Characterization of an *Ex vivo* Femoral Head Model Assessed by Markers of Bone and Cartilage Turnover

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Abstract

Objective: The pathophysiology of osteoarthritis involves the whole joint and is characterized by cartilage degradation and altered subchondral bone turnover. At present, there is a need for biological models that allow investigation of the interactions between the key cellular players in bone/cartilage: osteoblasts, osteoclasts, and chondrocytes. *Methods:* Femoral heads from 3-, 6-, 9-, and 12-week-old female mice were isolated and cultured for 10 days in serum-free media in the absence or presence of IGF-I (100 nM) (anabolic stimulation) or OSM (10 ng/mL) + TNF-α (20 ng/mL) (catabolic stimulation). Histology on femoral heads before and after culture was performed, and the growth plate size was examined to evaluate the effects on cell metabolism. The conditioned medium was examined for biochemical markers of bone and cartilage degradation/ formation. *Results:* Each age group represented a unique system regarding the interest of bone or cartilage metabolism. Stimulation over 10 days with OSM + TNF-α resulted in depletion of proteoglycans from the cartilage surface in all ages. Furthermore, OSM + TNF-α decreased growth plate size, whereas IGF-I increased the size. Measurements from the conditioned media showed that OSM + TNF-α increased the number of osteoclasts by approximately 80% and induced bone and cartilage degradation by approximately 1200% and approximately 2600%, respectively. Stimulation with IGF-I decreased the osteoclast number and increased cartilage formation by approximately 30%. *Conclusion:* Biochemical markers and histology together showed that the catabolic stimulation induced degradation and the anabolic stimulation induced formation in the femoral heads. We propose that we have established an explant whole-tissue model for investigating cell-cell interactions, reflecting parts of the processes in the pathogenesis of joint degenerative diseases.

Keywords

osteoarthritis, growth plate, ex vivo, femoral head, cytokines, IGF-I

Introduction

Osteoarthritis (OA) is a degenerative joint disease leading to cartilage degradation and subchondral bone changes. Many factors contribute to the onset of OA, including both metabolic and biomechanical mechanisms, but it is still debated in which tissue the disease initiates.[1] In 1986, Dr. Radin and Dr. Rose[2] were the first to suggest that the subchondral bone plays an important role in the initiation and progression of OA. Physical characterizations of OA include cartilage degradation, subchondral bone sclerosis and thickening, and osteophyte formation.[3-5] In addition, trabecular bone in the subchondral region is thinned, and its elasticity is lost.[6-8]

An increasing line of evidence suggests that there is a strong interrelationship between subchondral bone and articular cartilage. Subchondral bone is separated from the articular cartilage by a thin layer of calcified cartilage.[9]

How much do bone and its interaction with cartilage contribute to the causality of OA? It has been suggested that bone turnover increases in patients with OA.[3,4] In a canine model, subchondral bone loss was seen in the early OA and bone sclerosis in the late OA.[10,11] A murine OA model, an anterior cruciate ligament transaction (ACLT) model, showed that bisphosphonates, approved as antiresorptive

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therapy for osteoporosis, effectively retarded the progression of cartilage degeneration and osteophyte formation, indicating the importance of bone remodeling in the pathogenesis of OA.[12] This and other studies suggest the existence of important interactions between bone and cartilage, which might be key events in OA pathogenesis.[13-17] However, human cartilage explants cultured in the presence of isolated osteoblasts from OA patients have shown increased proteoglycan degradation compared to cartilage cultured with osteoblasts from healthy patients.[13] Another group suggested that osteoblasts from sclerotic subchondral bone could initiate chondrocyte hypertrophy.[14] The communication between osteoblasts and osteoclasts via PTH/RANKL in normal bone turnover, where PTH stimulates osteoblasts to release RANKL that in turn stimulates bone resorption via osteoclasts, is well acknowledged.[16] However, this communication via PTH/RANKL is altered in joint tissues from OA patients.[17] This lack of correlation suggests that the communication between the cells is very important for maintaining a healthy joint, and more attention should be directed toward investigating this communication.

Bone and cartilage degradation can be measured by biochemical markers, such as fragments of C-terminal telopeptide of type I collagen (CTX-I), reflecting bone resorption,[18] and fragments from C-terminal telopeptide of type II collagen (CTX-II), reflecting cartilage degradation.[19,20] The turnover of aggrecan, the predominating proteoglycan in cartilage, can be measured by the release of sulfated glycosaminoglycans (sGAGs) from the extracellular matrix (ECM). The formation of cartilage can be measured by the biomarker, PIINP, which is the collagen type II propeptide at the N-terminal.[21] Biochemical markers for measuring biological processes, especially in OA, are valuable descriptive tools.

A model that not only identifies the different changes in cartilage degradation but simultaneously changes adjacent bone may be useful for investigating interactions between these 2 tissues. An *in vitro* model like this would allow us to investigate and evaluate potential effects of a given compound before *in vivo* experiments. Thus, such a model may be an important tool for finding potential joint disease–modifying drugs with "dualaction" targeting both bone and cartilage. Femoral heads from mice are small, enclosed compartments, which comprise both bone and cartilage. Thus, the interaction between osteoblasts, osteoclasts, and chondrocytes remains intact in the closed system when isolated from mice. The femoral heads are an open opportunity for investigating the signaling and communication between cells of bone and cartilage in near-normal conditions and under stimulation from a compound of interest.

In this study, we developed and characterized a murine femoral head *ex vivo* model in which we were able to induce a catabolic or anabolic response. As controls, we chose oncostatin M (OSM) + tumor necrosis factor (TNF)-α and insulin-like growth factor (IGF)-I, which we have previously established in other *ex vivo* models as catabolic and anabolic controls, respectively.[21,22] The idea for this model has emerged from a previously reported murine femoral head model, where only the cartilage compartment was cultured as part of investigating the enzymatic proteoglycan degradation.[23]

Methods

The Murine Femoral Head Explant Model

Femoral heads from 3-, 6-, 9-, and 12-week-old NMRI (inbred) female mice (Charles River, Sulzfeld, Germany) were isolated by cutting at the femoral neck. The femoral heads were cultured in the media DMEM:F12 (Invitrogen, Taastrup, Denmark) + penicillin and streptomycin (P/S) (Lonza, Vallensbaek Strand, Denmark) + 10% serum (Invitrogen) for 2 hours to adjust to the isolation. The femoral heads were washed in serum-free DMEM:F12 + P/S 5 times and randomly placed in doublets in each well in a 24-well plate. Some femoral heads were saved directly in 4% formaldehyde (Merck, Hellerup, Denmark) to be able to evaluate the femoral heads at the point before culturing (T = 0). The rest of the femoral heads were cultured for 10 days in DMEM:F12 + P/S in the absence (nonstimulated control named W/O) or presence of the anabolic factor, IGF-I (100 nM) (Sigma-Aldrich, Copenhagen, Denmark), or catabolic cytokines, OSM (10 ng/mL) (Sigma-Aldrich) + TNF-α (20 ng/mL) (R&D Systems, Abingdon, UK). Each treatment was repeated 5 times. The conditioned media were changed and saved in –20 °C at days 4, 7, and 10, except for experiments with 12-week-old mice, which only were saved at day 10 (the conditioned media at day 10 comprises the total release from the whole culture period). Overall cell viability was monitored using the dye Alamar Blue (Invitrogen) at day 10. The femoral heads were washed 3 times in PBS (Lonza) and saved in 4% formaldehyde. All culturing of femoral heads was at 37°C and 5% CO_2. For experiments with 3-, 6-, and 9-week-old mice, the conditioned media from days 4, 7, and 10 were assessed by biochemical markers of bone and cartilage. All 3 days were accumulated into one graph, to represent the total release of the respective biochemical marker from the femoral heads, during the 10-day culture period. For the experiments with 12-week-old mice, the total releases were measured at day 10. The microscopic changes were assessed by histology.

