcription (RT) of equal quantities of RNA (25 ng per μl RT volume) from each sample was performed using the Amplitaq-Gold RT kit (Applied Biosystems, CA).

Real-time PCR

Expression levels were quantified using the MJ Research Opticon2 instrument and SYBR Green Master Mix (Applied Biosystems, CA). Bovine primers were designed for matrix molecules (collagen II, aggrecan, link protein, fibronectin, fibromodulin, and collagen I), proteases (MMP-1,-3,-9,-13, ADAMTS4,5), protease inhibitors (TIMP-1,-2), cytokines (TNF-α, IL-1β), housekeeping (β-actin, GAPDH), transcription factors (c-fos, c-jun), and growth factor (TGF-β) using Primer Express software (Applied Biosystems, CA). All bovine primer sequences are as published [35]. Standard curves for amplification showed that all primers demonstrated approximately equal efficiency with standard curve slopes ~1, indicating a doubling in cDNA quantity each cycle.