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Evidence of a Direct Role for Bcl-2 in the Regulation of Articular Chondrocyte Apoptosis Under the Conditions of Serum Withdrawal and Retinoic Acid Treatment

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Abstract The regulation of chondrocyte apoptosis in articular cartilage may underlay age-associated changes in cartilage and the development of osteoarthritis. Here we demonstrate the importance of Bcl-2 in regulating articular chondrocyte apoptosis in response to both serum withdrawal and retinoic acid treatment. Both stimuli induced apoptosis of primary human articular chondrocytes and a rat chondrocyte cell line as evidenced by the formation of DNA ladders. Apoptosis was accompanied by decreased expression of aggrecan, a chondrocyte specific matrix protein. The expression of Bcl-2 was downregulated by both agents based on Northern and Western analysis, while the level of Bax expression remained unchanged compared to control cells. The importance of Bcl-2 in regulating chondrocyte apoptosis was confirmed by creating cell lines overexpressing sense and antisense Bcl-2 mRNA. Multiple cell lines expressing antisense Bcl-2 displayed increased apoptosis even in the presence of 10% serum as compared to wild-type cells. In contrast, chondrocytes overexpressing Bcl-2 were resistant to apoptosis induced by both serum withdrawal and retinoic acid treatment. Finally, the expression of Bcl-2 did not block the decreased aggrecan expression in IRC cells treated with retinoic acid. We conclude that Bcl-2 plays an important role in the maintenance of articular chondrocyte survival and that retinoic acid inhibits aggrecan expression independent of the apoptotic process. J. Cell. Biochem. 71:302–309, 1998.

Key words: cartilage; aging; osteoarthritis; programmed cell death; cell culture; human

During skeleton development, the fetal cartilage is replaced by bone through a process that involves transdifferentiation of chondrocytes into bone cells [Galotto et al., 1994] and chondrocyte apoptosis [Hatori et al, 1995; Zenmyo et al., 1996] within the growth plate. In contrast, the chondrocytes residing in articular cartilage survive long after endochondral ossification has ceased. However, there is recent evidence that the apoptosis of articular chondrocytes does occur and increases with age in rats and mice [Adams et al., 1998]. Furthermore, it has been suggested that apoptosis of articular chondrocytes may be related to decreased collagen II in the matrix [Yang et al., 1997] and/or cartilage disease [Blanco et al., 1998]. Many studies have reported a decline in the number of articular chondrocytes with aging in animals and humans [Mitrovic et al., 1983] and it is well established that the incidence of degenerative cartilage disease increases with age [Lethbridge-Cejku et al., 1994]. Therefore, studies into the regulation of articular chondrocyte apoptosis may be critical to our understanding of cartilage aging and disease.

Members of the Bcl-2 family are involved in the regulation of apoptosis in a variety of cell types [Reed, 1995; Korsmeyer, 1996]. There is also evidence that Bcl-2 may regulate cellular function beyond the control of apoptosis, for example, the regeneration of neuronal axons [Chen et al., 1997]. The upregulation of Bcl-2 by parathyroid hormone related-protein delays maturation of growth plate chondrocytes towards hypertrophy and subsequent apoptosis [Amling et al., 1997]. This finding, and the report that Bcl-2 expression may decline in the articular cartilage of collagen II knock out mice [Yang et al., 1997], suggests that Bcl-2 may have a role in regulating articular chondrocyte

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survival as well as maturation of chondrocytes in the growth plate.

Here we report direct evidence that Bcl-2 regulates articular chondrocyte apoptosis using primary human articular chondrocytes and a rat cell line that resembles articular chondrocytes in that it expresses collagens II, IX, and XI, as well as link protein, and aggrecan but not collagen X [Horton Jr et al., 1988; Oxford et al., 1994]. In addition, we demonstrate that the expression of aggrecan, a chondrocyte specific gene, may be regulated both in concert with or independent of apoptosis, depending on the signal.