Biochemical Markers

The biochemical marker CTX-I was used to detect type I collagen degradation fragments in the conditioned medium. The competitive ELISA used, RatLaps (IDS Ltd., Herlev, Denmark), quantified cathepsin K–mediated degradation

products of type I collagen. The primary antibody was directed toward the neoepitope [1196]EKSQDGGR.[24] Serum Pre-clinical CartiLaps (IDS Ltd.) was used to quantify MMP-mediated degradation products from type II collagen. The primary antibody was directed toward the neoepitope [1230]EKGPDP.[24] PIINP (IDS Ltd.) was a biochemical marker for cartilage formation. The primary antibody was directed against an internal epitope of the N-terminal propeptides of collagen type II: [122]GPQGPPAGEQGPRGDR[136].[25] All 3 ELISAs were preformed accordingly to the manufacturer's protocols.

Measurement of Collagen Turnover

The amino acid hydroxyproline was used as a measurement of total collagen release since it is a major component of collagen. The 20 uL sample or standard (prepared from 1-hydroxyproline diluted in 1 mM HCl) was diluted 5 times in 7.5 M HCl and hydrolyzed overnight (20 hours) at 110 °C. Samples were centrifuged for 2 minutes at 3000 g/min and then evaporated at 50 °C until samples were completely dry. Isopropanol/H_2O (2:1) of 25 uL (Merck) was used to dissolve samples, and 10 uL of dissolved sample was transferred to a new plate, to which 20 uL isopropanol was added. This was oxidized by the addition of 10 uL of solution I (1 part of 24 M chloramines [Sigma-Aldrich] to 4 parts of 1 M Na-acetate [Sigma-Aldrich], 0.33 M Na_3 citrate [Merck] and 0.07 M citric acid [Merck] dissolved in isopropanol) incubated for 4 ± 1 minutes. There was 130 uL of solution II (3 parts of 4.5 M 4-dimethylamino-benzaldehyde [Sigma-Aldrich] dissolved in 60% perchloric acid [Merck] to 13 parts of isopropanol) added and incubated at 60 °C for 25 ± 5 minutes. The absorbance was measured at 558 nm on an ELISA reader.

Quantification of Osteoclast Number

TRAP was a quantitative measurement of osteoclast number. The reaction buffer (1.0 M sodium-acetate, 0.5% Triton X-100, 1 M NaCl, 10 mM EDTA [pH 5]) (Merck), 50 mM ascorbic acid (Sigma-Aldrich), 0.2 M disodium tartrate (Merck), 82 mM 4-nitrophenylphosphate (Sigma-Aldrich), and milli = Q were mixed 2:1:1:1:3 into a final TRAP solution buffer. Sample and final TRAP solution buffer was mixed (1:4) and incubated for 1 hour at 37 °C. There was 0.3 M NaOH (Merck) added to stop the color reaction, and the absorbance was measured at 405 nm with 650 nm as reference.

Quantification of Proteoglycans by Measurement of Sulfated Glycoaminoglycans

Proteoglycans were used to quantify cartilage turnover by measuring sulfated glycosaminoglycans. An assay using the dye 1.9-dimethylmehylene blue chloride (Sigma-Aldrich)

in a solution of sodium formate (Sigma-Aldrich) and formic acid (Merck) was used. A standard curve was prepared with chondroitin sulfate (Sigma-Aldrich) ranging from 0 to 100 ng/mL. The 40 uL standard or sample was transferred to a microtiter plate, followed by 250 uL 38.5 uM 1.9-dimethylmethylene blue chloride. The absorbance was immediately read at 650 nm on an ELISA reader to avoid precipitation.

Histological Analysis

Tissue preparation. After the culture period, femoral heads were decalcified in 15% EDTA at room temperature for approximately 1 week. Femoral heads were embedded separately in paraffin blocks and cut into 5-μm-thick sections. Excessive paraffin was removed by placing sections at 60 °C for 1 hour and dried at 37 °C overnight before staining.

Safranin O and fast green staining. To visualize bone and cartilage, safranin O (Sigma-Aldrich) stained proteoglycans red, and fast green (Sigma-Aldrich) stained collagens green. Cartilage was stained red due to the excess of proteoglycans compared to collagens. Sections (5 μm) were stained according to Bay-Jensen *et al.*[26] Digital histographs were taken using an Olympus BX60F-3 microscope and an Olympus DP71 camera (Tokyo, Japan).

Annotations of the femoral head to measure size of the growth plate. Digital histographs of femoral heads from 12-week-old mice were evaluated by standardized annotations, ensuring anatomical correspondence. A line connects the black dots where the cartilage starts on the femoral neck. The center of this line is perpendicularly connected to the cartilage surface, and 2 lines are placed at an angle of 30° from this perpendicular line. The lengths of the section of the 3 lines where they cross the growth plate are calculated to find the average length of the growth plate. The growth plate was evaluated in 4 different femoral heads (from 4 different experiments) from each treatment.

Statistical Analysis

Results are shown as mean ± standard error of the mean (SEM). Differences between mean values were compared by the Student *t* test for unpaired observations, assuming normal distribution where 5 replicates were used. Differences were considered statistically significant if $P < 0.05$.

