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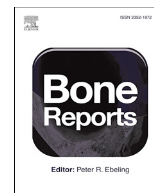

Expression of pro-apoptotic markers is increased along the osteochondral junction in naturally occurring osteochondrosis☆

Stacy A. Semevolos*, Katja F. Duesterdieck-Zellmer, Maureen Larson, Marc A. Kinsley[1]

Department of Clinical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, USA

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ABSTRACT

Osteochondrosis (OC) is a naturally occurring disease of the articular-epiphyseal cartilage and subchondral bone layers, leading to pain and decreased mobility. The objective of this study was to characterize gene and protein expression of apoptotic markers in chondrocytes surrounding cartilage canals and along the osteochondral junction of osteochondrosis (OC)-affected and normal cartilage, using naturally occurring disease in horses. Paraffin-embedded osteochondral samples (6 OC, 8 normal controls) and cDNA from chondrocytes captured with laser capture microdissection (4 OC, 6 normal controls) were obtained from the lateral trochlear ridge of femoropatellar joints in 14 immature horses (1–6 months of age). Equine-specific caspase-3, caspase-8, caspase-10, Fas, Bcl-2, BAG-1, TNFα, cytochrome *C*, thymosin-β10, and 18S mRNA expression levels were evaluated by two-step real-time quantitative PCR. Percentage of cell death was determined using the TUNEL method. Protein expression of caspase-10, Fas, cytochrome *C*, and thymosin-β10 was determined following immunohistochemistry. Statistical analysis was performed using the Wilcoxon rank sum test or two-sample *t*-test ($p < 0.05$). In OC samples, there was significantly increased gene expression of caspase-10, Fas, cytochrome *C*, and thymosin-β10 in chondrocytes along the osteochondral junction and increased Fas gene expression in chondrocytes adjacent to cartilage canals, compared to controls. In OC samples, higher matrix Fas and cytochrome *C* protein expression, lower mitochondrial cytochrome *C* protein expression, and a trend for higher cytoplasmic caspase-10 protein expression were found. Collectively, these results suggest that both extrinsic and intrinsic apoptotic pathways are activated in OC cartilage. Increased apoptosis of osteochondral junction chondrocytes may play a role in OC, based on increased gene expression of several pro-apoptotic markers in this location.

1. Introduction

Apoptosis is a form of programmed cell death that is typically initiated via the extrinsic or the intrinsic apoptotic pathway (Atasoy et al., 2003). In the extrinsic pathway, a ligand of the tumor necrosis factor (TNF) superfamily binds the extracellular domain of a death receptor, such as Fas or other receptors of the TNF receptor family (Atasoy et al., 2003). This binding results in intracellular activation of one of the apical caspases (caspase-8 and -10), which in turn activate downstream effector caspases (caspase-3, -6, and -7), leading to cell death (Wachmann et al., 2010). In the intrinsic pathway, mitochondrial release of cytochrome *C* into the cytosol stimulates the formation of apoptosomes, which activate caspase-9 (Kawamoto et al., 2016), followed by activation of downstream effector caspases (caspase-3, -6, and -7), leading to cell death (Wachmann et al., 2010). A multitude of anti-apoptotic proteins aid in regulation of apoptosis, among them B-cell lymphoma 2 (Bcl-2) (Opferman and Kothari, 2018) and Bcl-2 associated athanogene-1 (BAG-1) (Takayama et al., 1995), both of which inhibit the intrinsic pathway (Kinkel et al., 2004). In addition, some proteins play a role in regulation of apoptosis such as thymosin-β 10, a member of a group of actin monomer-sequestering proteins that inhibit actin polymerization (Shiotsuka et al., 2013; Viglietto et al., 1999).

