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LIDOCAINE INDUCES ROCK-DEPENDENT MEMBRANE BLEBBING AND CELL DEATH IN RABBIT ARTICULAR CHONDROCYTE

Purpose: Local anesthetics are administered intraarticularly for pain control in orthopedic clinics and surgeries. Although previous studies have shown that local anesthetics can be toxic to chondrocytes, the underlying mechanism remains unclear. The present study was undertaken to investigate the cellular mechanisms associated with lidocaine-induced toxicity to articular chondrocytes.

Methods: Isolated rabbit articular chondrocytes were exposed for 30 minutes to lidocaine at concentrations of 1, 3, 10 and 30 mM (360 mosmol/l; pH 6.8, 7.4 and 8.7) at 36 °C. To evaluate the lidocaine-induced blebbing, morphological changes of chondrocytes were monitored using time-lapse microscopy and then the percentage of blebbing cells was evaluated every minute. The levels of guanosine triphosphate (GTP)-bound RhoA and caspase-3 were determined using a RhoA G-LISA assay and a Caspase-Glo 3/7 assay. The viability of chondrocytes treated with 30 mM lidocaine for an hour was determined using a LIVE/DEAD® assay kit. To evaluate the percentage of living cells, the number of living cells (green) and dead cells (red) of each image were counted by an ImageJ software.

Results: Treatment of chondrocytes with lidocaine caused spherical protrusions on the cell surface (so called ‘membrane blebbing’) in a time- and concentration-dependent manner. After 30-min exposure, lidocaine (pH 7.4) at the concentrations of 1, 3, 10 and 30 mM induced membrane blebbing in 9,6±1.1%, 35,5±2.2% and 100±0.0% of chondrocytes, respectively, compared with the controls (1,2±2.1%) (mean±s.d., n=3). These effects of lidocaine could be fitted by the Hill equation giving an EC50 of 4,13 mM, which is lower than clinical use concentrations. When lidocaine was applied under alkaline (pH8.7) or acidic (pH 6.8) condition, the EC50s were shifted to 1,37 or 11,72 mM, respectively, as is expected for the pH-dependent change in the concentration of non-ionized lidocaine. ROCK (Rho-kinase) inhibitors Y-27632 (10 µM) and fasudil (100 µM) completely inhibited the lidocaine-induced membrane blebbing, suggesting that ROCK activation is required for the blebbing. Although the apoptotic blebbing is typically caused by the caspase-3-induced cleavage of ROCK-I, the caspase-3 level was unchanged by 20-min treatment of chondrocytes with 10 mM lidocaine. Furthermore, although it was reported that apoptotic blebbing was abrogated by z-VAD-fmk, a caspase inhibitor, this drug did not prevent the lidocaine-induced blebbing. These results suggest that lidocaine-induced membrane blebbing is independent of caspase-3 activity. The GTP-bound RhoA level was significantly increased (3,01±0,76 folds, P<0,0001) by 20-min treatment of chondrocytes with 10 mM lidocaine. However, Rho inhibitor-1 rather enhanced the membrane blebbing, suggesting that the lidocaine-induced membrane blebbing might be partially mediated through the pathway other than canonical Rho/ROCK signaling. Chondrocyte viability was significantly decreased to 17,6±5,7% after 1-h exposure to 30 mM lidocaine, compared with the control viability of 94,8±2,4% (P<0,0001). Pretreatment with Y-27632 or fasudil for 1-hour attenuated the lidocaine induced-cytotoxicity (49,4±12,5% and 47,2±9,1% viability respectively, P<0,0001), demonstrating that the lidocaine-induced hypertrophy of ROCK leads to cell viability.

Conclusions: These findings show that lidocaine induces a cytotoxic effect on chondrocytes through the mechanism involving membrane bleb formation and ROCK activation and that caution should be taken when administering lidocaine intraarticularly.

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INTERLEUKIN-1BETA IS ESSENTIAL FOR BLOOD-INDUCED CARTILAGE DAMAGE IN VITRO

Purpose: Exposure of joint cartilage to blood can occur after joint trauma, during or after major joint surgery, or due to hemophilia. This ultimately leads to joint damage, having both the inflammatory characteristics of rheumatoid arthritis and the degenerative characteristics of osteoarthritis. We previously reported that blocking interleukin(IL)-1β with a recombinant monoclonal antibody (IL-1βmAb) protects cartilage from the damaging effects of blood exposure in a dose-dependent way (Abstract 155; 2014 World Congress on Osteoarthritis). To further unravel the role of IL-1β in blood-induced cartilage damage, we investigated whether blocking IL-1 with a receptor antagonist (IL-1RA) is also able to prevent blood-induced cartilage damage in vitro, and whether blocking IL-1β with either IL-1RA or IL-1βmAb after onset of a bleed is still beneficial.

Methods: Healthy human cartilage explants were cultured for 4 days in presence/absence of 50% whole blood. IL-1RA was added in a broad concentration range (10-1000 ng/mL) during blood exposure (n=7). Furthermore, IL-1βmAb (100 ng/mL) or IL-1α (1000 ng/mL) was administered directly or after a delay of several hours up to 2 days (n=7). Proteoglycan turnover was determined after a recovery period of 12 days.

Moreover, in 4-day whole blood cultures (n=6) the effects of IL-1βmAb (100 ng/mL) and IL-1α (1000 ng/mL) on the levels of IL-1β, IL-6, and TNF-α were determined.

