Potent inhibition of cartilage biosynthesis by coincubation with joint capsule through an IL-1-independent pathway

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Abstract

The reason for the increased risk for development of osteoarthritis (OA) after acute joint trauma is not well understood, but the mechanically injured cartilage may be more susceptible to degradative mediators secreted by other tissues in the joint. To establish a model for such interactions, we coincubated bovine cartilage tissue explants together with normal joint capsule and found a profound (~70%) reduction in cartilage proteoglycan biosynthesis. This reduction is due to release by the joint capsule of a heat-labile and nontoxic factor. Surprisingly, while cultured synovium is a canonical source of IL-1, blockade by either soluble IL-1 receptor (sIL-1r) or IL-1 receptor antagonist (IL-1RA) had no effect. Combined blockade of IL-1 and TNF-alpha also had no effect. To support the clinical relevance of the findings, we harvested joint capsule from post-mortem human knees. Human joint capsule from normal adult knee also released a substance that caused a ~40% decrease in cartilage proteoglycan biosynthesis. Furthermore, this inhibition was not affected by IL-1 blockade with either sIL-1r or IL-1RA. These results suggest that joint capsule tissue from a normal knee joint can release an uncharacterized cytokine that potently inhibits cartilage biosynthetic activity by an IL-1 and TNF-independent pathway.

Keywords

joint injury; synovium; joint capsule; coincubation; interleukin-1

Introduction

A traumatic joint injury leads to a substantial increase in risk for subsequent development of OA in the joint, yet the reason is not clear (Davis et al., 1989; Gelber et al., 2000; Wilder et al., 2002). Injuries to joint ligaments and tendons cause mechanical joint instability, which is well-known to contribute to OA. However, the risk for OA remains even after stabilization of the joint by surgical repair (Lohmander et al., 2007). This suggests that some acute effects of injury are irreversible, and long-term biological effects of the injury continue despite mechanical stabilization.

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Observations such as these have led to the view that OA is a disease of the whole joint with disturbances in cell-mediated anabolic, catabolic, and inflammatory processes (Felson et al., 2000). Multiple joint tissues are often acutely damaged by a joint injury (Buckwalter, 2002), and cytokine levels are elevated in the synovial fluid of patients after an anterior cruciate ligament injury (Irie et al., 2003). Thus, even injuries causing gross damage only to the articular cartilage will be affected by substances released by other joint tissues such as synovium. Mesenchymal stem cells are also increased in synovial fluid after injury (Morito et al., 2008), further suggesting that synovium senses and responds to joint injury with a coordinated remodeling and repair program. Finally, investigations of OA at the molecular level suggest a pivotal role for inflammatory mediators and signaling pathways (Pelletier et al., 2001; Attur et al., 2002; Goldring and Goldring, 2007).

To investigate the events after joint injury which lead to the development of OA, in vitro models that apply injurious mechanical loading to the cartilage have been developed. Injurious compression damages the cartilage matrix, causing loss of proteoglycan to the conditioned medium (Jeffrey et al., 1997; Torzilli et al., 1999; Quinn et al., 2001) and increased levels of denatured collagen (Chen et al., 2001; Thibault et al., 2002). Injurious compression also causes chondrocyte apoptosis, suggesting cell death may be an important irreversible effect of injury in vivo (Loening et al., 2000; D'Lima et al., 2001). In addition, injurious compression may cause the remaining chondrocytes to lose their ability to respond to dynamic compression by increasing biosynthesis (Kurz et al., 2001).

While these models have focused on explants of cartilage alone or cartilage attached to subchondral bone, we hypothesized that modeling interactions with other joint tissues would be important for understanding the role of inflammatory mediators after joint injury. We have previously reported that injurious compression of cartilage explants followed by treatment with the cytokines IL-1 or TNFα results in a significant synergistic loss of tissue proteoglycans (Patwari et al., 2003). In addition, we have begun to investigate a model in which cartilage is coincubated with excised joint capsule tissue from a normal joint. In this model, the excision and culture of capsule tissue could be considered as a traumatic event for the joint capsule and allow for exploration of interactions between joint capsule and cartilage tissues that occur in a traumatic injury in vivo. Our initial observation from this model was that coincubation of cartilage and joint capsule tissue from a normal joint resulted in a profound inhibition of cartilage biosynthetic activity (Patwari et al., 2001). The objectives of the present study were to characterize this effect and to study the role of IL-1 and TNFα using cytokine blockade.

Materials and Methods

Materials

Etanercept was from Immunex Corp. (Seattle, WA); the recombinant human proteins interleukin-1α (IL-1), soluble IL-1 type II receptor (sIL-1r), interleukin-1 receptor antagonist (IL-1RA), and tumor necrosis factor α (rh TNF) were from R&D Systems (Minneapolis, MN). Recombinant bovine TNF α (rb TNF) was a generous gift of Dr. Theodore H. Elsasser (United States Department of Agriculture, Beltsville, MD). Radiolabeled ^{35}S-sulfate and ^{3}H-proline was from New England Nuclear (now PerkinElmer Life Sciences, Boston, MA). Additional supplies were from Sigma Chemical Co. (St. Louis, MO) where not otherwise noted.

Tissue Harvest

Articular cartilage explant disks were taken from femoropatellar grooves of 1-to-2-week-old calves obtained from a local abbatoir on the day of slaughter (Research '87, Hopkinton, MA), as previously described (Sah et al., 1989). In brief, cartilage-bone cylinders (9 mm in diameter) were drilled perpendicular to the cartilage surface and placed in a microtome holder. After

creating a level surface by removal of the most superficial ~100 µm, the next 2 mm of cartilage were sliced by a microtome, producing two 1-mm-thick slices. Finally, four explant disks were punched out of each slice, resulting in cartilage disks that were 1 mm thick and 3 mm in diameter. Treatment groups were matched for location and depth of the cartilage on the joint surface by distributing one disk from a single slice to each of the different treatment groups. Cartilage was then left in culture to equilibrate for 3 days in low-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 0.1 mM nonessential 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 (which we refer to as "standard medium"), in a 37°C, 5% CO_2 environment.

