Phosphatase-1 and -2A inhibition modulates apoptosis in human osteoarthritis chondrocytes independently of nitric oxide production

M J López-Armada, B Caramés, B Cillero-Pastor, M Lires-Deán, E Maneiro, I Fuentes, C Ruiz, F Galdo, F J Blanco

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 10^4 cells (the baseline level is 100%).

Abbreviations: DAPI, 4',6-dianidino-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-diamidino-2-phenylindole dihydrochloride (DAPI, 2 μg/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 5×10⁴ 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 5×10⁴ 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 anti-human 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 nitrite amounts using the Greiss reagent as previously described.⁸ The absorbance at 570 nm was measured and compared with a standard solution of NaNO₂. The production of NO was expressed as μmol of NO₂⁻/5×10⁴ cells.

Statistical analyses
The data were 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 (OKA) at 100 nmol/l caused a reduction in chondrocyte survival time dependently (fig 1B). Cells treated with OKA (100 nmol/l) for 24 hours showed changes in the cytoplasmic membrane (budding off) and apoptotic bodies (arrows). Panel (d) shows the typical morphology of an apoptotic nucleus: condensation and fragmentation (arrows; ×40). DNA fragmentation detected by ELISA. Human OA chondrocytes were incubated in a 96 well plate with different doses (10, 20, 50, and 100 nmol/l) of OKA for 48 hours. DNA fragmentation was determined in human OA chondrocytes by cell death detection ELISA in 96 well plates. Data are expressed as percentages with respect to basal conditions, and represent the mean (SE) of four independent experiments in triplicate (*p<0.01 v basal).

Table 1: Effects of caspase inhibitors on viability in OKA treated cultured OA chondrocytes
<table>
<thead>
<tr>
<th>Stimulus</th>
<th>48 Hours</th>
<th>72 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Basal+Z-VAD-FMK</td>
<td>93 (4.4)</td>
<td>94 (5.7)</td>
</tr>
<tr>
<td>Basal+Z-DEVD-FMK</td>
<td>97 (2.8)</td>
<td>96 (5.2)</td>
</tr>
<tr>
<td>OKA</td>
<td>60 (2.5)</td>
<td>43 (1.8)*</td>
</tr>
<tr>
<td>OKA+Z-VAD-FMK</td>
<td>87 (5.0)*</td>
<td>78 (4.5)*</td>
</tr>
<tr>
<td>OKA+Z-DEVD-FMK</td>
<td>73 (4.2)*</td>
<td>58 (2.5)*</td>
</tr>
</tbody>
</table>

Confluent chondrocytic cells were preincubated for 2 hours in the absence or presence of caspase inhibitors at 100 μmol/l (Z-VAD-FMK, a caspase general inhibitor or Z-DEVD-FMK, a caspase 3 inhibitor) both in basal conditions or with OKA (50 nmol/l) for an additional 48 or 72 hours. The data are expressed as percentages with respect to basal conditions, and are the mean (SE) of six independent experiments in triplicate (*p<0.01 v untreated chondrocytes; †p<0.01 v OKA; ‡p<0.05 v OKA).
respectively. Furthermore, the specific caspase-3 inhibitor Z-DEVD-FMK, increasing the viability to 87% and 78%, and 72 hours blocked the effect of OKA (50 nmol/l) on apoptosis induced by OKA, experiments with caspase inhibitors were performed (table 1). Results showed that administering the pan-caspase inhibitor Z-VAD-FMK for 48 and 72 hours blocked the effect of OKA (50 nmol/l) on chondrocyte viability, increasing the viability to 87% and 78%, respectively. Furthermore, the specific caspase-3 inhibitor Z-DEVD-FMK also partially blocked the effect of OKA (50 nmol/l) on chondrocyte viability (table 1).

### Modulation of NO synthesis by OKA

It has been reported that OKA induces NO production in 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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