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Growth Factors, Cytokines, Cell Cycle Molecules

Transforming Growth Factor-β2 Suppresses Collagen Cleavage in Cultured Human Osteoarthritic Cartilage, Reduces Expression of Genes Associated with Chondrocyte Hypertrophy and Degradation, and Increases Prostaglandin E_2 Production

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Articular cartilage degeneration in osteoarthritis (OA) involves type II collagen degradation and chondrocyte differentiation (hypertrophy). Because these changes resemble growth plate remodeling, we hypothesized that collagen degradation may be inhibitable by growth factors known to suppress growth plate hypertrophy, namely transforming growth factor (TGF)-β2, fibroblast growth factor (FGF)-2, and insulin. Full-depth explants of human OA knee articular cartilage from arthroplasty were cultured with TGF-β2, FGF-2, and insulin in combination (growth factors) or individually. In cultured explants from five OA patients, collagenase-mediated type II collagen cleavage was significantly down-regulated by combined growth factors as measured by enzyme-linked immunosorbent assay. Individually, FGF-2 and insulin failed to inhibit collagen cleavage in some OA explants whereas TGF-β2 reduced collagen cleavage in these 5 explants and in 19 additional explants. Moreover, TGF-β2 effectively suppressed cleavage at low concentrations. Together or individually these growth factors did not inhibit glycosaminoglycan (primarily aggrecan) degradation while TGF-β2 occasionally did. Semiquantitative reverse transcriptase-polymerase chain reaction of articular cartilage from six OA patients revealed that TGF-β2 suppressed expression of *matrix metalloproteinase-13* and *matrix metalloproteinase-9*, early (*PTHrP*) and late (*COL10A1*) differentiation-related genes, and proinflammatory cytokines (*interleukin-1β*, *tumor necrosis factor-α*). In contrast, TGF-β2 up-regulated *PGES-1* expression and prostaglandin E_2 release. These observations show that TGF-β2 can suppress collagen resorption and chondrocyte differentiation in OA cartilage and that this may be mediated by prostaglandin E_2. Therefore TGF-β2 could provide therapeutic control of type II collagen degeneration in OA. ***(Am J Pathol 2006, 168:131–140; DOI: 10.2353/ajpath.2006.050369)***

The pathology of osteoarthritis (OA) involves the whole joint and is associated with focal and progressive hyaline articular cartilage loss, concomitant sclerotic changes in the subchondral bone, and the development of osteophytes. Soft tissue structures in and around the joint including synovium, ligaments, and muscles are also affected. Cartilage erosion is a fundamental pathological feature of OA. It involves damage to and resorption of the extracellular matrix, composed mainly of type II collagen and the proteoglycan aggrecan as well as many other matrix molecules. There is excessive cleavage of type II collagen in OA[1,2] associated with the up-regulation of the synthesis and activities of collagenases,[3] in particular matrix metalloproteinase (MMP)-13.[1,2] Although OA is traditionally defined as a noninflammatory arthropathy, degenerative changes in OA cartilage are accompanied by the up-regulation of proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α,[4] which are involved in cartilage resorption.[5] IL-1β has

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been shown to weakly induce prostaglandin (PG) E_2 production in OA articular cartilage.[6] Others have suggested that prostaglandins may augment IL-1β-mediated degradation of OA cartilage *ex vivo*.[7]

Cartilage pathology in OA is associated with an overall up-regulation of anabolic activity in articular cartilage, which may accompany changes in cellular phenotypes of articular chondrocytes.[3,8–10] These phenotypic changes may also involve chondrocyte terminal differentiation (hypertrophy), associated with the expression of *type X collagen* in superficial, mid, and deep zones,[11–13] and other alterations in phenotype involving onset of synthesis of type IIA collagen[14] and dedifferentiation, accompanied by expression of *type I and III collagens*[15–17] and synthesis of type III collagen.[15]

We have shown recently that the formation of very early age-related OA-like lesions in the knee[18] is accompanied by the up-regulation of chondrocyte differentiation-related genes implicated in the regulation of chondrocyte hypertrophy in endochondral bone formation. These include *parathyroid hormone-related peptide (PTHrP)*, *fibroblast growth factor 2 (FGF-2), transforming growth factor (TGF)-β1* and *-β2*, *Indian hedgehog (Ihh), MMP-13, MMP-9, cyclin B2,* and *Sox 9*. All of these genes are expressed in the bovine growth plate[19] where early and late chondrocyte differentiation is accompanied by the sequential expression of genes responsible for extracellular matrix remodeling and regulatory growth factors. Thus, the up-regulation of *PTHrP* and *FGF-2*, *TGF-β2* in the early proliferative zone is followed by a hypertrophy-related increase in expression of *type X collagen*, *MMP-13*, *MMP-9*, *TGF-β1*, and *Ihh*. These observations suggest that chondrocyte differentiation in articular cartilage is an early event in the development of OA.[18] Moreover it is now well established that chondrocyte hypertrophy results in increased expression of the collagenase *MMP-13*, the same protease that is up-regulated in OA cartilage.[20–22] Also, inhibition[22] or deletion[23] of *MMP-13* expression suppresses chondrocyte hypertrophy as well as collagenase activity.[22] In view of these observations, the destruction of the collagen network in OA by collagenases may involve the regulatory mechanisms of extracellular matrix resorption that govern chondrocyte differentiation in development in endochondral bone formation.

