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Bcl-2 Regulates Chondrocyte Morphology and Aggrecan Gene Expression Independent of Caspase Activation and Full Apoptosis

Lixin Feng,[1] Richard Balakir,[1] Patricia Precht,[1] and Walter E. Horton, Jr.[2]*

[1]Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224
[2]Department of Anatomy, Northeastern Ohio Universities College of Medicine, Rootstown, Ohio 44272-0095

Abstract Bcl-2 is widely expressed in a variety of cell types and is known to block apoptosis through a conserved pathway. However, recent reports have demonstrated that Bcl-2 regulates cell behavior independent of its control of apoptosis. Chondrocytes express a unique set of matrix proteins, including the proteoglycan aggrecan, and have been widely used to study the relationship between trophic factors and apoptosis. In this article, we report that Bcl-2 affects the morphology and regulates the expression of aggrecan in a rat chondrocyte cell line (IRC). Endogenous Bcl-2 and aggrecan mRNA were both down-regulated in response to serum withdrawal in parental IRC cells, while constitutive expression of Bcl-2 maintained aggrecan levels under conditions of serum withdrawal. In addition, expression of anti-sense Bcl-2 resulted in decreased aggrecan mRNA and produced a fibroblastic morphology compared with parental cells. The caspase inhibitor ZVAD-fmk effectively blocked full apoptosis of IRC cells in response to serum withdrawal or anti-sense Bcl-2 but did not prevent the down-regulation of aggrecan expression from either signal. These results suggest a novel role for Bcl-2 in regulating the differentiated phenotype of chondrocytes and the expression of a differentiation-specific gene independent of its control of apoptosis. J. Cell. Biochem. 74:576–586, 1999.

Key words: apoptosis; chondrocyte; gene regulation; cartilage; aging

Bcl-2 functions in an evolutionarily conserved cell death pathway [Hawkins and Vaux, 1994], protecting a variety of cell types from apoptosis [Korsmeyer, 1995]. Bcl-2 exists as a homodimer or as a heterodimer with other members of the Bcl-2 superfamily, including Bax, which promotes apoptosis [Oltvia et al., 1993; Korsmeyer, 1995; Reed, 1995]. Bcl-2 is thought to function, in part, by blocking the release of cytochrome c from mitochondria into the cytosol and/or inhibiting the activation of caspases, which are cysteine proteases that mediate the downstream apoptotic signal [Patel et al., 1996; Shimizu et al., 1996]. Through its anti-apoptotic function, Bcl-2 affects the morphogenesis of developing limbs [Novack and Korsmeyer, 1994] and the nervous system [Abe-Dohmae et al., 1993; Novack and Korsmeyer, 1994], as well as regulating the homeostasis of the hematopoietic and lymphatic systems [Novack and Korsmeyer, 1997; Hawkins and Vaux, 1997].

However, recent studies have suggested that Bcl-2 may play a role in regulating cell function beyond its control of cell death. For example, the expression of Bcl-2 in developing central and peripheral neurons and its selective retention in the adult peripheral nervous system is consistent with a role in regulating neuron survival, but its expression in some neuronal populations beyond the recognized period of cell death is suggestive of an additional role for Bcl-2 [Merry et al., 1994]. It also has been reported that Bcl-2 promotes the growth and regeneration of retinal axons through a mechanism independent of its anti-apoptotic activity [Chen et al., 1997]. Bcl-2 expression is responsive to extracellular signals and plays a role in regulating proliferation and maturation in some tissues [Bullock et al., 1996; Torcia et al., 1996]. In human mammary epithelial cells, Bcl-2 af-

*Correspondence to: Walter E. Horton, Jr., Department of Anatomy, Northeastern Ohio Universities College of Medicine, 4209 State Route 44, P.O. Box 95, Rootstown, OH 44272-0095.

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fects the phenotype of original epithelial cells and promotes epithelial-mesenchymal conversion [Lu et al., 1995].

