cells were studied at passage 2. Immunohistochemical staining for alkaline phosphatase, osteocalcin, and collagen I confirmed the maintenance of osteocytic phenotype. Cells were treated at either normoxia (20%) or hypoxia (1%) for 2-16 hours. Hypoxia was achieved with a Biospherix Environmental Chamber. qPCR and ELISA were done for gene expression and protein concentration respectively for plasminogen, urokinase and tissue plasminogen activator (uPA and tPA) and PAI-1. Plasmin activity was assessed with a functional assay with the specific substrate S2251.

**Results:** By two hours of hypoxia, plasmin expression increased from $4.3 \pm 0.4$ to $8.9 \pm 0.3$ ($P < 0.001$). Plasminogen, uPA and tPA gene expression increased significantly (Fig. 1a, b). However, PAI-1 gene expression was not increased at any time point (Fig. 2). Protein concentration followed the same pattern at 2-4 hours of hypoxia (Table 1). PAI-1 protein was not significantly increased.

**Table 1. Protein Expression**

| Protein     | Normoxia | Hypoxia | P <
|-------------|----------|---------|-------
| Plasminogen | 1.5±0.3  | 2.5±0.3 | 0.03  |
| uPA         | 0.3±0.06 | 1.0±0.3 | 0.04  |
| tPA         | 2.5±0.4  | 6.1±1.4 | 0.03  |
| PAI-1       | 15.4±3.7 | 23.0±4.4 | 0.20  |

hypoxia in other tissues such as lung. We suggest that defective PAI-1 response to hypoxia in OA bone, permitting increased plasmin generation, is a feature in the pathogenesis of OA and is associated with an altered physicochemical environment in bone. If so, this describes a specific defect that may provide insight into linkages between physical and chemical pathways that could be useful in early diagnosis or therapy.

**Conclusions:** Decreased perfusion and hypoxia have been observed both in human and experimental OA bone. This study demonstrates that hypoxia activates the fibrinolytic pathway in human OA osteocytes resulting in increased plasmin generation but reveals a defective response in PAI-1. The lack of PAI-1 expression in OA osteocytes is at variance with increases in PAI-1 by 455

**SHORT-TERM GLUCOCORTICOID TREATMENT ABOLISHED THE COMBINED CATABOLIC EFFECTS OF TNF-α AND MECHANICAL INJURY**

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**Purpose:** Traumatic joint injury damages cartilage and releases inflammatory cytokines from adjacent joint tissues, increasing the risk of developing osteoarthritis. Previously, we reported that mechanical injury increased the catabolic effects of TNF-α in immature bovine and adult human articular cartilage, and that endogenous IL-6 played a role in proteoglycan loss caused by injury+TNF-α treatment. Glucocorticoids such as dexamethasone can inhibit expression of inflammatory genes. In this study we hypothesized that the combination of mechanical injury and TNF-α would up-regulate expression of proteins involved in catabolic and inflammatory processes, and that short-term dexamethasone treatment would prevent cartilage degradation by suppressing the production of matrix-degrading enzymes and cytokines.

**Methods:** Cartilage disks (3 mm diam., 1 mm thick) were harvested from the middle zone of the femoropatellar grooves of 1-2-week old calves. Location-matched disks were either injuriously compressed (50% strain, 100%/second strain rate) or maintained free-swelling, cultured in medium with or without rhTNF-α (25 ng/ml), and in the presence or absence of dexamethasone (10nM). Culture was terminated after 6 days of treatment. DMMB dye was used to quantify sGAG released into the medium. Disks were radiolabeled during days 4-6 with $5 \mu$Ci/ml $^{35}$SO-4 and $10 \mu$Ci/ml $^{3}$H-proline to assess proteoglycan and total protein synthesis. In parallel tests, cartilage disks from 6 animals were flash-frozen after 4 days of treatment. RNA was extracted, reverse transcribed and the mRNA levels for 24 genes of interest (proteases, matrix molecules, cytokines, growth and transcription factors) were measured using qPCR (ABI 7900HT). Within each condition, RNA copy numbers of each gene were normalized to

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that of 18S from the same condition. To examine the effects of treatments, each gene was normalized to its level in the no-treatment control. Statistical analysis was performed using a linear mixed-effect model, followed by Tukey’s pairwise comparison.

