Osteoarthritis and Cartilage (2008) **16,** 90–97

doi:10.1016/j.joca.2007.05.009

Osteoarthritis and Cartilage

International Cartilage Repair Society

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Differential regulation of proteoglycan 4 metabolism in cartilage by IL-1α, IGF-I, and TGF-β1

T. A. Schmidt Ph.D., N. S. Gastelum B.S., E. H. Han M.S., G. E. Nugent-Derfus Ph.D., B. L. Schumacher B.S. and R. L. Sah M.D., Sc.D.*

Department of Bioengineering and Whitaker Institute of Biomedical Engineering, University of California-San Diego, La Jolla, CA 92093-0412, United States

Summary

Objectives: To determine (1) if interleukin-1 alpha (IL-1α), insulin like growth factor I (IGF-I), and transforming growth factor-beta 1 (TGF-β1) regulate proteoglycan 4 (PRG4) metabolism in articular cartilage, in terms of chondrocytes expressing PRG4 and PRG4 bound at the articular surface, and (2) if these features of cartilage PRG4 metabolism correlate with its secretion.

Methods: Articular cartilage explants were harvested and cultured for 6 days with or without 10% fetal bovine serum (FBS), alone, or with the addition of 10 ng/ml IL-1α, 300 ng/ml IGF-I, or 10 ng/ml TGF-β1. PRG4 expression by chondrocytes in the cartilage disks was assessed by immunohistochemistry (IHC). PRG4 bound to the articular surface of disks was quantified by extraction and enzyme-linked immunosorbent assay (ELISA). PRG4 secreted into culture medium was quantified by ELISA and characterized by Western Blot.

Results: PRG4 expression by chondrocytes near the articular surface was markedly decreased by IL-1α, stimulated by TGF-β1, and not affected by IGF-I. The level of PRG4 accumulation in the culture medium was correlated with the number of chondrocytes expressing PRG4. The amount of PRG4 bound at the articular surface was modulated by incubation in medium including FBS, but did not correlate with levels of PRG4 secretion.

Conclusions: Cartilage secretion of PRG4 is highly regulated by certain cytokines and growth factors, in part through alteration of the number of PRG4-secreting chondrocytes near the articular surface. The biochemical milieu may regulate the PRG4 content of synovial fluid during cartilage injury or repair.

Key words: Proteoglycan 4, Cartilage biology, Cytokines, Growth factors.

Introduction

Articular cartilage is a multifunctional tissue at the ends of long bones that allows for smooth articulation within diarthrodial joints[1]. Cartilage consists of three zones (superficial, middle, and deep), each with distinct matrix structure and composition[2,3] that impart specialized mechanical properties to these regions[4,5]. Chondrocytes of the middle and deep zones synthesize aggrecan at high rates, and thereby contribute to the ability of cartilage to bear compressive load. The chondrocytes of the superficial zone exhibit a number of specific functions including the synthesis and secretion of superficial zone protein (SZP)[6]. SZP is a ~345 kDa protein present at the surface of articular cartilage[6], and in synovial fluid[7], encoded for by the proteoglycan 4 (PRG4) gene[8].

PRG4 molecules play an important role in the lubrication of articular cartilage by synovial fluid[9–14]. In addition to encoding for SZP, the PRG4 gene encodes for a highly homologous protein termed lubricin[15], a ~220 kDa product of synovial fibroblasts originally isolated from synovial fluid[16]. Mutations of the PRG4 gene can cause camptodactyly-arthropathy-coxa vara-pericarditis (CACP) disease in humans[17], which results in joint failure associated with non-inflammatory synoviocyte hyperplasia and subintimal fibrosis of the synovial capsule[18]. Both SZP and lubricin have boundary lubrication properties for native cartilage or certain synthetic surfaces[9–14]. Since the Human Genome Organization Gene Nomenclature Committee assigned the name PRG4 for the gene (GenBank accession no. AF056218 for bovine partial sequence and U70136 for complete human sequence) encoding various immunoreactive proteins[8,17,19], including megakaryocyte stimulating factor, we refer collectively these molecules, herein, as PRG4[6,20]. PRG4 therefore plays an important role in joint mechanics.

In joint injury and arthritis, the synovial fluid has impaired friction-lowering lubrication qualities[21,22], possibly due to altered *in vivo* metabolism of PRG4 under regulation of the altered cytokine environment. *In vitro* studies have demonstrated cytokines transforming growth factor-beta 1 (TGF-β1) and interleukin-1 alpha (IL-1α), which are present at elevated levels in joint injury and arthritis[23], up-regulate and down-regulate PRG4 secretion into culture medium, respectively, from superficial zone chondrocytes in explant culture, as assessed by enzyme-linked immunosorbent assay (ELISA)[24], and by chondrocytes in agarose culture, as assessed by Western Blot[19]. Preliminary studies have

*Address correspondence and reprint requests to: Dr Robert L. Sah, M.D., Sc.D., Department of Bioengineering, Mail Code 0412, 9500 Gilman Drive, University of California-San Diego, La Jolla, CA 92093-0412, United States. Tel: 1-858-534-0821; Fax: 1-858-822-1614; E-mail: rsah@ucsd.edu