Joint capsule was harvested medial and lateral to the patella and suprapatellar fat pad (Figure 1). The joint capsule tissue, which varied from 0.5- to 3-mm-thick, was punched and cultured in standard medium. Adjacent capsule punches were distributed evenly among treatment groups to prevent bias from variations in thickness and location of the capsule. For coincubation, each cartilage disk was incubated together with a 3-mm-diameter sample of capsule tissue in a separate well of a 96-well plate.

Human Donor Tissue

Normal adult human donor knee joints were obtained from the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL). All research was approved by the Committee on the Use of Humans as Experimental Subjects at the Massachusetts Institute of Technology. Post-mortem knee joints were washed, placed in DMEM, and harvested within 48 hours as described previously (Patwari et al., 2003). 0.5-mm-thick, 3-mm-diameter cartilage disks were obtained from femoropatellar, femoral condylar, and tibial plateau articular cartilages and placed into standard medium. In addition, portions of joint capsule tissue adjacent to the attachment site of the capsule to the tibia were dissected and punched to form 3-mm-diameter disks.

Radiolabel Incorporation

After coincubation for 3 days, the cartilage disks were radiolabeled by incubation in fresh standard medium containing 10 µCi/ml each of ^{35}S-sulfate and ^{3}H-proline (all capsule tissue was removed before the label) for 12 to 24 hours. The disks then were washed of unincorporated label, digested in protease K, and analyzed by liquid scintillation for incorporated radiolabel. Control studies in the newborn calf cartilage used here have demonstrated that >95% of incorporated ^{35}S-sulfate label is in aggrecan, and ~60% of incorporated ^{3}H-proline is in collagen (Sah et al., 1989; Sah et al., 1991).

Statistical Analysis

Most experiments used a 2×2 factorial design, where one treatment was coincubation, and the purpose of the experiment was to test whether a second treatment (such as IL-1 blockade) modified the effect of coincubation. Therefore, in each experiment our primary hypothesis of interest was whether there was a significant interaction between the effects of the two treatments. This was tested in a two-way analysis-of-variance (ANOVA) model. Otherwise, comparisons were made by two-tailed t-tests.

Results

Characterization of cartilage-inhibitory substance in coincubation with joint capsule

To test whether the joint capsule itself releases a substance that inhibits cartilage synthesis, we tested the effect of culture medium conditioned by incubation with the joint capsule tissue. We

found that capsule-conditioned medium was sufficient to strongly inhibit cartilage sulfate incorporation (data not shown).

We therefore proceeded to characterize the inhibitory effect of the capsule-conditioned medium. To test whether the inhibition was reversible, we performed a recovery experiment. Radiolabel incorporation into cartilage was measured both immediately after incubation in capsule-conditioned medium and after recovery for five days in standard medium (Fig. 2A). In cartilage labeled immediately after incubation in capsule-conditioned medium, sulfate incorporation was decreased by 63% compared to control cartilage incubated in standard medium. In contrast, in cartilage cultured for five days in standard medium after incubation with capsule-conditioned medium the sulfate incorporation rate had significantly recovered (p < 0.001 for interaction). The recovery of cartilage biosynthetic activity suggests that the inhibitory effect of capsule-conditioned medium is not attributable to chondrocyte toxicity.

Standard medium and conditioned medium were boiled for 10 minutes, cooled, and sterile filtered. Cartilage disks were then incubated for 3 days in standard medium, conditioned medium, boiled standard medium, or boiled conditioned medium. After the 3-day incubation, radiolabel incorporation was measured and normalized to the mean incorporation of the standard medium group (Fig. 2B). Conditioned medium decreased sulfate incorporation by 59%, and this effect was significantly reversed by boiling (p < 0.001 for interaction). Inhibition of proline incorporation by conditioned medium was also significantly reversed by boiling (p < 0.001 for interaction, data not shown). The loss of the inhibitory activity of the boiled conditioned medium suggests that the factor released from capsule tissue is heat-labile, such as a protein that is denatured on boiling.

IL-1 blockade had no effect on the inhibitory factor

The cytokines IL-1 and TNFα are potent inhibitors of cartilage biosynthetic activity (Saklatvala et al., 1984; Saklatvala, 1986). Furthermore, IL-1 can be released by excised normal synovial membrane in culture (Dingle et al., 1979; Jubb and Fell, 1980), and appears to be responsible for much of the proteoglycan-degrading activity of medium conditioned by inflamed synovium (Yodlowski et al., 1990). We therefore proceeded to test whether the effect of joint capsule coincubation on cartilage was abolished by blockade of IL-1 and TNFα.

First, we used recombinant human soluble IL-1 type II receptor (sIL-1r) to block IL-1 (Fig. 3A). While coincubation inhibited sulfate incorporation into the cartilage by 80%, addition of 5 μg/ml sIL-1r to the medium had no more effect on coincubated cartilage than on control cartilage (p = 0.32 for interaction). As a positive control for the activity of sIL-1r blockade, the effect of 1 ng/ml rhIL-1α was significantly reversed by sIL-1r (p < 0.001 for interaction). The results were similar for proline incorporation (p = 0.87 for interaction between coincubation and sIL-1r treatment, data not shown).