Results

Femoral Heads Exhibit Different Bone and Cartilage Morphology during Development

Each individual age has a unique bone and cartilage morphology. In 3-week-old mice, the cartilage compartment is dominating (red stain) compared to the bone compartment
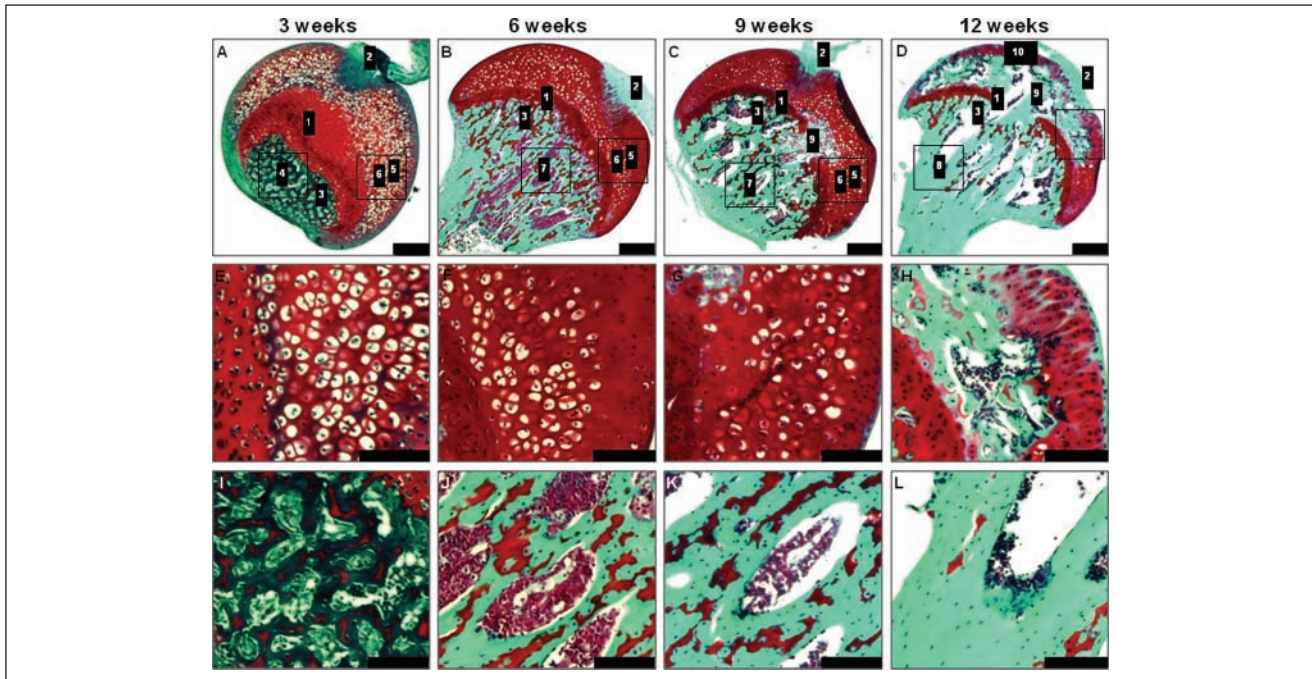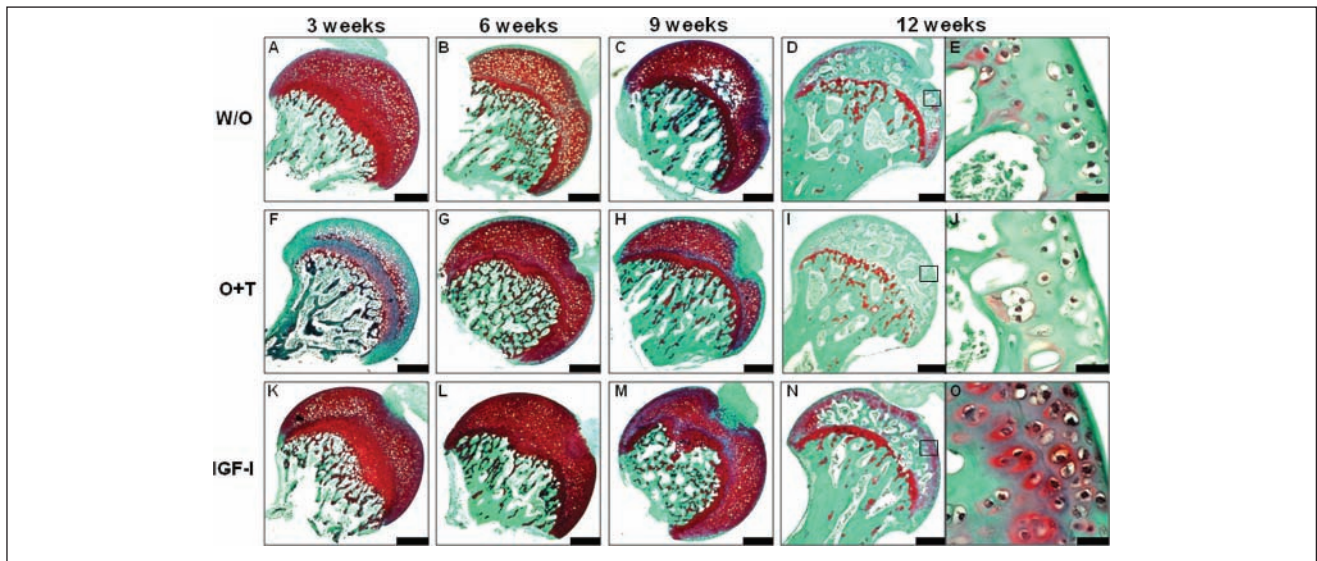
Figure 1. Characterization of the development in murine femoral heads. Sections from femoral heads from 3-, 6-, 9-, and 12-week-old mice were stained with safranin O and fast green, which color the proteoglycans red and the collagens green. The black bars represent 250 µM (**A-D**) and 100 µM (**E-L**). The numbers represent growth plate (1), ligamentum teres femoris (2), endochondral ossification (3), immature bone embedded with calcified cartilage (4), prehypertrophic and hypertrophic chondrocytes (5), calcified cartilage (6), woven bone (unmineralized) (7), lamellar bone (mineralized) (8), secondary ossification center (9), and articular cartilage (10). The black squares in the whole femoral heads (**A-D**) represent the magnification of either the cartilage compartment (**E-H**) or the bone compartment (**I-L**), which is divided in columns after age.

(green stain); however, the cartilage is not fully materialized (**Fig. 1A**). The cartilage-bone ratio changes over time due to the development of the secondary ossification center at the age of 9 weeks (**Fig. 1C**). The growth plate is visible as a thick line of dense red staining separating bone from cartilage in the 3-week-old mouse, which decreases concurrently with increasing age (**Fig. 1A-D**). In femoral heads of 3-week-old mice, the cartilage predominantly consists of proliferating, prehypertrophic, and hypertrophic chondrocytes (**Fig. 1E**), whereas the 6- and 9-week-old mice have less proliferating chondrocytes, which are located in the reduced growth plate (**Fig. 1F and G**). Additionally, the extracellular matrix of the cartilage compartment in 3- to 9-week-old mice is calcified (**Fig. 1E-G**), preparing itself for the secondary ossification center, whereas the cartilage compartment is mainly articular cartilage (noncalcified) at 12 weeks of age (**Fig. 1H**). The bone compartment in the 3-week-old mice consists of immature bone embedded with calcified cartilage (**Fig. 1I**). As the mice get older, the calcified cartilage is replaced by unmineralized bone (normal endochondral ossification process), resulting in woven bone with a high proportion of osteocytes (**Fig. 1J and K**). The mechanically weak woven bone is replaced by more

resilient lamellar bone (mechanically strong) with a low proportion of osteocytes when the mice reach the age of 12 weeks (**Fig. 1L**). Overall, these results suggest that the different ages represent different developmental stages, as expected, especially with regards to ratio and quality of bone and cartilage, and thus, these can individually be used in our *ex vivo* model for specific purposes.

Anabolic and Catabolic Stimulations Induce Morphological Changes

The viability of the cells of the cultured femoral heads is not impaired by the 10 days of anabolic or catabolic stimulation (data not shown). Femoral heads stimulated with OSM + TNF-α show a major loss of proteoglycans from the cartilage compartment at the age of 3 weeks (**Fig. 2F**) compared to the nonstimulated control (W/O) (**Fig. 2A**). In 9-week-old femoral heads, OSM + TNF-α stimulation increases the loss of proteoglycans from the cartilage surfaces (**Fig. 2H**) compared to the nonstimulated controls (W/O) (**Fig. 2C**). However, in femoral heads from 6-week-old mice, both nonstimulated (**Fig. 2B**) and OSM + TNF-α–stimulated (**Fig. 2G**) femoral heads have lost proteoglycans
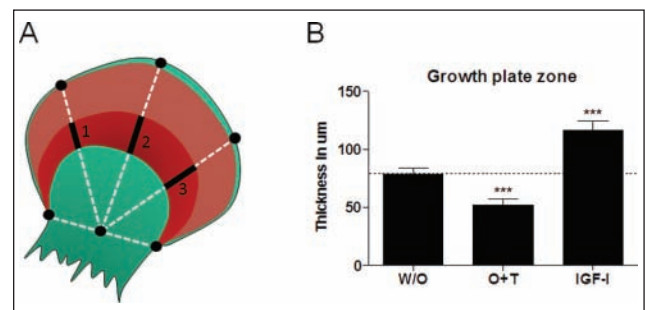
Figure 2. Microscopic changes after catabolic and anabolic stimulation. Sections from femoral heads from 3-, 6-, 9-, and 12-week-old mice were stained with safranin O (proteoglycans) and fast green (collagens) after culture for 10 days in the absence (W/O) or presence of IGF-I (anabolic) and OSM + TNF-α (catabolic) stimulation. The black bars represent 250 μM (**A-D, F-I, K-N**) and 20 μM (**E, J, O**). The black squares in the 12-week-old femoral heads (**D, I, N**) represent the magnification of the cartilage surface (**E, J, O**), which is divided in rows after treatment.

