Role of Apoptosis in Development of Early Osteochondrosis

Stacy Semevolos, Katja Duesterdieck-Zellmer, Maureen Larson.
Oregon State University, Corvallis, OR, USA.


Introduction: Osteochondrosis (OC) is a disease of articular cartilage development involving abnormal endochondral ossification along the osteochondral junction. Associated etiological factors of OC have included rapid growth rate, biomechanical trauma, abnormal collagen turnover, aberrant paracrine signaling, and altered blood supply involving cartilage canals. In addition, cell death of terminally differentiated chondrocytes is a critical process occurring along the osteochondral junction and is likely to be involved in OC disease pathogenesis. However, the mode of cell death has yet to be fully explored in early OC lesions. The objective of this study was to characterize expression of markers involved in apoptosis in cartilage canal and osteochondral junction chondrocytes of early OC and normal cartilage, using naturally-occurring disease in horses. Our hypothesis was that OC develops as a result of aberrant cell death along the cartilage-bone junction during development and that markers of apoptosis will be increased in OC cartilage canal and osteochondral junction chondrocytes compared to normal controls.

Methods: Osteochondral samples were obtained (IACUC-approved) from femoropatellar joints of 15 euthanized immature horses (1-6 months old). Disease status was determined based on histology of osteochondral junctions (7 early OC, 8 normal controls). Osteochondral sections were frozen in OCT for laser capture microdissection (LCM) or fixed in 4% paraformaldehyde and paraffin-embedded for immunohistochemistry and TUNEL staining. Chondrocytes surrounding cartilage canals and osteochondral junctions were captured using LCM. RNA isolation and reverse transcription were performed, followed by logarithmic pre-amplification (14 cycles) of target genes. Equine-specific Caspase-3, Caspase-8, Caspase-10, Bcl-2, BAG-1, Fas, TNFα, Cytochrome C, Thymosin-β10, and 18S mRNA expression was evaluated by two-step real-time qPCR. Percentage of cell death was determined using the TUNEL method. Spatial protein expression was determined by immunohistochemistry using rabbit polyclonal (Fas, Caspase-10, Thymosin-β10) or mouse monoclonal (Cytochrome C) primary antibodies. Statistical analysis comparing gene expression and percentage cell death between early OC vs. normal controls was performed using Wilcoxon rank sum test (p<0.05).

Results: Chondrocytes along the osteochondral junction (Fig. 1) had significantly increased gene expression of Caspase-10 (p=0.04), Fas (p=0.02), Cytochrome C (p=0.01), and Thymosin-β10 (p=0.04) in OC samples compared to normal controls. OC chondrocytes adjacent to cartilage canals (Fig. 2) had significantly increased gene expression of Fas (p=0.002) and Thymosin-β10 (p=0.04) compared to normal controls. There was no significant difference in percentage of cell death of chondrocytes surrounding cartilage canals (p=0.20) or along the osteochondral junction (p=0.43) between OC and normal controls. Fas immunostaining was mainly territorial surrounding hypertrophic chondrocyte lacunae in the deep cartilage layer, with minimal expression in small chondrocytes surrounding cartilage canals. No Caspase-10 immunostaining was apparent along the osteochondral junction or cartilage canals. Moderate Cytochrome C cytoplasmic immunostaining was visible in a few hypertrophic cells in the deep layer, but was minimal surrounding cartilage canals. Strong Thymosin-β10 cytoplasmic protein expression was
apparent throughout all layers of cartilage, with less expression in chondrocytes surrounding cartilage canals and the osteochondral junction.

**Discussion:** Caspase-10 is an apical caspase responsible for triggering the extrinsic death pathway of apoptosis. This apical caspase, in conjunction with Fas, a cell-surface receptor that mediates apoptotic signals, form part of the death-inducing signalling complex (DISC). Cytochrome C release from the mitochondrial inner membrane results in activation of the caspase cascade and apoptotic cell death. Thymosin-β10 is an intracellular G-actin binding protein that has been shown to induce apoptosis in certain cancer lines. In addition, Thymosin-β10 is a homolog of Thymosin-β4, which has been linked to articular cartilage degeneration in osteoarthritis. In normal articular-epiphyseal cartilage, studies suggest non-apoptotic mechanisms of physiological cell death in terminally differentiated epiphyseal chondrocytes. However, chondrocyte apoptosis has been shown to be involved in disease processes such as osteoarthritis or osteochondropathy. In this study, increased gene expression of Caspase-10, Fas, Cytochrome C and Thymosin-β10 in OC chondrocytes along the osteochondral junction suggests apoptosis may play an important role in osteochondrosis. Given these findings, it is tempting to speculate that increased expression of Thymosin-β10 may initiate Cytochrome C release and trigger the caspase cascade in osteochondral junction chondrocytes, although no difference was found in TUNEL positive cells in this location.

**Significance:** Aberrant apoptosis of osteochondral junction chondrocytes may play a role in early osteochondrosis based on increased gene expression of several apoptotic markers in this location.

![Osteochondral junction gene expression](image)
Cartilage canal gene expression

Relative gene expression/18S RNA (log scale)

- OC
- Normal

Gene expression levels for different genes:
- Caspase-3
- Caspase-8
- Caspase-10
- Fas
- Bcl-2
- BAG-1
- Cytochrome C
- TNF-alpha
- Thymosin-beta 10

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