Chondrocyte hypertrophy is a process that can be arrested at various checkpoints. The arrest may be temporary, such as to attenuate endochondral ossification, but it also can be more permanent, as in healthy uncalcified hyaline cartilage.[24] The latter is characterized by a very low expression of *collagen type II*; no expression of *type I, III,* or *X collagens*; a relatively high turnover rate for aggrecan; and very restricted chondrocyte replication.[25–27] It is also characterized by a limited expression of *TGF-β1*; a complete lack of expression of *TGF-β2*, *PTHrP, FGF-2*, *insulin growth factor (IGF)-1*, and *Ihh*;[28,29] and a negligible rate of chondrocyte apoptosis.[30–32]

A number of growth factors are capable of arresting chondrocyte hypertrophy. FGF-2 suppresses hypertrophy.[33,34] Defects in FGF-2 receptor result in a loss of suppression.[35,36] TGF-β2 in synergy with FGF-2 can suppress maturation of chondrocytes in culture.[37]

Insulin and its structural and functional analog IGF-1[38] are strong stimulators of the synthesis of aggrecan and type II collagen.[39–41] Also IGF-1 favors chondrocyte hypertrophic development inducing *type X collagen* and *alkaline phosphatase* in avian sternal chondrocytes.[37,42] However, in combination with TGF-β2 and FGF-2, IGF-1 can increase synthesis of cartilage matrix molecules without induction of *type X collagen* up-regulation.[37] Because TGF-β2, FGF-2, and insulin acting synergistically can suppress normal terminal differentiation in chondrocytes,[42,43] and because excessive type II collagen resorption by collagenase[1,2,22] and chondrocyte hypertrophy[11,12] are key components of the pathology of OA we hypothesized that these growth factors may be capable of suppressing increased type II collagen cleavage by collagenase in OA, if the increased cleavage is associated with chondrocyte hypertrophy as in the growth plate.[21,22]

We describe in this report how type II collagen cleavage by collagenase in OA articular cartilage can indeed be arrested by growth factors that also suppress expression of *IL-1β*, *TNF-α*, and genes characteristic of chondrocyte hypertrophy. This suppression is accompanied by an up-regulation of PGE_2 production and expression of *PGES-1*. This suggests that expression of these cytokines and development of chondrocyte hypertrophy are important components of the pathology of OA and that up-regulation of PGE_2 production favors the control of collagen degradation.

Materials and Methods

Cartilage Isolation and Preparation

Human femoral condylar cartilages were obtained at total knee arthroplasty from 30 patients (18 men, mean age, 72.2 $\pm$ 7.05 years; range, 52 to 82 years; 12 women, mean age, 73.5 $\pm$ 7.14 years; range, 58 to 82 years) with OA diagnosed according to the criteria of the American College of Rheumatology.[44] Cartilages were prepared as previously described.[45] In brief, OA articular cartilages were washed three times with Dulbecco's modified Eagle's medium A (Life Technologies, Inc., Grand Island, NY) containing 20 mmol/L HEPES buffer, pH 7.4 (Life Technologies, Inc.), 45 mmol/L $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, and 150 μg/ml gentamicin sulfate. Full-depth cartilage slices from a single site, $\sim 20 \times 20$ mm^2, were cut vertical to the articular surface and then into cubes of $\sim 2 \times 2$ mm^2. All of the articular cartilage available from each patient, regardless of the degree of degeneration (Mankin grades 4 to 12) as reported previously,[46] was used. Five to seven cubes were randomly obtained and wet weights of $\sim$60 mg were distributed in each culture well (48-well Costar 3548 plate; Corning Inc., Corning, NY). Samples were maintained before culture for 48 hours at 37°C in 1 ml per well of medium A in 95% air and 5% CO_2.

Cartilage Explant Culture

Media were changed after 48 hours (day 0) and thereafter were replaced every 4 days. The growth factor mixture (final concentrations) of 10 ng/ml TGF-β2 (R&D Systems, Minneapolis, MN), 10 ng/ml FGF-2 (R&D Systems), and 100 ng/ml insulin (Sigma Chemical Co., St. Louis, MO) was based on the concentrations used by Szuts and colleagues[42] in studies of chondrocyte hypertrophy. In some dose-response studies, TGF-β2 was used in the range 0.1 to 10 ng/ml. Final concentrations of growth factors were freshly added to medium A from day 0 at each medium change. The cartilage (triplicate cultures for each analytical point) was cultured for a total of 16 days and analyzed at day 16 for collagen cleavage and proteoglycan release. The conditioned media were collected every 4 days at each medium change from day 4 to day 16 and stored at −20°C until analyzed. For analyses of gene expression, cultures were maintained for up to 48 hours and analyzed as described below.