from the cartilage surface. In 12-week-old femoral heads, the proteoglycans seem to be almost depleted from the cartilage compartment in the nonstimulated (**Fig. 2D**) and the OSM + TNF-α–stimulated (**Fig. 2I**) explants. Anabolic stimulation with IGF-I protects against the proteoglycan loss seen in W/O (**Fig. 2A-D and K-N**). Additionally, in 12-week-old mice, anabolic stimulation shows proteoglycan staining around the chondrocytes (**Fig. 2O**) compared to the W/O (**Fig. 2E**). Next, we measured the size of the growth plate to investigate whether morphological changes could be induced (**Fig. 3A**). The size of the growth plate in IGF-I–stimulated 12-week-old femoral heads is increased by approximately 48% ($P < 0.001$) compared to W/O, whereas OSM + TNF-α stimulation decreases the growth plate size by approximately 34% ($P < 0.001$) compared to W/O (**Fig. 3B**). This indicates that we are able to induce alteration to the femoral heads *ex vivo* with catabolic (degenerative) or anabolic (generative) factors.

Catabolic Stimulation Increases Collagen and Proteoglycan Turnover

Levels of hydroxyproline, an indicator of total collagen turnover, in the conditioned medium indicate that stimulation by OSM + TNF-α increases the total release of collagens by approximately 49% ($P < 0.05$) to approximately 190% ($P < 0.001$) in femoral heads aged 6, 9, and 12 weeks, but not in femoral heads from 3-week-old mice (**Fig. 4A**). The longitudinal effects from the different stimulations are shown in **Table 1**. OSM + TNF-α increases the release of collagens

Figure 3. Quantification of growth plate zone in femoral heads. Sections from femoral heads from 12-week-old mice were stained with safranin O (proteoglycans) and fast green (collagens) after culture for 10 days in the absence (W/O) or presence of IGF-I (anabolic) and OSM + TNF-α (catabolic) stimulation. The growth plate size was measured in one femoral head per treatment from 4 different experiments using the annotation system (see Methods). The growth plate thickness (μm) is calculated by the average of the black lines in the growth plate (**A**). The catabolic stimulation decreased the growth plate size, whereas the anabolic stimulation increased the size of the growth plate (**B**). ***$P < 0.001$.

over time in 6- and 9-week-old mice. In femoral heads from 12-week-old mice, the anabolic stimulation also increases total collagen release by approximately 49% ($P < 0.01$) compared to W/O (**Fig. 4A**). OSM + TNF-α stimulation increases the total release of sulfated glycosaminoglycans (sGAG) to the conditioned media by approximately 22% ($P < 0.05$) to approximately 40% ($P < 0.01$), except from the experiment with 6-week-old mice (**Fig. 4B**); however, similar trends were observed.
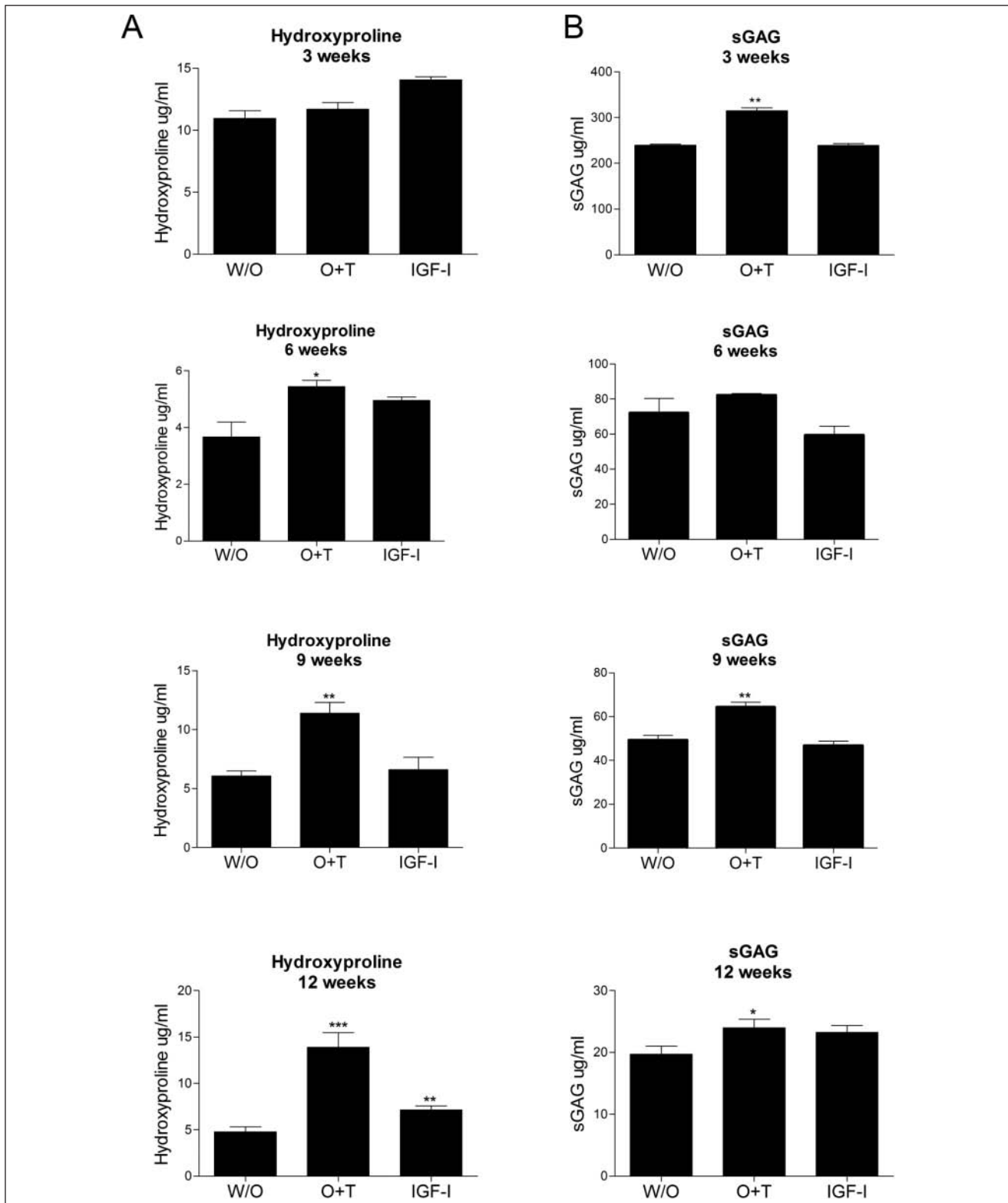
Figure 4. The catabolic control induces a release of collagens and proteoglycans. The experiments were assessed by the collagen degradation marker, hydroxyproline (**A**), and the proteoglycan turnover marker, sGAG (**B**). Each graph represents the accumulated measurements from days 4, 7, and 10 except from 12-week-old mice (only day 10). Cytokine stimulation increases the collagen release in experiments with 6-, 9-, and 12-week-old mice (**A**). Cytokine stimulation also increases proteoglycan release in experiments with 3-, 9-, and 12-week-old mice (**B**). Asterisks indicate statistical significance: *P < 0.05; **P < 0.01; ***P < 0.001.

