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Coordinate Down-Regulation of Cartilage Matrix Gene Expression in Bcl-2 Deficient Chondrocytes is Associated With Decreased SOX9 Expression and Decreased mRNA Stability

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Abstract The anti-apoptotic protein Bcl-2 has been shown to function in roles unrelated to apoptosis in a variety of cell types. We have previously reported that loss of Bcl-2 expression alters chondrocyte morphology and modulates aggrecan expression via an apoptosis-independent pathway. Here we show that Bcl-2 is required for chondrocytes to maintain expression of a variety of cartilage-specific matrix proteins. Using quantitative, real-time PCR, we demonstrate that Bcl-2-deficient chondrocytes coordinately down-regulate genes coding for hyaline cartilage matrix proteins including collagen II, collagen IX, aggrecan, and link protein. The decrease in steady-state level of these mRNA transcripts results, in part, from decreased mRNA stability in Bcl-2-deficient chondrocytes. Transcriptional regulation is also likely involved because chondrocytes with decreased Bcl-2 levels show decreased expression of SOX9, a transcription factor necessary for expressing the major cartilage matrix proteins. In contrast, chondrocytes constitutively expressing Bcl-2 have a stable phenotype when subjected to loss of serum factor signaling. These cells maintain high levels of SOX9, as well as the SOX9 targets collagen II and aggrecan. These results suggest that Bcl-2 is involved in a pathway important for maintaining a stable chondrocyte phenotype. J. Cell. Biochem. 88: 941–953, 2003.

Key words: Bcl-2; Sox9; cartilage; chondrocyte; gene expression; quantitative RT-PCR

Chondrocytes are the only cell type found in cartilage and they are solely responsible for the formation, maintenance, and repair of cartilage. Therefore, it is important to the health of cartilage to maintain a stable chondrocyte phenotype in which the cells secrete the appropriate matrix molecules, including collagens II, IX, and XI, aggrecan, and link protein [Poole et al., 2001]. Identifying factors that stabilize and regulate the chondrocyte phenotype is critical for understanding age-related degenerative changes, such as osteoarthritis.

The majority of chondrocytes that form cartilaginous anlagen during development are programmed to terminally differentiate and undergo apoptosis during endochondral ossification [Bronckers et al., 1996; Zenmyo et al., 1996]. However, chondrocytes of the growth plates persist through puberty and chondrocytes at the articular surfaces are maintained throughout life. In the prehypertrophic zone of the growth plate, chondrocytes express the anti-apoptotic protein Bcl-2 [Amling et al., 1997; Wang et al., 1997] and secrete matrix proteins typical of hyaline cartilage [Vornehm et al., 1996]. However, in the hypertrophic phase of chondrocyte differentiation Bcl-2 expression is lost [Amling et al., 1997] and this coincides with terminal differentiation of the chondrocytes which includes expression of type X collagen [Nerlich et al., 1992; Amling et al., 1997], up-regulation of bone-related matrix proteins

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[Gerstenfeld and Shapiro, 1996], and apoptosis [Hatori et al., 1995]. Chondrocyte apoptosis is also increased in osteoarthritis [Blanco et al., 1998; Hashimoto et al., 1998; Heraud et al., 2000] and this is associated with an overall decrease in Bcl-2 expression compared to healthy cartilage [Kim et al., 2000]. We have previously demonstrated that Bcl-2 regulates articular chondrocyte apoptosis in vitro [Feng et al., 1998] and that there is an increase in apoptosis in articular cartilage with aging in vivo [Adams and Horton, 1998]. Significantly, the decline of Bcl-2 expression in osteoarthritic cartilage is temporally associated with changes in matrix gene expression that are similar to the changes in the hypertrophic zone of the growth plate when Bcl-2 expression is lost. This suggests that in both cases there may be a role for Bcl-2 in preventing chondrocyte apoptosis and in maintaining the production of typical hyaline cartilage matrix proteins.

In a variety of cell types it has been demonstrated that Bcl-2 has a role in regulating processes beyond its function as an anti-apoptotic molecule [Delia et al., 1992; Merry et al., 1994; Lu et al., 1995; Chen et al., 1997; Hilton et al., 1997; Lee et al., 1998], and there is some evidence that this is the case for chondrocytes as well. Specifically, chondrocytes expressing low levels of Bcl-2 have decreased aggrecan expression even when the full apoptotic pathway is blocked by inhibiting caspase activity, which demonstrates a novel role for Bcl-2 in regulating the chondrocyte phenotype [Feng et al., 1999]. Additionally, in chondrocytes that are constitutively expressing Bcl-2, aggrecan mRNA expression is maintained when the cells are deprived of serum factors [Feng et al., 1999]. We, therefore, hypothesize that a minimal level of Bcl-2 expression may be important not only in preventing apoptosis, but in maintaining the chondrocyte phenotype so that the correct hyaline cartilage matrix proteins are expressed. It is currently not known whether the modulation of aggrecan expression by Bcl-2 levels is specific for aggrecan or whether there is a coordinate effect on additional cartilage matrix proteins. Nor has the mechanism been identified by which Bcl-2 levels influence aggrecan expression.