We also tested the effect of blockade with recombinant human IL-1 receptor antagonist (IL-1RA). IL-1RA binds directly to the IL-1 receptor, so cross-reactivity with the bovine receptor would be required in order to block the effect of rhIL-1. The results for blockade with IL-1RA (Fig. 3B) were similar to those observed for blockade with sIL-1r. Addition of 250 ng/ml IL-1RA had no significant effect on the inhibition of sulfate incorporation by coincubation (p = 0.34 for interaction) but significantly reversed the effect of 1 ng/ml rhIL-1α (p < 0.001 for interaction). Similarly, IL-1RA had no significant effect on inhibition of proline incorporation by coincubation (p = 0.46 for interaction, data not shown). These data suggest that the factor responsible for the inhibitory activity of capsule-conditioned medium is not IL-1.

Inhibition is not affected by combined blockade of IL-1 and TNF-α

To test the effect of blocking TNF in our system, we used etanercept, a recombinant TNF receptor-Fc fusion protein. Since etanercept is based on the human TNF receptor, we first confirmed whether etanercept blocks the effect of bovine TNFα. After incubating cartilage for three days with 100 ng/ml bovine recombinant TNFα, sulfate incorporation was reduced by 21% compared to controls. This effect was significantly reversed by addition of 25 μg/ml of etanercept to the culture medium ($p < 0.001$ for interaction).

We then tested the effect of combined blockade with 25 μg/ml of etanercept and 250 ng/ml IL-1RA (Fig. 4). After three days of culture, coincubation reduced sulfate incorporation by 72%. Addition of IL-1RA and etanercept had no significant effect on coincubation ($p = 0.36$ for interaction). Similarly, inhibition of proline incorporation by coincubation was not significantly affected by combined blockade with IL-1ra and etanercept ($p = 0.75$ for interaction, data not shown). As a positive control for the blockade, inhibition of sulfate incorporation by 1 ng/ml hrIL-1α and 25 ng/ml hrTNFα was significantly affected by addition of IL-1ra and etanercept to the medium ($p < 0.001$ for interaction).

Human joint capsule inhibits cartilage biosynthesis through an IL-1-independent Pathway

To further support the clinical relevance of these results, we tested whether human joint capsule also releases an inhibitory substance in culture. Joint capsule and cartilage from post-mortem donor joints, cut into 3-mm-diameter disks, were harvested and equilibrated for 3 days in standard medium. The cartilage was then transferred to serum-free medium (standard medium without FBS), and either incubated alone or coincubated with joint capsule. After 3 days, the cartilage was radiolabeled for 24 hours. Radiolabel incorporation of coincubated cartilage disks was decreased by $39 \pm 8\%$ for 35S-sulfate and $21 \pm 2\%$ for 3H-proline, relative to the mean value of cartilage incubated alone ($N = 10$ per group; $p < 0.01$ for both comparisons by t-test) (Figure 5).

Finally, we tested whether the inhibitory factor released from human joint capsule is affected by IL-1 blockade. Conditioned medium was collected from joint capsule incubated in standard medium for 7 days. The conditioned medium, along with medium treated the same way but incubated without tissue in it (control medium), was collected and frozen. Bovine cartilage was then incubated in the conditioned medium or control medium, with or without IL-1 blockade, for 3 days and radiolabeled (Fig. 6). Blockade with sIL-1r had no significant effect (Fig. 6A): cartilage incubated in capsule-conditioned medium had a $45 \pm 4\%$ decrease in 35S-Sulfate incorporation, compared to a $49 \pm 4\%$ decrease with the addition of 5 ug/ml sIL-1r ($N = 8$; $p = 0.44$ by t-test). Furthermore, a similar experiment showed no significant effect of blockade with IL-1RA (Fig. 6B): cartilage incubated in capsule-conditioned medium had a $49 \pm 3\%$ decrease in 35S-sulfate incorporation, compared to a $39 \pm 4\%$ decrease with the addition of 200 ng/ml IL-1RA ($N = 8$; $p = 0.09$ by t-test). As a control in both experiments, the inhibitory effect of 2 ng/ml IL-1 was significantly reversed ($p < 0.001$ for the comparison by t-test in both experiments).

Discussion

With the long-term goal of developing an in vitro injurious mechanical compression model that could reveal interactions between the joint capsule tissues and the cartilage, we coincubated cartilage tissue with joint capsule tissue excised from normal femorotibial joints. We report here that chondrocyte biosynthesis is strongly inhibited by a substance released from both bovine and human joint capsule tissue in culture. Surpisingly, blockade of IL-1 and TNF-a had no effect on the inhibitory substance, suggesting that an important factor released from normal joint tissue has yet to be identified.

Despite the isolation of IL-1 ("catabolin") from minced porcine synovial tissue from a normal MCP joint (Jubb and Fell, 1980; Saklatvala et al., 1984), and blockade of proteoglycan loss from cartilage coincubated with inflamed synovium by anti-IL-1 antibodies (Yodlowski et al., 1990; Neidhart et al., 2000), to our knowledge the inhibition of cartilage biosynthesis by excised capsule tissue from a normal joint has not been characterized. Although inflamed synovium is now recognized to secrete a wide range of cytokines, the proteins released by normal synovium are far less characterized. Beyond IL-1, one group has previously noted that a potentially novel cytokine is secreted from normal rabbit synovial fibroblasts that causes release of prostaglandin E2 and matrix metalloproteases from cartilage (termed "chondrocyte activating factor") (Bandara et al., 1992). Chondrocyte activating factor was found to be a heat-labile, 20 kDa substance, and may be the same as the inhibitory factor we describe here: our preliminary size-exclusion chromatography data support a weight of 20–25 kDa (unpublished observation).