Table 1. Biochemical Markers Measured from the Conditioned Medium at Days 4, 7, and 10 in the Different Age Groups

Age of Mice, wk	Culture Day	Stimulated with	HP, µg/mL	sGAG, µg/mL	TRAP	CTX-I, ng/mL	PIINP, ng/mL	CTX-II, pg/mL
	4	W/O	5.22 ± 0.81	113.12 ± 3.46	0.47 ± 0.06	2.97 ± 0.82	93.61 ± 4.65	48.69 ± 3.73
		O + T	6.21 ± 1.22	143.84 ± 8.45**	0.81 ± 0.12*	3.23 ± 0.27	54.92 ± 9.58**	74.96 ± 19.19
		IGF	5.16 ± 0.59	92.61 ± 10.11*	0.63 ± 0.06	3.79 ± 0.84	100 ± 0.00	26.10 ± 3.87**
3	7	W/O	2.59 ± 0.46	49.51 ± 8.51	0.27 ± 0.02	4.61 ± 0.54	68.78 ± 5.55	6.30 ± 1.40
		O + T	2.41 ± 0.35	87.18 ± 5.04**	0.33 ± 0.01*	11.72 ± 3.54	40.83 ± 3.02**	17.44 ± 3.83*
		IGF	4.38 ± 0.46*	58.96 ± 4.34	0.26 ± 0.01	4.49 ± 0.39	74.38 ± 1.76	15.28 ± 1.86**
	10	W/O	3.56 ± 0.53	70.74 ± 4.85	0.22 ± 0.001	2.55 ± 0.56	46.01 ± 3.05	0.48 ± 0.38
		O + T	3.09 ± 0.29	83.18 ± 9.70**	0.25 ± 0.01**	3.62 ± 0.47	28.95 ± 2.11**	7.90 ± 1.93**
		IGF	4.53 ± 0.55	89.71 ± 3.88*	0.21 ± 0.01	2.26 ± 0.17	69.24 ± 1.38***	8.51 ± 1.50**
	4	W/O	1.34 ± 0.16	40.52 ± 5.05	0.29 ± 0.03	3.21 ± 0.39	54.55 ± 6.48	10.70 ± 0.86
		O + T	1.69 ± 0.11	50.53 ± 0.68	0.36 ± 0.03	5.29 ± 0.76*	64.01 ± 4.38	29.66 ± 4.14**
		IGF	1.27 ± 0.09	24.16 ± 3.10	0.21 ± 0.01*	4.26 ± 0.16*	82.18 ± 6.23*	8.38 ± 2.30
6	7	W/O	1.48 ± 0.28	24.22 ± 4.24	0.24 ± 0.01	3.11 ± 0.65	74.48 ± 2.48	16.26 ± 1.68
		O + T	1.57 ± 0.13	20.86 ± 1.01	0.46 ± 0.05**	13.77 ± 4.18*	59.43 ± 3.58**	139.18 ± 27.05**
		IGF	1.77 ± 0.06	24.86 ± 2.79	0.18 ± 0.002***	4.21 ± 0.47	64.86 ± 5.31	14.81 ± 2.50
	10	W/O	1.53 ± 1.02	6.42 ± 1.32	0.19 ± 0.01	2.08 ± 0.14	56.22 ± 1.54	12.94 ± 1.70
		O + T	2.37 ± 0.38	10.81 ± 1.00	0.45 ± 0.06**	6.02 ± 1.07**	48.12 ± 4.02	160.84 ± 45.55*
		IGF	2.02 ± 0.45	10.35 ± 0.51	0.19 ± 0.02	4.58 ± 0.29***	44.61 ± 5.05	14.47 ± 2.19
	4	W/O	2.26 ± 0.40	25.56 ± 1.35	0.33 ± 0.03	10.82 ± 0.84	65.45 ± 3.70	68.75 ± 11.89
		O + T	1.61 ± 0.38	28.26 ± 2.72	0.36 ± 0.04	14.46 ± 2.72	54.89 ± 3.22	59.61 ± 9.14
		IGF	2.05 ± 0.37	22.08 ± 2.46	0.24 ± 0.004**	4.80 ± 0.29***	78.57 ± 2.69*	36.53 ± 8.24
9	7	W/O	1.49 ± 0.35	9.70 ± 1.93	0.36 ± 0.04	14.86 ± 2.50	53.16 ± 4.19	32.18 ± 3.13
		O + T	3.01 ± 0.88	15.95 ± 1.56*	0.48 ± 0.06	18.71 ± 1.98	46.87 ± 2.82	309.83 ± 34.52***
		IGF	1.49 ± 0.27	11.39 ± 2.18	0.23 ± 0.01**	14.87 ± 2.68	66.78 ± 2.62*	18.63 ± 5.09
	10	W/O	2.34 ± 0.41	14.12 ± 2.34	0.30 ± 0.02	9.09 ± 1.07	29.98 ± 3.36	30.86 ± 6.86
		O + T	6.76 ± 0.13***	20.10 ± 0.91*	0.55 ± 0.07**	24.13 ± 3.33**	33.73 ± 2.81	361.52 ± 2.09***
		IGF	3.05 ± 0.81	13.32 ± 1.35	0.23 ± 0.03	6.25 ± 0.49*	33.09 ± 1.78	37.77 ± 13.16
		W/O	4.79 ± 0.53	19.74 ± 1.31	0.19 ± 0.008	27.84 ± 8.69	20.31 ± 3.61	62.88 ± 28.45
12[a]	10	O + T	13.89 ± 1.59***	24.00 ± 1.39*	0.30 ± 0.037*	341.39 ± 78.25***	0.80 ± 0.80*	1714.80 ± 379.31**
		IGF	7.15 ± 0.43**	23.67 ± 1.11	0.17 ± 0.011	42.60 ± 16.09	67.78 ± 0.73***	54.07 ± 22.04

Note: Values are significantly different from W/O.
[a]The biochemical markers released from 12-week-old mice at day 10 represent total release from the entire culture period.
*$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