Here we demonstrate, in a rat articular chondrocyte Immortalized Rat Chondrocytes (IRC), cell line that a reduction in Bcl-2 level resulting from antisense mRNA expression is associated with a coordinated down-regulation of mRNAs that code for a variety of cartilage matrix proteins including aggrecan, link protein, collagen type II, and collagen type IX. We also show that for aggrecan and collagen II, this down-regulation is effected, in part, by decreased message stability. Conversely, chondrocytes that stably express Bcl-2 generally maintain the expression of collagen type II and aggrecan in the absence of serum factors. Additionally, we analyzed expression of SOX9, a transcription factor that has been identified as critical for maintaining collagen II expression levels and thereby maintaining a stable chondrocyte phenotype. Collagen XI, aggrecan, and cartilage-derived retinoic acid-sensitive protein (CD-RAP) have binding sites for SOX9 as well [Bridgewater et al., 1998; Xie et al., 1999; Sekiya et al., 2000], and it has, therefore, been proposed that SOX9 acts as a master transcription factor for chondrocyte matrix genes [Huang et al., 2001]. We demonstrate that the SOX9 transcription factor is down-regulated in IRC cells that have decreased Bcl-2 expression resulting either from serum factor withdrawal or expression of the antisense transcript, and we demonstrate that SOX9 mRNA shows no difference in stability compared to control IRC cells. These data suggest that Bcl-2 is part of a signaling pathway involved in the coordinate regulation of chondrocyte phenotype.

METHODS

Cell Culture

The IRC cell lines stably transfected with sense or antisense Bcl-2 have been described previously [Feng et al., 1998]. In general, IRC cells were transfected with a plasmid that contained the cytomegalovirus (CMV) promoter upstream of the Bcl-2 coding sequence inserted in the reverse orientation (antisense construct). Following transfection, clonal lines were isolated by antibiotic selection and screened for one of two phenotypes. Antisense clonal lines were isolated that displayed Bcl-2 protein expression at 10–50% of the wild-type cells even when grown in medium containing 20% serum. In addition, sense clonal lines were isolated that had abundant Bcl-2 protein expression that was not altered to a significant extent by serum factor withdrawal [Feng et al., 1998, 1999]. The cells were maintained in Ham's F-12 medium (Gibco BRL, Grand Island, NY) with either 10%

fetal bovine serum (FBS) in the case of wild-type cells and cell lines transfected with sense Bcl-2, or 20% FBS for cell lines with reduced Bcl-2 expression. Prior to experiments, cell lines were cultured in monolayer in Ham's F-12 medium containing 20% FBS for at least 24 h. For mRNA stability experiments transcription was inhibited by culturing the cells for up to 24 h in F-12 medium containing 2 μg/ml actinomycin D (Sigma, St. Louis, MO). For each experiment, RNA samples were derived from two 100-mm culture dishes per time point per cell line. The half-life analysis was performed twice in independent studies and the results were averaged. For serum withdrawal experiments, cells were cultured for up to 48 h in serum-free F-12 medium.

Bcl-2 Protein Expression

Bcl-2 expression level was determined by Western blotting as previously described [Feng et al., 1998]. In general, protein was isolated using standard procedures and quantitated using the BCA protein assay (Pierce, Rockford, IL). The Bcl-2 primary antibody (Transduction Laboratories, Lexington, KY) was diluted 1:1,000 in Blotto (PBS containing 0.05% Tween and 5% non-fat milk). The anti-mouse IgG-HRP conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:2,000 in Blotto. The β-actin primary antibody (Santa Cruz Biotechnology) was diluted 1:1,000 in Blotto, and the anti-goat IgG-HRP conjugated secondary antibody (Santa Cruz Biotechnology) was diluted 1:1,000 in Blotto. Secondary antibodies were visualized by chemiluminescence using Luminol and following the manufacturer's protocol (Santa Cruz Biotechnology). The expression of Bcl-2 was quantitated using IPLab Gel software (Scanalytics, Fairfax, VA) and normalized to the level of expression of β-actin.

Northern Blotting

Total RNA was isolated using Trizol reagent (Gibco). Ten microliters RNA per lane was electrophoresed through a 1% agarose gel containing formaldehyde and transferred to a Nytran SuPerCharge nylon membrane (Schleicher & Schuell, Keene, NH). The following probes were used: (1) an 872-bp cDNA from the 5′ end of the rat aggrecan mRNA [Doege et al., 1986a]; (2) a 778-bp rat link protein cDNA [Doege et al., 1986b]; (3) a 600-bp cDNA from the 5′ region of the rat collagen II mRNA [Kohno et al., 1984]; (4) a 1,179-bp rat Bcl-2 cDNA [Feng et al., 1998]; (5) a 905-bp mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (Ambion, Austin, TX). The probes were labeled with ^{32}P using Multiprime Labeling System (Amersham, Piscataway, NJ). With the exception of the collagen II blot, hybridization was performed as previously described [Feng et al., 1998]. The collagen II blot was pre-hybridized overnight in Denhardt's solution at 42°C. Hybridization was carried overnight and the blot was washed three times, 10 min each, at 56°C using $1 \times SSC +$ 0.1% SDS. Washed blots were exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) overnight and imaged using IPLab Gel software (Scanalytics) or ImageQuant v5.0 software (Molecular Dynamics).

cDNA Synthesis

RNA was treated with DNase (Gibco) to remove genomic DNA, following the manufacturer's protocol. RNA was reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Reverse transcription was performed at 37°C for 60 min

Primer Design and Validation

Primers for quantitative real-time PCR were designed for aggrecan, link protein, collagens II and IX, SOX9, 18S, and GAPDH (Table I) using Primer Express software (Applied Biosystems) and following the manufacturer's recommended parameters. Primer specificity was confirmed using BLAST and primers were synthesized by Fisher Scientific. The utility of 18S as an internal control was confirmed by observing no differences in 18S expression among the cell lines and following experimental treatments. Each primer pair was validated for use with the relative quantitation procedure by performing a standard curve analysis to confirm that the amplification efficiency for each primer pair was equivalent to the 18S amplification efficiency [Applied Biosystems, 1997]. Primer efficiencies were validated across three orders of magnitude using 60ng, 6ng and 0.6ng of input cDNA. The analysis showed that within this range, 18S is a valid normalizer for calculating relative expression levels and beyond this range, calculations are approximate. Efficiencies were validated for the primer concentrations shown in Table I.