Received 20 July 2006; revision accepted 14 May 2007.

qualitatively characterized PRG4 expression to be regulated in a similar manner, using immunohistochemistry to identify chondrocytes secreting PRG4[24]. Insulin like growth factor I (IGF-I), a potent physiological regulator of proteoglycan metabolism by chondrocytes[25,26], may also regulate PRG4 metabolism. These cytokines may also modulate the amount of PRG4 bound at the surface of articular cartilage, where it is positioned to impart boundary lubrication function[27]. The relationships between the amount of PRG4 bound at the articular surface, PRG4 expression within chondrocytes at or near the articular surface, and PRG4 secretion from cartilage explants are unknown. Collectively, these features of cartilage PRG4 metabolism may be key aspects of PRG4 lubrication function.

The objectives of this study were therefore to determine (1) if IL-1α, IGF-I, and TGF-β1 regulate PRG4 metabolism in articular cartilage, in terms of chondrocytes expressing PRG4 and PRG4 bound at the articular surface, and (2) if these features of cartilage PRG4 metabolism correlate with its secretion.

Methods

MATERIALS

Materials for tissue harvest and culture were obtained as described previously[24,28]. Recombinant human IL-1α, porcine TGF-β1, and recombinant human IGF-I were obtained from R&D Systems (Minneapolis, MN).

CARTILAGE EXPLANT HARVEST AND CULTURE

Cartilage explants were harvested and cultured as described previously[24]. Briefly, osteochondral cores were harvested from the patellofemoral groove of immature (1–3 week old) bovine stifle joints. These cores were cut in a sledge microtome to obtain slices (0.3 mm thick) containing the superficial zone, with the articular surface intact. These slices of cartilage were then punched into smaller 3 mm diameter disks. Disks were then incubated for 6 days in a basal medium (Dulbecco's modified Eagle medium [DMEM], 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] buffer, 0.1 mM nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) at 37°C in an atmosphere of 5% CO_2, supplemented with 25 μg/ml ascorbic acid, 0.01% bovine serum albumin (−FBS) or 10% fetal bovine serum (+FBS), as well as 10 ng/ml IL-1α, 300 ng/ml IGF-I, or 10 ng/ml TGF-β1. Every 2 days, medium (0.360 ml per disk) was replaced, and the spent medium was collected for subsequent analysis.

PRG4 ANALYSIS IN CARTILAGE EXPLANTS

PRG4 immunolocalization

PRG4 expression within chondrocytes of cartilage disks was visualized by immunohistochemistry (IHC) using the monoclonal antibody (mAb) 3A4 (a gift from Dr Bruce Caterson[29]), as described previously[24]. Briefly, after 6 days of culture, disks were incubated overnight in medium supplemented with 0.1 μM monensin, and then rinsed with phosphate buffered saline (PBS). Five micrometer thick cryosections were prepared, reacted with mAb 3A4, and detected with a peroxidase-based system. As negative controls, some samples were probed with a non-specific isotype-matched (IgG) antibody. The stained samples were viewed and documented by photomicroscopy to identify immunoreactive cells, indicating synthesis of PRG4.

The depth-associated variation in chondrocyte PRG4 expression was determined from these micrographs, essentially as described previously[30]. Briefly, a representative 740 μm wide $\times$ 300 μm deep region of each section was analyzed using a custom program written in Matlab 6.5 software (The Mathworks, Inc.). Chondrocytes expressing PRG4 (PRG4+) were identified manually by one reader, in a blinded manner. (This was confirmed as being reasonably independent of the reader in these studies since eight micrographs were also read by a second reader, and the number of PRG4+ cells identified were significantly correlated to those identified by the first reader, $P < 0.001$ and $R^2 = 0.97$.) Then, the total number of PRG4+ cells, and the number of PRG4+ cells as a function of depth from the articular surface were counted. Results are presented as the number of PRG4+ cells in successive 50 μm bins below the articular surface. The total number of cells counted in each section was 300 ± 98 (mean $\pm$ standard deviation (SD).

PRG4 surface concentration

PRG4 bound to the articular surface of freshly isolated disks, and those cultured for 6 days, was determined by extraction and ELISA, as described previously[31]. Briefly, four 3 mm disks were nutated in 0.4 ml of 4 M GuHCl, 0.02 M Tris, pH 8.2 containing protease inhibitors (0.005 M benzamidine HCl, 0.01 M *N*-ethylmaleimide, 0.005 M disodium ethylenediamine tetraacetic acid , and 0.001 M phenylmethylsulfonylfluoride) for 24 h. Portions (200 μl) of extracts or purified bovine standards[6] were then applied to a nitrocellulose membrane in a Bio-Dot apparatus (Bio-Rad, Hercules, CA), blocked with 5% non-fat dry milk, incubated with mAb 3A4, and then with an anti-mouse antibody conjugated to horseradish peroxidase, with rinses in PBS between each step. The sample areas were removed and reacted with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) substrate. Control studies, where a known amount of PRG4 (in the form of medium conditioned by explants containing the superficial zone and quantified by ELISA) was added to serial dilutions of sample extracts, indicated the extracted cartilage components in samples caused negligible interference in this assay of PRG4 content. Amounts of PRG4 were expressed as $\mu g/cm^2$ area of articular surface (since PRG4 has been immunolocalized at or near the articular surface of intact disks, both freshly isolated and cultured[24].)