OSM + TNF-α Increases the Osteoclast Number and Bone Resorption

Measurements of the conditioned media show that stimulation with OSM + TNF-α increases the tartrate-resistant acid phosphatase (TRAP) activity for all the age groups in a range from approximately 41% ($P < 0.05$) to approximately 78% ($P < 0.01$) compared with their respective W/O, indicating increased osteoclast numbers (**Fig. 5A**). OSM + TNF-α increases the osteoclast number over time in 6- and 9-week-old mice. However, the osteoclast number decreases over time in 3-week-old mice (**Table 1**). Conversely to OSM + TNF-α stimulation, IGF-I stimulation shows approximately 17% ($P < 0.05$) to approximately 29% ($P < 0.01$) decrease in the osteoclast number

in 6- and 9-week-old mice (**Fig. 5A**), which results from constant low values measured at all 3 days (**Table 1**). The collagen type I resorption measurements reflect the respective osteoclast number during catabolic stimulation. The resorption increases approximately 65% ($P < 0.05$) to approximately 1200% ($P < 0.001$) in the presence of OSM + TNF-α stimulation compared to W/O (**Fig. 5B**). However, in 6-week-old mice, the collagen type I resorption measurements do not reflect the TRAP activity when stimulated with IGF-I. In 3- and 6-week-old mice, the resorption of collagen type I peaks at day 7, whereas it peaks at or after day 10 in 9-week-old mice (**Table 1**). These measurements show that it is possible to measure osteoclast number and activity via biochemical markers in our *ex vivo* model.
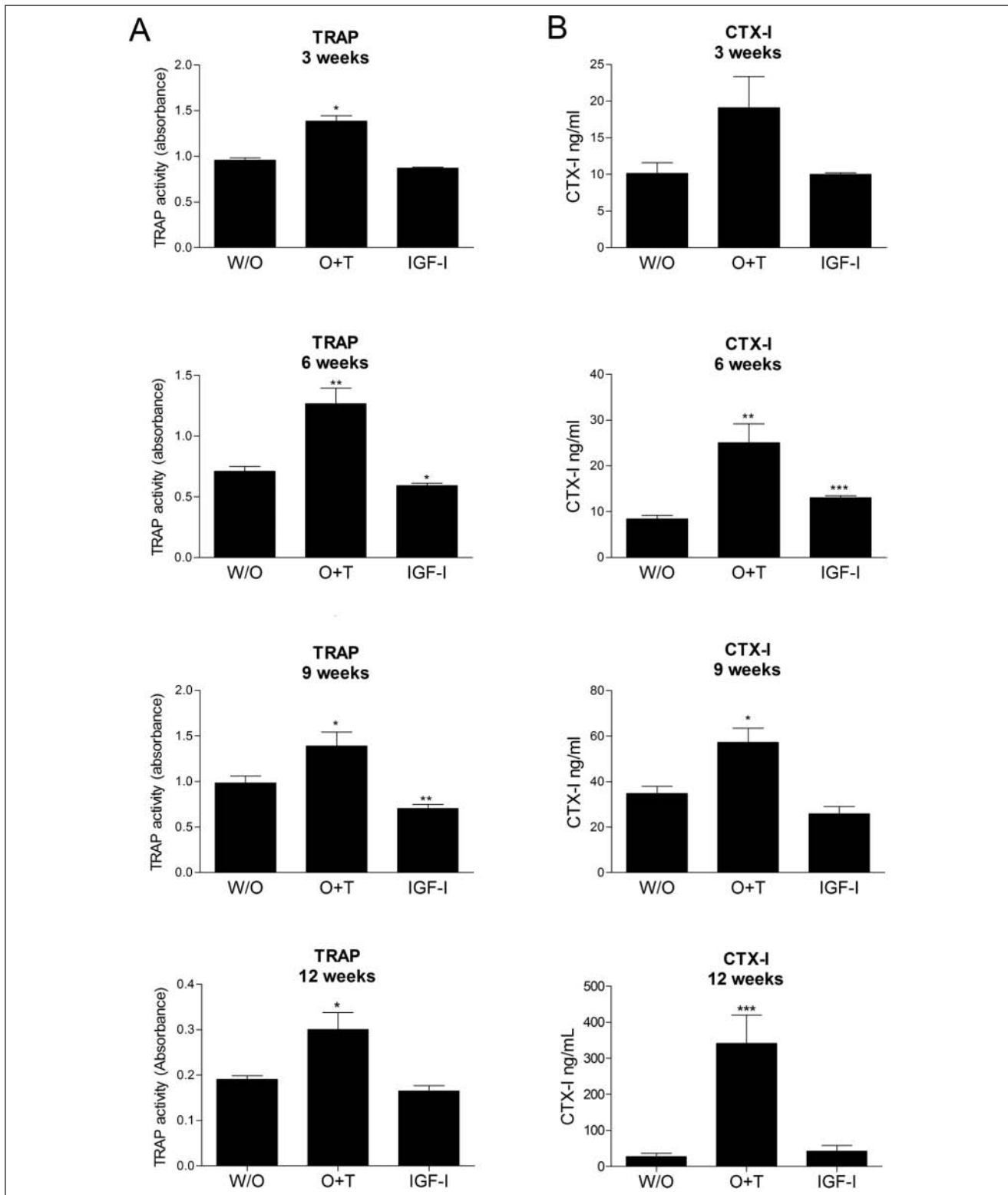
Figure 5. Cytokines increase the osteoclast number and resorption of bone. The conditioned media from the 4 experiments with 4 individual age groups were assessed by the osteoclast number measured by TRAP activity (**A**) and the collagen type I resorption marker, CTX-I (**B**). Each graph represents the accumulated measurements from days 4, 7, and 10 except from 12-week-old mice (only day 10). Catabolic stimulation increases the osteoclast number in all 4 experiments, whereas the anabolic stimulation decreases the osteoclast number in the experiments with 6- and 9-week-old mice (**A**). Cytokines (O + T) also increase the total collagen type I resorption in the experiments with 6-, 9-, and 12-week-old mice (**B**). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

IGF-I and OSM + TNF-α Stimulation Mediate Collagen Type II Formation and Degradation, Respectively

Collagen type II formation, measured by the PIINP released into the conditioned medium, decreases over time (**Table 1**). However, the total collagen type II formation increases by approximately 22% ($P < 0.05$), approximately 21% ($P < 0.05$), and approximately 230% ($P < 0.001$) in anabolic stimulated (IGF-I) femoral heads from 3-, 9-, and 12-week-old mice, respectively, when compared to W/O (**Fig. 6A**). OSM + TNF-α decreases collagen type II formation by approximately 38% ($P < 0.01$) and approximately 96% ($P < 0.05$) compared to W/O in 3- and 12-week-old mice, respectively (**Fig. 6A**).

Stimulation with OSM + TNF-α increases the degradation of collagen type II by approximately 450% ($P < 0.001$) to approximately 2600% ($P < 0.01$) in femoral heads from mice aged 6, 9, and 12 weeks, while femoral heads from 3-week-old mice show only a nonsignificant increase (~80%) compared with W/O (**Fig. 6B**). The longitudinal release pattern of CTX-II shows that OSM + TNF-α increases the collagen type II degradation over time in 6- and 9-week-old mice (**Table 1**). In 3-week-old mice, the release of CTX-II decreases over time (**Table 1**). These results indicate that we are able to induce cartilage formation and degradation in our *ex vivo* model.

Discussion

OA is a complex disease of the entire joint affecting both bone and cartilage.[12,27] Currently, there are no effective disease-modifying OA drug (DMOAD) treatments.[28] We have developed an *ex vivo* murine femoral head model, comprising both bone and cartilage in one closed compartment system that allows us to identify changes in bone and cartilage simultaneously. This model allows interactions between cartilage and bone cells—chondrocytes, osteoblasts, and osteoclasts—in a manner resembling their *in situ* conditions and microenvironment. However, the availability for humoral communication, due to the direct contact between the culture media and the different tissues, far exceeds what is possible *in vivo*. Furthermore, the *ex vivo* model does not mimic the biomechanical communication between bone and cartilage that is part of both normal physiology and pathophysiology. Nevertheless, the model is an important fundamental tool, allowing us to find preliminary results for potential therapies targeting one or both tissues. These preliminary results could aid the selection of which future *in vivo* experiments, concerning joint diseases, should be investigated.