The reason that the joint capsule tissue from a normal joint releases a factor that inhibits chondrocyte biosynthesis is not clear. The release of this factor could be related to the trauma from excision of the tissue, the change in its mechanical and biochemical environment, and the loss of blood supply when placed in culture. It may be that trauma and environmental stress induced by excision of the capsule tissue mimics aspects of intact joint injury. That is, like the chondrocytes, the capsule fibroblasts and/or synoviocytes may be sensitive to mechanical forces that occur during a joint injury, even in the absence of a gross tear or puncture of the capsule. On the other hand, it is also possible that release of the inhibitory factor is not dependent on cell signaling. For example, recent studies have shown that cutting cartilage releases bFGF that was already secreted and held in the tissue by binding to extracellular matrix proteoglycans (Vincent et al., 2007). Finally, we note that release of the inhibitory factor may not be specific to joint capsule tissue, as with previously identified cartilage-degrading factors (Decker and Dingle, 1982).

Perspective

The increased risk of OA after joint injury, even in surgically stabilized joints, suggests that both mechanical and biological factors interact to cause joint pathology. Ligament or cartilage injuries are often accompanied by subchondral bone injury and synovial release of inflammatory mediators. We have hypothesized that synovium may also sense and respond to the mechanical effects of joint injury even in the absence of gross trauma to the capsule, and begun to characterize the response of synovium in an in vitro model. Surprisingly, we found that normal joint capsule, once in culture, strongly inhibits cartilage biosynthesis by an IL-1-independent pathway. Identification of the factor released by joint capsule may be important for understanding the response of the joint to traumatic injury.

