

EXTENDED REPORT

Increased Bcl-2/p53 ratio in human osteoarthritic cartilage: a possible role in regulation of chondrocyte metabolism

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Ann Rheum Dis 2005;64:217–221. doi: 10.1136/ard.2004.022590**Objective:** To determine whether Bcl-2, p53, and Fas/CD95 help to control cartilage metabolism.**Methods:** Six normal and 14 osteoarthritic (OA) cartilage samples were examined, and two zones from each sample showing the least (Min) and most (Max) anatomical damage were selected. Chondrocytes were isolated by sequential enzymatic digestion and freshly processed. Bcl-2, p53, and Fas/CD95 expression was evaluated by immunofluorescence and FACS analysis; the cell cycle was analysed using propidium iodide, and chondrocyte proliferation assessed by [³H]thymidine incorporation.**Results:** Intracellular levels of Bcl-2 were significantly higher in Max (27.5%) than in Min (21%, $p < 0.01$) OA or normal chondrocytes (18.5%, $p < 0.01$). Intracellular p53 expression was significantly decreased in Max (25.5%) compared with Min (37%, $p < 0.01$) OA or normal cartilage (41.5%, $p < 0.05$). Fas/CD95 receptor expression on surface chondrocytes did not significantly differ between OA and normal cartilage. Cell cycle analysis showed that the proportion of activated chondrocytes in the S phase was significantly higher in Max (69%) than in Min (49%) OA or normal cartilage (43%). The prevalence of proliferating chondrocytes progressively increased according to the degree of OA damage (mean (SEM) Min 1247 (260), Max 2423 (460), $p < 0.05$). Chondrocyte [³H]thymidine uptake correlated positively with Bcl-2 ($r_s = 0.62$, $p = 0.009$) and correlated inversely with p53 levels ($r_s = -0.55$, $p = 0.02$).**Conclusions:** Bcl-2 and p53 play a part in apoptosis, but also help to regulate chondrocyte growth and differentiation. Whereas Bcl-2 promotes cell survival, p53 can arrest cell cycle. The data confirm that chondrocyte activity is enhanced in OA and suggest that the increased Bcl-2/p53 ratio sustains the metabolic boost of chondrocytes.

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Accepted 6 July 2004

Osteoarthritis (OA) is generally defined as a degenerative disease of cartilage, leading to progressive loss of chondrocytes and failure of cartilage functions. Chondrocytes, attempting to repair the cartilage breakdown, undergo metabolic activation.¹ Whether chondrocyte activation precedes or follows cartilage damage is matter of debate. Several studies have provided evidence that chondrocyte activation occurs very early in OA. In a canine experimental model of OA,² it has been shown that transection of the anterior cruciate ligament induces first an increase of proteoglycan content of cartilage; then the turnover of cartilage matrix is enhanced, resulting in proteoglycan depletion; and, finally, chondrocytes are lost. Incorporation of radiolabelled substrates *in vitro* is enhanced in human OA chondrocytes,³ and correlates directly with the severity of the disease process.^{4–5} Conceivably, chondrocyte hyperactivity is promoted by an altered local production of factors that modulate cell survival, and several studies have shown that growth factors are increased in OA cartilage.^{6–7}

Among the array of intracellular signals that modulate cell activity, we focused on some transcription factors such as p53, Bcl-2, Fas/CD95, as these proteins both regulate cell survival and programme cell death. The factor p53 has a key role in controlling the cell cycle, in promoting DNA repair, and in causing apoptosis.⁸ Bcl-2 is a mitochondrial protein that prolongs cell survival and prevents apoptosis, which can be induced by downstream events after cell surface Fas/CD95 activation.⁹

It is likely that under physiological conditions the reciprocal balance of these proteins modulates chondrocyte growth and differentiation, whereas their imbalance in OA can drive chondrocyte metabolism towards activation and apoptosis.