MATERIALS AND METHODS

Cell Isolation and Culture

The protocol for maintaining Immortalized Rat Chondrocytes (IRC) has been described previously [Horton Jr et al., 1988]. Rabbit articular chondrocytes were obtained from cartilage chips scraped from the tibial plateaus and femoral condyles of 12–24-month-old New Zealand white rabbits. The chips were washed with PBS and the cells were released by sequential digestion at 37°C with 2 mg/ml hyaluronidase (Worthington Biochemical Corp., Freehold, NJ) for 45 min, 2 mg/ml trypsin (Biofluids, Inc., Rockville, MD) for 45 min, and 4 mg/ml collagenase (class 2, Worthington Biochemical) for 3 h. Primary cultures were plated in Ham's F-12 medium (Biofluids, Inc.) containing 10% fetal bovine serum (FBS) and 10 mg/ml gentamicin. Articular cartilage was obtained from the knees of osteoarthritis patients over 70 years of age who are undergoing knee replacement surgery (kindly provided by The Department of Orthopedic Surgery, BayView Medical Center, Baltimore, MD). No patient names or identifiers were provided with the samples. The chondrocytes were isolated and primary cultures established as described above for rabbit chondrocytes. Apoptosis was induced by culturing the cells in serum free F-12 medium or in F-12 medium containing 10% FBS with the addition of 10^{-6} M all-transretinoic acid.

Identification of Apoptosis by DNA Ladder Formation

Adherent cells were collected with a cell scraper and pooled with the floating cells. The genomic DNA was prepared and analyzed by agarose gel electrophoresis as described previously [Adams et al., 1998].

Northern Blotting

Total RNA was isolated by Trizol reagent (GIBCO/BRL, Gaithersburg, MD) following the manufacturers protocol. The total RNA (20 ug/lane)was size fractionated by electrophoresis through a 1.0% agarose gel containing formaldehyde. The following cDNA probes were utilized: 1) a Rat cDNA of Bcl-2 (provided by Dr. John Kusiak, Genebank accession L14680); 2) a rat Bax cDNA (provided by Dr. John Kusiak, Genebank accession L22473); 3) a 1.9 kb cDNA for human Bcl-2 (provided by Dr. R. Srivastava (NIA, NIH); and, 4) an oligonucleotide for human Bax (provided by Dr. Myriam Gorospe, NIA, NIH). The oligonucleotide probe was end-labeled using terminal transferase (Boehringer-Mannheim Biochemicals, Indianapolis, IN). All cDNA probes were labeled by Prime-a-Gene-Labeling System (Promega, Madison, WI). Hybridization with the various probes was carried out overnight at 65°C with Church hybridization buffer. The filters were washed at 65°C with Church wash buffers A and then B, twice for 15 min each, and exposed to X-ray film.

Construction of Cell Lines Overexpressing Sense and Anti-Sense Bcl-2

To construct a plasmid expressing antisense Bcl-2, the pcDNA3 plasmid carrying a 980 bp cDNA of rat Bcl-2 between Hind III and EcoR (provided by Dr. John Kusiak, NIA, NIH) was digested with Hind III/BamH I. The Hind III/BamH I fragment (representing 600 bp of the 5' portion of the rat Bcl-2 cDNA) was isolated and subcloned into the pBluescript II KS+ plasmid (Stratagene, La Jolla, CA). Isolated positive recombinant DNA was digested with Xho I/BamH I to produce a fragment that contained the Hind III/BamH I rat Bcl-2 cDNA. This fragment was subcloned into pcDNA3 between BamH I and Xho I resulting in the insertion of the rat cDNA in reverse orientation downstream from the CMV promoter. The sense and anti-sense constructs were transfected separately into IRC cells using SuperFect transfection reagent (Qiagen, Chatsworth, CA), and cell lines containing integrated plasmid DNA were selected using Geneticin, G418 Sulfate (GIBCO/BRL). Clonal lines were established from isolated colonies for each of the sense and anti-sense expressors. The Bcl-2 protein level was determined by Western blotting. Briefly, collected cells were lysed in SDS Buffer (500 mM

Tris.Cl, pH 6.8, 10% SDS, and 10% glycerol), and the concentration of protein was measured using the BCA protein assay reagent (Pierce, Rockford, IL). The protein was size fractionated through a 12.5% SDS-page gel and transferred to PVDF membrane (NOVEX, San Diego, CA). The Bcl-2 antibody (Transduction Laboratories, Lexington, KY) was diluted 1:250 in block solution (1X TBST containing 5% BSA), and a mouse IgG chemiluminescent Immunoblotting System (Oncogene Science, Manhasset, NY) was used to detect the Bcl-2 following the manufacturer's protocol.