TABLE I. Primers Used for Quantitative, Real-Time PCR

Primer	Orientation	Sequence	Concentration (nM)	GenBank accession number
Aggrecan	Forward	5′-CTAGCTGCTTAGCAGGGATAACG-3′	100	NM022190
	Reverse	5′-TGACCCGCAGAGTCACAAAG-3′		
Link protein	Forward	5′-GCTGGATTGGTGCAATGCT-3′	100	NM019189
	Reverse	5′-AGGGCTCACGTGGTTTGG-3′		
Collagen IIα1	Forward	5′-GAGTGGAAGAGCGGAGACTACTG-3′	100	L48440
	Reverse	5′-CTCCATGTTGCAGAAGACTTTCA-3′		
Collagen IXα1	Forward	5′-CTGATGGATTAACAGGACCTGATG-3′	300	S67620
	Reverse	5′-CAGGCTCACCAGGTTCTCCTT-3′		
SOX9	Forward	5′-CGTCAACGGCTCCAGCA-3′	100	NM011448
	Reverse	5′-TGCGCCCACACCATGA-3′		
18S	Forward	5′-AGTCCCTGCCCTTTGTACACA-3′	50	XO1117
	Reverse	5′-GATCCGAGGGCCTCACTAAAC-3′		
GAPDH	Forward	5′-CGGATTTGGCCGTATTGG-3′	300	NM017008
	Reverse	5′-CAATGTCCACTTTGTCACAAGAGAA-3′		

Quantitative PCR

The ABI Prism 7700 Sequence Detection System (Applied Biosystems) was used to run the PCR reactions and collect fluorescence data. Reactions were performed using SYBR Green PCR core reagents (Applied Biosystems) following the manufacturer's protocol. Experiments were run in triplicate using 96-well PCR plates (Applied Biosystems). To confirm the absence of genomic DNA, an aliquot of each RNA sample that had not been reverse transcribed was amplified using each primer pair. This was performed in duplicate or triplicate. To confirm the absence of contamination in the reaction, controls were run (in duplicate or triplicate), for each primer set, in which H_2O was substituted for template. Following the PCR, melting curve analyses were performed using Dissociation Curves v1.Ob1 software (Applied Biosystems) to confirm the absence of primer dimers and to confirm that the correct product was amplified. Amplification data were analyzed using Sequence Detector v1.7 software (Applied Biosystems). Relative expression levels were calculated using the mathematical formulas recommended by Applied Biosystems [Applied Biosystems, 1997].

Statistical Analyses

To report statistics for quantitative PCR, mean normalized cycle threshold (ΔCT) values and the pooled standard deviation of the mean ΔCT were analyzed by one-way ANOVA followed by Dunnett's test [Zar, 1984]. The CT is the fractional cycle number at which fluorescence levels of the cDNA crosses a threshold that is within the linear phase of amplification and that is significantly above baseline fluorescence. The ΔCT is the CT of the target gene normalized by the CT of 18S. The mean ΔCT was calculated from independent experiments, and mean relative expression was calculated from the mean ΔCTs. To analyze quantitative PCR measures of mRNA stability, ΔCT values from two independent experiments were averaged, and mean relative expression was calculated from mean ΔCT values. Decay curves were fitted, and half-lives calculated, using nonlinear regression analysis with a one-phase exponential decay model [Motulsky, 1996]. Regression analysis was performed using GraphPad Prism v3.0 software.

RESULTS

Bcl-2 Protein Expression and Cell Morphology

To determine the effects of Bcl-2 expression levels on cartilage matrix gene expression, we used previously isolated IRC clonal lines with stably integrated plasmids expressing sense or antisense transcripts of Bcl-2 downstream of the CMV promoter [Feng et al., 1998]. These IRC cell lines constitutively express either full-length mRNA coding for Bcl-2, termed "sense" lines, or a 600-bp Bcl-2 sequence from the 5′ end of the Bcl-2 cDNA fragment in reverse orientation, termed "antisense" lines. We have previously reported Bcl-2 expression levels from multiple clonal lines [Feng et al., 1998, 1999], and here we use a subset of those cell lines. Prior to utilizing specific cell lines in the following experiments, we confirmed the expression level of Bcl-2 and the cell morphology. Western blotting showed that in two independent cell lines expressing antisense

Bcl-2, labeled AS-3 and AS-8, the Bcl-2 protein expression was reduced to 56 and 29% of the wild-type IRC value, respectively. Conversely, in three independent cell lines expressing sense Bcl-2 (sense lines 1–3), the Bcl-2 protein expression was increased by 29, 567, and 629%, respectively, compared to a wild-type IRC clonal line. These values were determined densitometrically after normalizing Bcl-2 expression to β-actin. Wild-type IRC cells and cells over-expressing Bcl-2 exhibited a polygonal morphology with abundant extracellular matrix whereas IRC cells expressing antisense Bcl-2 were spindle-shaped and secreted little extracellular matrix (Fig. 1). This morphology was consistent with previous studies [Feng et al., 1999].