PATIENTS AND METHODS

Cartilage specimens

Articular cartilage was obtained, after written informed consent, from 14 patients with OA (aged 58–71 years) undergoing surgery knee replacement. Articular cartilage was subdivided into two zones showing the least (Min) and most (Max) OA damage, and samples were taken from each zone. The articular cartilage in the Min zone resembled normal cartilage, with a translucent, smooth, integer surface; in the Max zone the cartilage surface was yellowish, softened, and fibrillated.

Macroscopic findings were validated by a histological study performed on full-thickness specimens biopsied from each zone and stained with safranin O (fig 1). The degree of microscopic cartilage damage was evaluated using the Mankin grading scheme.¹⁰

Healthy knee cartilage, obtained post mortem after approval of the ethical committee, from six human donors (18–33 years) was also studied.

Chondrocyte isolation

Chondrocytes were isolated as described elsewhere.¹¹ Briefly, chondrocytes were released from the cartilage matrix by hyaluronidase (0.2%, 30 minutes, 37°C; Sigma, Milano, Italy), pronase (0.25%, 90 minutes, 37°C; Sigma), and collagenase (0.2%, 3 hours, 37°C; Sigma) enzymatic digestion. The number of chondrocytes obtained from each zone was variable, ranging from 0.5×10^6 to 1.2×10^6 depending on the amount of cartilage available. More than 95% of the

Abbreviations: IGF, insulin-like growth factor; mAb, monoclonal antibody; OA, osteoarthritis; PBS, phosphate buffered saline

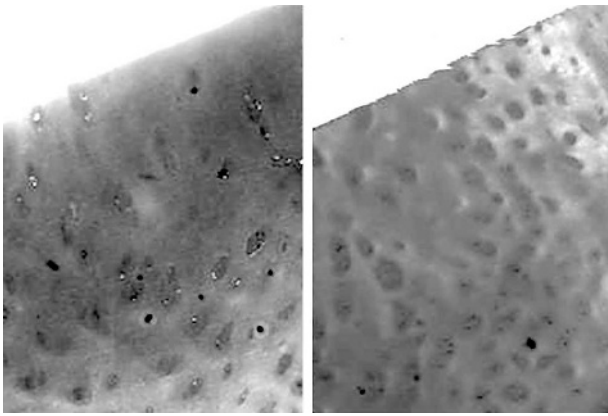


Figure 1 Photomicrographs of safranin O stained histological sections of zones with macroscopically defined minimum (left) and maximum (right) damage in typical OA cartilage (original magnification $\times 60$). A diffuse staining of the cartilage, almost entirely masking the chondrocytes, is present in the minimum zone, while in the maximum zone there is evident hypocellularity together with some clusters of chondrocytes and severe reduction of the safranin O staining.

chondrocytes were viable (trypan blue exclusion test) after their isolation.

Cell cycle analysis

A modification of a procedure previously described¹² was used for cell cycle analysis. After two washes in phosphate buffered saline (PBS), chondrocytes were fixed in paraformaldehyde (0.01% in PBS) for 15 minutes at 4°C. The cells were washed twice and permeabilised by 5 minutes' exposure to 0.6% n-octylglucoside (Sigma, St Louis, USA) at 4°C. Finally, cells were incubated in 200 μ l of DNA staining solution, containing 25 μ g/ml of propidium iodide (Calbiochem) and 2 mg/ml (100 000 U/ml) of RNase (Calbiochem), for 20 minutes at 4°C. Cell cycle analysis was performed by flow cytometry (FACScan, Becton Dickinson, Erembodegem-Aalst, Belgium) using a linear setting for fluorescence 2, and the results were analysed by the CellFIT software program (Becton Dickinson).

Chondrocyte proliferation assay

Chondrocytes from Min and Max OA cartilage were resuspended in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and cultured in 96 well flat bottom microtitre plates (Nunc) at 10×10^3 cells/well at 37°C in 5% CO₂. At 0, 2, and 4 days they were pulsed with 0.5 μ Ci [³H]thymidine (Amersham) and harvested 24 hours later. Proliferation was assessed and [³H]thymidine incorporation expressed as disintegrations per minute (dpm). Each experiment was performed in quadruplicate.