RESULTS

Serum Withdrawal Induced Apoptosis in Primary Human Articular Chondrocytes

Chondrocytes, present in articular cartilage are unique from those in the growth plate in that they survive as a resident cell population throughout the lifespan of the organism. However, there is evidence that articular chondrocytes also undergo apoptosis. In order to study the mechanisms regulating apoptosis of articular chondrocytes, we first examined whether primary cells isolated from the articular cartilage of adult rabbits and aged humans could be induced to undergo apoptosis by the classic signal of serum withdrawal. Human articular chondrocytes cultured for 48 h in medium lacking serum underwent apoptosis as evidenced by the formation of a DNA ladder (Fig. 1). The cells also displayed morphological evidence of apoptotic cells such as cell shrinkage and surface blebbing and similar results were obtained with primary chondrocytes isolated from the articular cartilage of adult New Zealand White rabbits (data not shown).

Bcl-2 and Bax Expression During Apoptosis of Human Articular Chondrocytes Induced by Serum Withdrawal

Members of Bcl-2 family regulate apoptosis in a variety of cell types through a balance of pro-apoptotic molecules such as Bax and anti-apoptotic molecules such as Bcl-2. We next asked if this mechanism was operating in human articular chondrocytes exposed to serum withdrawal. Primary human articular chondrocytes maintained in medium containing 10% serum expressed the expected 8.5 kb and 5.5 kb transcripts of Bcl-2 (Fig. 2 , lane 1). Following 48 h of culture in serum-free medium, the steady state level of both Bcl-2 transcripts was down-regulated (Fig. 2, lane 2). In contrast, the steady-state level of mRNA encoding Bax was expressed at a similar level in cells cultured in the presence or absence of serum (Fig. 2). This result supports the hypothesis that the ratio of Bcl-2 to Bax expression is important for controlling the viability of human articular chondrocytes.

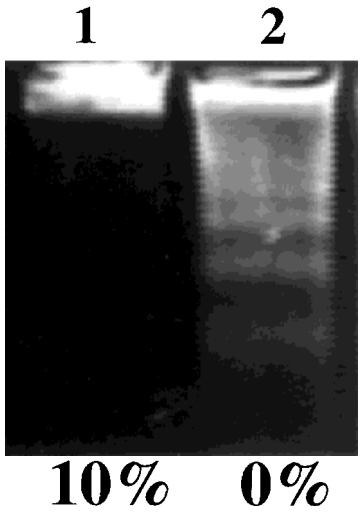

Fig. 1. Induction of apoptosis in human articular chondrocytes by serum withdrawal as evidenced by DNA laddering. **Lane 1:** Primary human articular chondrocytes cultured for 48 h in medium containing 10% serum. **Lane 2:** Primary human articular chondrocytes cultured for 48 h in serum-free medium.

Induction of Apoptosis in a Rat Chondrocyte Cell Line by Serum-Withdrawal or Retinoic Acid

IRC cells have a phenotype similar to articular chondrocytes. Having established that the ratio of Bcl-2 to Bax expression was altered in primary articular chondrocytes undergoing apoptosis, we wanted to determine if apoptosis of IRC cells was regulated in a similar manner. IRC cells cultured for 48 h in serum-free medium also underwent apoptosis as evidenced by the formation of a DNA ladder (Fig. 3, lane 4). Confirming a previous result [Adams et al., 1998], treatment of IRC cells with 10^{-6}M all-transretinoic acid also induced apoptosis (Fig. 3, lane 6).

Bcl-2 and Bax Expression During Apoptosis of IRC Cells Induced by Serum withdrawal

IRC cells maintained in medium containing 10% serum expressed abundant levels of the mRNA for both Bax and Bcl-2 (Fig. 4, lane 1). However, the steady state level of Bcl-2 mRNA