Enzyme-Linked Immunosorbent Assay of Collagenase-Cleaved Type II Collagen

The OA cartilage explants from day 16 of culture were digested and extracted with α-chymotrypsin to cleave and solubilize denatured collagen, including the carboxyterminal neo-epitope COL2–3/4C short (C1,2C) epitope generated by cleavage of type II collagen by collagenase. This was measured by enzyme-linked immunosorbent assay as described previously in α-chymotrypsin extracts and conditioned media.[1,2] Total cleavage neo-epitope in cartilages and media was calculated by summation of the data from each medium change and cartilage analysis. Results were expressed as pmol of epitope/mg wet weight of cartilage, based on a molecular weight of the standard peptide epitope of 608 d.

Determination of Proteoglycan Content and Release

This was determined in cartilage extracts and conditioned media as sulfated glycosaminoglycans (GAG), which is primarily a measure of proteoglycan aggrecan content, using a modification of the colorimetric 1,9-dimethylmethylene blue dye-binding assay.[47] Cumulative proteoglycan release (GAG in the media) and its content in cartilages (GAG in the cartilage) were expressed as μg GAG/mg wet weight of cartilage. GAG release into medium was represented as a percentage of the total cumulative GAG in the cartilage plus cumulative GAG released into medium. This provides an accurate measure of release accounting for the marked variation in cartilage GAG content in OA cartilage.[2,48]

Quantification of PGE_2 Release

PGE_2 concentrations were determined in the undiluted conditioned medium collected at each media change using a commercially available competitive enzyme-linked immunosorbent assay kit (Cayman Chemical Company, Ann-Arbor, MI) according to the manufacturer's instructions. Results were expressed in pg/mg of cartilage wet weight. PGE_2 standards provided with the kit were diluted with Dulbecco's modified Eagle's medium A.

Total RNA Isolation

Total RNA was isolated from OA cartilage explants after culturing for 6, 24, or 48 hours by a modification of the method described previously.[19] Fresh cartilage tissue in solution D (4 mol/L guanidine isothiocyanate, 25 mmol/L sodium citrate, pH 7.0, 0.5% laurylsarcosine, and 0.1 mol/L 2-mercaptoethanol) was immediately frozen in liquid nitrogen and kept at −80°C until all of the samples were collected. Samples were defrosted at room temperature and vortexed vigorously for 30 minutes. The debris was removed by centrifugation at 5000 × *g* for 10 minutes at 4°C. Proteins and nucleic acids in the supernatant were precipitated with 1 vol of isopropanol overnight at −20°C. The precipitate was removed at 10,000 × *g* for 20 minutes at 4°C and resuspended in digestion buffer (10 mmol/L Tris-HCl, 5 mmol/L ethylenediaminetetraacetic acid, 1% sodium dodecyl sulfate, pH 8.0, with 2 mg/ml of proteinase K (Life Technologies, Inc.) and incubated at 50°C until the pellet disappeared. After extraction with 1 vol of phenol, 0.2 vol of chloroform and 0.1 vol of 2 mol/L sodium acetate, pH 4.0, the aqueous phase was recovered by centrifugation (10,000 × *g* for 30 minutes at 4°C). An equal volume of 70% ethanol was added to each sample's aqueous phase and loaded onto an RNeasy spin column (Qiagen, Valencia, CA). Further RNA purification was performed using RNeasy kit (Qiagen) according to the manufacturer's instructions.

Reverse-Transcriptase (RT) and Polymerase Chain Reaction (PCR)

The RT reaction was performed using total RNA isolated from OA cartilage explants and SuperScript TMII H^- reverse transcriptase as recommended by Gibco BRL-Invitrogen, Ontario, Canada, and as described previously.[19] Genes analyzed and oligo sequences used for PCR are shown in Table 1. PCR was performed in a total volume of 25 μl as described previously.[19] PCR products were visualized by ethidium bromide staining. 18S RNA was used as reference for gel loading. The 18S RNA amplicon levels were weak because the PCR reaction was performed using equally diluted samples to ensure that band intensities were below saturation. Each analysis was performed at least three times at different dilutions of each sample of the original cDNA. The result of single dilution for all of the samples in a given set that most clearly showed differences in expression is presented in Figure 4. Care was taken to ensure that the number of cycles used was standardized for each comparative analysis and accurately represented the amount of PCR product in relationship to the cycle number.