We were able to induce catabolic and anabolic responses by stimulation with catabolic and anabolic factors, respectively.

Catabolic stimulation resulted in increased bone resorption and degradation of cartilage, whereas anabolic stimulation had a protective effect against proteoglycan loss and resulted in increased cartilage formation.

Even though the different developmental stages of endochondral ossification, including that of the femoral head, are known from the literature already,[29] it was important to determine the individual stages in our particular murine model for comparison with the available literature. The microscopic evaluation of femoral heads from mice aged 3 to 12 weeks revealed unique bone and cartilage morphologies.

In 3-week-old mice, the cartilage had a high metabolic rate due to the ongoing endochondral ossification process. The cartilage compartment was dominating compared to the bone compartment and mainly consisted of proliferating and hypertrophic chondrocytes, which included a large and active growth plate. Hypertrophic chondrocytes are a hallmark of early OA,[30] so this murine age group is highly relevant for preliminary studies, for finding potential DMOADs, by investigating the complicated nature of this phenotype. This age group is also relevant for investigating the metabolism of very active cartilage *in vitro*.

In mice aged 6 and 9 weeks, the ratio of bone to cartilage was more even, and an emerging secondary ossification center had started to penetrate the cartilage compartment in 9-week-old mice. The bone compartment was deposited as woven bone with a high proportion of osteocytes. Woven bone forms quickly when osteoblasts produce osteoid, which occurs initially in all fetal bones but is weak due to a disorganized collagen structure. The growth plate was reduced by more than 70%, separating the woven bone from the indefinable mix of prehypertrophic and hypertrophic chondrocytes (in calcified cartilage) in an even-looking arc. These 2 age groups are unique for investigating the metabolism of hypertrophic chondrocytes in the presence of woven bone (comprised of osteoclasts, osteoblasts, and osteocytes) or for studying the initial development of the secondary ossification center.

In 12-week-old mice, the bone compartment had become larger than the cartilage compartment, in direct contrast to the situation with 3-week-old mice. The calcified cartilage had been replaced by woven bone during the secondary ossification, which was enclosed by noncalcified cartilage, resembling mature articular cartilage. The woven bone from the endochondral ossification had been replaced by the stronger and more resilient lamellar bone, which was highly organized in concentric collagen sheets with a low proportion of osteocytes. A small growth plate was still present and separated the endochondral ossification (lamellar bone) from the secondary ossification (woven bone). This older age group is unique for investigating mature bone and subchondral bone metabolism in the presence of
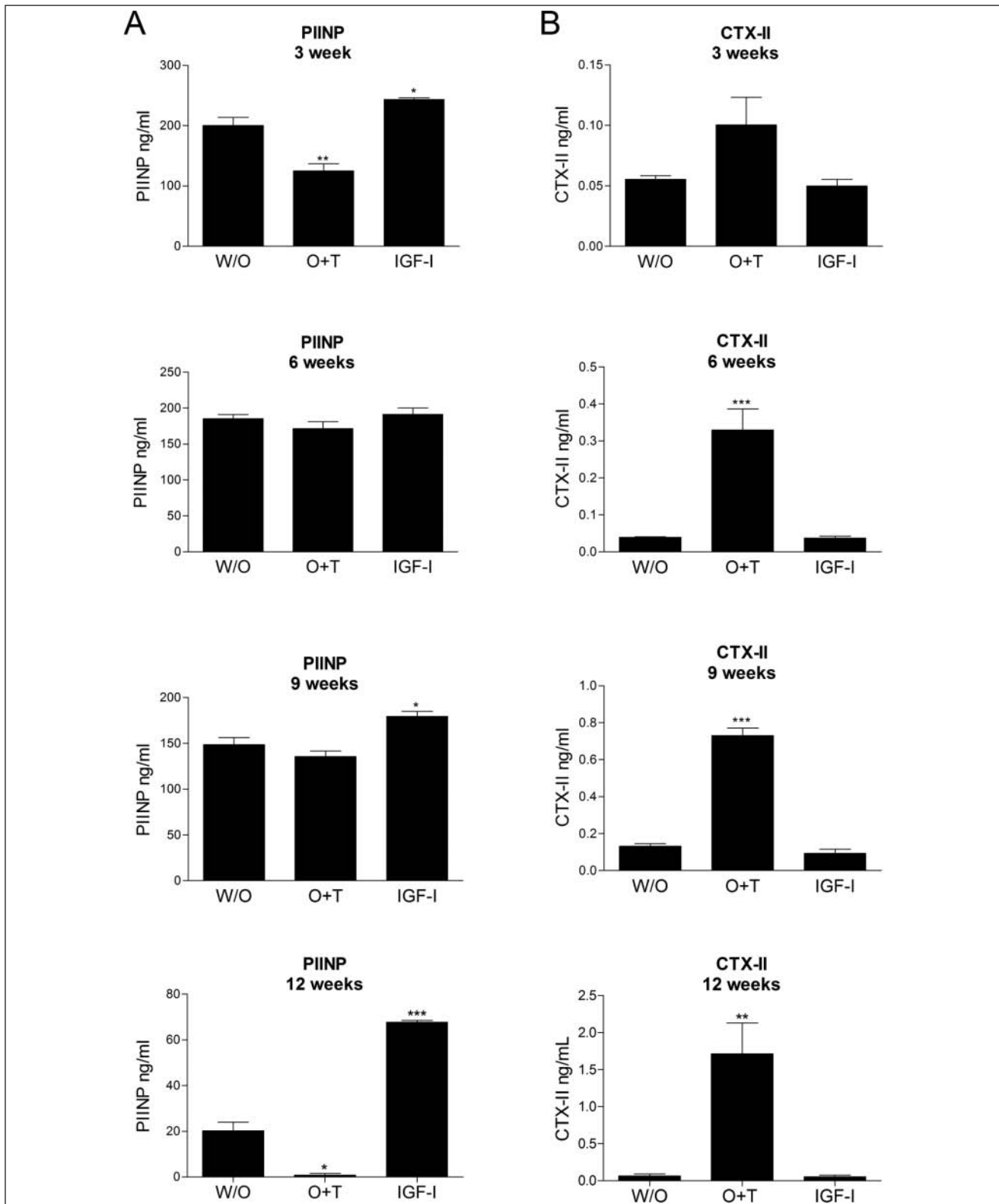
Figure 6. The anabolic and catabolic controls induce cartilage formation and degradation, respectively. The experiments were assessed by the collagen type II formation marker, PIINP (**A**), and the degradation marker, CTX-II (**B**). Each graph represents the accumulated measurements from days 4, 7, and 10 except from 12-week-old mice (only day 10). IGF-I induces formation in the experiments with 3-, 9-, and 12-week-old mice (**A**). Cytokines (O + T) increased the total cartilage degradation in experiments with 6-, 9-, and 12-week-old mice (**B**). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$.

a reduced quantity of mature articular cartilage. Importantly, 12-week-old mice resemble a human joint compared to the other age groups. Furthermore, the model system enables us to induce anabolic or catabolic responses, which mimic parts of the processes from different bone and cartilage diseases in an adult individual.