Intracellular Bcl-2 and p53 detection

Freshly isolated chondrocytes were resuspended in PBS containing 0.1% sodium azide and 0.2% bovine serum albumin, and blocked by incubating with 2% normal human serum (Advanced Protein Products, UK). After fixation with paraformaldehyde and permeabilisation with saponin (Fix & Perm Cell Permeabilisation Kit, Caltag Laboratory, Burlingame, CA), chondrocytes were first incubated (20 minutes at 4°C) with 5 μ l of unconjugated mouse antihuman p-53 monoclonal antibody (mAb) IgG1 (Serotec, UK) or mouse antihuman Bcl-2 mAb IgG1 (Serotec, UK), and then washed and incubated with 5 μ l of fluorescein conjugated affinity goat antimouse immunoglobulin F(ab')₂ fragments (GAM-FITC, Becton Dickinson) for 20 minutes at

4°C. Control samples were incubated with mouse IgG1-FITC/IgG2-PE (DAKO, Glostrup, Denmark) and GAM-FITC alone. Stained cells were analysed on a FACScan (Lysis 2, Becton Dickinson). The FACS setting was identical throughout the study and 10 000 chondrocytes were acquired for each sample.

Fas/CD95 chondrocyte expression

CD95 expression on the cell surface was assessed by flow cytometry on non-permeabilised chondrocytes. The cell suspension was first allowed to react (20 minutes at 4°C) with 5 μ l of unconjugated mouse antihuman CD95 mAb IgG1 (Serotec, UK), and then incubated with 5 μ l of fluorescein conjugated affinity goat antimouse immunoglobulin F(ab')₂ fragments (GAM-FITC, Becton Dickinson) for 20 minutes at 4°C.

Statistical analysis

Results are shown as median and range. Results of cell proliferation are shown as mean (SEM). The statistical difference among the distinct specimens of cartilage was assessed with a Mann-Whitney U test. The Spearman test was used for correlation analysis. Significance was set at $p < 0.05$.

RESULTS

Cell cycle analysis

Analysis of the cell cycle carried out on chondrocytes freshly isolated from Min and Max zones of OA cartilage (five samples) and normal cartilage (three samples) showed that the proportion of activated chondrocytes in the S phase (synthesis) was significantly higher in Max chondrocytes (69%, range 52–85) than in Min chondrocytes (49%, range 41–60, $p < 0.001$) or normal chondrocytes (43%, range 33–54, $p < 0.001$). In contrast, resting chondrocytes in the G0/G1 phase were significantly higher in normal (40%, range 29–51) and in Min chondrocytes (31%, range 25–34) than in Max chondrocytes (13%, range 8–21, $p < 0.001$). A similar and not statistically different percentage of G2/M phase chondrocytes was detected in Min (20%, range 10–30), Max (18% 12–31), and normal (17%, range 12–29) chondrocytes.

Chondrocyte proliferation

[³H]Thymidine incorporation of normal (three samples), Min, and Max OA (five samples) chondrocytes was evaluated by short term cultures in vitro. The proliferation was significantly higher in Max than in Min or normal chondrocytes at all times. At day 1, [³H]thymidine uptake was significantly higher in Max (2109 (381)) than in normal (1280 (450), $p < 0.05$) or Min chondrocytes (1160 (402), $p < 0.05$). A similar pattern was present at day 3 (Max 2622 (410), normal 1560 (390), Min 1858 (365), $p < 0.05$) and day 5 (Max 2423 (460), normal 1100 (340), Min 1247 (260), $p < 0.05$).

p53, Bcl-2, CD95 expression on chondrocytes

This set of experiments was performed on six normal and 14 OA cartilage samples. Expression levels of p53, Bcl-2, and CD95 were studied on normal and Min and Max OA chondrocytes by flow cytometry and fig 2 summarises the results. Intracellular levels of p53 were significantly lower in Max OA chondrocytes (25.5%, range 12–35) than in Min OA chondrocytes (37%, range 19–56, $p < 0.01$) or normal chondrocytes (41.5%, range 28–52, $p < 0.05$), but they did not differ significantly between normal and Min OA chondrocytes. Bcl-2 levels were significantly higher in Max OA chondrocytes (27.5%, range 20–45) than in Min OA chondrocytes (21%, range 10–30, $p < 0.01$) or normal chondrocytes (18.5%, range 5–31, $p < 0.01$), and again no difference was detected between normal and Min OA