PRG4 ANALYSIS OF SPENT CULTURE MEDIUM

PRG4 secretion

PRG4 secreted into culture medium was quantified by ELISA using mAb 3A4, as described previously[28]. Briefly, medium samples pooled from the 6 day culture were diluted serially, adsorbed to ELISA plates, and then reacted with mAb 3A4, horseradish peroxidase-conjugated secondary antibody, and ABTS substrate, with three washes with PBS + 0.1% Tween between each step. PRG4 levels were calculated using purified bovine standards[29].

Western Blot

PRG4 in spent culture medium samples, pooled from the 6 day culture, was characterized by Western Blot, essentially as described previously[30]. PRG4 was purified from equal

volumes (60 ml) of pooled samples by anion exchange chromatography with diethylaminoethyl (DEAE) Sepharose™ gel, collecting the 0.3–0.6 M NaCl eluate, concentration with a Centricon® Plus 100 kDa MW cutoff filter, dialysis against distilled water, and then lyophilization. Samples containing the same amount of recovered dry mass (10 μg) for each condition were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 3–8% gradient polyacrylamide gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with mAb 3A4, with ECL-Plus detection. Luminescence from the membrane was digitized with a STORM 840 Imaging System (Molecular Dynamics, Fairfield, CT).

STATISTICAL ANALYSIS

Data are expressed as the mean ± S.E.M.. Effects of medium components (FBS and cytokines) on PRG4 expression by chondrocytes were assessed by analysis of variance (ANOVA) with depth as a repeated measure; a square root transformation was applied to improve the uniformity of variance amongst the experimental groups. Effects of medium components on PRG4 surface concentration were assessed by ANOVA, with Tukey's *post hoc* tests for comparisons between experimental groups. The effect of culture on PRG4 surface concentration was also assessed by ANOVA with Dunnett's *post hoc* tests for comparisons to freshly isolated disks. Effects of medium components on PRG4 secretion were assessed by ANOVA, after a log transformation to improve uniformity of variance, with Tukey's *post hoc* tests. The relationships between the total number of PRG4+ cells and PRG4 secretion, and PRG4 surface concentration and PRG4 secretion, were analyzed by linear regression.

Results

PRG4 ANALYSIS IN CARTILAGE EXPLANTS

PRG4 immunolocalization

PRG4 was localized differentially in cartilage disks, depending on the culture conditions (Fig. 1). In vertical sections from all groups, PRG4 staining was evident at the articular surface, and within chondrocytes near the articular surface. Control samples using the non-specific IgG primary antibody were appropriately PRG4 negative.

The total number of PRG4+ cells and depth-associated variation in chondrocyte expression was modulated by culture condition (Fig. 2). The total number of PRG4+ cells varied with cytokine treatment ($P < 0.001$), but not with FBS ($P = 0.78$) or an interaction ($P = 0.61$). Samples incubated without additives, i.e., without and with FBS, had 51 ± 10 PRG4+ cells, which was similar to the number of PRG4+ cells in samples treated with IGF-I (41 ± 8, $P = 0.74$). In comparison, samples treated with TGF-β1 had significantly more (228 ± 20, $P < 0.001$) PRG4+ cells, and those treated with IL-1α had significantly less (6 ± 3, $P < 0.001$). The number of PRG4+ cells also varied with depth from the articular surface ($P < 0.001$), with significant interactions between depth and FBS or cytokines (each $P < 0.001$). Untreated samples had many PRG4+ cells in the top 200 μm and very few below 200 μm, in medium −FBS [Fig. 2(A)] and +FBS [Fig. 2(B)]. Samples treated with IL-1α, IGF-I, TGF-β1 appeared to have less, similar, and more PRG4+ cells, respectively, in the top 200 μm of tissue compared to untreated samples. Additionally, samples treated with TGF-β1 consistently had PRG4+ cells below a depth of 200 μm, whereas all other groups did not.

PRG4 surface concentration

The amount of PRG4 bound to the articular surface of disks at the end of the culture period varied with FBS ($P < 0.01$), but not with cytokine ($P = 0.92$), and without an interaction effect ($P = 0.08$, Fig. 3). Samples incubated in medium without FBS had 0.72 ± 0.05 μg/cm^2 PRG4, which was similar to the amount bound to samples treated with IL-1α, 0.49 ± 0.07 μg/cm^2 ($P = 0.51$), IGF-I, 0.65 ± 0.11 μg/cm^2 ($P = 0.99$), and TGF-β1, 0.52 ± 0.08 μg/cm^2 ($P = 0.66$). These samples did, however, have significantly more PRG4 bound than those samples incubated in medium +FBS (0.33 ± 0.12 μg/cm^2, $P < 0.05$). Samples incubated in medium +FBS had bound PRG4 levels that were unaffected ($P = 0.96$–1.00) by additional treatment with IL-1α (0.46 ± 0.03 μg/cm^2), IGF-I (0.32 ± 0.10 μg/cm^2), or TGF-β1

