CASPASE MEDIATED NECROSIS IN MITOMYCIN-C TREATED CHONDROCYTES

Introduction:
A loss of chondrocyte viability may play a significant role in the progression of OA. Cyclic mechanical impacts on cartilage explants cause local damage to the collagen network, an increase in fibronectin and cell death at the site of impact (1,2) similar to the changes seen in OA lesions in our colony of Labrador Retrievers. Previously, we reported that when 4mm cartilage disks were loaded with a 2mm indenter, initially, cell death was located in the regions of direct impact, but after 21 days in culture post impact, cell death had spread both radially and transversely from the loaded core region into the unloaded surrounding ring. Furthermore, we showed that this spread of cell death was prevented if the ring was separated from the core immediately after loading and cultured separately from the core (3). This suggests release of intercellular signals which propagate a wave of cell death (and possibly matrix damage) after an initial injury.

We have previously reported the fact that the increase in cell death in the cartilage over time is not associated with an increase in the release of glycosaminoglycans or nitric oxide into the culture media (4). We are looking to determine the extent to which the spread of cell death from loaded core to unloaded ring represents apoptosis or necrosis or both. We hypothesise that caspases are the effectors of cell death in our model. To begin testing this hypothesis we induced apoptosis in chondrocyte cultures and tried to prevent death using the caspase inhibitors Z-VAD FMK, a broad spectrum caspase inhibitor and a selective non-peptide inhibitor of the effector caspases 3 and 7.

Methods:
Cartilage was obtained from canine shoulders using a No.10 scalpel blade. After washing in Gey’s balanced salt solution explants were cultured in Ham’s F12 culture medium containing 10% foetal bovine serum for 48 hours at 37°C, 95% humidity and 5% CO2 prior to extraction. Cartilage was cut into small pieces and chondrocytes were extracted in 25 mls of culture medium containing 15mg of collagenase (CLS-1, Worthington). Extraction was overnight at 37°C with constant stirring. The digested cartilage was filtered through a sterile funnel. The filtrate was collected and spun at 1,000 rpm for 10 minutes. Cells were washed once in media, spun a second time and resuspended in 1 ml. Cells were counted and seeded at 1x10^6 cells per T25 flask or at 100,000 cells per well of a 96 well plate. Cells used in this experiment were passage 2 cells. Cells were allowed to adhere to the plate (usually 48 hours) before treatment. Treatments were as follows: (i) media alone (ii) 50µg/ml mitomycin-c (MMC) alone (iii) MMC + 50µM Z-VAD FMK (iv) MMC + 100µM Z-VAD FMK (v) MMC + 50µM caspase 3/7 inhibitor (vi) MMC + 100µM caspase 3/7 inhibitor (vii) DMSO toxicity control (viii) MMC + non-specific inhibitor control peptide. Cells were observed after 24 and 48 hours. In a second set of experiments, Z-VAD FMK was added to chondrocytes that were treated with 50µg/ml MMC and a two-fold dilution series of caspase 3/7 inhibitor (from 100µM – 0.19µM), to test the ability of Z-VAD FMK to rescue the cells from the effects of the caspase 3/7 inhibitor.

Results:
After 24 hours post-treatment, cells cultured in both medium alone and medium + DMSO seemed healthy and spindle-like in shape, as typical for passaged chondrocytes in monolayer cultures. Those treated with MMC, and MMC + the irrelevant inhibitor peptide, were small and rounded in appearance. These cells stained positive in the TUNEL assay and showed nuclear characteristics of apoptosis.

Cells treated with MMC and Z-VAD FMK showed reduced levels of apoptotic cells, in a dose-dependent manner, with a combination of spindle-like and small, rounded apoptotic cells (Figure 1). Those cells treated with the MMC and caspase 3/7 inhibitor behaved differently. They appeared as large, rounded cells (Figure 2). A dose-response curve of the caspase 3/7 inhibitor (two-fold dilution from 100µM - 0.19µM) showed that after 24 hours, concentrations down to 1.56µM induced this large, rounded shape. Cells treated with the lower concentrations appeared as MMC alone (apoptotic). The caspase 3/7 inhibitor alone at all of the above concentrations had no effect on the cells and they behaved as with media alone.

Discussion:
In chondrocyte cultures we have shown that apoptosis induced by MMC can be prevented by using the general caspase inhibitor, Z-VAD FMK, as expected. However, when only the terminal (effector) caspases 3 and 7 were inhibited, the cells were not protected but the morphology was not classical apoptosis. Electron microscopy showed that these large, rounded cells appeared necrotic. This necrotic phenotype was inhibited using Z-VAD FMK indicating that necrosis, in this case, is caspase mediated. Initial studies showed that inhibition of both caspase 8 and 9 can prevent this necrosis, suggesting that caspase 8 and 9 are able to cause necrosis as well as apoptosis and that necrosis may occur if the apoptotic program cannot be completed.


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50th Annual Meeting of the Orthopaedic Research Society
Poster No: 0604