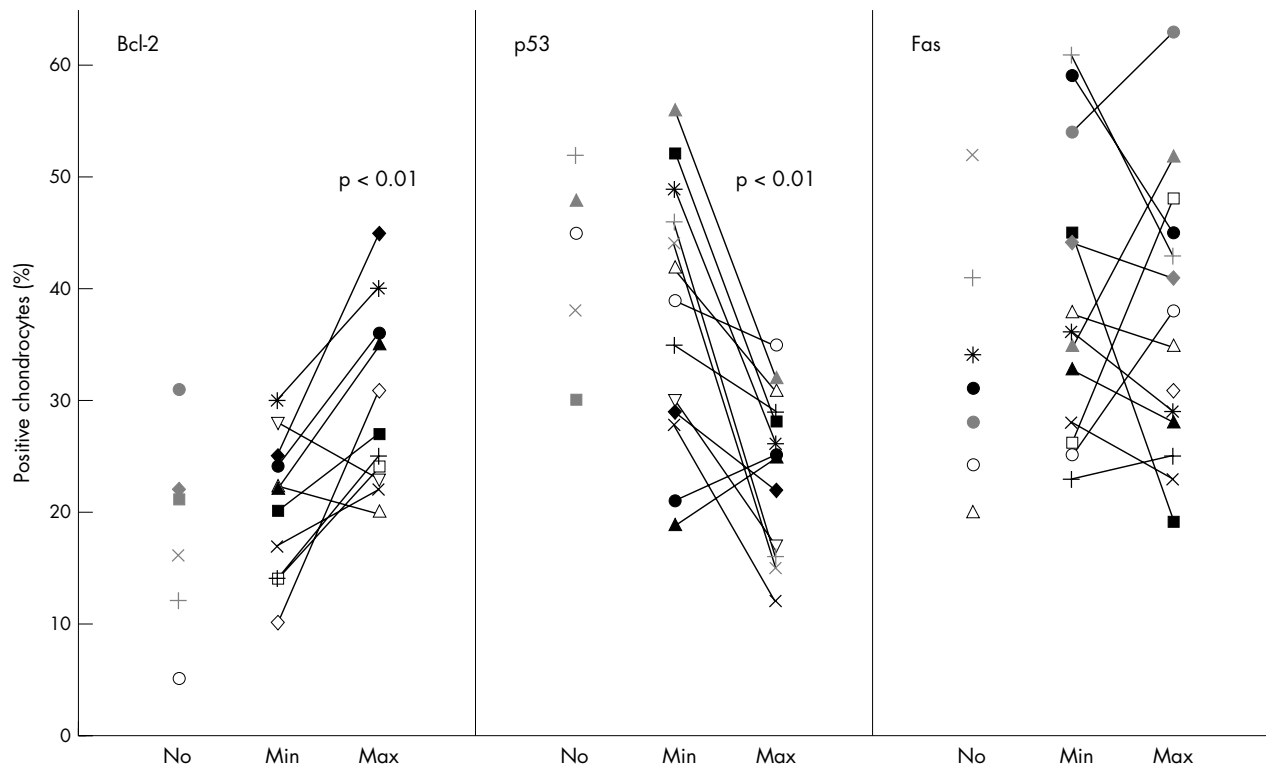


Figure 2 Percentage of chondrocytes expressing intracellular p53 and Bcl-2, and cell surface Fas/CD95 in six normal cartilages (No), and in the lowest (Min) and highest (Max) damaged zones of 14 OA cartilages.

chondrocytes. CD95 was similarly expressed on normal (31%, range 20–52), Min OA (41%, range 23–61), and Max OA (39.5%, range 19–93).