Coordinated Down-Regulation of Matrix Gene Expression With Decreased Bcl-2

We have previously demonstrated that decreased Bcl-2 expression, either from serum withdrawal or expression of antisense transcripts, resulted in a decrease in aggrecan expression via a pathway that is independent of full apoptosis [Feng et al., 1999]. Here we determined whether this effect on aggrecan expression was limited to aggrecan or whether down-regulation of Bcl-2 was associated with a general down-regulation of cartilage matrix genes. We isolated total RNA from IRC cells expressing antisense Bcl-2, and probed Northern blots for two additional matrix protein mRNAs, collagen type II and link protein. We also probed for aggrecan to confirm that the cell lines used here continue to show decreased aggrecan expression, as reported earlier [Feng et al., 1999]. Figure 2 demonstrates the observed decrease in the steady-state level of mRNA coding for aggrecan, collagen II, and link protein in both IRC cell lines expressing antisense Bcl-2 compared to wild-type IRC cells, with the AS-8 line (lane 3) showing lower steady-state levels than the AS-3 line (lane 2).

These Northern blot data were confirmed and extended by quantitating expression levels

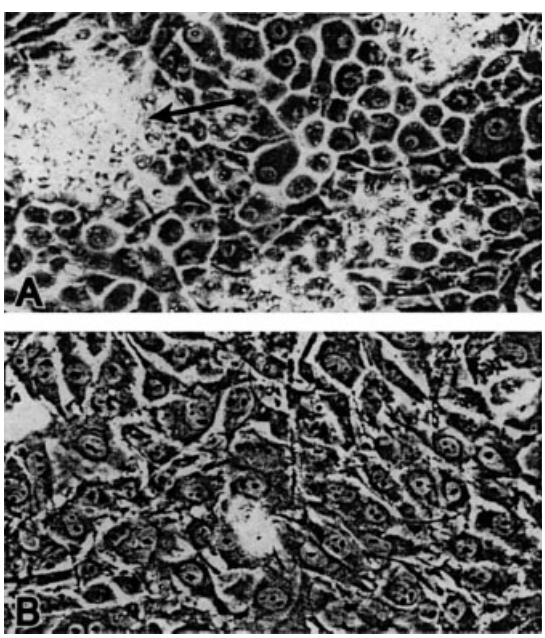

Fig. 1. Morphology of Bcl-2 cell lines. **A**: Representative picture of a sense Bcl-2 cell line: chondrocytes over-expressing Bcl-2 are morphologically indistinguishable from wild-type IRC cells. Arrow indicates a refractile area of intense matrix synthesis. **B**: Representative picture of an antisense Bcl-2 cell line. Cells are fibroblastic in appearance and there is less matrix surrounding individual cells than in A.

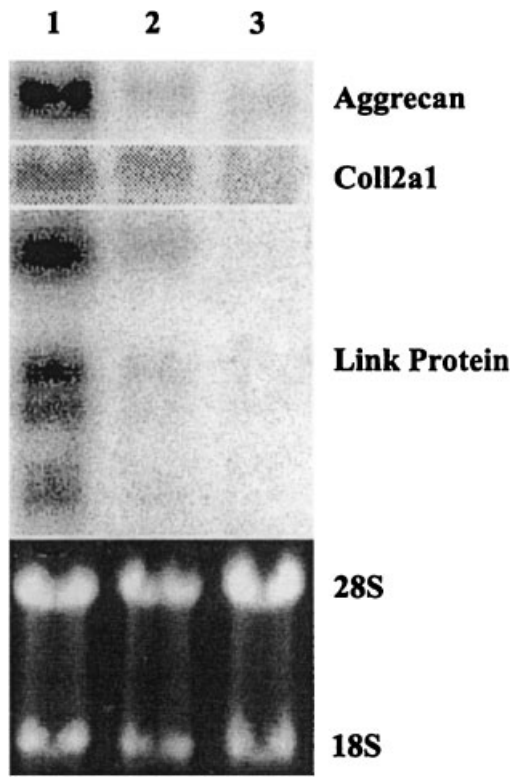

Fig. 2. Chondrocytes expressing antisense Bcl-2 down-regulate cartilage matrix mRNA expression. Antisense Bcl-2 cell lines show decreased mRNA expression of aggrecan, collagen IIα1, and link protein compared to wild-type IRC cells, determined by Northern blotting. Note that link protein has multiple transcripts. A representative picture of 18S and 28S rRNA shows similar loading for each sample. **Lane 1**: Wild-type IRC; **lane 2**, AS-3; **lane 3**, AS-8.

using quantitative real-time PCR. RNA was taken from the same cell lines used for the Northern analyses, and reversed transcribed. We performed PCR using primers for aggrecan, link protein, collagens II and IX, and GAPDH, and collected relative fluorescence data in real-time (during amplification). The expression levels for the two antisense Bcl-2 cell lines were calculated relative to the expression levels of the wild-type IRC cells, which were arbitrarily set to 1 for each gene (Table II). The AS-3 line showed an approximately fivefold decrease in expression level of aggrecan, link protein, and collagen II relative to wild-type IRC cells. The AS-8 line showed an eightfold decrease in aggrecan expression, a 100-fold decrease in collagen type II expression, and a 14-fold decrease in link protein expression, relative to control IRC cells. In addition, both antisense Bcl-2 cell lines expressed collagen type IX at decreased levels relative to wild-type IRC cells. The housekeeping gene GAPDH showed no significant difference among the cell lines.

