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Joint instability leads to long-term alterations to knee synovium and osteoarthritis in a rabbit model

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SUMMARY

Objectives: Joint instability is believed to promote early osteoarthritic changes in the knee. Inflammatory reactions are associated with cartilage degradation in osteoarthritis (OA) but their possible synergistic or additive effects remain largely unexplored. The goal of the present study was to investigate the *in vivo* effects of Botulinum Toxin A (BTX-A) induced joint instability on intraarticular alterations in an otherwise intact rabbit knee joint model.

Methods: Ten 1-year-old female New Zealand White rabbits (average 5.7 kg, range 4.8–6.6 kg) were randomly assigned to receive three monthly unilateral intramuscular injections of BTX-A (experimental group), or no treatment (control group). After 90 days, all knees were analyzed for specific mRNA levels using RT-qPCR. The synovium and cartilage tissue was assessed for histological alterations using the OARSI scoring system.

Results: Cartilage and synovial histology showed significant higher OARSI scores in the BTX-A group animals compared to the untreated controls and contralateral limbs. There were no differences between the untreated control and the contralateral experimental limbs.

Gene expression showed significant elevations for collagen I, collagen III, nitric oxide, TGF-β, IL-1 and IL-6 compared to the healthy controls.

Conclusion: BTX-A induced joint instability in a muscle weakness model uniquely leads to alterations in gene expression and histological changes in the synovial membranes and cartilage in otherwise intact knee joints. These results lead to the conclusion that joint instability may promote an inflammatory intraarticular milieu, thereby contributing to the development of OA.

Introduction

Osteoarthritis (OA) is a slow, but progressive disease with the potential for disabling patients in their daily activities, with painful joint movements and mobility limitations. Furthermore, the socio-economic impact of OA is steadily growing with an expected rise of medical treatment costs to quadruple in the next 20 years[1]. Despite this substantial socio-economic impact, the onset, natural history, or progression of OA is still poorly understood. Scientific research over the last decade has increasingly described OA as a whole joint disease and the result of a complex interplay between several variables and risk factors[2–5].

Muscle weakness and atrophy is one of the earliest signs in patients with symptomatic OA[4,6]. Muscle weakness has also been shown to be an independent risk factor for OA leading to increased loads in the lower limb, whereas neuromuscular training protocols appear to have a protective effect against disease progression[7–9]. Intramuscular administration of BTX-A has been used in the past to induce controlled and reversible muscle paralysis in patients with muscle spasms such as cerebral palsy, and has been used for achieving joint destabilization in studies aimed at investigating the

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role of muscles forces on joint kinematics and OA[10,11]. In previous studies, muscle weakness has been shown to result in joint instability and subsequent cartilage degradation and promotion of OA[4,7,9,12].

Non-mechanical risk factors for OA are more difficult to define but include genetics, obesity, age, sex and inflammation[13]. Especially inflammation and subsequent synovitis have become an intensively studied area in the pathogenesis of OA[14]. Although OA is historically defined as a non-inflammatory disease, it is generally accepted that inflammatory reactions mediated by the release of proinflammatory cytokines result in cartilage degradation, impaired tissue repair, and act as a trigger for symptomatic OA[5,15–17]. Cytokines, such as interleukins (IL-1β, Il-6) or tumor necrosis factor (TNF-α, TNF-β) are thought to activate mast cells, macrophages and neutrophils, as well as chondrocytes and fibroblasts, to further enhance the production of proinflammatory cytokines[18,19]. This cascade of events creates an inflammatory joint milieu resulting in the production of matrix metalloproteinases (MMP's) and other catabolic genes[20]. Excessive expression of proteolytic enzymes has been associated with the breakdown of collagen type II and subsequent cartilage degradation. Other proteinases, such as cathepsin K, nitric oxide synthetase (iNOS) and members of the ADAMTS family, have also been implicated in OA, and are detectable in synovial fluid samples[21]. Recently, Erhart-Hledik and colleagues linked mechanical stimuli, and changes in serum cartilage oligomeric matrix protein (COMP), to cartilage thinning within 5 years, likely indicating that proinflammatory cytokines contribute to levels of soluble COMP that may be released through a trigger event, and subsequently have a detectable impact on the long term progression of OA[22]. However, to date there is no clear evidence if joint inflammation represents a primary cause for the onset of OA, or if inflammation is merely a secondary effect resulting from mechanical alterations that are associated, but not a direct cause of OA.