Figure 3 shows the results of a representative FACS experiment demonstrating the intracellular expression of p53 and Bcl-2 and the surface expression of CD95/Fas by

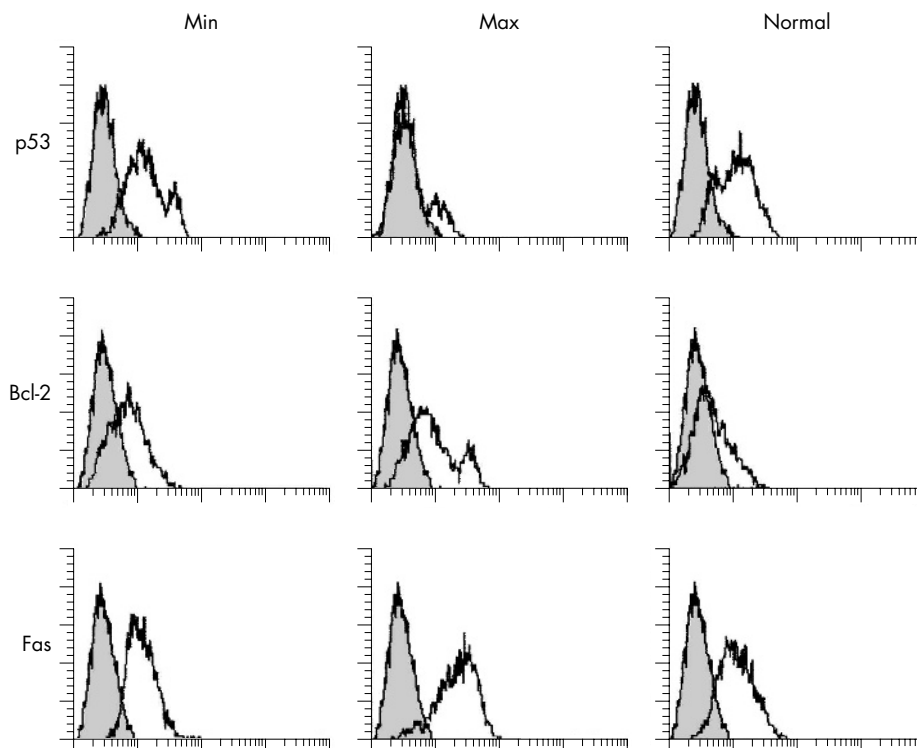


Figure 3 Fluorescence intensity for intracellular p53 and Bcl-2 and surface CD95/Fas from an OA cartilage with minimal (Min) and maximum (Max) anatomical damage, and from a normal cartilage. Negative controls (a non-binding mAb) are shown as grey overlapping histograms. The y axis shows the percentage of positive chondrocytes and the x axis the mean channel fluorescence. Protein expression of p53 is definitely lower in Max than in Min or normal cartilage, and Bcl-2 was higher in Max than in Min or normal cartilage.

Table 1 Correlation between intracellular levels of p53 and Bcl-2 and chondrocyte proliferation assessed by [³H]thymidine uptake in five patients with OA and three normal controls

	[³ H]Thymidine uptake					
	1 Day		3 Days		5 Days	
	OA	Normal	OA	Normal	OA	Normal
<i>p53</i>						
<i>r_s</i>	-0.51	-0.39	-0.25	-0.48	-0.55	-0.41
<i>p</i> Value	0.03	0.2	0.3	0.04	0.02	0.06
<i>Bcl-2</i>						
<i>r_s</i>	0.47	0.35	0.43	0.52	0.62	0.37
<i>p</i> Value	0.05	0.1	0.07	0.03	0.009	0.3

Spearman correlation test: *r_s*, correlation coefficient; *p*, significance level.

normal chondrocytes and chondrocytes isolated from Min and Max damaged zones of the same OA cartilage.

Expression levels of p53 and Bcl-2 correlated with chondrocyte [³H]thymidine incorporation in five patients with OA, irrespective of the degree of OA lesions, and in three normal samples. Table 1 shows that Bcl-2 had a tendency to correlate positively, whereas p53 correlated inversely with chondrocyte proliferation.