Table 1. Oligosequences for PCR

Collagenase 3 (MMP-13)	D (1241–1259) GATAAAGACTATCCGAGAC	R (1369–1386) GAGTAACCGTATTGTTCG
Gelatinase B (MMP-9)	D (139–156) GCAGAGGAATACCTGTAC	R (361–377) CACAACATCACCTACTG
Parathyroid hormone-related peptide (PTHrP)	D (233–253) GAAATCAGAGCTACCTCGGAG	R (317–336) GATGAGGGCAGATACCTAAC
Procollagen type X(α1) (COL10A1)	D (1655–1680) CAGGAATGCCTGTGTCTGCTTTTAC	R (1957–1980) GACCAGGTGTGGCTCCAGCTTCCC
Interleukin 1β (IL-1β)	D (710–728) GGAAAAGCGATTTGTCTTC	R (844–863) GCCAGGATATAACTGACTTC
Tumor necrosis factor-α (TNF-α)	D (633–650) GTCTCCTACCAGACCAAG	R (816–837) GAGTCTGGGCAGGTCTACTTTG
Prostaglandin E synthase (PGES)-1	D (311–329) CTTCGTCTACTCCTTTCTG	R (577–595) GACTTGATGTTCCTTCCAG
18sRNA	D (29–52) CTACTTGGATAACTGTGGTAATTC	R (181–197) GACTCTAGATAACCTTCG

The isolated clones of each amplified cDNA fragment were sequenced to verify the identity of the cDNA products. Only RNA samples having no DNA contamination were used. The lack of chromosomal DNA contamination in RNA samples was confirmed by amplification in PCR aliquots of nonreverse-transcribed RNA as a template. To avoid potential variation in efficiency between experiments, all samples were simultaneously subjected to reverse transcription, and all samples of cDNA were simultaneously amplified in PCR.

Statistical Analysis

Quantitative data were expressed as the mean ± SD. Assays were run at least in triplicate. A normality test showed that data are distributed according to a Gaussian distribution curve. To analyze treatment effects, Mann-Whitney and paired *t*-test analyses were used. *P* values less than 0.05 were considered significant.

Results

Inhibition of Collagenase Activity by Growth Factors

Growth factors TGF-β2 (10 ng/ml), FGF-2 (10 ng/ml), and insulin (100 ng/ml) alone and in combination (GF), previously used to suppress hypertrophy in growth plate chondrocytes,[42] were capable of inhibiting type II collagen cleavage by collagenase in cultured OA articular cartilages (Figure 1a). Collagenase activity varied, as revealed by C1,2C values in control explants. Inhibition by growth factors also varied among specimens. As shown in five representative individual studies, the mean combined growth factor inhibition was 56.5%, and individual results varied from 45 to 70.3% inhibition.

TGF-β2 alone inhibited collagenase activity in all five individual studies (Figure 1a) and in 19 other explants (Figure 2). Analysis of the five individual cases revealed a mean inhibition of 59.2% (range, 54.6 to 62.4%), which was as effective as the growth factors. It was more potent than insulin (mean, 48.5%; range, 25.8 to 64.5%), which significantly inhibited collagen cleavage in four of five explant cultures. FGF-2 alone down-regulated collagen cleavage by only 26.5% (mean) (range, 0 to 61%) and showed significant inhibition in only two of five explants.

Although we used a TGF-β2 concentration of 10 ng/ml based on its previous usage for growth plate chondrocytes,[42] collagen degradation in some OA articular car-