Acknowledgments

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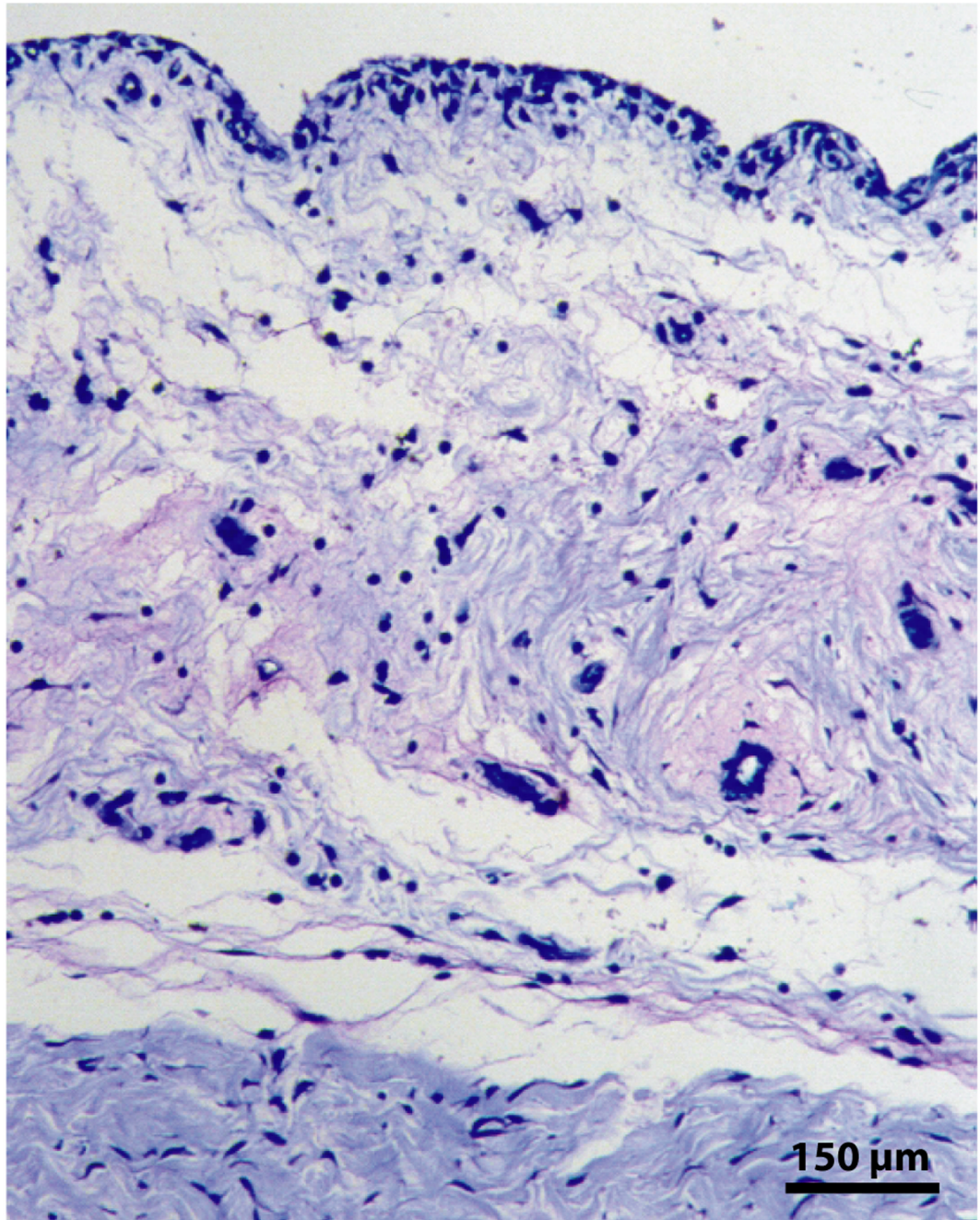
Figure 1.
Histology of bovine joint capsule explants. Joint capsule was excised from the bovine stifle joint medial and lateral to the patella and punched out to form 3-mm-diameter tissue cores ranging in thickness from 0.5 to 3 mm. The joint capsule tissue was then coincubated with cartilage disks that were 3 mm in diameter and 1 mm thick. Histology demonstrated that the tissue was a full-thickness core through the capsule containing synoviocytes in the intimal layer (top); a subintimal layer of mixed areolar and adipose connective tissue (middle); and an outer layer of dense fibrous connective tissue (bottom).
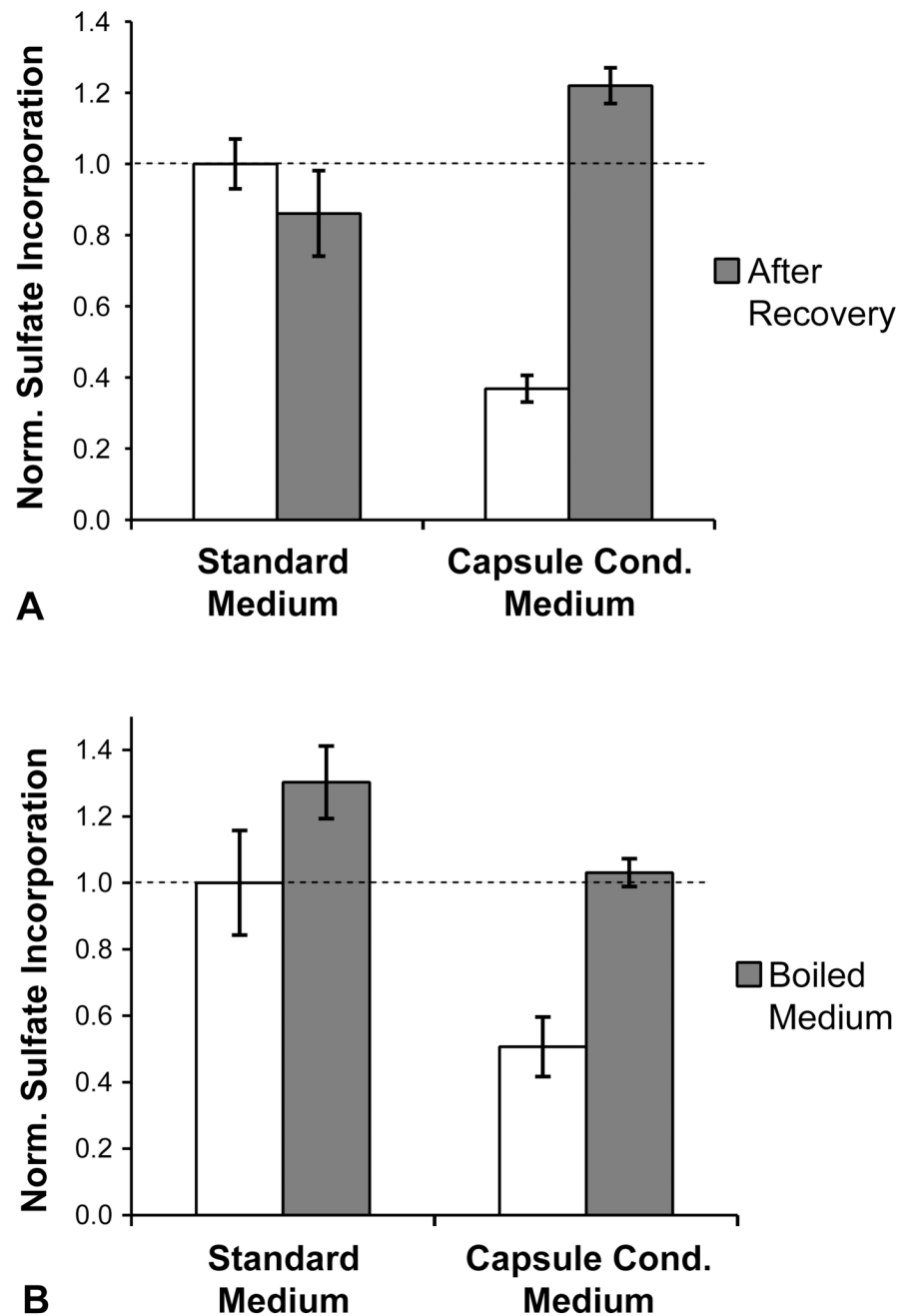
Figure 2.
Characterization of joint-capsule-conditioned medium A) Recoverability: bovine cartilage was incubated for 3 days in either standard medium or capsule-conditioned medium and then either radiolabeled immediately (white), or allowed to recover for 5 days in standard medium before labeling (grey). Sulfate incorporation was normalized to the mean value of disks that were labeled after incubation for 3 days in standard medium. The inhibition of sulfate incorporation by capsule-conditioned medium was significantly reversed after recovery in standard medium (p<0.001 for interaction by ANOVA, N=5 per treatment group). B) Heat lability: cartilage was radiolabeled after incubation for 3 days in either unheated or boiled medium. Sulfate incorporation was normalized to the mean value of disks that were incubated in standard