Figure 4 shows the global correlation of intracellular levels of p53 and Bcl-2 with chondrocyte proliferation at day 5.

DISCUSSION

The crucial point emerging from our study is that the Bcl-2/p53 ratio is increased in OA cartilage owing to the increase in Bcl-2 and concurrent decrease in p53 levels in chondrocytes. The increased Bcl-2/p53 ratio may represent a reactive attempt to heal the damaged cartilage. These findings correlated with the degree of chondrocyte metabolism as suggested by the increased uptake of [³H]thymidine in vitro and by the high percentage of chondrocytes in the S phase of the cell cycle detected in OA chondrocytes. Although enzymatic digestion to loosen articular chondrocytes may affect the expression of these proteins, minimally (Min) and maximally (Max) damaged cartilage were simultaneously processed and, herein, the differences detected can be still considered significant.

Increasing chondrocyte activity in OA has been already reported by Lippiello *et al*, who showed that the severity of OA correlates positively with the rate of incorporation of [³H]proline in collagen⁴ and proteoglycan synthesis.⁵ These findings are consistent with our results. We found that the

proportion of S phase chondrocytes is significantly higher in Max than in Min OA cartilage. Likewise in our study, the rate of cell proliferation assessed by [³H]thymidine incorporation is significantly increased in Max chondrocytes compared with Min chondrocytes in a 5 day in vitro culture.

The suggestion that during the early stage of OA chondrocytes undergo a metabolic boost has been further corroborated by the findings showing enhanced synthesis of matrix and increase in matrix content preceding the lesional stage in an experimental model of OA.² It appears that a crucial event in this early stage of OA may be an excessive activation of chondrocyte metabolism induced by several growth factors.^{6, 7, 13-15} These growth factors reciprocally interact and their expression is regulated by several signals and molecules. Some molecules, such as p53, Bcl-2, and CD95/Fas, are intriguing, being involved in the regulation of both cell survival and programmed cell death.^{8, 9}

The tumour suppressor gene, p53, is a transcription factor that enhances the rate of transcription of at least six or seven genes involved in controlling cell proliferation and DNA repair.⁸ After DNA injury, p53 is first up regulated and activated, and then it switches on the transcription of the p53 dependent genes to arrest the cell cycle in the G0 phase, to repair DNA damage and, when the latter is irreversible, to commit cell death by activating *bax* and insulin-like growth factor (IGF)-binding protein 3.⁸ *Bax* binds to Bcl-2 and reduces its ability to prevent apoptosis, and IGF-binding protein 3 blocks the mitotic activity of IGF.⁸ Obviously, reduced p53 transcription enhances the cell metabolism; knockout p53 mice are prone to develop cancer as result of the extreme cell activation and loss of the ability to repair

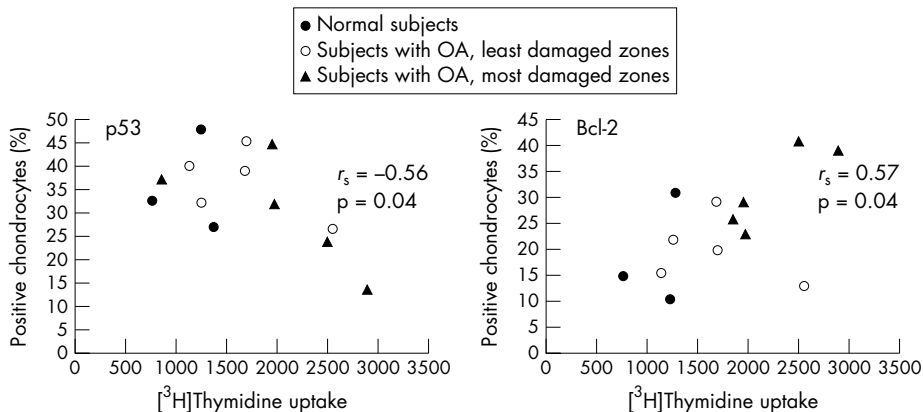


Figure 4 Global correlation of intracellular levels of p53 and Bcl-2 with chondrocyte proliferation at day 5 assessed in three normal subjects and in the least (open circles) and most (closed triangles) damaged zones of 14 OA cartilage specimens. Spearman correlation test: *r_s*, correlation coefficient; *p*, significance level.