The microscopic evaluation of femoral heads cultured for 10 days in the absence or presence of IGF-I (anabolic) or OSM + TNF-α (catabolic) stimulation showed that femoral heads stimulated with OSM + TNF-α lost more proteoglycans from the cartilage compartment than the W/O at almost all age groups. Nonstimulated femoral heads from 6- and 12-week-old mice also lost proteoglycans from the cartilage, as seen in the OSM + TNF-α stimulation. This suggests that the proteoglycan loss may be a spontaneous effect from the culturing rather than a catabolic effect from the OSM + TNF-α stimulation. The femoral heads stimulated with the anabolic factor (IGF-I) showed that this loss and depletion of proteoglycans during culture were protected compared to the W/O for all 4 age groups. Furthermore, in 12-week-old mice, anabolic stimulation showed far more proteoglycan staining around the chondrocytes compared to the W/O, indicating a regenerative effect of IGF-I, as previously observed in bovine cartilage explants.[31] We also showed that IGF-I stimulation in 12-week-old mice increased the growth plate size significantly, whereas the OSM + TNF-α stimulation significantly decreased the size. These findings corroborate previous findings in the literature; IGF-I is very important for the growth plate during bone growth as it increases proliferation of resting and proliferative chondrocytes, and OSM + TNF-α has, on the contrary, shown to decrease the rate of endochondral bone growth.[32-34]

Biochemical markers are valuable, longitudinal, and dynamic tools, which in combination with histology can assess the systematic effects of specific compounds. The histology shows the end result of the effect of a specific compound on the femoral head, whereas biochemical markers reveal which protein processes are up-regulated and down-regulated and in which order they are released. We have chosen bar graphs to present the total release of the biochemical markers to the conditioned media as accumulated data from all media changes. **Table 1** presents all the details for the individual days.

Hydroxyproline measurements showed that the release of collagen fragments increased in the presence of OSM + TNF-α stimulation, except from 3 weeks of age. However, IGF-I also increased the release of collagens in 12-week-old mice. If this collagen release results from increased degradation, increased turnover or lack of incorporation into the extracellular matrix after synthesis may be an irrelevant discussion for our use. We can use the results of "collagen release" in combination with other biochemical markers of collagens in our model. We can furthermore not exclude the fact that some portion of the hydroxyproline measured is released from the ligamentum teres.

The release of proteoglycans from the femoral heads decreased proportionally with the increasing age of the mice, indicating that the release of proteoglycans mainly comes from the cartilage compartment and not the bone. However, a limitation of the current study is that we were not able to analyze the specific proteolytic mechanisms responsible for sGAG release. Future studies should thus aim to determine the longitudinal patterns of MMP- and ADAMTS-mediated aggrecan degradation in the femur head model and compare to other *in vitro* and *in vivo* models of bone and cartilage diseases. To evaluate the results further, we then measured more protein-specific biochemical markers.

We found that OSM + TNF-α stimulation for 10 days increased the number of osteoclasts and increased the resorption of collagen type I. Even though the total release of TRAP increased in the presence of OSM + TNF-α (in 3-week-old mice), the osteoclast number decreased over time for both nonstimulated and stimulated treatments. However, the TRAP concentrations at day 4 were higher than the concentration at day 10 for the other age groups. This incoherence might be explained by a peak at day 4 for the 3-week-old mice, in which we did not find a large amount of bone. The increased resorption seen in this model is consistent with the literature, which reports that subchondral bone was lost in the early stage of OA, whereas bone sclerosis was present at the late stage of OA.[10,11] Furthermore, TNF-α can induce osteoclast differentiation *in vitro* through a mechanism independent of RANKL.[35,36] Additionally, in murine osteoblastic MC3T3-E1 cells, M-CSF expression is constitutive and can be further increased by TNF-α.[37-39] OSM can, *in vitro*, induce the formation of osteoclasts, leading to bone resorption. However, OSM can also influence differentiation and proliferation of osteoblasts, leading to bone formation.[40-42]

The anabolic stimulation with IGF-I generally showed, in all the age groups, a tendency to decrease the number of osteoclasts, indicating a protective effect on the bone by reducing bone resorption. However, the collagen type I resorption did not decrease but seemed to be more or less unaffected by the anabolic stimulation. Interestingly, collagen type I resorption data did not reflect the anabolic TRAP data from mice aged 6 weeks. The femoral heads have decreased osteoclast number but increased bone resorption when treated with IGF-I. This ambiguity between the different age groups should be investigated further but may primarily result from the different developmental stages of the femoral head, suggesting an increased activity of osteoclasts in 6-week-old mice, where the secondary ossification center might initiate. However, the literature

supports the increased resorption in 6-week-old mice. One study indicates that IGF-I regulates osteoclastogenesis, which subsequently results in increased bone resorption, and that IGF-I is required for maintaining the normal interaction between osteoclasts and osteoblasts through RANKL and RANK.[43] Another study indicates that IGF-I stimulates both resorption and formation of bone.[44]

The concentration of CTX-I, as a function of OSM + TNF-α stimulation, increased with increasing age, which is consistent with the increasing size of the bone compartment shown by the histology. We expect the majority of CTX-I to come from bone resorption because it is mediated by cathepsin K, which is produced by the osteoclasts in this model. Correspondingly, the cartilage compartment decreased proportionally with the increasing age of the mice. However, our study showed that it was still possible to measure the anabolic and catabolic pattern of total collagen type II formation (PIINP) in 9- and 12-week-old mice, as we saw in 3-week-old mice. The formation of collagen type II decreased over time for all 3 treatments. However, IGF-I slowed the drop of the formation. This indicates that the total increase in collagen type II formation, seen by IGF-I in the bar graph (**Fig. 6A**), was mediated by a protective effect instead of an anabolic effect. The same protective effect of IGF-I stimulation has been published for bovine cartilage explants.[21] Another possibility is that the formation of collagen type II peaks at day 4; thus, we only detect a decrease over time. OSM + TNF-α stimulation increased the degradation of collagen type II (CTX-II) in femoral heads of all 4 age groups, which correlates with earlier *ex vivo* experiments stimulated with OSM + TNF-α, which showed that collagen type II degraded.[22,45] The concentration of CTX-II fragments released to the conditioned medium from the femoral heads decreased over time in 3-week-old mice, probably due to a peak at day 4 since the OSM + TNF-α stimulation was very high at that particular day. Conversely, the concentrations of CTX-II in the other age groups increased over time and proportionally with the age, even though the ratio of cartilage to bone became smaller. This suggests that the peaks for CTX-II release come later in mice older than 3 weeks. Furthermore, this indicates that the secondary ossification center plays an important part in the release of CTX-II. Collagen type II is the predominant collagen of cartilage. Thus, we expect the biochemical markers, PIINP and CTX-II, to primarily come from the cartilage compartment and not from the bone or ligamentum teres.

In the present study, the biochemical markers in combination with histology have shown that we now have a simple *ex vivo* model that henceforth can be used for screening compounds of interest. Furthermore, this model could be essential as a preliminary study to *in vivo* animal studies to narrow down expenses and time spent on big animal studies with futile outcomes.

In conclusion, the murine femoral head model we developed comprises both bone and cartilage in one unit and may be of particular relevance for investigating the communication between the different cell types in these 2 tissues. This might aid the understanding of diseases dealing with miscommunication or improve the efficacy for finding new therapies targeting either cartilage or bone or both. Our model enables observations not only of the cells in *in situ*-like conditions but also monitoring of changes in bone and cartilage as mice age and in response to catabolic and anabolic stimulation. As the morphology of the femoral head changes with age, our study indicates it is critical to select the appropriate age of mice to investigate a specific question.

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Declaration of Conflicting Interests

All authors declare that the affiliation reveals full disclosure. In addition, Dr. Karsdal owns stock in Nordic Bioscience.

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