Matrix Gene Expression Under Serum Withdrawal

We have previously reported that for IRC cells constitutively expressing Bcl-2, aggrecan mRNA expression remains high even when the cells are cultured for 48 h in medium without serum [Feng et al., 1999]. The fact that down-regulating Bcl-2 is associated with a general down-regulation of the major cartilage matrix genes, led us to the hypothesis that constitutively expressing Bcl-2 would have a general protective effect on matrix gene expression in the absence of serum-derived trophic factors. We cultured wild-type IRC cells and three independent IRC cell lines constitutively expressing sense Bcl-2 for 48 h in medium containing 20% serum or no serum. The wild-type IRC cells showed decreased expression of aggrecan and collagen II in the absence of serum (Fig. 3). In contrast, when the sense Bcl-2 cell lines were cultured in 0% serum, they generally maintained expression of the mRNAs coding for aggrecan and collagen II similar to the expression levels observed when cultured in 20% serum. Northern blotting confirmed that all three sense Bcl-2 cell lines maintained expression of Bcl-2 mRNA in the absence of serum at or above the level observed in the presence of 20% serum. In contrast, serum withdrawal resulted in a 64% decrease in Bcl-2 expression in wild-type IRC cells (Fig. 3C).

Decreased Bcl-2 Expression is Associated With Decreased Message Stability of Aggrecan and Collagen Type II

The steady-state level of mRNA transcripts encoding cartilage proteins is regulated by both transcriptional and post-transcriptional mechanisms [Horton et al., 1991; Pearson and Sasse, 1992; Goldring et al., 1994; Murray et al., 2000]. Therefore, we tested whether the down-regulation of the mRNA levels observed with

TABLE II. Quantitation of Matrix Protein mRNA Expression

		WT-IRC	AS-3	AS-8
Aggrecan	Expr	1	0.21	0.12
	ΔCT	7.96 ± 0.40	$10.20 \pm 0.20^{**}$	$11.06 \pm 0.25^{**}$
		n = 6	n = 6	n = 6
Collagen II	Expr	1	0.20	0.01
	ΔCT	7.46 ± 0.02	$9.78 \pm 0.02^{**}$	$14.68 \pm 0.02^{**}$
		n = 4	n = 4	n = 4
Link protein	Expr	1	0.31	0.22
	ΔCT	4.99 ± 0.43	$6.60 \pm 0.21^{**}$	$6.85 \pm 0.38^{**}$
		n = 4	n = 4	n = 4
Collagen IX	Expr	1	0.0002	0.03
	ΔCT	9.36 ± 0.52	$21.31 \pm 0.98^{**}$	$14.49 \pm 0.42^{**}$
		n = 4	n = 4	n = 4
SOX9	Expr	1	0.37	0.38
	ΔCT	6.39 ± 0.21	$7.79 \pm 0.86^{**}$	$7.78 \pm 0.18^{**}$
		n = 6	n = 6	n = 6
GAPDH	Expr	1	1.00	1.79
	ΔCT	10.11 ± 0.30	10.10 ± 0.81^{ns}	9.27 ± 0.59^{ns}
		n = 2	n = 2	n = 2

Relative expression levels of cartilage matrix mRNAs were quantitated using real-time PCR. Antisense Bcl-2 cell line expression levels were calculated relative to wild-type IRC expression levels, which were set at 1. Statistical significance was determined by ANOVA and Dunnett's post-test. Expr, expression level; ΔCT, mean normalized CT value; $^{**}P < 0.01$; ns, not significant.

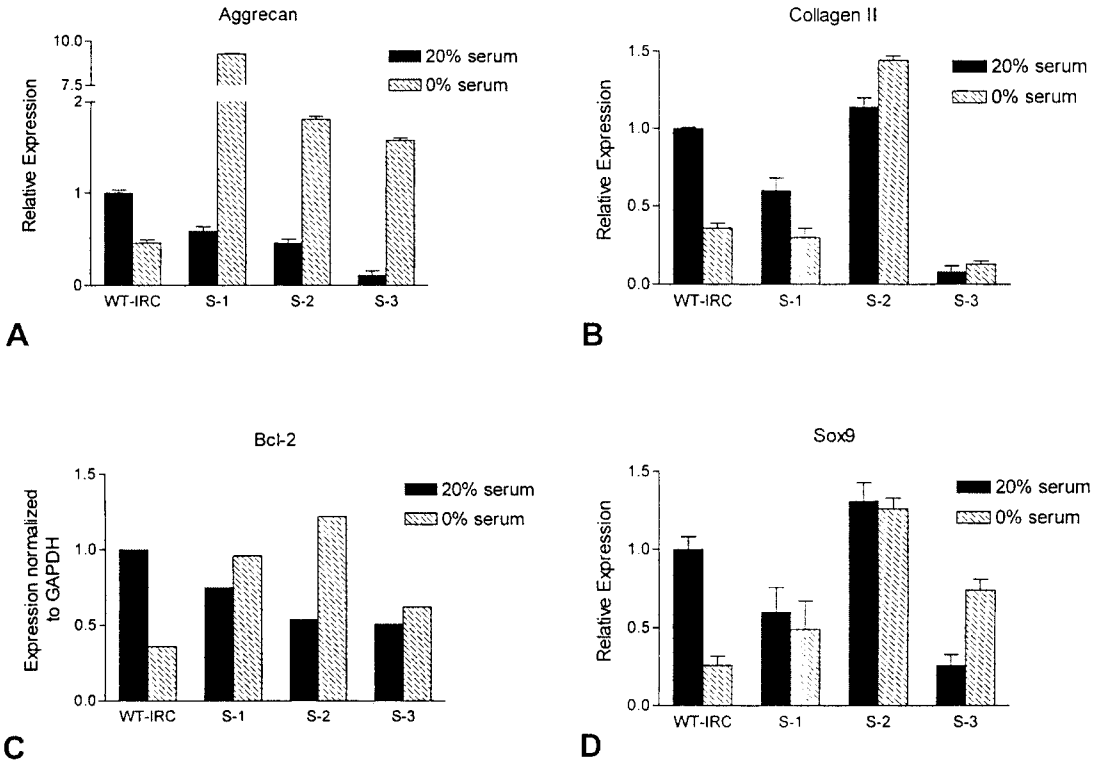

Fig. 3. Chondrocytes stably-expressing Bcl-2 maintain matrix protein and SOX9 mRNA expression in the absence of trophic signaling. IRC cells were cultured for 48 h in medium with 20 or 0% serum prior to RNA isolation. **A**, **B**, **D**: Expression levels of mRNAs were quantitated using real-time PCR and calculated relative to the expression by wild-type IRC cells cultured in 20% serum. Data shown are the mean ± SD of two independent experiments. **C**: Relative mRNA expression was assessed densitometrically following Northern blotting. Data shown are representative of three independent experiments.