DNA damage.⁸ Bcl-2 is a cytoplasmic protein that promotes cell survival and prevents apoptosis.⁹ Its modulation plays a key part in controlling cell growth and differentiation; an abnormal expression of Bcl-2 has been detected in various rheumatic diseases.¹⁶ Fas/CD95 is a cell membrane surface receptor that, when bound to its ligand (FasL), activates apoptosis.⁹

In recent years many authors have shown that the rate of apoptosis is increased in OA cartilage^{17–23} and speculated on the role of apoptosis in the pathogenesis of OA, suggesting possible targets for new therapeutic strategies. In general, decreased Bcl-2²⁴ and high Fas/CD95^{18, 24} expression in OA cartilage have been reported. In our study intracellular levels of Bcl-2 are significantly increased in chondrocytes of Max OA cartilage, but we detected no difference for Fas/CD95 expression. In contrast, p53 levels were significantly lower in OA Max chondrocytes than in OA Min or normal chondrocytes. The imbalance of the ratio p53/Bcl-2 may prompt chondrocytes to activate synthesis processes. This hypothesis is consistent with cell cycle analysis and cell [³H]thymidine uptake, which showed a high rate of synthesis and proliferation in OA Max chondrocytes. Furthermore, in short term chondrocyte cultures, [³H]thymidine incorporation correlated inversely with p53 and correlated directly with Bcl-2 levels.

However, this discrepancy between our data and those reported is only apparent. Kim *et al* reported lower Bcl-2 levels in OA than in normal cartilage by immunohistochemistry,²⁴ but its expression was significantly higher in OA lesional cartilage than in non-lesional cartilage, as we found in our study by flow cytometry. Also, Erlacher *et al* found increased staining of Bcl-2 in OA severe cartilage both by competitive polymerase chain reaction analysis and by immunohistochemistry and flow cytometry.²⁵ Kim *et al*, in the same study,²⁴ detected by immunohistochemistry a higher expression of Fas in the OA lesional cartilage than in the non-lesional cartilage, but no differences between OA and normal cartilage were found. Yet, Hashimoto *et al* reported that OA chondrocytes are more susceptible than normal chondrocytes to apoptosis after stimulation with anti-Fas antibody in vitro, but Fas expression, assessed by flow cytometry, was similar in normal and OA chondrocytes,¹⁸ as in our study.

The diversity of results can be ascribed to the different age of patients and donors, because expression of these proteins by chondrocytes depends on age,²⁶ the different degree of OA progression, different methodologies, and may be intrinsic to the functions of Fas/CD95, p53, and Bcl-2 themselves. These proteins and other proto-oncogenes control both cell proliferation and apoptosis. The mechanisms promoting cell proliferation and leading to apoptosis are strictly coupled and when cell metabolism is activated, the pathway of programmed cell death is already primed to prevent uncontrolled cell proliferation that would lead to an increase of organ size and tumour. Thus, searching for Fas, Bcl-2, p53 expression in active OA chondrocytes will lead to conflicting results depending on the relative prevalence of proliferation or apoptosis in that particular stage of the disease and in that defined area of cartilage. Perhaps apoptosis is not as relevant as previously believed in induction of the OA process. In a recent review, Aigner and Kim highlight the possibility that apoptosis occurs in OA cartilage, but at a very low rate.²⁷ We think that p53, Bcl-2, and Fas/CD95 modulate chondrocyte metabolism, (Bcl-2 has been also reported to regulate chondrocyte phenotype independently of its control of apoptosis^{28, 29}) and that during OA their imbalance can activate cell proliferation and consequently apoptosis, which causes chondrocyte death and hypocellularity of cartilage.

ACKNOWLEDGEMENT

This work was supported by grants from the University of Bari.

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