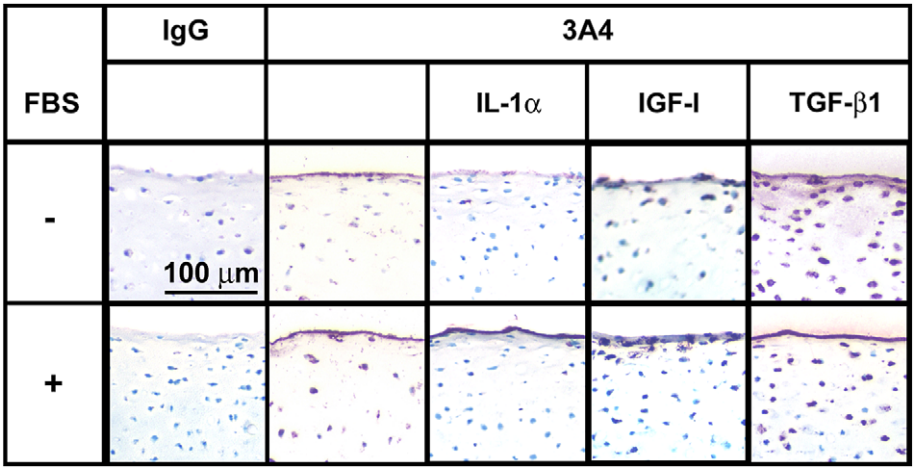

Fig. 1. Effect of culture conditions on PRG4 presence and expression by chondrocytes in cartilage explants containing the superficial zone. Disks were cultured in medium supplemented with 25 μg/ml ascorbic acid, ±10% FBS, as well as 10 ng/ml IL-1α, 300 ng/ml IGF-I, or 10 ng/ml TGF-β1. PRG4 was probed with mAb 3A4 in disks after 6 days of culture. A non-specific IgG was used for the control.

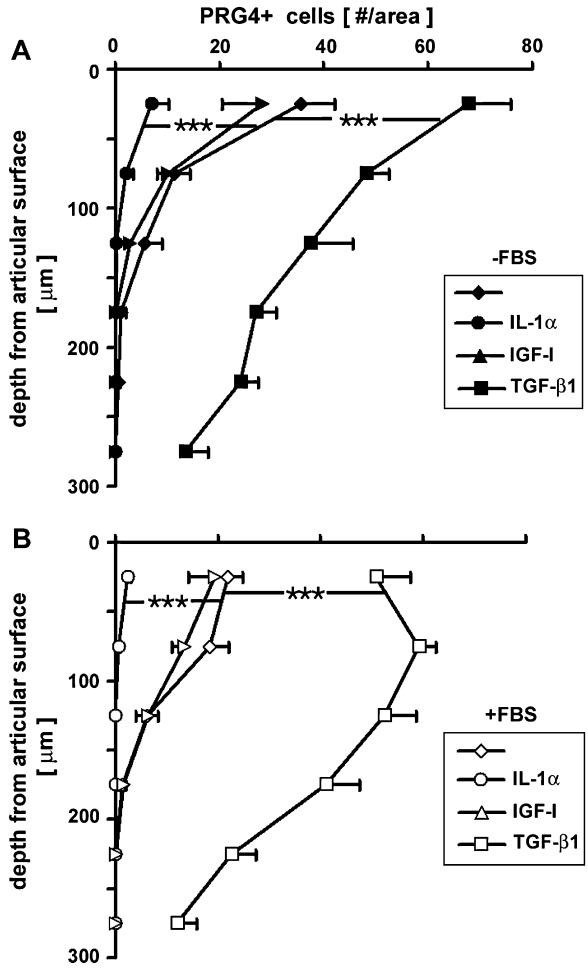

Fig. 2. Effect of culture conditions on depth-dependent PRG4 expression by chondrocytes in cartilage explants containing the superficial zone, represented as cells per area. PRG4 was probed with mAb 3A4 in disks after 6 days of culture. Data are mean ± S.E.M. ($n = 6–7$). *** = $P < 0.001$.

(0.45 ± 0.05 μg/cm^2). The amount of PRG4 bound to the articular surface of freshly isolated samples (Day 0), 0.85 ± 0.08 μg/cm^2, was significantly more than the amount bound to the surface of samples incubated in medium −FBS and treated with IL-1α or TGF-β1 ($P < 0.05$), and also more than every sample group incubated in medium +FBS ($P < 0.001–0.05$).