Results: Exposure of cartilage to blood severely impacted proteoglycan turnover (PG synthesis -76%; P<0.018 see figure A; PG release +125%; P<0.018; PG content -18%; P<0.028, all compared to control). Addition of IL-1RA resulted in a clear dose-dependent improvement in proteoglycan turnover (see figure A for proteoglycan synthesis). For PG release and PG content, the highest concentration led to normalisation as compared to control (PG release +33% of control (p=0.063); PG content +2% of control (p=0.866)). The protective effect of IL-1βmAb or IL-1α was most pronounced when administered within 8 hours after the bleed (see figure B for proteoglycan synthesis upon IL-1RA addition). Blocking IL-1β reduced IL-6 (21 347 pg/mL in whole blood culture versus 27 pg/mL and 289 pg/mL after addition of IL-1β and IL-1βmAb (both p=0.028)) and IL-1β (74 pg/mL in whole blood culture versus undetectable after addition of IL-1RA, p=0.028) levels in whole blood cultures, but did not affect the levels of TNF-α (35 pg/mL in whole blood culture versus 37 pg/mL after addition of IL-1RA or IL-1βmAb (both p=0.753)).

Conclusions: This study demonstrates that IL-1β is a critical factor in the development of blood-induced cartilage damage in vitro. Blocking it with either a monoclonal antibody or receptor antagonist protects cartilage from the damaging effects of blood exposure in a dose- and time-dependent way. Early administration after blood-exposure is most beneficial. This key role of IL-1β results at least partly from regulating the production of other pro-inflammatory cytokines, including IL-1β itself and IL-6. As therapeutic agents opposing the activity of IL-1β are readily available, further research is warranted to investigate its in vivo capacity in prevention and treatment of joint damage upon joint bleeding.

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THE ROLE OF SODIUM HYDROGEN EXCHANGER REGULATORY FACTOR 1 IN OSTEOARTHRITIS
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Purpose: In a pilot study, selected gene expression in tissue samples from patients with OA and osteoporosis (OP) were compared. The gene SLC9A3R1 which encodes for a member of the sodium/hydrogen regulatory factor protein family (NHERF1) was found to be differentially expressed in osteoelasts. NHERF1 plays a role in tumour suppression, the production of osteoblasts. NHERF1 which encodes for a member of the sodium/hydrogen exchanger (NHE). We are investigating whether a decrease in NHERF1 causes a change in the pH of chondrocytes and leads to...
chondrocyte proliferation. The aim of the project is to test the hypothesis that dysregulated expression of NHERF1 is a key part of the abnormal behaviour of articular chondrocytes.

**Methods:** Full depth cartilage explants or shavings were removed from osteoarthritic femoral heads or knee joints, and from healthy controls (amputees or osteoporotic femoral heads). Explants from 17 patients (4 OA and 4 non-OA femoral heads, 4 OA knees and 5 amputees) were decalcified and fixed in formalin for paraffin embedding and sectioning, whilst shavings were placed in Dulbecco’s Modified Eagles Medium (DMEM) and washed prior to enzymatic chondrocyte isolation. Sections were placed in Dulbecco’s Modified Eagles Medium (DMEM) and washed prior to enzymatic chondrocyte isolation. Sections were then deparaffinised and either stained with Fast-Green-ECF and Safranin O’, or treated with Target Retrieval Solution and hyaluronidase prior to incubation with glycine, blocking with non-immune serum and overnight incubation with either rabbit anti-human NHE3 (pAb, C-20), mouse anti-human NHERF1 (mAb, IgG2b), or goat anti-human PTEN (pAb, N19) diluted in Dako Antibody Diluent (1:50; PTEN 1:100). Bound NHE3, NHERF1 or PTEN antibodies were detected with goat anti-rabbit (488 nm), goat anti-mouse (555 nm) or donkey anti-goat (488 nm) secondary antibodies (1:100, PTEN 1:200) respectively. Sections were then incubated with glycine, washed in DMEM without phenol red, aliquoted (14 x 104) and placed in a sterile Ibidi µ-Slide I (0.4) Flow Chamber (Thistle Scientific, UK) for 20-30 min (37 °C) to attach. They were then placed in the appropriate saline.Af ter saline washing, and BCFE was then alternately excited at 488 nm, 495 nm, and 594 nm, and emission collected at 505 nm using a CLSM (Zeiss LSM 710) from individual cells. The background from each wavelength was subtracted and the 498:458 nm ratio (R) converted to pH using a calibration curve constructed by permeabilizing the chondrocytes to H+ in high-potassium (K+) solutions of different pH containing nigericin (2 µM).

**Results:** NHE3, NHERF1 and PTEN were expressed in all zones of OA and non-OA articular cartilage. Image analysis showed that NHERF1 co-localised with both proteins in the cytoplasm and perinuclear region, but little was found near the cell membrane. It was also expressed in the nucleus in 50% of chondrocytes in OA and non-OA samples. The median diameter of the nuclei of OA chondrocytes was also found to be significantly larger (7.47 µm) than in non-OA chondrocytes (6.78 µm, P=0.001777). The pH of normal, healthy chondrocytes was 6.9 ± 0.2 (n = 3) but OA chondrocytes appeared to be more acidic. The pH of these chondrocytes was too low to be recorded successfully and reliably using BCECF (pKa 6.98) which is used for measuring near neutral pH, so this necessitated exploring the use of another fluorescein derivative, DCFDA (pKa 5.5) for pH measurements from pH sensitive dyes. A high proportion of the measured pH values were below 5.0.

**Conclusions:** This is the first study to identify NHE3, NHERF1 and PTEN expression and co-localisation in human articular cartilage, and to measure pH in single isolated chondrocytes using confocal microscopy and the fluorescent dyes, BCECF-AM and DCFDA. NHERF1 was expected at the cell membrane but, surprisingly, location was predominantly cytoplasmic. Detection of NHERF1 expression and function in articular cartilage may open new approaches for OA therapy in the future.