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NEUROTRANSMITTER FROM THE SYMPATHETIC AND SENSORY NERVOUS SYSTEM ALTER PROLIFERATION AND MEATBOLIC ACTIVITY OF CHONDROCYTES IN VITRO

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Purpose: The peripheral nervous system plays an important role in fracture healing and bone remodelling. Nerve fibres of sympathetic and sensory origin innervate bone and fracture callus thereby influencing callus size and bone formation, additionally chondrocytes themselves express substance P (SP) and its receptor NK1-R. We employed a 3-D micromass pellet culture in vitro model of murine primary chondrocytes which focuses on the role of neurotransmitter from the sympathetic and sensory nervous system for the organization and differentiation of the cartilaginous callus.
Methods: All experiments were carried out with expanded (1 passage) costal chondrocytes isolated from newborn animals. To determine the influence of neurotransmitters on proliferation and metabolic activity, micromass pellets were cultured in minimal medium containg DMEM/F12, 1mM cystein, 1mM pyruvate, 50μg/ml ascorbate, and 1% penicillin/streptomycin. Micromass pellets were stimulated daily with substance P (10^{-9}, 10^{-10}, 10^{-11} M) and norepinephrine (10^{-6}, 10^{-7}, 10^{-8} M NE). Pellets were harvested after 1, 4, and 7 days and gene and protein expression was analyzed histologically and with quantitative PCR.
Results: Alcian blue staining and collagen II and IX immunohistochemistry revealed a regular cartilage-like extracellular matrix development in the proliferation phase, unaffected by substance P and norepinephrine. However, after 7 days of stimulation with norepinephrine Col1a1 and Col9a1 gene expression was suppressed compared to non-stimulated controls. PCNA staining after 7 days showed undisturbed proliferation, but after stimulation with substance P and norepinephrine proliferation activity decreased from day 4 to day 7. Gene expression of MMPs (MMP-13) and cytokines (TNF-α) was altered by norepinephrine, resulting in higher MMP13 and lower TNFα levels after 4 days compared to controls.
Conclusions: Sympathetic and sensory nerve fibres invading the fracture callus release neurotransmitters which in turn affect proliferation rate and metabolic activity of chondrocytes. These data suggest an inhibitory effect of NE and SP on chondrocyte metabolic activity, possibly resulting in a delay of callus maturation and remodelling activity. Chondrocytes themselves stain positive for substance P and express the NK1 receptor on the cell surface indicating an autocrine loop may exist in parallel.

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EVIDENCE FOR AN ESSENTIAL ROLE OF SPHINGOSINE KINASE IN CHONDROCYTE PROLIFERATION

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Purpose: Chondrocyte proliferation is a common feature in areas of cartilage damage. Furthermore the sites of chondrocyte proliferation (termed chondrocyte clusters) are known to be foci of cartilage matrix degeneration.
We have identified the endogenous lipid mediator sphingosine-1-phosphate (S1P) as an inducer of chondrocyte proliferation. S1P is generated by phoshorylation of sphingosine by sphingosine kinase (SphK) and has been implicated in inflammation, proliferation and anti-apoptotic processes.
The aim of the study was to investigate expression of SphK in osteoarthritis (OA) cartilage and evaluate the effect of SphK inhibition on chondrocyte proliferation.
Methods: Human cartilage specimens from macroscopically damaged and undamaged areas were obtained from patients undergoing total knee joint replacement. Specimens were formalin fixed and paraffin embedded. Safranin O stained sections were graded according OARSI histological grading score and SphK was detected by immunohistochemistry using the labelled streptavidin biotin method. For cell culture bovine Chondrocytes from metacarpo-phalangeal joints of adult animals were isolated using collagenase B. Cells grown in monolayer were cultured in Ham's F-12/DMEM (1:1) and 10% FCS to 80% confluence. Chondrocytes were then serum starved for 24 hours and incubated with 10μM of L-threo Dihydrosphingosine (DHS), a specific SphK inhibitor, 1-10mg/l of bovine insulin or 1-10% FCS. Proliferation was assessed using tritium incorporation.
Results: SphK expression was detected in 9-33% of chondrocytes. Particularly intense staining was observed in chondrocyte clusters and in areas of cartilage damage. The percentage of SphK positive cells was significantly higher in macroscopically damaged compared to undamaged cartilage of the same patient (mean 23.9% vs. 16.6%, $P<0.05$). Furthermore in cartilage sections with high histological grading a significantly higher percentage of cells stained positive for SphK.
Treatment of cultured chondrocytes with the SphK inhibitor DHS significantly reduced chondrocyte proliferation (mean 437 vs. 8182cpm, $P>0.01$), without signs of cell death. Interestingly increasing doses of insulin but not of FCS partially reversed DHS induced proliferation stop
Conclusions: SphK is expressed particularly by proliferating chondrocytes in areas of cartilage damage. Inhibition of SphK almost completely abrogates chondrocyte proliferation in response to FCS but not to insulin. These data suggest that SphK plays an essential role in chondrocyte proliferation.