PRG4 ANALYSIS OF SPENT CULTURE MEDIUM

PRG4 secretion

The average rate of PRG4 secretion over the 6 days of culture varied with FBS ($P < 0.01$) and cytokine treatment ($P < 0.001$), without an interaction effect ($P = 0.60$) (Fig. 4). Samples incubated in medium −FBS secreted 1.3 ± 0.3 μg/cm^2/day, which was similar to the amount secreted by samples treated with IGF-I (1.9 ± 0.7 μg/cm^2/day, $P = 1.0$). Compared to untreated samples during incubation, those treated with TGF-β1 secreted significantly more PRG4 (65 ± 18 μg/cm^2/day, $P < 0.01$), while those treated with IL-1α secreted significantly less (0.05 ± 0.04 μg/cm^2/day, $P < 0.001$). For samples incubated in medium +FBS, the trends in regulation of PRG4 secretion

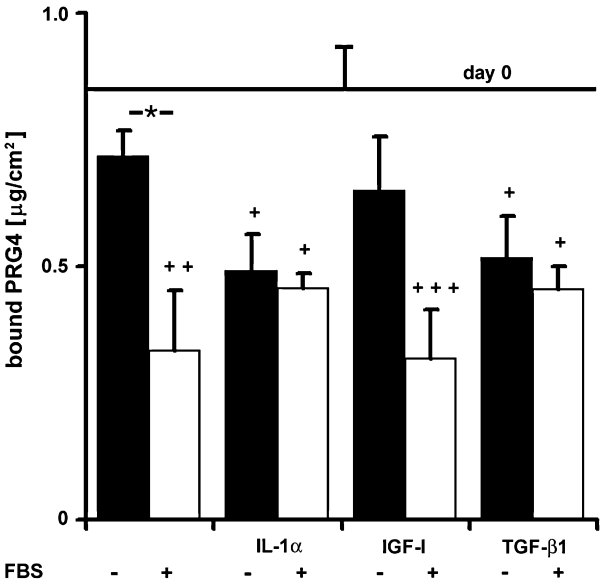

Fig. 3. Effect of culture conditions on the amount of PRG4 bound to the surface of cartilage explants containing the superficial zone after 6 days of culture. Data are mean ± S.E.M. ($n = 5$). * = $P < 0.05$; + = $P < 0.05$, ++ = $P < 0.01$, +++ = $P < 0.001$ compared to day 0.

were similar, although the rates were significantly higher. Samples incubated with FBS secreted 4.3 ± 1.3 μg/cm^2/day, while those additionally treated with IL-1α, IGF-I, TGF-β1 secreted 0.21 ± 0.05 μg/cm^2/day, 4.8 ± 0.8 μg/cm^2/day, and 124 ± 15 μg/cm^2/day, respectively.

Western Blot

A major band immunoreactive with mAb 3A4 appeared at an apparent molecular weight of ~345 kDa in all samples,

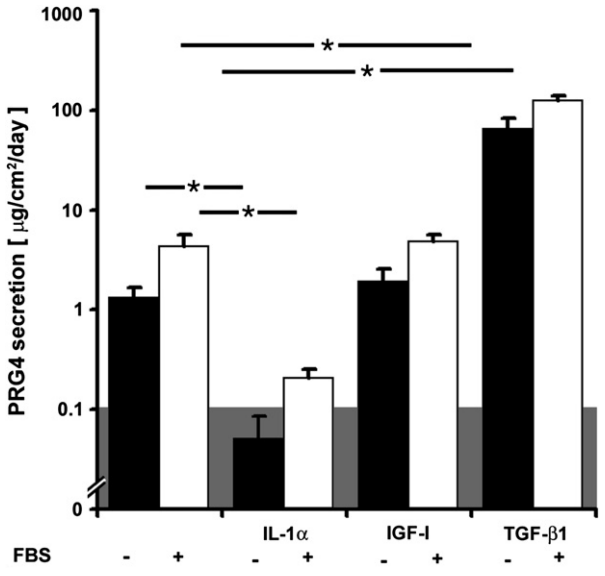

Fig. 4. Effect of culture conditions on PRG4 secretion by chondrocytes in cartilage explants containing the superficial zone. PRG4 secretion rates are shown averaged over 6 days of culture. Shaded regions indicate levels at or below assay sensitivity. Data are mean ± S.E.M. ($n = 8$). * = $P < 0.05$.

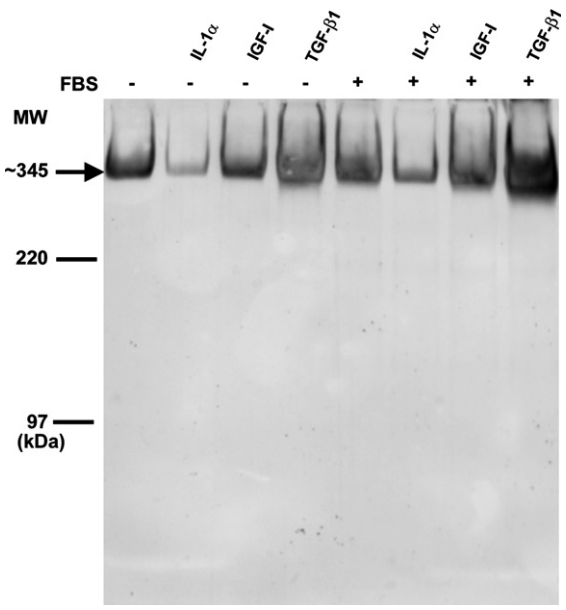

Fig. 5. Western Blot of PRG4 secreted by chondrocytes in cartilage explants containing the superficial zone under various culture conditions probed with mAb 3A4 after separation on a 3−8% gradient polyacrylamide gel.

except those treated with TGF-β1, in which a band appeared slightly above, and another slightly below the ~345 kDa band (Fig. 5). Considering the relative proportion of sample applied to each lane, the differences in band intensities between the groups (Fig. 5) appeared consistent with the differences in secretion observed by ELISA (Fig. 4).