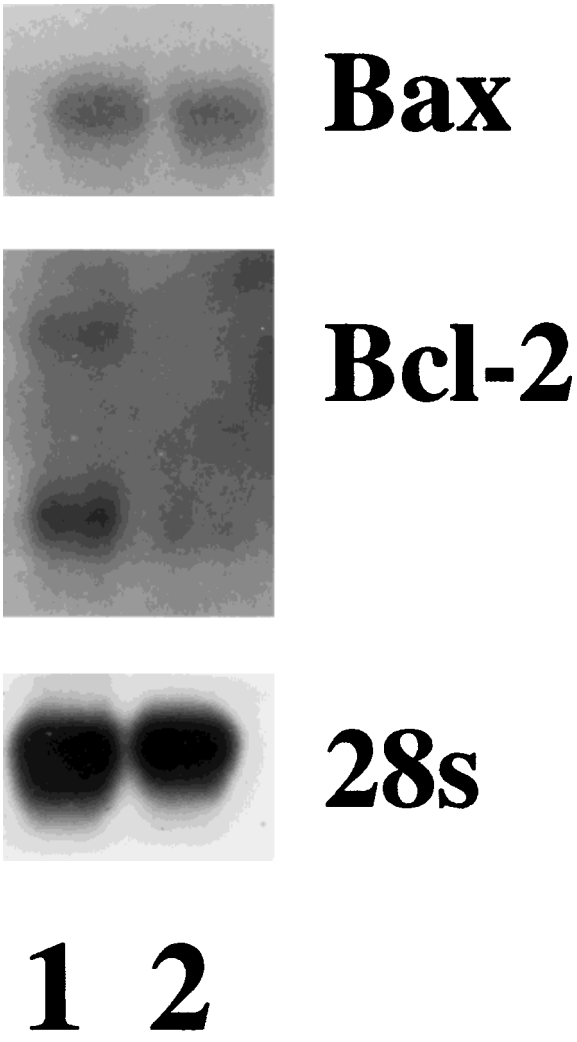

Fig. 2. Bcl-2 and Bax mRNA expression in primary human articular chondrocytes. **Lane 1:** RNA isolated from cells cultured for 48 h in medium containing 10% serum. **Lane 2:** RNA isolated from cells cultured for 48 h in serum-free medium.

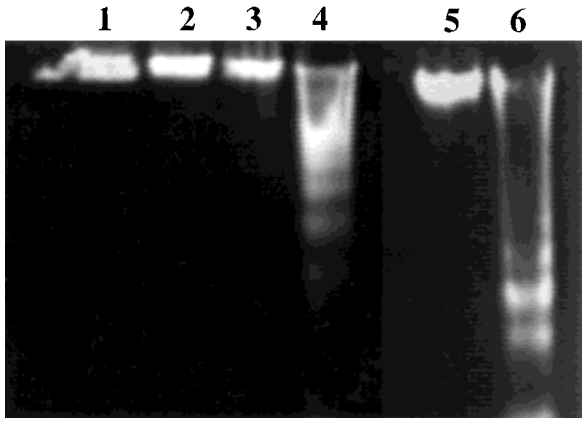

Fig. 3. Induction of apoptosis in IRC cells by serum withdrawal and retinoic acid as evidenced by DNA laddering. **Lane 1:** Cells cultured for 24 h in medium containing 10% serum. **Lane 2:** Cells cultured for 24 h in serum-free medium. **Lane 3:** Cells cultured for 48 h in medium containing 10% serum. **Lane 4:** Cells cultured for 48 h in serum-free medium. **Lane 5:** Cells cultured for 48 h in medium containing 10% serum. **Lane 6:** Cells cultured for 48 h in medium containing 10% serum + 10^{-6} M all-transretinoic acid.

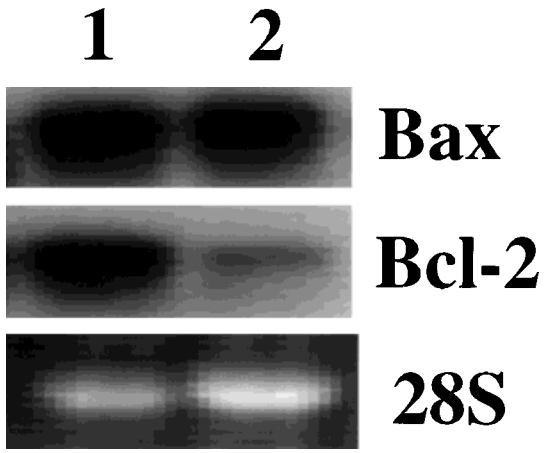

Fig. 4. Bcl-2 and Bax expression in IRC cells in response to serum-withdrawal. **Lane 1:** RNA isolated from IRC cells cultured for 48 h in medium containing 10% serum. **Lane 2:** IRC cells cultured for 48 h in serum-free medium.

was downregulated in IRC cells cultured for 48 h in serum-free medium, while the level of expression of Bax mRNA was unchanged (Fig. 4). This result supports the findings using primary articular chondrocytes and suggests that the IRC line is a good model for further studies on the regulation of articular chondrocyte apoptosis.

