CONCISE REPORT

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 Ruíz, F Galdo, F J Blanco

Ann Rheum Dis 2005;64:1079–1082. doi: 10.1136/ard.2004.034090

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.¹ 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.²

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.¹ 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.¹ One of these compounds is okadaic acid (OKA) (C₄₄H₄₄O₁₃), 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 PP2C.¹

Clearly, PPase are potential targets for investigation in many diseases, including cancer and inflammatory and degenerative diseases.^{3–4} 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.⁵ 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.⁶ 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-VAD-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-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

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; $\times 40$). (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).

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 μ g/ml, Sigma) for 30 minutes at 37°C, mounted in 90% glycerol/PBS, and observed by fluorescence microscopy.⁷

DNA fragmentation ELISA

To measure DNA fragmentation, chondrocytes were seeded at 5×10^4 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 5×10^4 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 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

Table 1 Effects of caspase inhibitors on viability in OKA treated cultured OA chondrocytes

Stimulus	48 Hours	72 Hours
Basal	100	100
Basal +Z-VAD-FMK	93 (4.4)	94 (5.7)
Basal +Z-DEVD-FMK	97 (2.8)	96 (5.2)
OKA	60 (2.5)*	43 (1.8)*
OKA+Z-VAD-FMK	87 (5.0)†	78 (4.5)†
OKA+Z-DEVD-FMK	73 (4.2)‡	58 (2.5)‡

Confluent chondrocytic cells were preincubated for 2 hours in the absence or presence of caspase inhibitors at 100 μ mol/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. * $p \leq 0.001$ v untreated chondrocytes; † $p \leq 0.001$ v OKA; ‡ $p \leq 0.05$ v OKA.

and compared with a standard solution of NaNO_2 . The production of NO was expressed as $\mu\text{mol/l}$ of $\text{NO}_2^-/5 \times 10^4$ 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

Figure 2 Role of NO in apoptosis induced by OKA. (A) Western blot of iNOS in chondrocytes stimulated with OKA (10, 20, 50, and 100 nmol/l) for 72 hours. Interleukin (IL) 1 β was used as a positive control. One representative blot of two experiments is shown. (B) Production of NO by human OA chondrocytes incubated in medium alone or with different doses (10, 20, 50, and 100 nmol/l) of OKA. At the indicated times, the production of NO in the supernatants was analysed by the Greiss reaction. Nitrite production (μ mol/l/50 000 cells) is expressed as the mean (SE) of four experiments performed in triplicate. Of all the stimuli employed, only IL1 was able to induce NO synthesis (* p <0.01).

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% *v* 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 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, 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.⁹⁻¹¹ 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.¹² 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- κ B and the induction of iNOS.¹³ As it has been reported that the inhibition of PP1/2A stimulates the expression of iNOS and the production of NO in rat astrocytes,¹⁴ and as NO is implicated in the apoptosis of human chondrocytes,⁷ 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.¹⁴

Recently, it has been reported that the protein Irod/Ian5 protected Jurkat T cells against OKA-induced apoptosis.¹⁵ 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.

ACKNOWLEDGEMENTS

We thank Dr M Ramallal and Ms Lourdes Sanjurjo and all members of the Department of Orthopaedics and the Tissue Bank of the Complejo Hospitalario Universitario Juan Canalejo for providing cartilage samples. We also thank Beatriz Lema Costa for excellent technical assistance and Ms Pilar Cal Purriños for her expert secretarial assistance.

This study was supported by grants from the Fondo Investigación Sanitaria-Spain; Expediente: 02/1635; 02/1700 and a grant from Secretaria I+D+I. Xunta de Galicia PGIDIT03SAN91605PR. MJ López-Armada and C Ruíz were supported by Ramón y Cajal and Juan de la Cierva postdoctoral programmes, respectively (Ministerio de Educación). E Maneiro was supported by the Ministerio de Sanidad (Contratos Investigadores SNS). B Caramés and B Cillero-Pastor are the recipients of a grant from Secretaria Xeral I+D+I (PGIDIT03PXIC91601PN and PGIDIT02PXIC91604PN). M Lires-Deán is the recipient of a grant from the Fondo Investigación Sanitaria (BEFI-2003).