In normal articular-epiphyseal cartilage, chondrocytes differentiate from proliferative to hypertrophic phenotypes, followed by ossification of the cartilage. Data is inconsistent on whether or not chondrocytes in this last step towards bone formation undergo apoptosis (Ahmed et al.,

Abbreviations: OC, Osteochondrosis; TNF, tumor necrosis factor; Bcl-2, B-cell lymphoma 2; BAG-1, Bcl-2 associated athanogene-1; QH, Quarter Horse; TB, Thoroughbred; POA, Pony of the Americas; LCM, laser capture microdissection; OCT compound, optimal cutting temperature compound

☆ Declaration of interest: none.

* Corresponding author.
E-mail address: stacy.semevolos@oregonstate.edu (S.A. Semevolos).
[1] Present address: Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, MI, 48824.

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2007; Roach and Erenpreisa, 1996; Yang et al., 2014). In postnatal physeal cartilage, it has been suggested that hypertrophic chondrocytes undergo transformation into osteoblasts (Yang et al., 2014). However, another study (Roach and Erenpreisa, 1996) describes asymmetric cell division of hypertrophic chondrocytes leading to apoptosis of one cell and differentiation of the other cell into an osteogenic cell. Yet another study (Ahmed et al., 2007) identifies the presence of dark and light hypertrophic chondrocytes, each dying by different non-apoptotic means.

However, expression patterns of proteins involved in regulation of apoptosis in physeal cartilage suggest that this form of controlled cell death may well play a role in endochondral ossification. For example, late proliferative and prehypertrophic chondrocytes in growth cartilage appear to resist apoptotic cell death, as anti-apoptotic proteins such as Bcl-2 and BAG-1 are expressed prominently in these cells (Kinkel et al., 2004). Furthermore, expression of both proteins decreases with age in articular cartilage (Kinkel et al., 2004).

Osteochondrosis (OC) is a naturally occurring disease of the articular-epiphyseal cartilage and subchondral bone layers, with the hallmark feature of delayed ossification of epiphyseal cartilage, leading to pain and decreased mobility. One proposed etiology includes failure of cartilage canals due to crushing or shearing, resulting in cartilage necrosis and subsequent OC lesions (Carlson et al., 1995; Olstad et al., 2008; Olstad et al., 2011; Olstad et al., 2013). Other studies support a delay in endochondral ossification due to abnormal differentiation of chondrocytes into bone without apparent crushing or shearing injury (Kinsley et al., 2015; Laverty and Girard, 2013; Mirams et al., 2009; Riddick et al., 2012). Whether by necrosis or abnormal differentiation, OC may involve altered chondrocyte death, including altered apoptosis, along the osteochondral junction. Support for this premise includes the finding that thymosin-β4 is increased in experimentally-induced osteochondrosis (Mirams et al., 2016) and the fact that thymosins play a significant role in regulating apoptosis (Hall, 1995; Shiotsuka et al., 2013).

The objective of this study was to characterize gene and protein expression of apoptotic markers in chondrocytes surrounding cartilage canals and along the osteochondral junction of osteochondrosis (OC)-affected and normal cartilage, using naturally occurring disease in horses. Our hypothesis was that OC is associated with abnormal apoptotic cell death along the osteochondral junction during development and that expression of pro-apoptotic proteins increase in OC cartilage compared to normal controls.

2. Materials and methods

2.1. Samples

Archived paraffin-embedded osteochondral samples (6 OC, 8 normal controls) and cDNA from chondrocytes captured with laser capture microdissection (4 OC, 6 normal controls) were previously obtained from the lateral trochlear ridges of femoropatellar joints of 14 immature horses (Kinsley et al., 2015; Riddick et al., 2012). Institutional animal care and use committee approval was obtained for the previous study (Riddick et al., 2012). Foals were 1–6 months of age, including 4 intact males and 10 females (see Table 1).