Evidence for a Direct Role of Bcl-2 in the Regulation of Chondrocyte Apoptosis

The above data showed that Bcl-2 expression was downregulated by signals that induced apoptosis of both primary human articular chondrocytes and IRC cells suggesting that loss of Bcl-2 is an important factor in triggering apoptosis in articular chondrocytes. However, the expression of other proteins mediating the apoptotic signal could be changing as well. In order to confirm that altered Bcl-2 expression alone was enough to control apoptosis, we created IRC cell lines expressing either sense or antisense Bcl-2 mRNA. Cell lines expressing antisense Bcl-2 grew slowly with a more elongated phenotype as compared to the wild-type IRC cells (data not shown). We determined the level of Bcl-2 expression in four separate cell lines by Western blotting, and found that expression of

antisense Bcl-2 mRNA blocked Bcl-2 expression in all cases (Fig. 5A, lanes 4–6). However, cell lines expressing sense Bcl-2 mRNA appeared similar to the wild-type cells with sustained growth and a rounded morphology. Six separate cell lines were analyzed for the expression of Bcl-2 by Western blotting (Fig. 5B, lanes 3–8), and three cell lines were found to have a high constitutive level of expression of Bcl-2 protein as compared to the wild-type IRC cells (Fig. 5B, lanes 6–8 compared to lanes 1,2). The four-cell lines expressing antisense Bcl-2 all showed spontaneous apoptosis as evidenced by DNA ladder formation even when cultured in medium containing 10% serum (Fig. 6, lanes 1–3 correspond to lanes 4–6 in Fig. 5A; the data for the fourth cell line is not shown). In contrast, two cell lines overexpressing the sense mRNA of Bcl-2 were resistant to the induction of apoptosis by either serum withdrawal (Fig. 6, lanes 7,8) or treatment with RA (Fig. 6, lanes 12,13). These findings support the hypothesis that Bcl-2 plays a direct role in regulating apoptosis of articular chondrocytes.

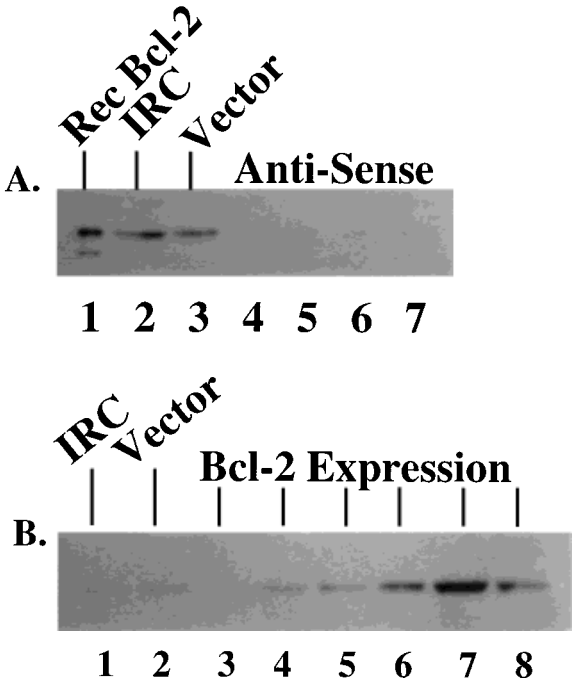

Fig. 5. Western blot analysis of Bcl-2 protein level in IRC cell lines overexpressing sense or anti-sense Bcl-2 mRNA. **A: Lane 1**: Purified recombinant Bcl-2. **Lane 2:** Protein isolated from wild-type IRC cells. **Lane 3:** Protein isolated from IRC cells transfected with the CMV promoter vector containing no insert and selected by resistance to G-418. **Lanes 4–7:** Protein from four different clonal IRC lines obtained by transfection of IRC cells with a construct expressing anti-sense mRNA for Bcl-2 followed by selection with G-418. Note that the development time for the blots used to examine the effects of antisense Bcl-2 was extended in order to detect low level of expression of the endogenous Bcl-2 protein. **B: Lane 1:** Protein isolated from wild-type IRC cells. **Lane 2:** Protein isolated from IRC cells transfected with the CMV promoter vector containing no insert followed by selection with G-418. **Lanes 3–8:** Protein isolated from five different clonal IRC lines obtained by transfection of IRC cells with a construct expressing sense mRNA for Bcl-2 followed by selection with G-418. Note that the development time for the blots used to examine the effects of antisense Bcl-2 was extended in order to detect low level of expression of the endogenous Bcl-2 protein.

