Objective: To characterise the role of phosphatase-1 and -2A (PP1/2A) in the modulation of apoptosis in human osteoarthritis (OA) chondrocytes.

Methods: Human OA chondrocytes were isolated from cartilage obtained from the femoral heads of patients undergoing joint replacement surgery. Cell viability was evaluated by MTT assay. Apoptosis was quantified by ELISA, which measures DNA fragmentation. Nitric oxide (NO) production was evaluated by the Greiss method, and inducible nitric oxide synthase (iNOS) protein synthesis was studied by western blotting.

Results: Inhibition of PP1/2A by the specific inhibitor okadaic acid (OKA) dose and time dependently caused a reduction of cell viability (OKA at 50 nmol/l: a reduction to 60% and 43% at 48 and 72 hours, respectively). Genomic DNA from chondrocytes treated with OKA at 50 and 100 nmol/l for 48 hours displayed increased internucleosomal DNA fragmentation by 11 and 13 fields, respectively. Light microscopy and DAPI studies showed that OKA induced DNA condensation and fragmentation, typical of death by apoptosis. The caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK increased cell viability, reduced by OKA at 50 nmol/l to 87% and 73%, respectively. OKA did not increase iNOS protein synthesis or NO production.

Conclusion: PP1/2A modulate apoptosis in human OA chondrocytes; this is independent of NO production but dependent on caspases.

Cell viability

Cell viability was evaluated in a 96 well culture plate using a colorimetric assay based on the MTT assay (Roche Diagnostics, Mannheim, Germany). Quantification was conducted with an enzyme linked immunosorbent assay (ELISA) reader at 570 nm (Amersham, Buckinghamshire, UK). Both pools of cells, floating and attached chondrocytes, were employed in these experiments as well as in the experiments to analyse apoptosis. The results are expressed as the percentage of MTT cleaved to form a formazan dye per 10⁴ cells (the baseline level is 100%).

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; ELISA, enzyme linked immunosorbent assay; iNOS, inducible nitric oxide synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; OA, osteoarthritis; OKA, okadaic acid; PBS, phosphate buffered saline; PP1/2A, phosphatase-1 and -2A; PPase, protein phosphatases.
Morphological evidence of apoptosis

For morphological studies, chondrocytes were cultured in eight well slides (Costar) and treated with OKA at 100 nmol/l for 24 hours. The cells were then washed with phosphate buffered saline (PBS), fixed in 10% formalin buffered saline for 10 minutes, stained with 4,6-dianidino-2-phenylindole dihydrochloride (DAPI, 2 mg/ml, Sigma) for 30 minutes at 37˚C, mounted in 90% glycerol/PBS, and observed by fluorescence microscopy.

DNA fragmentation ELISA

To measure DNA fragmentation, chondrocytes were seeded at 5x10^4 per well in a 96 well culture plate. After incubation, the nucleosomes were detected in the sample by immunoassay using the cell death detection ELISA (Roche Diagnostics) according to the manufacturer’s instructions. The results are expressed as a percentage of optical density units per 5x10^4 cells (the baseline level is 100%).

Western blot

After appropriate stimulation, the chondrocytes were washed in ice cold PBS, pH 7.5, and harvested in hot lysis buffer. The membranes were incubated overnight with anti-NO synthase (rabbit antihuman inducible nitric oxide synthase (iNOS), 1:1000, BD) in a freshly prepared blocking solution at 4˚C. After washing, detections were made by incubation with peroxidase conjugated secondary antibodies and developed using an ECL chemiluminescence kit (Amersham).

Quantification of nitrates

The NO production of chondrocyte cells was measured by estimating nitrate amounts using the Greiss reagent as previously described. The absorbance at 570 nm was measured and compared with a standard solution of NaNO_3. The production of NO was expressed as μmol of NO_2⁻/5x10^4 cells.

Statistical analyses

The data are expressed as the mean (SEM) from n determinations or as representative results, as indicated. The statistical software program, SPSS, was used to perform analysis of variance or Tukey tests. Differences were considered to be significant at p<0.05.

RESULTS

Inhibition of PP1/2A causes death by apoptosis

The inhibition of PP1/2A by OKA, dose and time dependently caused a reduction in chondrocyte survival (fig 1A). Okadaic...
acid (>20 nmol/l for 48 and 72 hours) caused a decrease in chondrocyte viability. Light microscopy studies showed cells with small bodies that were surrounding the chondrocytes or were budding off from the cell membranes (fig 1B). The staining of the cell with DAPI showed that OKA induced apoptosis. The fragmentation of DNA was confirmed and some cell types. However, in human OA chondrocytes, the inhibition of PP1/2A for 72 hours did not modify the production of iNOS protein in chondrocytes or the production of NO (fig 2).

**DISCUSSION**

As far as we know, this is the first study analysing the role of PPase on the apoptosis of human OA articular chondrocytes. Herein, we elucidate the pathways involved in the OKA triggered apoptosis of human OA articular chondrocytes in culture. We report that OKA-induced apoptosis involves the activation of caspases and DNA fragmentation independently of NO production.

It has been reported that OKA induces apoptosis in most, if not all, animal cells. We have shown that incubation for 48 hours with OKA (50 or 100 nmol/l) induced a large fraction of the cell population to undergo nucleus condensation, cellular fragmentation, and increases in hypoploid cellular populations, all of which are compatible with apoptosis. The death induced by OKA can be caspase dependent or independent. This study demonstrated that OKA induces the mRNA expression of caspase-3, as well as their protein synthesis. Furthermore, both Z-VAD.FMK (pan-caspase inhibitor) and Z-DEVD-FMK (a specific caspase-3 inhibitor) partially prevented the death induced by OKA.

The serine/threonine phosphatases may modulate the phosphorylation state of critical phosphoproteins associated with the activation of NF-κB and the induction of iNOS. It has been reported that the inhibition of PP1/2A stimulates the expression of iNOS and the production of NO in rat astrocytes and as NO is implicated in the apoptosis of human chondrocytes, we decided to evaluate whether the apoptosis induced by OKA in chondrocytes is mediated by NO. Our results showed that the inhibition of PP1A/2A did not induce iNOS expression or NO production. Similar results have been reported in other types of cells, such as rat macrophages, suggesting that different intracellular signalling events may be involved in the induction of iNOS.

Recently, it has been reported that the protein Irod/Ian5 protected Jurkat T cells against OKA-induced apoptosis. Irod/Ian-5 is widely expressed in human tissues and its effect is specific. These data support the idea that serine/threonine PPase are potential targets for new therapeutic agents with applications in some diseases such as OA. In particular, Irod/Ian5 could become a promising candidate for treatment of OA.

In summary, our data support the view that the decreased activity of PP1/2A is an important signalling event in the apoptotic process of human articular chondrocytes. Apoptosis induced by OKA is caspase dependent and NO independent.

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Authors’ affiliations
MJ López-Armada, B Caramés, B Cillero-Pastor, M Lires-Dea´n,
E Maneiro, C Ruiz, F Galdo, F J Blanco, Laboratory of Investigation,
Rheumatology Division, Juan Canalejo Hospital, Xubias 84. 15006-A
Coruña, Spain
I Fuentes, F Galdo, Department of Medicine, Universidade da Coruña,
Spain
Presented in part at the 67th Annual Scientific Meeting of the American
College of Rheumatology, Orlando, FL, October 2003.
Correspondence to: Dr F J Blanco, fblagar@canalejo.org
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M J López-Armada, B Caramés, B Cillero-Pastor, M Lires-Deán, E Maneiro, I Fuentes, C Ruiz, F Galdo and F J Blanco

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