The aim of this study was to determine the effects of a BTX-A induced joint instability on the intraarticular milieu of the rabbit knee. Knee joint instability was produced by paralysis of the knee extensor mechanism, which left the tissues of the knee completely intact. We hypothesized that muscle paralysis and subsequent joint instability leads to synovial alterations, subsequently leading to measurable increases in proinflammatory markers, and possibly enhancing cartilage degradation and symptomatic OA.

Methods

Experimental design

10 one-year-old skeletally mature female New Zealand White rabbits (weight: average 5.65 kg, range 4.8–6.6 kg; Riemen's Furriers, St. Agatha, Ontario, Canada) were used in accordance with an experimental protocol approved by the University of Calgary Animal Care Committee. Animals were housed in pairs in floor pens to permit normal activity. Rabbits received a standard diet and water *ad libitum*. For the study duration of 3 months, the animals were divided into two experimental groups; an untreated control group (1) and an experimental group (2).

Group 1: Five animals were assigned to the experimental group. One hind limb was randomly designated as the experimental limb and was injected with BTX-A (BOTOX, Allergan Inc., Toronto, Ontario, Canada). Rabbits were anesthetized for the intervention procedures using a 3.5% isoflurane (Benson Medical) to oxygen mixture.

On day 0, 30, and 60, 3.5 U/kg BTX-A was injected along three lines of the thigh, identifying the medial, central, and lateral aspect of the thigh and corresponding to the vastus medialis muscle, rectus femoris muscle, and vastus lateralis muscle, respectively. The contralateral side received the same amount of fluid as the experimental limb (0.5 ml) in the form of sterile saline.

Group 2: five animals were assigned to be the untreated control group. They were kept and fed in the same manner as the experimental group animals.

After 90 days, the animals were euthanized by an intravenous injection of Euthanyl (Bimeda-MTC). The following tissues were harvested for molecular analysis: the medial and lateral collateral ligaments, the medial and lateral menisci and the synovial membrane. Cartilage tissue of the tibia and femur and a piece of synovial tissue was processed for histology.

Total RNA isolation

For molecular analysis, tissues were immediately harvested after sacrifice, weighed, flash-frozen in liquid nitrogen, and subsequently stored at −80°C. The experimental and contralateral knees of all group 1 and group 2 animals were collected. All tissues except the cartilage were subjected to RNA isolation using the TRI spin method and total RNA was quantified using the SYBR Green reagent (Molecular Probes, Eugene, Oregon, USA)[23].

Reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA (1ug) of each sample (assessed with a NanoVue; GE Health Care, Quebec, Canada) was initially reverse transcribed with random RT primers using a Qaigen Omniscript kit (Qaigen Inc., Mississauga, Ontario, Canada). All samples for a given tissue were subjected to RT at the same time to avoid potential variability. mRNA levels for specific molecules were assessed using validated primer sets for rabbits[24], qPCR using a BioRad CTX Connect iCycler (BioRad, CA, USA), and the Sybr Green method. Validation of the qPCR amplification was confirmed using the delta CT software that was built into the equipment. All samples for a given tissue were subjected to optimized PCR conditions at the same time to avoid potential variability. Assessment of all no RT controls were negative for genomic DNA contamination of RNA preparations. A subset of RNA samples from each tissue was subjected to RT a second time and re-analysis confirmed the original findings. The mRNA levels for the following molecules were assessed: Biglycan, Decorin, Collagen I, Collagen III, Matrix Metalloproteinases (MMP-1, MMP-3, MMP-13), connective tissue growth factor (CTGF), transforming growth factor (TGF-β), Interleukin-1β, IL-6, Versican, Proteoglycan 4, Nitric Oxide Synthase (iNOS) and vascular endothelial growth factor (VEGF). All results were normalized to the housekeeping gene 18S as described previously[24]. The molecules assessed were a subset of anabolic and catabolic/inflammatory molecules that are relevant for the maintenance of the mechanical integrity of the tissues of the knee.

Histology

A specimen of the synovial membrane was harvested from the patellar fat pad of all 10 animal knees (experimental, contralateral side and sham controls). Cartilage tissue was harvested and the articular surfaces of the tibia and femur and processed for histology, as described previously[9]. The knee joints were harvested, fixed in a 10% neutral buffered formalin solution for 10 days (Fisher Scientific, Wohlen, Switzerland) and then decalcified with Cal-Ex II decalcifying solution (10% formic acid solution in 4% formaldehyde, Fisher Scientific) at room temperature. The solution was changed daily. After 2 weeks, joints were opened and returned to a fresh decalcifying solution for an additional 2–3 weeks. Decalcification was considered complete when joints could be cut smoothly and

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