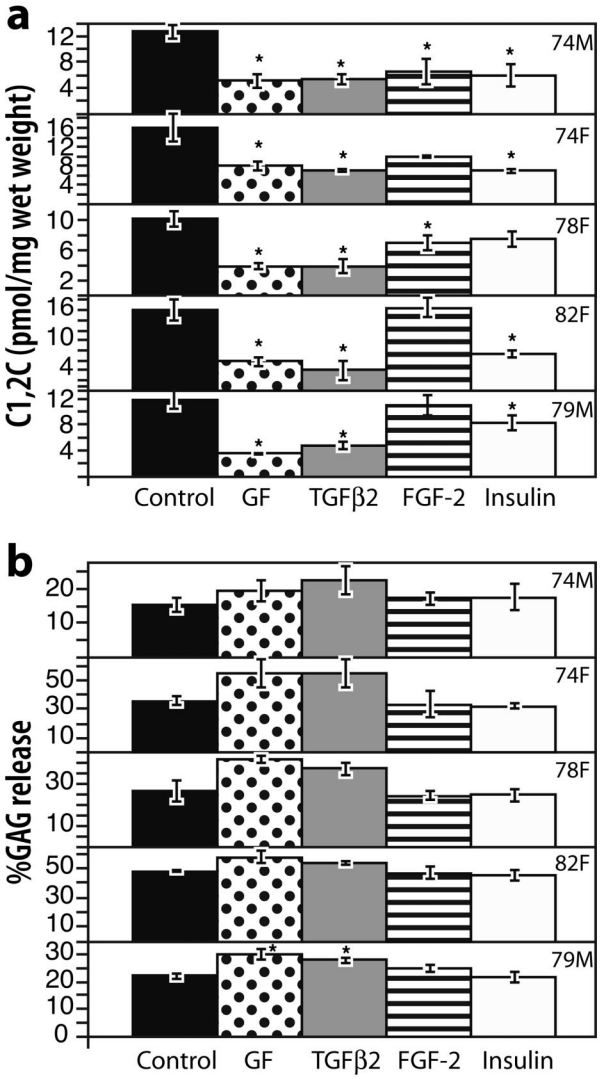

Figure 1. Inhibition of collagen cleavage by collagenase (**a**) and release of total proteoglycan (GAG release) in conditioned media (**b**) in human OA cartilage explants treated with combined growth factors (10 ng/ml TGF-β2, 10 ng/ml FGF-2, and 100 ng/ml insulin) and individually: 10 ng/ml TGF-β2, 10 ng/ml FGF-2, or 100 ng/ml insulin. The ages and sexes of patients are indicated. *P* value (paired *t*-test analysis) less than 0.05 is considered significant and is shown compared to control (*).

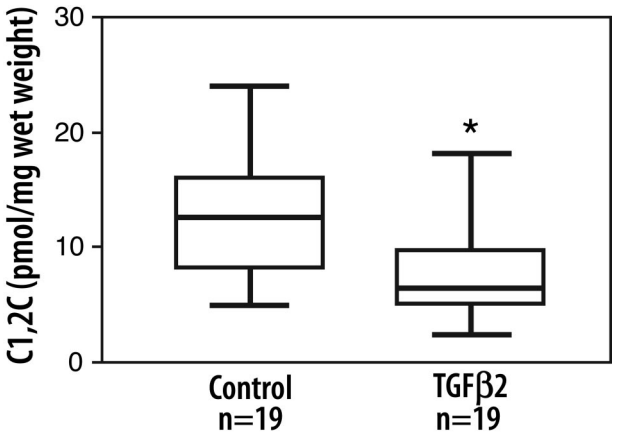

Figure 2. Inhibition of type II collagen cleavage by collagenase in human OA articular cartilage explants by 10 ng/ml TGF-β2 alone. *P* value (Mann-Whitney analysis) less than 0.05 is considered significant and is shown compared to control (*).

tilage samples was inhibited by as little as 0.1 or 1 ng/ml of TGF-β2 depending on the patient studied. Three representative examples of these dose-response studies are shown in Figure 3. However, as we did not routinely have sufficient cartilage from a single patient to examine this range of TGF-β2 concentrations, we ordinarily used the higher concentration (10 ng/ml) that was known to potently suppress normal chondrocyte hypertrophy.[42]

Proteoglycan Release Is Not Significantly Affected by Treatment with Growth Factors

Down-regulation of collagen cleavage by growth factors in combination or individually was never accompanied by inhibition in GAG release (Figure 1b). Treatment with the growth factor combination or TGF-β2 alone resulted in a significant increase in GAG release in one of five cases. There were no effects of the other growth factors on this GAG release.

Alterations of Gene Expression in OA Cartilage Explants by Growth Factors

Cartilage explants from six OA patients were examined in culture with or without TGF-β2 (10 ng/ml) for expression of genes characteristic of chondrocyte hypertrophy. Generation of C1,2C epitope was always significantly down-regulated in all cultures in the manner shown in Figure 2 (data not shown). The analysis of the effects of TGF-β2 at 10 ng/ml for these six patients was conducted throughout a 48-hour period, with changes in gene expression being recorded at 6, 24, and 48 hours. The individual results are presented in Figure 4 and summarized in Table 2 to permit a clear appreciation of this data. The summary was prepared by recording the total changes (down-regulation, up-regulation, no change, or no detectable expression) for either control or plus TGF-β2 at each time point and for each patient (total possible score is therefore 18 for the three time points and six patients).