The Relationship Between Retinoic Acid Induced Apoptosis and the Regulation of Chondrocyte-Specific Gene Expression

Here and in previous work [Adams et al., 1998] we have demonstrated that RA induces apoptosis of chondrocytes. Previous studies in our laboratory using chick sternal chondrocytes established that RA inhibits the transcription of genes coding for cartilage matrix proteins such as aggrecan [Horton Jr et al., 1987]. These results suggest the hypothesis that the regulation of chondrocyte-specific gene expression may be linked to apoptosis. This hypothesis was tested using the IRC cell line. We first demonstrated that treatment of IRC cells with RA for 24 and 48 h resulted in a decreased level of the aggrecan mRNA in concert with the decreased expression of Bcl-2 (Fig. 7). However, in two separate IRC lines overexpressing Bcl-2, RA treatment still resulted in the downregulation of aggrecan expression even though apoptosis was blocked (Fig. 8).

DISCUSSION

Here we report through two lines of study that Bcl-2 is an important survival factor for articular chondrocytes. First, we demonstrate that the endogenous level of Bcl-2 is downregulated by two diverse signals that initiate chondrocyte apoptosis, serum-withdrawal, and exposure to RA, while the level of the pro-apoptotic molecule, Bax remains unchanged. This result was true for both primary human articular chondrocytes and a rat cell line that is a model for the articular chondrocyte. Second, we used transfected cells overexpressing sense or antisense Bcl-2 to demonstrate a direct correlation between the level of Bcl-2 and chondrocyte survival. The third major point of this paper is the novel finding that the regulation of chondrocyte apoptosis may be separated from the regulation of chondrocyte-specific gene expression as evi-