At the time of harvest, osteochondral samples ($n = 2$ per trochlear ridge, 3–4 mm thick) were sharply dissected from mid-lateral trochlear ridges of both distal femurs. Cartilage was sharply cut with a scalpel down to bone and then a sharp thin osteotome was used to section the bone underneath. Osteochondral samples were either frozen in OCT medium (Tissue Tek OCT compound, VWR International, Radnor, PA, USA) and stored at −80 °C for laser capture microdissection, or fixed in 4% paraformaldehyde for 48 h and transferred to 10% EDTA solution for decalcification (2–4 weeks). Decalcified samples were embedded in paraffin and sectioned for immunohistochemistry and H&E staining (Histopathology Shared Resource Laboratory, Oregon Health & Science University, Portland, OR, USA).

Table 1
Age, sex, and breed characteristics of horses in OC and normal cartilage groups.

	OC-affected	Normal
Age	Median (range): 4.5 months (1–5)	Median (range): 4 months (3–6)
Sex	3 M, 3F	1 M, 7F
Breed	4 QH cross, 1 TB, 1 POA	7 QH cross, 1 POA

2.2. Sample evaluation and classification

All osteochondral samples were evaluated grossly at the time of harvest and histologically following H&E staining in order to classify them as normal or OC-affected, as previously described (Kinsley et al., 2015; Riddick et al., 2012). Normal cartilage was defined as having no gross or histologic abnormalities. OC was defined as samples having altered endochondral ossification (locally thickened cartilage only, loss of normal columnar arrangement of chondrocytes, chondrones) or separation (fissures, necrosis) along the osteochondral junction without concurrent superficial cartilage lesions (Weeren and Barneveld, 1999). Briefly, 6 foals were determined to have OC, and 8 were classified as normal. In OC samples, 5 foals had separation along the osteochondral junction and 3 foals had locally thickened cartilage (2 with concurrent osteochondral separation), all without concurrent superficial lesions.

2.3. Laser-capture microdissection (LCM)

Frozen osteochondral samples (4 OC, 6 normal) were sectioned using a cryomicrotome, mounted on slides using a tape transfer system (CryoJane, Instrumedics, Leica Biosystems, Inc., Buffalo Grove, IL, USA) and stored at −80 °C. Immediately prior to LCM, each slide was dehydrated in graded alcohol and xylene. LCM was performed using PIXCELL II Laser Capture Microdissection System (Arcturus Bioscience, Mountainview, CA, USA) and CapSure Macro LCM caps (Applied Biosystems, Foster City, CA, USA). Chondrocytes were captured immediately surrounding the cartilage canals, representing small rounded chondrocytes, and osteochondral junction, representing hypertrophic chondrocytes of each animal. Up to 8 caps from sequential sections were combined for each site (approximately 400–800 cells per site).

2.4. RNA isolation

PicoPure RNA Isolation Kit (Arcturus Bioscience, Mountainview, CA, USA) was used for RNA isolation of LCM samples with slight modifications. Briefly, cell lysate from up to 8 caps was loaded onto a pre-conditioned RNA-purification column. The column was then washed and treated with RNase-free DNase prior to RNA elution from the column. RNA quality control was performed using an Agilent 2100 Bioanalyzer and the RNA 6000 Pico LabChip kit (Agilent Technologies, Santa Clara, CA, USA).

2.5. Real-time quantitative RT-PCR

Two-step quantitative real time RT-PCR was performed as described in the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA) to evaluate expression of equine-specific caspase-3 (NM_001163961), caspase-8 (XM_001496753), caspase-10 (XM_001498075), Fas (GQ429290), Bcl-2 (XM_001490436), BAG-1 (XM_001917727), TNFα (EU438779), cytochrome *C* (NM_001164014), and thymosin-β10 (AF506973) mRNA expression levels, using a real-time PCR system (ABI Step One Plus instrument and software, Applied Biosystems, Foster City, CA, USA) (Kinsley et al., 2015; Riddick et al., 2012). First strand cDNA synthesis was accomplished with reverse transcription, using random hexamers as primers. Logarithmic pre-

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