PRG4 EXPRESSION CORRELATIONS

The average rate of PRG4 secretion over the 6-day culture was regressed linearly with the total number of PRG4+ cells and the PRG4 bound to the articular surface at the end of the culture period (Fig. 6). The average rate of PRG4 secretion showed a strong correlation with the total number of PRG4+ cells [$R^2 = 0.61$, $P < 0.001$, Fig. 6(A)]. Conversely, PRG4 secretion did not correlate with the amount of PRG4 bound to the articular surface [$P = 0.75$, Fig. 6(B)].

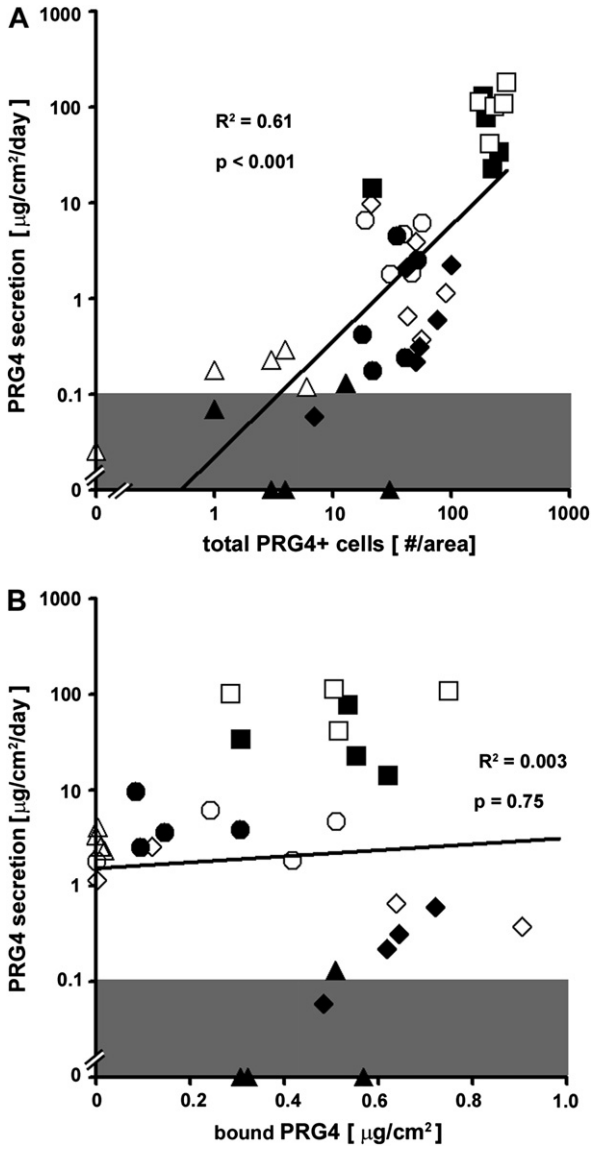

Fig. 6. Correlation of PRG4 secretion by chondrocytes in cartilage explants containing the superficial zone over 6 days of culture to (A) the total PRG4+ cells per area in the cartilage explants, and (B) the amount of PRG4 bound at the surface of the cartilage explants, at the end of the culture duration. Shaded regions indicate levels at or below assay sensitivity.

Discussion

These results indicate that (1) PRG4 expression in chondrocytes near the articular surface is highly regulated by IL-1α and TGF-β1, and is strongly correlated with PRG4 secretion, and (2) PRG4 bound at the articular surface is modulated by incubation in medium with FBS, in a way that does not correlate with PRG4 secretion. IL-1α had an inhibitory effect on PRG4 expression by chondrocytes, TGF-β1 had a stimulatory effect, whereas IGF-I had no detectable effect (Figs. 1 and 2). The amount of PRG4 bound to the surface of cultured disks was not significantly altered by cytokine supplementation, whereas incubation in medium +FBS did significantly reduce the amount of surface bound PRG4 compared to freshly isolated disks (Fig. 3). In all culture conditions, the PRG4 secreted into the medium (Fig. 4), with an apparent molecular weight (based on migration distance) at ~345 kDa on SDS-PAGE (Fig. 5). PRG4 secretion correlated strongly with PRG4 expression, but not the amount of PRG4 bound at the articular surface (Fig. 6). These results provide insight into chondrocyte metabolism of the boundary lubricant, PRG4, under conditions relevant to normal growth, homeostasis, and pathology of articular cartilage.

