**Hyperfibrinolysis and Deficient Regulation of PAI-1 in Human OA Subchondral Bone**

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**Disclosures:**


**Introduction:** Several studies have shown that osteoarthritic (OA) subchondral bone exhibits regional hypoxia that can stimulate OA osteoblasts to alter their expression of cytokines and structural proteins relevant to bone remodeling and cartilage degeneration. One cytokine of interest is plasmin which is generated by the activation of the fibrinolytic system and which has been shown to be present in high concentration in OA cartilage and bone. Plasmin activates matrix-degrading enzymes and is involved in cartilage degeneration. The major regulator of fibrinolysis is plasminogen activator inhibitor (PAI-1). It is the hypothesis of this study that hypoxia in subchondral bone fails to upregulate PAI-1 and this failure is a key factor by which fibrinolysis is activated, producing hyperfibrinolysis in OA bone and resulting in plasmin generation. However, hypoxia generally upregulates PAI-1 and produces hypofibrinolysis in blood and this has been observed in OA plasma as well. Understanding how hypoxia could have the opposite effect in OA bone, to produce local hyperfibrinolysis and generate plasmin, would help elucidate a potential pathophysiological signaling pathway in OA.

**Methods:** The study was performed with the approval of the Institutional Review Board of the Lifespan Academic Medical Center.

Clinical Studies: PAI-1 was measured in the serum of 40 subjects undergoing THR with histologically proven OA of the hip and was compared to 40 individuals without OA. Platelet-poor plasma was obtained from fasting blood samples and PAI-1 was measured by ELISA.

Cell Studies: Human OA osteoblasts were derived from tibial specimens removed at total knee replacement (n=10). Osteoblasts were isolated with trypsin/ dispase /collagenase digestion, were plated at 1M/ml, and were grown to confluence in Promocell osteoblast growth media. All cells were studied at passage 2. The mouse fibroblast cell line, RAW 264.7, was used as a positive control since these cells have been shown to upregulate PAI-1 in response to hypoxia. Cells grown under hypoxic conditions were exposed to 1% oxygen for 1-8 hrs. in a Biospherix Environmental Chamber and were compared to cells grown in a standard incubator with 21% oxygen.

Immunohistochemistry: Osteoblasts were fixed in zinc formalin and paraffin embedded. After blocking with 5% normal goat serum, sections were incubated with rabbit polyclonal antibodies to PAI-1 and its 3 transcription factors, hypoxia-inducible factor (HIF-1), early growth response gene (EGR-1) and CCAAT/enhancer binding protein (C/EBPα) and secondary goat anti-rabbit antibodies with biotintylated horseradish peroxidase.

Quantitative PCR (qPCR): Expression of genes for plasminogen, tissue and urokinase plasminogen activator (tPA and uPA), PAI-1, and EGR-1 was assessed with qPCR. Total RNA was isolated using TRIzol plus RNA purification system and concentrated by Spectramax M2. 1µl of total RNA was utilized for cDNA production using the Bio-Rad iScript cDNA synthesis kit. The qPCR primers were obtained from Superarray and assays were performed using the Superarray RT-PCR SYBR Green/Fluorescein master mix. Signal levels were normalized with β2 microglobulin after 35 cycles. Relative transcript levels were calculated as X = 2-ΔΔCt.

Protein Assay: Cell extracts were lysed in extraction buffer and protein concentration was carried out with Quick Start Bradford protein assay. The levels of plasminogen, tPA, uPA, and PAI-1 protein were determined by solid phase, double antibody ELISA using ImmunoBind kits.

Functional Assay for Plasmin: The measurement of plasmin activity was carried out with the specific synthetic peptide substrate S2251 (D-Val-Leu-Lys-O-MnA). This substrate has been extensively characterized and has been demonstrated to be specific to plasmin cleavage.

Statistics: Results are expressed as mean ± SEM. Data was tested for normality with the Kolmogoroff-Smirnoff equation. Parametric data were compared with Student’s T test; non-parametric data were compared with the Mann-Whitney U test. Results were considered statistically significant when p<0.05.

**Results:** After exposure of OA osteoblasts to hypoxia for 2 hrs., the fibrinolytic pathway was activated with the exception that no upregulation of PAI-1 was observed. Plasmin activity was increased from 4.3 ± 0.4 to 8.9 ± 0.3 (p<0.004) after 2 hours of hypoxia; plasminogen and uPA and tPA gene expression increased 2-fold (p<0.01). Protein levels increased for plasminogen from 1.5 ± 0.3 to 2.5 ± 0.3 (p=0.03); tPA from 2.5 ± 0.4 to 6.1 ± 1.4 (p=0.02); and uPA from 0.3 ± 0.1 to 1.0 ± 0.3 (p=0.03). PAI-1 protein did not increase (p=0.20). These results were confirmed histochemically (Fig. 1). PAI-1 gene expression was not upregulated by hypoxia in OA osteoblasts but was in RAW 264.7 cells (Fig. 2). The absence of PAI-1 elevation in OA bone is in contrast to the elevation...
observed in plasma from patients with OA in which PAI-1 was 55.7 ± 6.8 compared to 30.2 ± 3.6 in individuals without OA (p<0.003) (Figs. 3,4). The 3 transcription factors regulating PAI-1, HIF-1, EGR-1, and C/EBPα, were not expressed by hypoxic osteoblasts but were in RAW 264.7 cells (Fig. 5).

**Discussion:** Elevations of PAI-1 and hypofibrinolysis are highly conserved responses to hypoxia. Systemic elevations of PAI-1 are also seen in the plasma of patients with OA and obesity in the metabolic syndrome. Our data from the plasma of patients with OA is consistent with this observation. However, in response to a hypoxic stimulus, OA osteoblasts exhibit a deficient PAI-1 response together with unrestrained upregulation of other fibrinolytic peptides leading to the generation of plasmin.

Transcription factors that normally regulate PAI-1 in response to hypoxia are also not upregulated in OA osteoblasts. Elevated plasmin and fibrinolytic peptide levels have been reported in OA bone and cartilage and activate matrix-degrading enzymes. Regional hypoxia has been described in OA bone and the deficient PAI-1 response to hypoxia, unregulated hyperfibrinolysis, and plasmin generation may be a mechanism by which subchondral bone plays a role in the pathogenesis of OA.

**Significance:** These data explain the apparent paradoxical observations of plasmin secretion in the OA skeleton coincident with systemic hypofibrinolysis. Neither PAI-1 nor its transcription factors are upregulated by hypoxia in OA osteoblasts. The deficient regulation of PAI-1 in OA subchondral bone may contribute to the generation of the protease, plasmin, which participates in the degradative cascade of OA.
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Fig 1: Immunohistochemistry of plasminogen in hypoxic OA bone.

Fig 2: Gene expression of PAI-1 in OA osteoblasts and RAW 264.7.

Fig 3: Plasma PAI-1 protein.

Fig 4: PAI-1 protein in OA osteoblasts.

Fig 5: Immunohistochemistry of OA osteoblasts and RAW 264.7.

References:

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