decreased Bcl-2 expression results, at least in part, from decreased message stability. Cells were grown to confluency and then treated with actinomycin D, an inhibitor of RNA polymerases. Total RNA was isolated at intervals up to 24 h, reverse transcribed, and quantitated with real-time PCR using primers for aggrecan and collagen type II. Here the amount of cDNA for each gene represents the amount of RNA remaining in the cell following inhibition of transcription, and thus is a measure of message stability. The quantity of each cDNA at time zero was set at 1 for each cell line, and relative message decay rates were compared. The aggrecan message decayed to 50% of the starting level by 4–5 h in the antisense Bcl-2 cell lines as compared to a 9.7 h half-life in the wild-type IRC cells (Fig. 4A). The collagen type II message also showed decreased stability in the antisense Bcl-2 cell lines. In AS-3 and AS-8, the collagen type II mRNA half-life was 2.7 and 1 h, respectively, compared to 7.4 h in the wild-type IRC cells (Fig. 4B).

Down-Regulation of Bcl-2 is Associated With Down-Regulation of SOX9

From the previous experiments, we concluded that the down-regulation of steady-state mRNA levels coding for aggrecan and collagen II in chondrocytes expressing antisense Bcl-2 occurs, at least in part, post-transcriptionally via decreased message stability. However, this does not preclude a role for regulation at the level of transcription, especially since there is abundant evidence that the regulation of collagen II mRNA expression occurs primarily at the level of transcription [Chandrasekhar et al., 1990; Ghayor et al., 2000; Murray et al., 2000]. Several cartilage matrix proteins are known to be transcriptionally regulated by SOX9. The SOX9 transcription factor is required for collagen type II expression [Bronckers et al., 1996; Bell et al., 1997] and also acts on aggrecan [Sekiya et al., 2000], collagen XI [Bridgewater et al., 1998], and CD-RAP [Xie et al., 1999]. It has also been reported that decreased

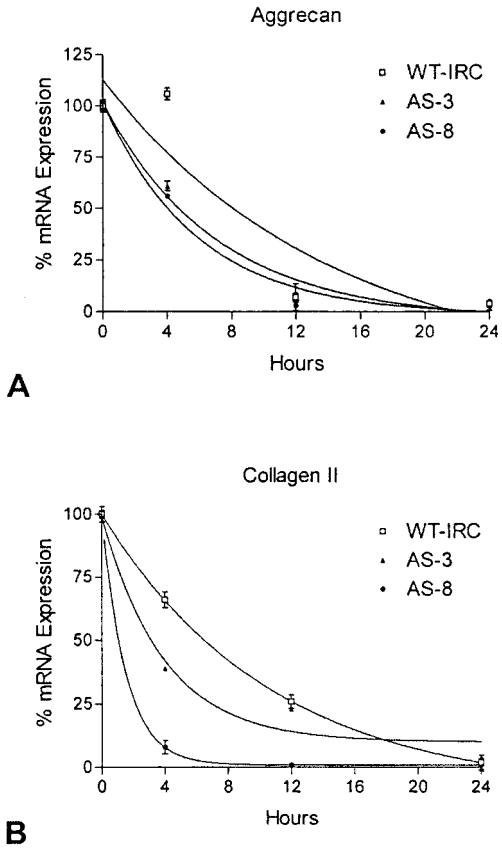

Fig. 4. Matrix protein mRNAs are down-regulated post-transcriptionally in antisense Bcl-2 cell lines. Chondrocytes were treated with 2 μg/ml actinomycin D to block transcription. Relative expression levels were quantitated using real-time PCR. Data points are means ± SD of two independent experiments. Half-lives were calculated by non-linear regression using a one-phase exponential decay equation. Aggrecan mRNA half-lives: WT-IRC, 9.7 h; AS-3, 4.9 h; AS-8, 4.0 h. Collagen II mRNA half-lives: WT-IRC, 7.4 h; AS-3, 2.7 h; AS-8, 1.0 h.

expression of SOX9 causes down-regulation of collagen IX [Bronckers et al., 1996] and link protein [Kolettas et al., 2001], although SOX9 binding sites for these genes have yet to be identified. We, therefore, hypothesized that SOX9 would be down-regulated in the antisense Bcl-2 cell lines. We isolated RNA from the IRC antisense Bcl-2 cell lines, reverse transcribed it, and performed quantitative real-time PCR using primers for SOX9. SOX9 mRNA levels are reduced in both antisense Bcl-2 cell lines compared to wild-type IRC cells (Table II).