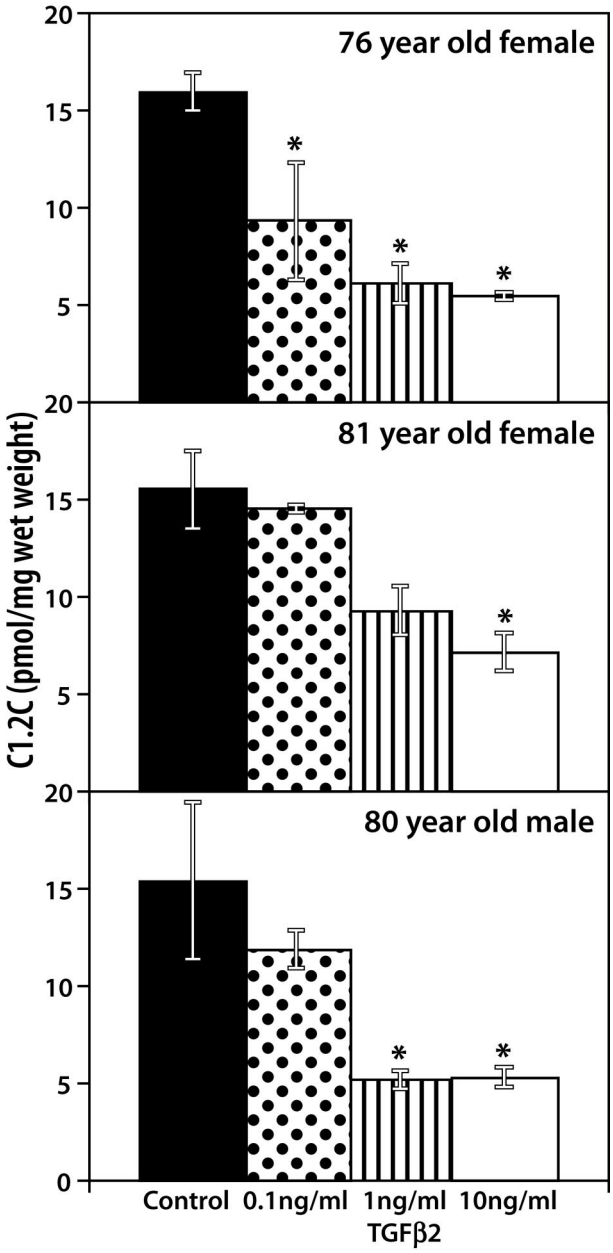

Figure 3. Concentration-dependent inhibition of collagen cleavage by TGF-β2 in human OA explants. The ages and sexes of patients are indicated. *P* value (paired *t*-test analysis) less than 0.05 is considered significant and is shown compared to control (*).

It can be seen that in a majority of cases gene expression was recorded. For all genes examined, with the exception of *PGE synthase-1* that was usually up-regulated (16 of 18), TGF-β2 down-regulated gene expression in a majority of cases (range, 10 to 18 of 18). Except for *PGES-1* only one other gene showed any evidence for a weak up-regulation. This was *PTHrP* (4 of 18), which has the capacity to suppress chondrocyte hypertrophy.[49–51] In a minority of cases (range, 0 to 3 of 18 for each gene) no changes were observed. Thus, TGF-β2 suppresses the expression of the cytokines *IL-1*β (15 of 16 detected) and *TNF-*α (11 of 12 detected) and suppresses expression of differentiation markers, namely

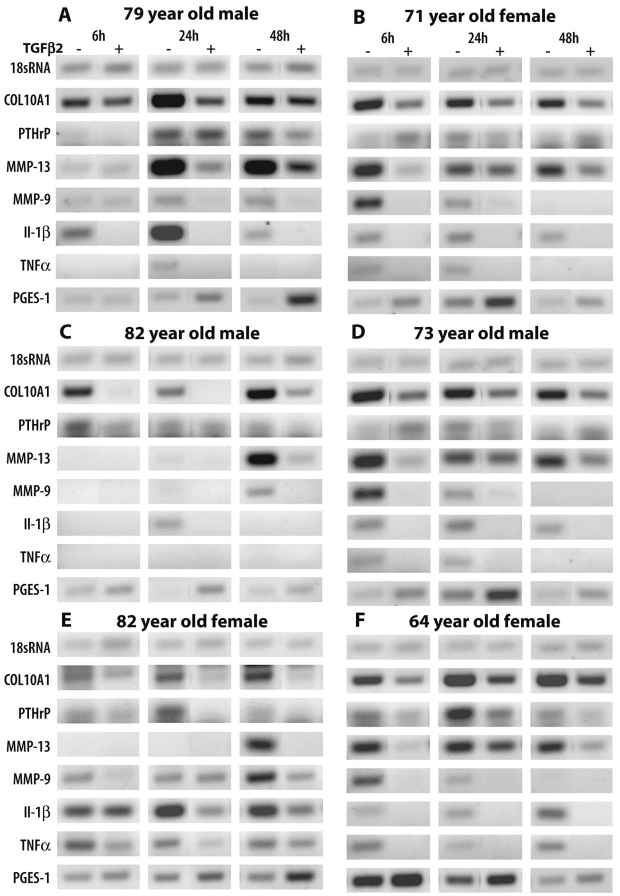

Figure 4. Gene expression, measured by RT-PCR in human OA articular cartilage of a 79-year-old male (**A**), an 82-year-old male (**C**), an 82-year-old female (**E**), a 71-year-old female (**B**), a 73-year-old male (**D**), and a 64-year-old female (**F**) cultured with (+) and without (−)10 ng/ml TGF-β2. 18sRNA is used as a gel loading reference.

COL10A1 (18 of 18 detected), *MMP-13* (10 of 12 detected), and *MMP-9* (11 of 13 detected). *PGE synthase-1* was increased in 16 of 18 cases.