medium. The inhibition of sulfate incorporation by capsule-conditioned medium was significantly reversed by boiling (p<0.001 for interaction by ANOVA, N=6 per treatment group).
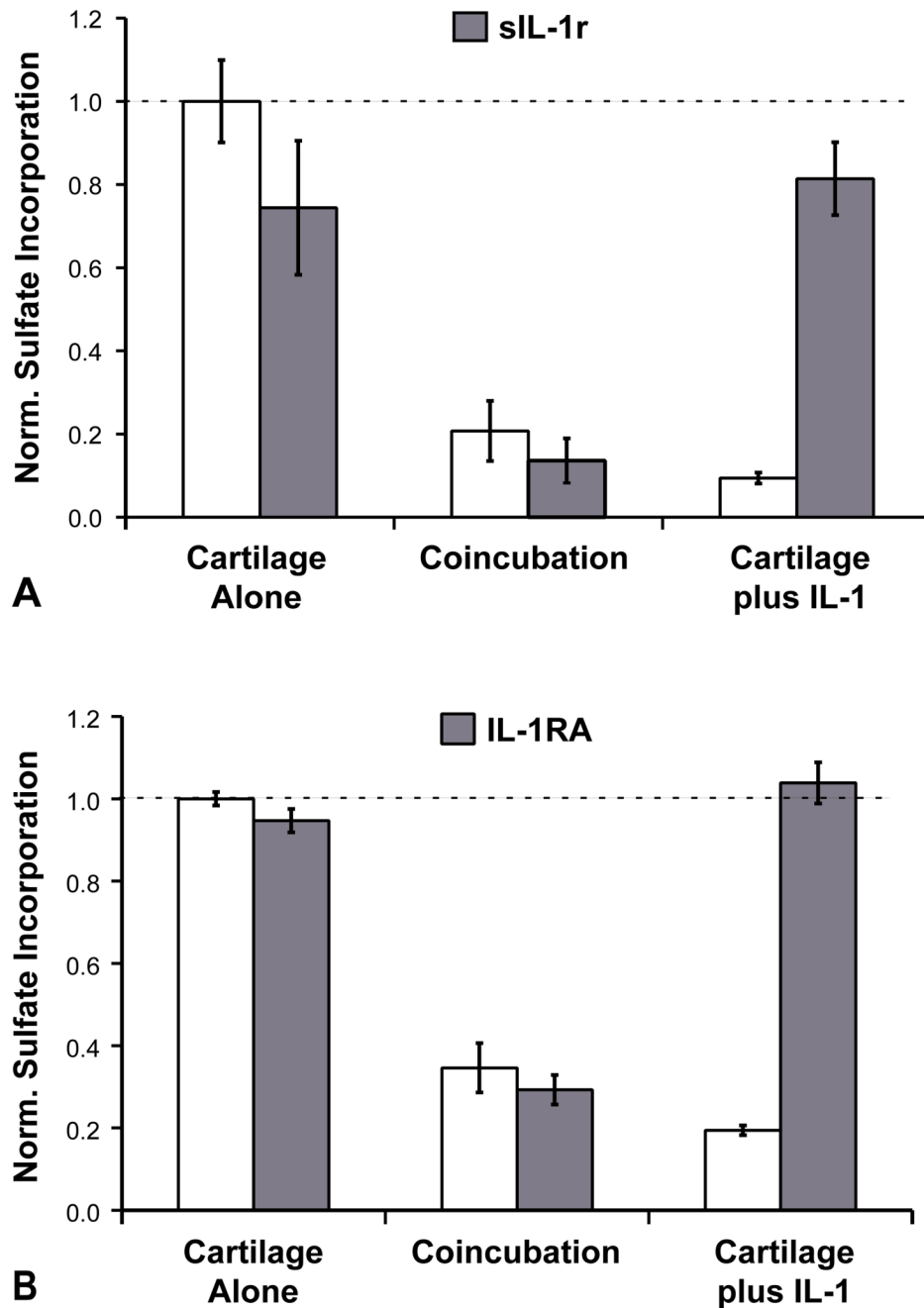
Figure 3.
IL-1 Blockade. Bovine cartilage disks were incubated for 3 days alone (control) or in the presence of joint capsule tissue (coincubation). IL-1 was blocked by A) addition of 5 µg/ml human recombinant soluble IL-1 receptor (sIL-1r) or B) by addition of 250 ng/ml of human recombinant IL-1 receptor antagonist (IL-1RA). IL-1 blockade had no significant effect on the inhibition of sulfate incorporation by coincubation in either experiment (A, $p = 0.32$ and B, $p = 0.34$ for interaction by ANOVA). As a positive control for IL-1 blockade, in both experiments IL-1 blockade had a significant effect on the inhibition of sulfate incorporation by 1 ng/ml rhIL-1 (both $p < 0.001$ for interaction by ANOVA). A, $N = 5$ and B, $N = 7$ per treatment group.
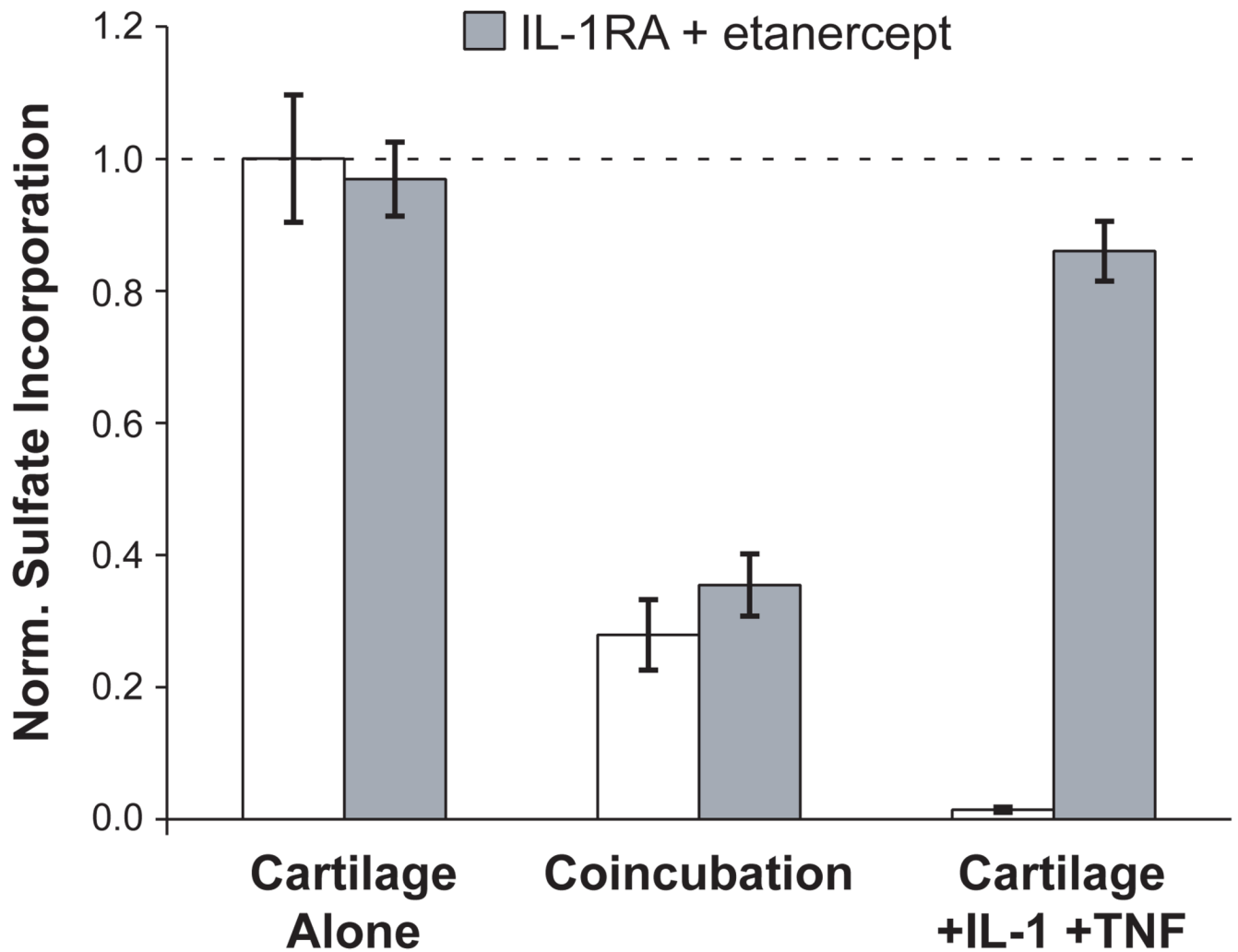