Under particular culture conditions, this study assessed PRG4 metabolism quantitatively in terms of number of chondrocytes staining positively by IHC, the amount bound at the articular surface and quantified in tissue extracts by ELISA (since the relative IHC staining intensity may not be directly related to the quantity of matrix-bound epitope), and that present in the medium by ELISA. Any apparent effects of IGF-I and TGF-β1 on surface bound PRG4 as assessed by IHC could be related to altered cartilage matrix metabolism of proteoglycans[25,26,32], thereby possibly affecting surface

staining. The overnight treatment of cartilage disks with 0.1 μM monensin prior to IHC inhibited PRG4 secretion to undetectable levels (<0.001 μg in 0.1 ml of medium[28]), suggesting that essentially all of the synthesized PRG4 was trapped within the chondrocytes. The accumulation of PRG4 molecules in culture medium represents molecules predominantly synthesized, since the amount present at the articular surface before and after culture was much less, as described previously[24,28,30]. The reported secretion levels could be slight underestimations of the total synthesis, under certain conditions where secretion rates are relatively low, depending on the as of yet unknown partition coefficients of PRG4 in cartilage. The serial dilution curves of medium samples obtained by ELISA for PRG4 were similar, irrespective of growth factor or cytokine supplementation, indicating non-PRG4 proteins or glycosaminoglycans in the samples did not interfere with the assay for PRG4 content. The concentration of TGF-β1 and IL-1α (10 ng/ml) was chosen because their respective stimulatory and inhibitory effects on PRG4 secretion by chondrocytes in explants containing the superficial zone was previously shown to be dose-dependent and plateaued at 10 ng/ml[24]. Similarly, the concentration of IGF-I (300 ng/ml) was chosen because the dose-dependent stimulatory effect on proteoglycan synthesis plateaued at 30–300 ng/ml[26]. Physiological levels have been reported to range from ~4 to 10 ng/ml in arthritic human SF[33] for TGF-β1, from ~45 to 103 ng/ml in osteoarthritic human SF[34,35] for IGF-I, and increase after severe acute injury in horse SF[36] to levels >0.005 ng/ml for IL-1. Therefore the concentrations used in this study are in the range of pathophysiological levels. Immature articular bovine cartilage was used in this study since its expression of PRG4 is known to be mechanically regulated[30,37]; its biosynthetic response to biochemical stimuli may be more vigorous than that of adult cartilage, since its density of cells is higher than that of mature tissue[38]. Three-dimensional imaging analysis[39] of the tissue used here has shown a thin layer (~50 μm) of flattened cells typical of the superficial zone of cartilage, followed by spheroidal cells in small groups, typical of the middle zone; the classical deep zone may not be present until later in the development process[40,41]. Although the boundary between these morphologically distinct zones cannot be sharply defined, the morphology of cells, structure of the extracellular matrix, and the PRG4+ phenotype (Fig. 1) contained in the region of tissue used here (top ~300 μm) suggest that a classical superficial zone exists in the immature tissue. Additionally, the up-regulation of PRG4 expression by chondrocytes in immature cartilage explants containing the top ~300 μm by chemical (TGF-β1)[24] and mechanical (shear)[30] stimuli, but not in explants without the top ~300 μm, also suggests a superficial zone exists in immature tissue.

The results of this study agree with and extend previous studies on the regulatory effects of TGF-β1 and IL-1α on PRG4 secretion by chondrocytes. TGF-β1 and IL-1α have been shown to qualitatively up- and down-regulate, respectively, chondrocyte secretion of PRG4 into culture medium by Flannery *et al.*[19]. Chondrocytes from the superficial zone of bovine calf articular cartilage were cultured in agarose for 10 days with DMEM + 5% FBS, alone, or with 2 ng/ml TGF-β1 or 10 ng/ml IL-α. Western Blots of the conditioned culture medium probed with mAb 3A4 indicated that TGF-β1 stimulated ~345 kDa PRG4 secretion while IL-1α markedly inhibited secretion. In a preliminary study[24], we found a dose-dependent (0.1, 1, and 10 ng/ml were examined) stimulatory and inhibitory effect of TGF-β1 and IL-1α, respectively, on PRG4 secretion into culture medium by chondrocytes in explants containing the superficial zone, both in the presence and absence of 10% FBS. Additionally, we demonstrated the time course of alteration of PRG4 secretion was fairly rapid, with differences in PRG4 secretion clearly distinguishable after 2 days of culture with 10 ng/ml of TGF-β1 or IL-1α, and changed gradually in subsequent days (days 2–4 and 4–6). In the present study, the effects of TGF-β1 and IL-1α at 10 ng/ml on PRG4 secretion by chondrocytes in explants containing the superficial zone were confirmed. Future studies may address whether regulatory effects are at the transcriptional, translational, or post-translational level. Additionally, Western Blots of the conditioned culture medium probed with mAb 3A4 indicated the secreted PRG4 had an apparent molecular weight at or near ~345 kDa (Fig. 5). This is consistent with several previous studies where PRG4 prepared from medium conditioned by superficial zone chondrocytes was characterized using similar methods of electrophoresis on SDS-PAGE gradient gels[6,19,29]. Additionally, the 3–8% polyacrylamide gels used here provided resolving capacity for high molecular weight molecules; some variations in migration distance near the apparent molecular weight of ~345 kDa was evident, in particular with samples treated with TGF-β1 where two immunoreactive bands were evident. The details of the structural alterations of PRG4 secreted under TGF-β1 stimulation, and their potential function significance, remain to be determined. The presence of an immunoreactive ~345 kDa band in the pooled conditioned medium supplemented with 10 ng/ml IL-1α is likely due to PRG4 secreted early on in the culture media before IL-1α essentially abolished PRG4 secretion. Other studies have also reported the inclusion of ascorbic acid in culture media[28], and mechanical stimuli in the form of dynamic shear[30], up-regulate PRG4 secretion by chondrocytes in explants. Collectively, these results support the hypothesis that chemo-mechanical processes govern chondrocyte metabolism[42], specifically secretion of PRG4, within articular cartilage.