Results: The combination of TNF-α+injury up-regulated the expression of ADAMTS-4,-5, MMP-3,-9,-13, TIMP-3, iNOS, Caspase-3 and IL-6 (Fig.1, *p<0.05 vs. no-treatment control). TNF-α, with or without injury, significantly increased GAG loss (Fig. 2, *p<0.05) and decreased chondrocyte biosynthesis (data not shown). However, the catabolic effects of TNF-α and TNF-α+injury were abolished in the presence of dexamethasone: both proteoglycan loss and biosynthesis remained at the levels similar to no-treatment controls (Fig. 2).

Matrix Biochemistry

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PUTATIVE 3D HUMAN TISSUE MODEL FOR OSTEOARTHRITIS

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Purpose: Osteoarthritis is a major cause of disability during aging. While the mechanisms involved in osteoarthritis are not yet clear, animal models and cell lines are most often used as disease models. However, inherent limitations with these approaches prompt the need for improved options using human tissue systems. A relevant tissue engineering osteoarthritis model in vitro would allow more insight into underlying mechanisms and would also provide a useful platform for drug screening.

Methods: Tissue engineered (TE) cartilage was prepared by using silk-derived porous 3D scaffolds seeded with primary human chondrocytes and culturing in chondrogenic medium for 4 weeks. The TE cartilage mimicked cartilage in terms of many aspects of cell and matrix features. An osteoarthritis-like phenotype was induced by culturing the cells with cytokines, a combination of IL-1β, and TNF-α, or culturing the cells with conditioned medium from THP-1 derived macrophages. The later served to generate a broad spectrum of cytokines to represent the infiltration of macrophages in vivo. The systems were studied for up to 5 weeks in a static culture.

Results: After 1 week with cytokines induction, matrix anabolism-related genes such as ACAN (GAG) and Col II were dramatically down-regulated; while at the same time genes for matrix catabolism-related enzymes, such as MMP13, ADAMTS4, were up-regulated. As a marker of aging and calcification in osteoarthri-

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ULTRASTRUCTURAL ANALYSIS OF COLLAGEN IN THE ARTHROPATHY OF ALKAPTONURIA IN VIVO AND IN VITRO

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Purpose: Alkaptonuria (AKU) is a rare autosomal recessive condition resulting from lack of homogentisate 1,2 dioxygenase (HGD), the enzyme responsible for the breakdown of homogentisic acid (HGA). HGA accumulates in body tissues resulting in ochronosis, the deposition of pigmented HGA polymers that have high affinity for collagenous tissues, primarily articular cartilage of the weight bearing joints. Over time, pigmentation leads to severe joint degeneration presenting as osteoarthritis. The aim of this study was to use light and transmission electron microscopy (TEM) to identify the location of ochronotic pigment in joint tissues in vivo and in an in vitro model.

Methods: Tissues were collected at the time of surgery from AKU patients undergoing joint surgery. C20-A4 transformed human chondrocytes and SaOS-2 osteosarcoma cells were cultured in DMEM in the presence or absence of 0.33×10^-4 M HGA. All samples were processed routinely for histology and TEM. Paraffin sections were stained with either H&E or nuclear fast red and Schmor’s reagent to highlight pigment. TEM samples were post stained using uranyl acetate and lead citrate.

Results: Macroscopic examination of AKU tissues revealed intense pigmentation of articular cartilage. Synovial and capsular tissues had pigmented and non-pigmented areas. Bone was almost devoid of pigmentation. H&E staining of surgical tissues demonstrated pigment intra- and extracellularly. Chondrocytes, fibroblasts and osteocytes all contained pigment. Ochronotic pigment was located among collagen fibres in articular cartilage, capsule and synovium. Pigment was absent in mineralised collagen fibres of bone. Schmor’s reagent demonstrated smaller amounts of pigment attached to the collagen fibres, undetected using H&E. Schmor’s staining of in vitro cultures revealed pigment deposition in articular collagen, but intra- and extracellularly. Ultrastructural examination of tissue samples revealed pigment deposition on collagen fibres in articular cartilage; in some regions pigment was easily identifiable bound to collagen, whereas others had no pigment. Ultrastructural distribution differed between capsule and cartilage. Pigment in capsule was associated with the collagen fibril periodicity and was also