Next we asked whether SOX9 is regulated by a post-transcriptional mechanism as was shown above for collagen II and aggrecan. We treated the control IRC and antisense Bcl-2 cell lines with actinomycin D to inhibit transcription and performed quantitative real-time PCR using primers for SOX9. The average half-life of SOX9 mRNA was 1.2 h in both antisense Bcl-2 cell lines and the wild-type IRC cells (Fig. 5). In fact, the mRNA decay curves for each cell line were nearly identical, indicating no difference in message stability between the different cell lines. Finally, we asked whether IRC cell lines with constitutive expression of Bcl-2 would maintain expression of SOX9 in the absence of serum. We grew the sense Bcl-2 cell lines to confluency for 48 h in medium containing no serum. Consistent with our earlier results for collagen II and aggrecan, the sense Bcl-2 cell lines maintained expression of SOX9 in the absence of serum at a level equal or greater to that observed for each cell line cultured in medium containing serum (Fig. 3). We, therefore, conclude that SOX9 is a component of Bcl-2 signaling in chondrocytes.

DISCUSSION

Studies on the growth plate have shown that the expression of both aggrecan mRNA and Bcl-2 mRNA are down-regulated during terminal chondrocyte differentiation [Mundlos et al., 1991; Chen et al., 1995; Amling et al., 1997; Wang et al., 1997]. We have previously demonstrated that down-regulation of aggrecan in chondrocytes with reduced Bcl-2 expression is independent of the apoptotic pathway [Feng et al., 1999]. Here we have shown that Bcl-2 is an upstream mediator of not only aggrecan

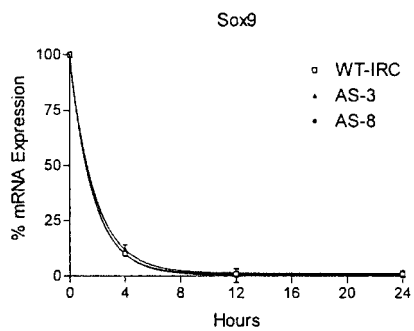

Fig. 5. SOX9 mRNA is not down-regulated post-transcriptionally in antisense Bcl-2 cell lines. Chondrocytes were treated with 2 μg/ml actinomycin D to block transcription. Relative expression levels were quantitated using real-time PCR. Data points are means ± SD of two independent experiments. Half-lives were calculated by non-linear regression using a one-phase exponential decay equation. For each cell line the half-life equals 1.2 h.

expression, but also expression of collagen II, collagen IX, and link protein. Furthermore, we have shown that the modulation of expression of these matrix molecules involves regulation of the transcription factor SOX9 as well as post-transcriptional regulation of matrix gene expression via altered message stability.

The initial hypothesis tested was that chondrocytes in which Bcl-2 protein expression was reduced would show coordinate down-regulation of a variety of cartilage matrix genes in addition to aggrecan. Northern blot analysis and quantitative RT-PCR demonstrated that IRC cells expressing antisense transcripts of Bcl-2, which resulted in a substantial reduction in the Bcl-2 protein level, showed a coordinate down-regulation of collagen II, collagen IX, and link protein mRNAs, along with aggrecan. Coordinated regulation of cartilage matrix molecules has been shown to take place in several different contexts including chondrogenesis within the limb anlagen [Kosher et al., 1986], chondrogenesis during fracture healing [Sakano et al., 1999], and in response to signaling by a variety of growth factors and cytokines [Yasui et al., 1986; Horton et al., 1987; Goldring et al., 1994; Yaeger et al., 1997]. Regulation of cartilage matrix proteins has also been shown to be non-coordinate in some cases, such as during de-differentiation of chondrocytes in culture [Benya et al., 1978; Zaucke et al., 2001] and during the initiation of matrix gene expression at the onset of chondrogenesis during development [Franzen et al., 1987]. Since both possibilities have been reported, the data reported here are important because they suggest that Bcl-2 is part of a pathway involved in coordinating the expression of cartilage matrix genes. It is likely that additional matrix protein mRNAs are down-regulated in chondrocytes with reduced Bcl-2 expression, for example, collagen XI. This collagen, along with collagen IX, is associated with collagen II to form a heterotypic collagen assembly in the cartilage matrix. Previous studies have shown that expression of these three collagens can be coordinately down-regulated by cytokines [Reginato et al., 1993], although studies on the growth plate have shown that collagen IX is independently down-regulated in the hypertrophic zone [Vornehm et al., 1996]. It is reasonable to predict that the expression of collagen XI may be regulated by a mechanism shared with collagens II and IX, and which may be influenced by Bcl-2.

Further evidence that Bcl-2 expression affects the chondrocyte phenotype was provided by experiments using IRC cells that constitutively expressed Bcl-2 due to stable integration of an expression cassette containing the CMV promoter upstream from the cDNA coding for Bcl-2. These cell lines were, therefore, able to maintain expression of Bcl-2 mRNA when challenged with serum-free medium, in contrast to wild-type cells which down-regulated Bcl-2 mRNA. This allowed us to test the hypothesis that maintaining an adequate level of Bcl-2 expression has a protective effect on matrix gene expression in the absence of trophic signaling. In fact, these sense Bcl-2 cell lines were able to maintain expression of collagen II and aggrecan mRNAs when challenged with serum-free medium, as compared to the wild-type IRC cells which responded to the loss of serum factors by down-regulating collagen II and aggrecan mRNAs. Chondrocytes are known to down-regulate matrix production as they age [Buckwalter et al., 1994; Bolton et al., 1999; Verbruggen et al., 2000] and this has been shown to be the result of a decreased responsiveness to trophic factor signaling [Dore et al., 1994; Guerne et al., 1995; Martin et al., 1997]. Our data raise the possibility that this decreased responsiveness is related to decreased Bcl-2 protein with age. Chondrocyte apoptosis increases with age in articular cartilage [Adams and Horton, 1998] and it has been shown in vitro that chondrocyte apoptosis is caused by a decrease in the Bcl-2/Bax ratio [Feng et al., 1998]. Currently, it is not known whether Bcl-2 expression in chondrocytes decreases with age specifically, but it has been shown that Bcl-2 does decrease in the age-related disease osteoarthritis [Kim et al., 2000]. A decline in Bcl-2 expression with age would be consistent with the observed increase in apoptosis as well as the loss of chondrocyte phenotype.

