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

CONCISE REPORT

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.

The importance of phosphorylation and dephosphorylation in intracellular signalling pathways has long been recognised, although attention has focused mainly on the kinases. Reversible phosphorylation is a key mechanism for regulating the biological activity of many human proteins that affect a diverse array of cellular processes, including protein-protein interactions, gene transcription, cell cycle progression, and apoptosis. Recent studies have made it eminently clear that protein phosphatases (PPase) are dynamic and highly regulated enzymes. For example, caspases are important proteins in cell death and the phosphorylation and/or dephosphorylation of caspases themselves, their targets, and their regulators modulates apoptotic pathways.

Phosphatase activity can be explained by the activity of only four principal catalytic subunits, designated PP1, PP2A, PP2B, and PP2C, with broad and overlapping substrate specificities. PP1 has been implicated in the regulation of actions as diverse as glycogen metabolism, synaptic plasticity, cell cycle progression, and smooth muscle contraction. PP2A can dephosphorylate many different substrates in vitro and in vivo. The use of agents that alter the activity of specific PPase seems especially promising for studying the function of PPase in cellular biology. One of these compounds is okadaic acid (OKA) (C_{44}H_{44}O_{13}), which is a potent inhibitor of the purified catalytic subunits of PP1 and PP2A, but is a weaker inhibitor of PP2B, and does not inhibit PPC.

Clearly, PPase are potential targets for investigation in many diseases, including cancer and inflammatory and degenerative diseases. Osteoarthritis (OA) is the most common joint disease. Several studies suggest that the apoptotic death of articular chondrocytes is implicated in the pathogenesis of human OA. However, the role of PP1/2A in the apoptosis of human articular OA chondrocytes has not been studied.

MATERIALS AND METHOD

Chondrocytic cell cultures and cell stimulation

Human OA chondrocytes were obtained, as previously described, from cartilages of patients who were undergoing hip joint replacement surgery. Cells were cultured at 37°C in 5% CO₂ and used within 2–3 weeks at confluency in primary culture. Chondrocytes were seeded into Petri dishes (Costar) for RNA or total protein extraction, or 96 well plates (Costar) for viability and DNA fragmentation studies, or for the measurement of nitric oxide (NO) released into the culture media. When the cells reached confluency, they were made quiescent by 48 hours of incubation in a medium containing 0.5% fetal calf serum. In some cases, cells were preincubated for 120 minutes with inhibitors of the caspases (Z-VD-FMK, a caspase general inhibitor or Z-DEVD-FMK, a caspase-3 inhibitor) and then were stimulated with OKA.

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-diamidino-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 5 x 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 x 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 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 nitrites

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 x 10⁴ 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

Figure 1 Effect of OKA on chondrocyte survival. (A) OKA reduces cell viability. Human OA chondrocytes were incubated in 96 well plates with different doses (10, 20, 50, and 100 nmol/l) of OKA. At the indicated times, cell viability was evaluated in both floating and attached cells using a colorimetric assay based on the MTT assay. Data are expressed as percentages with respect to control conditions, and represent the mean (SE) of four independent experiments in triplicate (*p < 0.05 v control). (B) OKA induces chondrocyte apoptosis. Cells in culture (a and b) and cells stained with DAPI (c and d) are shown and were analysed by light and fluorescence microscopy, respectively. (a and c) Untreated cells in culture show the normal morphology of chondrocytes with normal nuclei. (b and d) Cells treated with OKA (100 nmol/l) for 24 hours. Panel (b) shows 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). (C) 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>
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<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 (2.3)*</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>
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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 < 0.001 v untreated chondrocytes; †p < 0.001 v OKA; †p < 0.05 v OKA).
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 DNA condensation and fragmentation typical of death by apoptosis. The fragmentation of DNA was confirmed and quantified by ELISA. Genomic DNA from chondrocytes treated with OKA at 50 and 100 nmol/l for 48 hours displayed increases in internucleosomal DNA fragmentation by 11 and 13 fields, respectively (fig 1C).

Experiments carried out in our laboratory, but not included as figures in this report, showed that OKA at different concentrations and for different time intervals caused, in a time dependent manner, the up regulation of the executioner of mRNA for caspase-3. There was a rise at 48 hours after stimulation (caspase-3: 240% of basal levels 100%, n = 3, p<0.05). Furthermore OKA at 100 nmol/l for 72 hours reduced procaspase-3 protein (reduction by 48%).

To analyse the functional role of the caspases in the apoptotic process of human articular chondrocytes, apoptosis induced by OKA is caspase dependent and NO independent. 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-VA-D.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. 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-VA-D.FMK (pan-caspase inhibitor) and Z-DEVD-FMK (a specific caspase-3 inhibitor) partially prevented the death induced by OKA.

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