Figure 4.
Combined blockade of IL-1 and TNF. Bovine cartilage was incubated alone or with joint capsule tissue for three days. IL-1 and TNF were blocked by addition of 250 ng/ml IL-1RA and 25 µg/ml etanercept. Combined blockade had no significant effect on the inhibition of sulfate incorporation by coincubation ($p = 0.36$ for interaction by ANOVA; N = 6 per group). As a positive control, inhibition of sulfate incorporation by 100 ng/ml rhTNFα and 1 ng/ml rhIL-1α was significantly reversed by incubation with etanercept and IL-1RA ($p<0.001$ for interaction by ANOVA; N=4–6 per group).
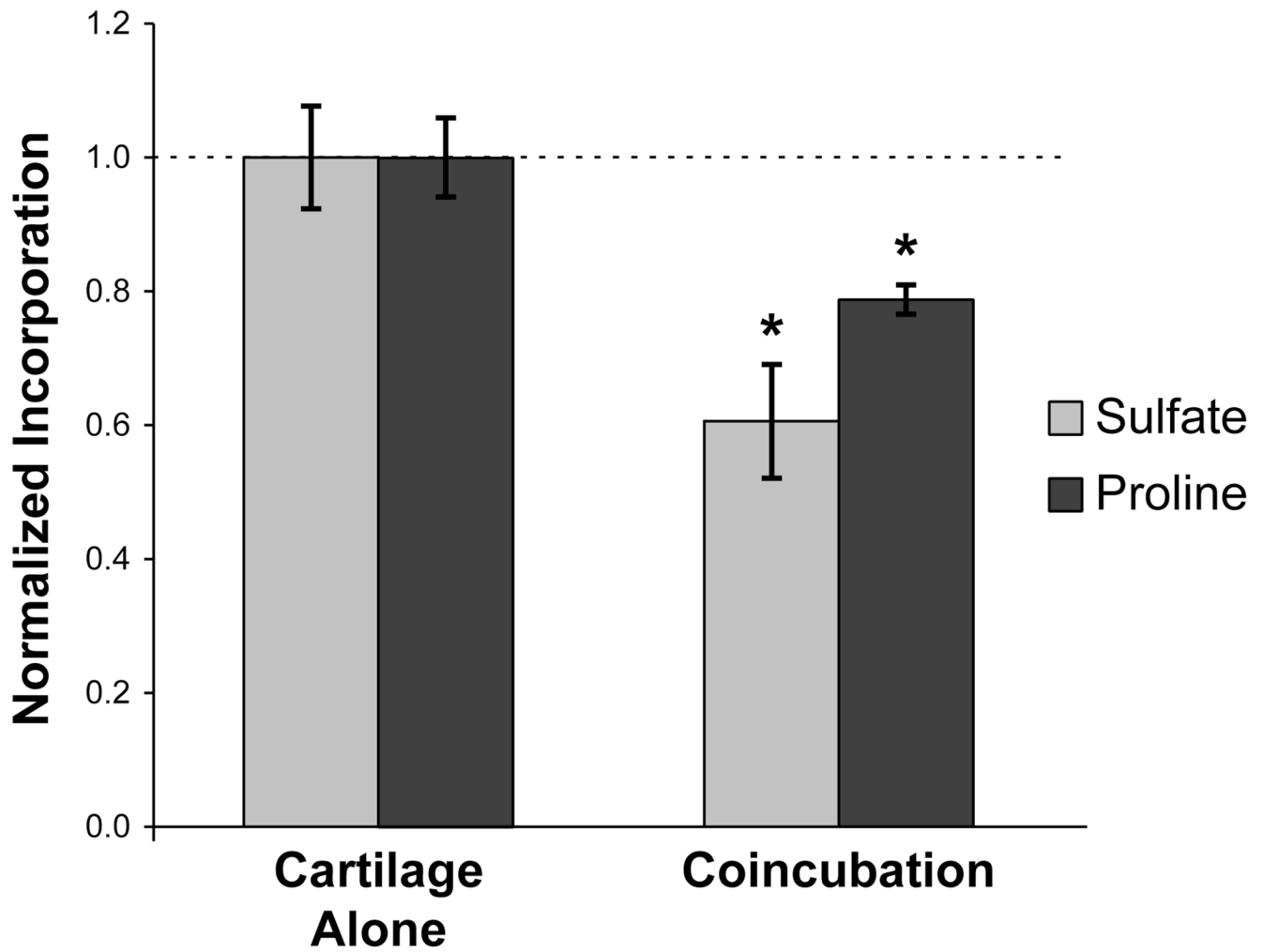
Figure 5.
Coincubation of human cartilage and joint capsule tissue. Cartilage and joint capsule were harvested from a post-mortem knee joint, cut into 3-mm-diameter disks, and equilibrated in standard medium. Cartilage disks were then incubated alone or coincubated with joint capsule in serum-free medium. After three days, the cartilage was radiolabeled with 