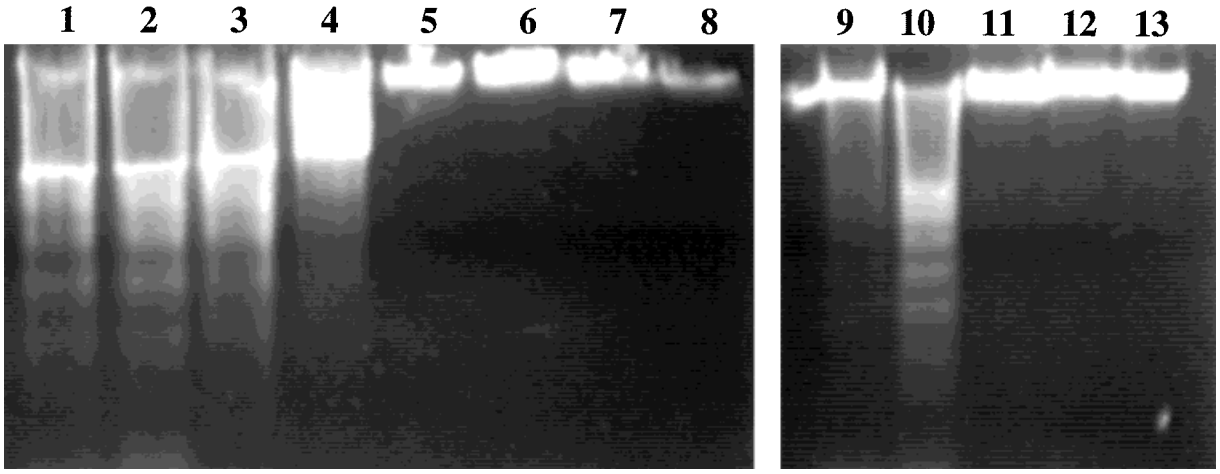

Fig. 6. Correlation between Bcl-2 expression and apoptosis in IRC cells as evidenced by DNA laddering. **Lanes 1–3:** DNA obtained from three different IRC lines over-expressing anti-sense mRNA for Bcl-2 cultured for 48 h in medium containing 10% serum. **Lane 4:** DNA obtained from wild-type IRC cells cultured for 48 h in serum-free medium. **Lane 5:** DNA obtained from wild-type IRC cells cultured for 48 h in medium containing 10% serum. **Lane 6:** DNA obtained from an IRC cell line with the integrated CMV vector containing no insert following culture for 48 h in medium containing 10% serum. **Lanes 7,8:** DNA obtained from two separate IRC lines over-expressing sense mRNA of Bcl-2 following culture for 48 h in serum-free medium. **Lane 9:** DNA from wild-type IRC cells cultured for 48 hrs. in medium containing 10% serum. **Lane 10:** DNA obtained from IRC cells cultured for 48 h in medium containing 10% serum + 10^{-6} M retinoic acid. **Lane 11**: DNA obtained from an IRC line with the integrated CMV vector containing no insert following culture for 48 h in medium containg 10% serum. **Lanes 12,13**: DNA obtained from two separate IRC cell lines over-expressing sense mRNA for Bcl-2 following culture for 48 h in medium containing 10% serum + 10^{-6} M all-transretinoic acid.

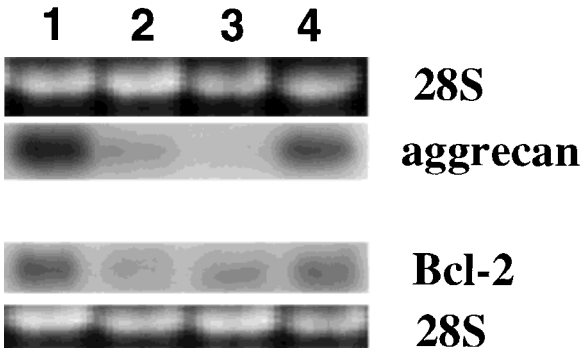

Fig. 7. Expression of Bcl-2 and aggrecan mRNA in IRC cells exposed to all-transretinoic acid. **Lanes 1,4:** RNA otained from IRC cells cultured in medium containing 10% serum for 24 and 48 h, respectively. **Lanes 2,3:** RNA obtained from IRC cells cultured in medium containing 10% serum + 10^{-6} M all-transretinoic acid are controls of IRC cultured for 24 and 48 h, respectively.

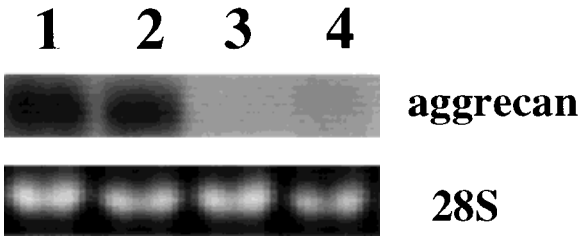

Fig. 8. Aggrecan mRNA expression in IRC cell lines overexpressing anti-sense mRNA for Bcl-2 in response to retinoic acid. **Lanes 1,2:** RNA isolated from two cell lines overexpressing Bcl-2 mRNA following culture for 48 h in medium containing 10% serum. **Lanes 3,4:** RNA isolated from the same two cell lines following culture for 48 h in medium containing 10% serum + 10^{-6} M retinoic acid.

denced by the experiments with RA. This finding has broader implications concerning the relationship between apoptosis and cell-type specific gene expression beyond the chondrocyte model.

One of the classic roles of apoptosis is as a mechanism to eliminate cells that function at one developmental stage but are not needed later in development or in the adult organism [Vaux et al., 1996; Raff et al., 1997]. For example, apoptosis is one mechanism whereby chondrocytes in the growth plate region of the developing skeleton are eliminated during the process of endochondral ossification [Hatori et al, 1995; Zenmyo et al., 1996]. However, articular cartilage is present throughout the life of the organism and it might be assumed that the viability of chondrocytes which support this tissue might be under different regulation than in the growth plate. Recent studies have demonstrated that a certain percentage of chondrocytes present in the articular cartilage die by