Up-Regulation of PGE_2 Production on Treatment with TGF-β2

Up-regulation of *PGES-1* expression by TGF-β2 in the same cartilages analyzed for gene expression was accompanied by the up-regulation of PGE_2 release into the conditioned medium (Figure 5). The treatment with the growth factor resulted in a significant increase in PGE_2 in four of six explants tested. The results for PGE_2 assay are not dissimilar to the results obtained for gene expression in which the latter was strongest for the 64-year-old female and weakest for the 82-year-old male, the same upper and lower limits being seen on immunoassay.

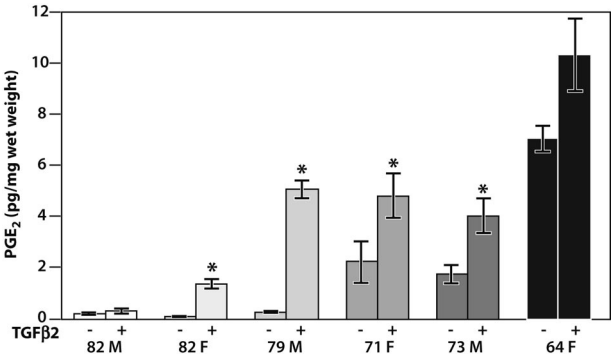

Figure 5. PGE_2 release induced by TGF-β2 in OA articular cartilage explants. The ages and sexes of patients are indicated. *P* value (paired *t*-test analysis) less than 0.05 is considered significant and is shown compared to control (*).

Discussion

Inhibition of articular cartilage degeneration is a priority in the effective treatment of OA because there are no disease-modifying therapies available. As excessive damage to type II collagen is considered an irreversible step of cartilage degradation in OA, in contrast to proteoglycan loss,[4] down-regulation of collagen cleavage is viewed as an important endpoint in the inhibition of cartilage degeneration. The similarity between type II collagen degradation in OA to that in the hypertrophic zone of the primary growth plate include up-regulation of cleavage of type II collagen by collagenases and the expression of differentiation-related genes associated with chondrocyte hypertrophy.[1,3,11,12] Therefore, it was very interesting to find that down-regulation of type II collagen cleavage in human cultured OA explants can be induced by the same growth factors that can suppress hypertrophy of growth plate chondrocytes in culture.[37,42] Of these three growth factors, TGF-β2 was the most potent in suppressing the cleavage of type II collagen. It also suppressed the expression of chondrocyte hypertrophy-associated genes such as *COL10A1*, *MMP-9*, and

Table 2. Summary of Gene Expression in OA Articular Cartilage Explants

	Gene expression response in patients treated with TGF-β*			
	Down-regulation	Up-regulation	No change	No expression
COL10A1	18	0	0	0
PTHrP	10	4	3	1
MMP-13	10	0	2	6
MMP-9	11	0	2	5
IL-1β	15	0	1	2
TNFα	11	0	0	7
PGES-1	0	16	2	0

*The data represents the results of TGF-β2 treatments shown in Figure 4 of all OA explants for all time points and for all six patients. A maximum score is 3 for each gene and for each patient. Thus the maximum score for six patients is 18.

PTHrP, as well as *MMP-13*, a collagenase believed to be mainly involved in matrix degradation in OA cartilage.[1,2] In the case of *PTHrP*, a weak up-regulation was observed in four cases. This would favor suppression of any progression in hypertrophy, besides being an early differentiation marker.[49–51] Therefore, TGF-β2 inhibition of collagen cleavage involves suppression of full chondrocyte differentiation.

The importance of molecules of the TGF-β superfamily in the maintenance of a healthy articular cartilage has been suggested by genetic manipulations in mice that produce either overexpression of a functionless type II receptor[52] or the deletion of a SMAD signaling component[53] used by ligands of the TGF-β family. In both cases these genetic alterations lead to the degeneration and loss of articular cartilage in aging mice resembling OA. This was accompanied by the extensive development of chondrocyte hypertrophy and the formation of large osteophytes. Moreover, TGF-β2-null mice exhibit multiple skeletal abnormalities that result in size reductions of normal tissues, reflecting abnormalities in the process of endochondral bone formation.[54] These mice differ from TGF-β1-null mice, which have an autoimmune-like inflammatory disease.[55]

Although it is difficult to predict whether the results from *in vitro* studies directly reflect the *in vivo* situation, the available evidence suggests that the presence of TGF-β2 in articular cartilage favors the formation and maintenance of articular cartilage *in vivo*. TGF-β2 is the most widely and highly expressed isoform in all zones of the developing human bone,[56] and its expression is reduced in adult rat articular cartilage when degenerative changes occur.[57] Because OA affects mostly the aging population, a deficiency of TGF-β2 would favor degeneration. This is supported by the observation that the expression of *TGF-β2* is significantly decreased in advanced stages of human or experimental OA articular cartilage.[58–60] In osteoarthritic cartilage there may be deficiency in TGF-β2 activation resulting from the lack of its activator plasmin, the activity of which may be limited by high concentrations of plasminogen activator inhibitor (PAI-1) seen in OA patients.[61,62]