Somewhat surprisingly, IGF-I, which stimulates proteoglycan synthesis in articular cartilage[26], did not modulate PRG4 secretion by chondrocytes in explants containing the superficial zone of articular cartilage. These results were different than those reported in Flannery *et al.*[19], which, found superficial zone chondrocytes in agarose culture with 30 ng/ml IGF-I to secrete PRG4 to a similar (i.e., stimulatory) level as with 10 ng/ml TGF-β1. In the present study, IGF-I at 300 ng/ml had no detectable effect on PRG4 secretion by chondrocytes in explants containing the superficial zone, although it did stimulate proteoglycan synthesis (data not shown), as described previously[26]. In addition, no detectable effect on PRG4 secretion was observed at 3 and 30 ng/ml IGF-I (data not shown). Whether superphysiological concentrations >300 ng/ml IGF-I[34,35] have effects remains to be determined. This difference may be attributable to the different culture systems used[43], and suggests that cell–cell or cell–matrix interactions in cartilage may have regulatory roles together with IGF-I.

The marked biochemical regulation of the number of chondrocytes expressing PRG4 within cartilage is a major new finding in the present study. Regulation by TGF-β1 and IL-1α of chondrocyte expression of PRG4 near the articular surface paralleled effects on secretion. The quantitative analysis (Fig. 2) indicated that TGF-β1 and IL-1α up- and down-regulate, respectively, the total number of PRG4+ chondrocytes in cartilage explants containing the superficial zone. Therefore, not only does TGF-β1 increase the number of PRG4+ chondrocytes, but also the apparent secretion rate of each individual PRG4+ chondrocyte as well (since

secretion rates from cartilage disks increased ~100-fold (Fig. 4) whereas the total number of PRG4+ cells increased only ~5-fold). (Conversely, TGF-β1 and IL-1α appeared to have a similar suppressive effect, in the absence of FBS, on the amount of surface bound PRG4 (Fig. 3).) Dynamic shear has also been shown to increase the total number of PRG4+ chondrocytes in cartilage explants containing the superficial zone[30]. Collectively, these results broaden the concept that chemo-mechanical processes govern chondrocyte metabolism of PRG4 within articular cartilage to include both secretion and chondrocyte expression. Such chemo-mechanical factors altering the depth from the articular surface to which chondrocytes express PRG4, e.g., below a depth of 200 μm, where chondrocyte do not normally express PRG4 (Fig. 2 and in previous studies[6,30]) may complicate the use of PRG4 synthesis as a phenotypic marker for chondrocytes of the superficial zone[6,19]. This would be especially so under pathophysiological conditions where the chemo-mechanical milieu may be altered. Such altered or enhanced chondrocyte expression of PRG4 may be part of a feedback mechanism within articular cartilage, or a repair type response of chondrocytes, in conditions where the biochemo-mechanical milieu is altered.

The accumulations of PRG4 within synovial fluid and at the articular surface, are likely key functional determinants of PRG4's boundary lubricating ability. PRG4 synthesis and secretion by chondrocytes could significantly contribute to the concentration of PRG4 within synovial fluid, in both homeostatic and pathological conditions where physiological regulators are present[23]. Although the amount of PRG4 bound to the surface does not appear to correlate with secretion rates, previous studies suggest surface bound PRG4 can exchange with endogenous PRG4 in synovial fluid[44], especially under the influence of mechanical perturbation[27,30]. Clarification of the spatial and temporal aspects of PRG4 metabolism within the joint, particularly at the articular surface, would further the understanding of PRG4's contribution to the low-friction properties of articular cartilage, and possibly lead to treatments to prevent loss of this function. More remains to be determined about the processing, and the potentially additional or alternative functions of various PRG4 molecules of different molecular weights[9,10,19,30,45]. Additional studies, including further purification and analysis of tissue and medium samples, may also clarify the basis for, and functional significance of, various PRG4 molecules, both secreted and present at the articular surface, of different molecular weights. Finally, the combination of chemical and mechanical factors to stimulate PRG4 expression in chondrocytes near the articular surface may be useful for creating tissue engineered cartilage from isolated sub-populations[46] with a surface that is bioactive and functional in lubrication.

Acknowledgments

This work was supported by grants from the National Institute of Health and the National Science Foundation, and an award to UCSD under the Howard Hughes Medical Institute Professors Program.

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