apoptosis during aging [Adams et al., 1998] or in response to altered matrix synthesis [Yang et al., 1997] or during cartilage disease [Blanco et al., 1998]. Given these reports it is reasonable to examine the molecular signals that mediate apoptosis in articular chondrocytes. The distinction between growth plate and articular chondrocytes is very important. The cells in the growth plate undergo a continuous differentiation process that results in cell hypertrophy, the production of type X collagen, and the eventual loss of chondrocytes either by apoptosis or transdifferentiation into osteoblasts. However, articular chondrocytes do not show classic hypertrophy and do not produce type X collagen. The finding that the primary human articular chondrocytes and the IRC cells die by apoptosis in response to serum withdrawal is consistent with studies using chondrocytes derived from the distal sternum of adult rats and chick embryos [Ishizaki et al., 1994]. In that study it was found that culture at high cell density inhibited the chondrocyte apoptosis suggesting that autocrine factors maintained cell viability. We have also observed that maintaining human articular chondrocytes at high cell density will prevent apoptosis as will the addition of basic fibroblast growth factor to serum-free medium (data not shown). However, the specific intracellular molecules mediating the apoptotic signal have not been identified in any of these cell models. Studies on the growth plate have demonstrated the importance of Bcl-2 in controlling chondrocyte maturation and apoptosis [Amil et al., 1997], although it is not a universal finding that Bcl-2 is the principal anti-apoptotic molecule in all cell types [Wang et al., 1997]. Therefore our finding that Bcl-2 mRNA expression was downregulated during serum withdrawal, while the expression of Bax remained unchanged, is important in that it establishes a molecular link between the loss of trophic signals present in serum and the induction of articular chondrocyte apoptosis. The report of increased apoptosis and decreased Bcl-2 expression in the articular cartilage of a transgenic mouse line lacking the expression of type II collagen also suggests a similar mode of regulation [Yang et al., 1997]. However, these findings only provide circumstantial evidence for the relationship between Bcl-2 expression and the prevention of apoptosis. In order to show directly that Bcl-2 can regulate chondrocyte apoptosis we utilized IRC cells. Multiple IRC clones with an integrated plasmid expressing Bcl-2 in a constitutive manner were prevented from undergoing apoptosis in response to serum withdrawal or treatment with RA. A consistent result was obtained with the expression of antisense Bcl-2 in IRC where multiple clones showed increased apoptosis in media with 10% serum as compared to wild-type cells. Thus, it appears that the level of Bcl-2, more specifically the ratio of Bcl-2 to Bax, is a direct and important regulator of apoptosis of articular chondrocytes in response to diverse signals as is the case for a variety of cell types [Reed et al., 1995].

The final experiments reported on in this paper address the relationship between the expression of cartilage matrix proteins and chondrocyte apoptosis. Here we report that RA stimulated apoptosis of both primary human articular chondrocytes and IRC cells, with an accompanying decrease in Bcl-2 expression. The induction of apoptosis by RA has been reported for several cell types, including leukemia cells, thymocytes, and neuroblastoma cell lines [Stagno et al., 1997; Szondy et al., 1997; Melino et al., 1997]. Retinoic acid is a very important factor influencing the phenotype of chondrocytes. For example, RA induces hypertrophy and mineralization of chick sternal chondrocytes [Pacifici et al., 1993, 1994]. In addition, previous studies have demonstrated that RA, at the concentration used in this study, also inhibits the expression of genes coding for cartilage matrix proteins such as type II collagen and aggrecan, suggesting that the regulation of these genes might be linked to apoptosis. However, in two separate IRC clones overexpressing Bcl-2, RA inhibited expression of aggrecan in the absence of apoptosis. This finding suggests that RA may have a direct effect on the expression of chondrocyte-specific genes and that the induction of apoptosis is secondary, perhaps through a separate pathway. This seems likely since the RA receptor family consists of cytosolic receptors that bind directly to regulatory sites in gene promoters and enhancers [Holdener et al., 1993; Stunnenberg, 1993; Meier, 1997]. However, the results do leave open the possibility that Bcl-2 may play a role in regulating the expression of genes coding for matrix proteins such as aggrecan in the condition of serum withdrawal where the expression of the two proteins are coordinate. This type of regulation would be consistent with the reported role of Bcl-2 in regulating axonal regeneration inde-

pendent of apoptosis, perhaps through an autocrine growth factor loop [Chen et al., 1997]. This hypothesis will be tested in future studies.

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