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RELATIONSHIP BETWEEN THE EXPRESSION OF TOLL LIKE RECEPTORS AND DEGRADATION OF MATRIX IN OSTEOARTHIRITIC CARTILAGE

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Purpose: Osteoarthritis (OA) is a complex disease involving response of chondrocytes to variable stimuli such as mechanical stress, degradative products and inflammatory cytokines. However, the exact pathways of initiation and development of OA remain largely unknown. Toll like receptor (TLR) is a phylogenetically conserved receptors involved in the innate immune response, and they recognize pathogen-associated molecular patterns. Recently, it was reported that TLRs were involved in OA development through induction by cytokines and degradative products in chondrocytes in vitro. The purpose of this study is to investigate the relationship between expression of TLRs and degradative enzymes as well as their specific matrix degradative products in OA cartilage immunohistochemically.
Methods: We collected OA cartilage samples (N=6, age 72-78 ys) at the operation of total knee arthroplasty. Control normal cartilage was obtained from the knee of amputated limbs (Male, age 52 ys). The cartilage samples were fixed with buffered folmalin. We observed cartilage degradation by Toluidin-Blue staining. Immunohistochemical analysis was performed to study the expression of TLR-2 and 4, MMP-1, 3, and 13, and urokinase type plasminogen activator (uPA) and uPA receptor (uPAR) as well as specific cleavage site of type II collagen by collagenases (C2C).
Results: In normal cartilage, we could detect TLR-2 expression but not TLR-4. MMP-1 and 3, C2C were only detected dispersedly.

We observed fibrillation, loss of proteoglycan and cluster formation in OA cartilage. Expression of MMP -1, 3 and uPAR was widely detected. C2C was co-localized with these enzymes especially in the superficial zone of OA lesion. Interestingly, both TLR 2 and 4 were abundantly expressed in the OA lesion and co-localized with MMPs and C2C.
As TLR-2 was expressed in normal cartilage, but not TLR-4, there is the possibility that initially TLR-2 is important for chondrocyte to recognized the osteoarthritic condition. In addition, it is reported that TLR-2 can be regulated by IL-1 and fibronectin fragments. Secondary, induced TRL-4 could have important role on the further catabolic response of chondrocyte.
Conclusions: It is important to study the degradative enzymes in including MMPs and serine proteinases such as uPA and specific degradative products by those enzymes together because the activation mechanism of enzymes was very complicated. We demonstrated these degradative process of cartilage matrix could be related to the expression TLR-2 and 4. The innate immunoresponse through TLRs could have important roles on development of OA.

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ROTENONE TREATMENT IMPAIRS BIOSYNTHETIC ACTIVITY IN ARTICULAR CARTILAGE