It is important to point out that these data do not support a direct correlation between the level of Bcl-2 mRNA and the expression level of matrix genes. For example, all three of the sense cell lines studied show similar or slightly elevated levels of Bcl-2 mRNA when cultured in serum-free medium compared to when they are cultured in control medium. However, the level of aggrecan mRNA expression is increased 3- to 15-fold following serum withdrawal, depending on the cell line. Likewise, it is important to note that the sense cells do not

necessarily over-express Bcl-2 mRNA as compared to wild-type cells, but do maintain a level of Bcl-2 expression in the absence of serum that is greater than that observed for the wild-type cells. Clearly, for the wild-type cells the only source of Bcl-2 is from endogenous gene expression, whereas the sense cell lines have a steady-state level of Bcl-2 mRNA that is derived from both the endogenous gene and the integrated expression plasmid. Overall, these data are consistent with the hypothesis that a minimal level of Bcl-2 protein is important for supporting the differentiated phenotype of the chondrocytes, especially under conditions of serum factor withdrawal.

We addressed the mechanism by which Bcl-2 expression level influences matrix protein mRNA expression first by examining the stability of the collagen II and aggrecan message in two independent antisense Bcl-2 chondrocyte lines. Studies in which we blocked transcription and calculated decay rates of the specific mRNA transcripts showed that collagen II and aggrecan transcripts in these cell lines have decreased message stability relative to wild-type IRC cells. In the wild-type IRC cells, we established that the half-life for the aggrecan mRNA was approximately 9.7 h. This value was similar to the aggrecan message half-life of 8.5 h reported for bovine articular chondrocytes [McQuillan et al., 1986]. Post-transcriptional regulation of matrix proteins is consistent with previous studies in which we showed that IRC cells down-regulated aggrecan expression post-transcriptionally in response to treatment with vitamin D [Horton et al., 1991]. Next, we examined whether transcriptional regulation was involved by studying expression of SOX9, an important chondrocyte transcription factor. Studies with SOX9 knockout mice have shown that SOX9 is required for chondrogenesis: cells that lack SOX9 fail to express cartilage-specific transcripts [Bi et al., 1999]. Additionally, SOX9 has been shown to be a down-stream target of parathyroid hormone-related protein (PTHrP) signaling in the growth plate, where PTHrP increases SOX9 activity but not its expression [Huang et al., 2001]. PTHrP signaling delays differentiation of prehypertrophic chondrocytes to the hypertrophic phenotype [Lanske et al., 1996; Lee et al., 1996; Vortkamp et al., 1996]. In the hypertrophic zone of the growth plate PTHrP signaling is down-regulated, as is SOX9 expression [Bronckers et al., 1996; Zhao et al., 1997; Eerola et al., 1998], although a direct effect of PTHrP on SOX9 expression has not been established. Significantly, Bcl-2 is also a down-stream target of PTHrP in the growth plate, and like SOX9, is not expressed by hypertrophic chondrocytes [Amling et al., 1997]. It is, therefore, reasonable to propose that Bcl-2 is part of a signaling pathway involved in regulation of cartilage matrix gene expression that includes SOX9 regulation. Here we have shown that decreased Bcl-2 protein levels in chondrocytes results in down-regulation of SOX9 mRNA. This suggests that down-regulation of matrix gene expression in these cell lines may be the result of decreased transcription via SOX9-dependent regulatory sequences in addition to the post-transcriptional regulation described above.

Further support for a role for Bcl-2 in maintaining matrix gene expression via modulation of SOX9 was provided by experiments in which IRC cells constitutively expressing Bcl-2 maintained high levels of SOX9 expression in the absence of serum. This was not surprising, given that these cells were also able to maintain expression of collagen II and aggrecan under the same conditions. We conclude that the influence of Bcl-2 protein expression level on cartilage matrix protein mRNA expression involves the modulation of SOX9 expression. Interestingly, the SOX9 mRNA showed no difference in stability in any of the IRC clones examined, indicating that SOX9 is down-regulated only at the level of transcription. Down-regulation of SOX9 at the level of transcription has been reported following retinoic acid treatment in chondrocytes [Sekiya et al., 2001]. The 1.2 h half-life that we calculated for SOX9 is similar to a recent report of a 2.5 h SOX9 half-life calculated from Northern blot data [Sekiya et al., 2001] and is consistent with the finding of relatively short half-lives of mRNA transcripts coding for transcription factors. Currently, it is known that several growth factors and cytokines can regulate SOX9 expression, but the intracellular mechanisms by which these modulations occur are largely unknown [Healy et al., 1999; Murakami et al., 2000a,b; Kolettas et al., 2001; Uusitalo et al., 2001]. There is evidence that down-regulation of SOX9 by IL-1 and TNF-α is mediated in both cases by the transcription factor NF-$\kappa\beta$ [Murakami et al., 2000b]. It is also possible that additional transcription factors are involved. For example, immediate

early genes such as c-fos, c-jun, and ATF-2 act on chondrocyte-specific genes, and ATF-2 has been shown to be serum-responsive [Beier et al., 2000; Seghatoleslami and Tuan, 2002]. Here we have shown that Bcl-2 is involved in a pathway that modulates SOX9 expression in chondrocytes and that Bcl-2 is required for maintaining a stable chondrocyte phenotype. These findings have implications for the regulation of chondrocyte gene expression during both normal aging and disease.

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