Authors' affiliations

M J López-Armada, B Caramés, B Cillero-Pastor, M Lires-Deán, E Maneiro, C Ruíz, 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

Accepted 16 December 2004

REFERENCES

- 1 **Honkanen RE**, Goleen T. Regulators of serine/threonine protein phosphatases at the dawn of a clinical era? *Curr Med Chem* 2002;**9**:2055–75.
- 2 **Klumpp S**, Kriegstein J. Serine/threonine protein phosphatases in apoptosis. *Curr Opin Pharmacol* 2002;**2**:458–62.
- 3 **Zakany R**, Szucs K, Bako E, Felszeghy S, Czifra G, Biro T, et al. Protein phosphatase 2A is involved in the regulation of protein kinase A signaling pathway during in vitro chondrogenesis. *Exp Cell Res* 2002;**275**:1–8.
- 4 **Miller C**, Zhang M, He Y, Zhao J, Pelletier JP, Martel-Pelletier J, et al. Transcriptional induction of cyclooxygenase-2 gene by okadaic acid inhibition of phosphatase activity in human chondrocytes: co-stimulation AP-1 and CRE nuclear binding proteins. *J Cell Biochem* 1998;**69**:392–413.
- 5 **Blanco FJ**, Guitián R, Vázquez-Martul E, J de Toro FJ, Galdo F. Osteoarthritis chondrocytes die by apoptosis. *Arthritis Rheum* 1998;**41**:284–9.
- 6 **Maneiro E**, Martín MA, De Andrés MC, López-Armada MJ, Fernandez-Sueiro JL, Del Hoyo P, et al. Mitochondrial respiratory activity is altered in OA human articular chondrocytes. *Arthritis Rheum* 2003;**48**:700–8.
- 7 **Blanco FJ**, Ochs RL, Schwarz H, Lotz M. Chondrocyte apoptosis induced by nitric oxide. *Am J Pathol* 1995;**146**:1–11.
- 8 **Blanco FJ**, Lotz M. IL-1 induced nitric oxide inhibits chondrocyte proliferation via PGE2. *Exp Cell Res* 1995;**218**:319–25.
- 9 **Kolb TM**, Chang SH, Davis MA. Biochemical and morphological events during okadaic acid-induced apoptosis of Tsc2-null ERC-18 cell line. *Toxicol Pathol* 2002;**30**:235–46.
- 10 **Romano E**, Cannata S, Di Bartolomeo S, Spinedi A. Caspase inhibition shifts neuroepithelioma cell response to okadaic acid from apoptosis to an apoptotic-like form of death. *Biochem Biophys Res Commun* 2003;**303**:469–74.
- 11 **Huynh-Delerme C**, Fessard V, Kiefer-Biasizzo H, Puiseux-Dao S. Characteristics of okadaic acid-induced cytotoxic effects in CHO K1 cells. *Environ Toxicol* 2003;**18**:383–94.
- 12 **Liu W**, Akhand AA, Takeda K, Kawamoto Y, Itoigawa T, Kato M, et al. Protein phosphatase 2A-linked and -unlinked caspase-dependent pathways for downregulation of Akt kinase triggered by 4-hydroxynonenal. *Cell Death Differ* 2003;**10**:772–81.
- 13 **Miskolci V**, Castro-Alcaraz S, Nguyen P, Vancura A, Davidson D, Vancurova I. Okadaic acid induces sustained activation of NfκappaB and degradation of the nuclear IκappaBα in human neutrophils. *Arch Biochem Biophys* 2003;**417**:44–52.
- 14 **Pahan K**, Sheikh FG, Namboodiri AM, Singh I. Inhibitors of protein phosphatase 1 and 2A differentially regulate the expression of inducible nitric oxide synthase in rat astrocytes and macrophages. *J Biol Chem* 1998;**273**:12219–26.
- 15 **Sandal T**, Aumo L, Hedin L, Gjertsen BT, Doskeland SO. Iro2/Ian5: an inhibitor of γ-radiation- and okadaic acid-induced apoptosis. *Mol Biol Cell* 2003;**14**:3292–304.