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Purpose: It has been reported that loss of mitochondria from articular cartilage chondrocytes is involved in the pathogenesis of osteoarthritis (OA). Reduced mitochondrial function is believed to promote cartilage degeneration by undercutting biosynthetic activity, but direct evidence of this relationship is lacking. To test this hypothesis we determined the effects of rotenone, an inhibitor of complex I of the mitochondrial electron transport chain, on protein biosynthesis and matrix stability in bovine osteochondral explants.
Methods: *Tissue.* Osteochondral explants were harvested from bovine lateral tibial plateaus and incubated in culture medium with 10% serum.
Rotenone Treatment. Samples were treated daily for 7 days with 2.5 μm rotenone.
Mitochondrial Activity. Explants were stained with calcein AM and Mitotracker Deep Red FM (Invitrogen) for 1 hour each prior to imaging. Confocal microscopy was used to obtain Z-series images (0 to ~200 μm deep in 20 μm intervals), which were projected and analyzed with *ImageJ* software. Numbers of live cells (calcein AM) and cells containing functional mitochondria (Mitotracker) were measured and ratios of Mitotracker positive cells to Calcein AM positive cells were calculated for each image.
DNA and Proteoglycan Content. The DNA and proteoglycan content were assayed by standard methods following Papain digestion and normalized to the wet weight of the samples (ng DNA/mg tissue and μg GAG/mg tissue).
Biosynthetic Activity. Protein synthesis was analyzed by measuring ^{3}H-Proline incorporation after 18 hours of radiolabeling. The results were normalized to the wet weight of the samples (CPM/mg tissue).
Statistical Analysis. Statistical analysis was performed using Student's t-tests ($p < 0.05$).
Results: Rotenone significantly reduced Mitotracker staining in the superficial and middle zones (Figure 1). ^{3}H-proline incorporation was also significantly inhibited by rotenone treatment (Figure 2). DNA content was significantly reduced by rotenone treatment (Figure 3), but no significant effect on proteoglycan content was observed (Figure 4).
Conclusions: Rotenone significantly inhibited mitochondrial function and suppressed biosynthetic activity. The DNA content of rotenone-treated explants was also reduced, indicating cell death. DNA content was reduced 1.6-fold whereas biosynthetic activity

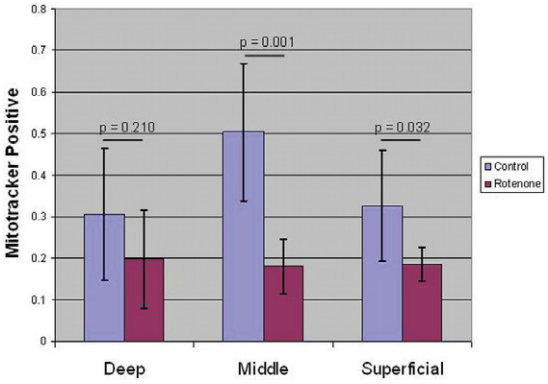

Figure 1. Rotenone treatment significantly reduced mitochondrial activity in chondrocytes in the superficial (<200 μm deep) and middle (400–200 μm deep) zones, but not in the deep zone (>400 μm deep).

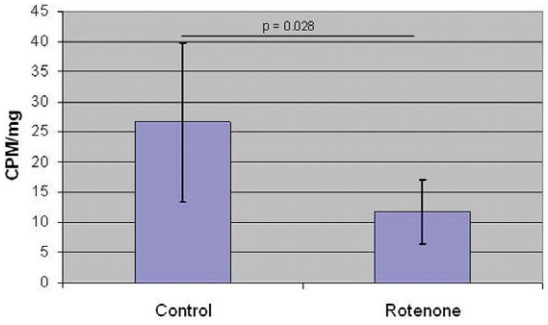

Figure 2. Rotenone treatment significantly reduced tritiated proline incorporation.

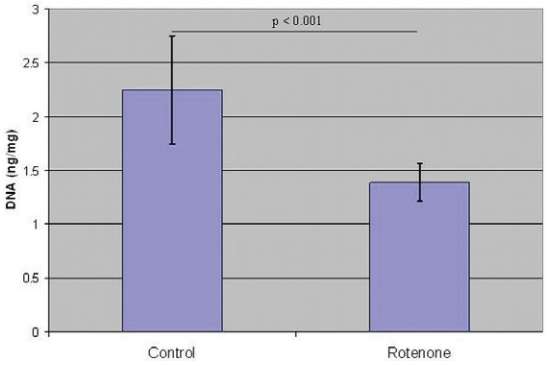

Figure 3. Rotenone treatment significantly reduced DNA content.

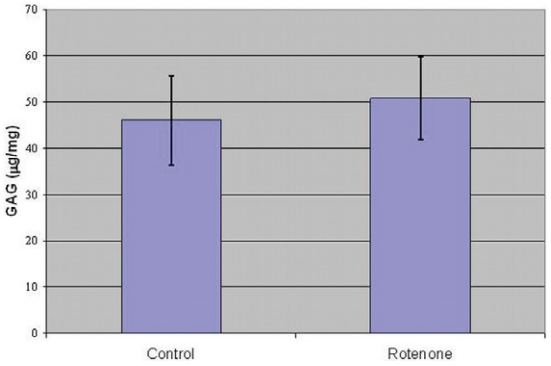

Figure 4. Rotenone treatment had no significant effect on glycosaminoglycan (GAG) content.