There is no evidence from our studies that TGF-β2 acts catabolically in OA articular cartilage. However it may be destructive for normal articular cartilage *in vivo* because intra-articular injections of TGF-β2 in high concentration produce catabolic effects in rabbit joints resulting in joint swelling, fibroblastic proliferation of synovial membrane, and profound loss of articular cartilage proteoglycan.[63]

The observed reduction in *MMP-13* expression and the reduced collagen cleavage produced by TGF-β2 is also in agreement with observations that TGF-β family members can individually down-regulate expression of the collagenase *MMP-13*.[28,64,65] In contrast others have shown that TGF-β1 and -2 can induce *collagenase* expression.[66–68] We never observed any evidence for an increase in type II collagen cleavage by TGF-β2. In fact the opposite was seen. The fact that proteoglycan degradation was unaffected suggests that collagen and aggrecan degradation in OA cartilage are differently regulated. When chondrocytes become hypertrophic in the growth plate, collagen cleavage and loss is not accompanied by a net loss of proteoglycan.[21]

Down-regulation of *MMP-9* by TGF-β2 may be important for the control of degradation of articular cartilage collagen after its primary cleavage by collagenase.[3] In OA cartilage increased *MMP-9* expression[69] and protein staining[70] has also been detected. MMP-9 may also be directly involved in chondrocyte differentiation because it is expressed in the hypertrophic zone of the bovine[19] and rabbit[71] growth plates and has been found in human[72,73] and chick[74] fetal hypertrophic chondrocytes.

Although TGF-β2 alone was effective in controlling collagen degradation and the other growth factors did not interfere with this property, a combination of growth factors such as we studied here could be more beneficial *in vivo* when it is desirable to combine an inhibition of excessive collagen cleavage with a stimulation of extracellular matrix repair. Thus, in responsive individuals insulin may facilitate tissue repair because it is a principal anabolic agent in the articular cartilage.[75] FGF-2 can also promote cartilage repair[76–79] by itself as well as induce local TGF-β or its own expression.[80] In addition combinations of these and other growth factors produce synergistic effects in maintaining synthesis of matrix molecules in articular and growth plate chondrocytes.[37,81,82] In contrast TGF-β2 alone may not be capable of restoring the anabolic functions of healthy articular cartilage since it has been reported to down-regulate type II collagen and aggrecan synthesis,[37,63] although these different effects of TGF-β2 may be very dose-dependent. The potency of TGF-β2 in the down-regulation of collagen cleavage may be associated with the increased responsiveness of OA chondrocytes to this growth factor.[83] In contrast, responsiveness to insulin-like growth factor 1 and FGF-2 decreases with age, joint inflammation, and OA.[75,84,85]

In our *in vitro* study, TGF-β2 was shown to suppress expression of the genes for *IL-1β* and *TNF-α*, which, like the other genes we studied, are not usually expressed in normal articular cartilage.[20,27,86] The down-regulation of cytokine *IL-1β* and *TNF-α* expression is noteworthy because these cytokines are considered to be important chondrocyte catabolic factors in OA,[9] and inhibition of these cytokines also suppresses collagenase activity and proteoglycan degradation in OA cartilage.[5] The ability of TGF-β1 to counteract the catabolic effects of paracrine IL-1β in rodent articular chondrocytes has been also shown.[28,87,88]

PGE_2 can produce both catabolic and anabolic effects in articular chondrocytes.[89] The up-regulation of *prostaglandin E synthase-1*, as well as PGE_2 release by TGF-β2 observed here, confirms earlier results that demonstrated PGE_2 release into the normal rabbit knee joint after intra-articular injections of the growth factor.[63] In this case it resulted in joint swelling and cartilage proteoglycan loss. In our explants TGF-β2 treatment did not produce evidence of proteoglycan release in the majority of tested cartilages. This may result from PGE_2 signaling through the EP2 receptor subtype, which has been shown to mediate anabolic responses of PGE_2 in human articular chondrocytes promoting growth, protecting against apo-

ptosis, and counteracting IL-1-induced production of IL-6.[89,90] Inhibition of chondrocyte terminal differentiation by PGE_2 has also been observed in animal studies.[91,92] Our results clearly demonstrate that increased expression of *PGE synthase-1* and PGE_2 production occur in association with the suppression of collagen cleavage. The significance of this observation awaits further study.

In conclusion, our results suggest that the excessive cleavage of type II collagen by collagenase observed in cultured OA articular cartilage can be suppressed by TGF-β2 and that this involves cellular events associated with suppression of chondrocyte hypertrophy and arrest of *IL-1*β and *TNF-*α expression, accompanied by increased PGE_2 production. Because TGF-β2 may play a role that favors the maintenance of a prehypertrophic phenotype, therapy aimed at controlling the activity of cytokines and chondrocyte differentiation may be of value in the management of cartilage degeneration in patients with OA.

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