35S-sulfate and 3H-proline. Incorporation values were normalized to the mean of cartilage incubated alone. Both sulfate and proline incorporation were significantly inhibited by coincubation with joint capsule (*p<0.01 by t-test, N=10 per group).
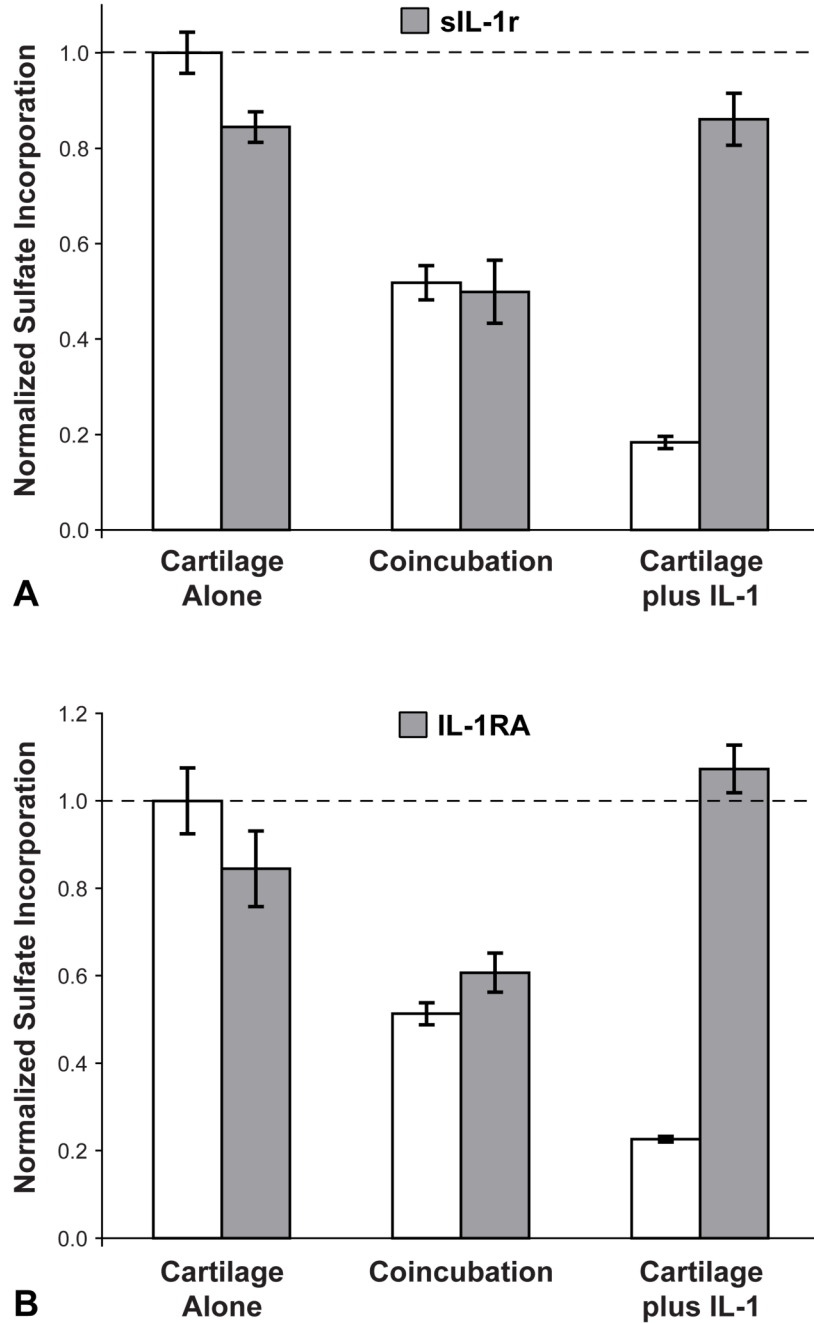
Figure 6.
Effect of IL-1 blockade on incubation with human joint capsule conditioned medium. Capsule-conditioned medium was incubated with bovine articular cartilage explants with or without IL-1 blockade. A) Capsule-conditioned medium inhibited sulfate incorporation, and addition of 5 µg/ml human recombinant soluble IL-1 receptor (sIL-1r) had no significant effect (N = 8 per group; p = 0.44 by t-test). B) Addition of 200 ng/ml human recombinant IL-1 receptor antagonist (IL-1RA) also had no significant effect (N = 8 per group; p = 0.09 by t-test). As a positive control, inhibition of sulfate incorporation by 2 ng/ml rhIL-1 was significantly reversed by IL-1 blockade (p < 0.001 by t-test in both experiments).