Chondrocytes are highly specialized cells that synthesize the extracellular matrix that forms the fetal skeleton and the articular cartilage in the adult [Muir, 1995]. It has been established that Bcl-2 is involved in a feedback loop that controls maturation of chondrocytes in the growth plate [Lee et al., 1995; Vortkamp et al., 1996; Wallis, 1996; Amling et al., 1997]. As cells pass from the proliferative zone into the hypertrophic zone, there is a down-regulation of Bcl-2 expression while Bax expression remains high [Amling et al., 1997] , and at least some of the chondrocytes in this region undergo apoptosis [Bronckers et al., 1996; Zenmyo et al., 1996], while other cells may transdifferentiate into osteoblasts [Roach et al., 1995]. It has been established that isolated chondrocytes require trophic signals to maintain viability in culture [Ishizaki et al., 1994], and we have further shown that Bcl-2 is important for regulating the survival of primary human articular chondrocytes and a rat chondrocyte cell line that resembles articular chondrocytes [Horton et al., 1998; Feng et al., 1998]. Associated with the decline in Bcl-2 expression and increased apoptosis in the hypertrophic zone of the growth plate is a decline in the expression of a chondrocyte-specific proteoglycan known as aggrecan [Mundlos et al., 1991; Chen et al., 1995]. Retinoic acid, which induces chondrocyte apoptosis, also down-regulates aggrecan expression [Feng et al., 1998], suggesting that there may be a functional link between Bcl-2 expression, apoptosis, and the regulation of chondrocyte-specific gene expression. In order to study this relationship, we used clonal isolates of a rat chondrocyte cell line (IRC) with stably integrated plasmids expressing either sense or anti-sense transcripts of Bcl-2. Parental IRC cells cultured in the absence of serum showed decreased aggrecan expression in parallel with the previously reported decline in Bcl-2 level. Further, it was observed that anti-sense Bcl-2, in the absence of any external apoptotic signal, also resulted in the down-regulation of aggrecan expression. This altered biochemical phenotype was accompanied by a dramatic change in cell morphology. Conversely, overexpression of Bcl-2 blocked the loss of aggrecan expression induced by serum withdrawal. Most significant was the fact that this inhibition of aggrecan expression occurred in the cell lines overexpressing anti-sense Bcl-2 transcripts or in parental cells exposed to serum withdrawal, even when full apoptosis was blocked by inhibiting the downstream caspases with ZVAD-fmk. These results provide evidence for a novel pathway operating in chondrocytes involving the regulation of aggrecan gene expression and chondrocyte morphology by Bcl-2.

MATERIALS AND METHODS

Cell Culture

The isolation and maintenance of the immortalized rat chondrocyte (IRC) line were been previously described [Horton et al., 1988]. For serum withdrawal experiments, 5×10^6 IRC cells were seeded in 100-mm dishes containing 10 ml F-12 medium with 10% fetal bovine serum (FBS). After 24 h, the cells were switched to serum free F-12 medium and cultured for an additional 48 h. Control cells received fresh F-12 medium with 10% FBS.

Confirmation of Bcl-2 Level in IRC Cell Lines Overexpressing Sense and Anti-Sense Transcripts of Bcl-2

We have reported on IRC cell lines established by stably integrating plasmids expressing either sense or anti-sense transcripts of Bcl-2 from the CMV promoter in IRC cells [Feng et al., 1998]. After extended passage, and before use in this study, the expression level of Bcl-2 in multiple clonal isolates was confirmed by western blotting as previously described [Feng et al., 1998].

RNA Preparation and Northern Blotting Analysis

Total RNA was isolated using the guanidinium isothiocyanate method [Chomczynski and Sacchi,1987]. Aliquots of 20 µg of each RNA sample was size-fractionated on 1% agarose gels in 1× MOPS buffer containing 4% formaldehyde, and blotted on GeneScreen Plus (NEN Research Products). The filters were incubated with an 872-base pair (bp) cDNA probe from the 3′ end of the mRNA for rat aggrecan [Horton et al., 1991] labeled by ^{32}P using a random primer labeling kit (Promega, Madison, WI). The hybridization was carried out in Church hybridization buffer [Church and Gilbert, 1984] at 65°C overnight, and the filter was then washed in wash buffer A, followed by wash buffer, B each

for 30 min. The filters were then exposed to Kodak XAR5 film.

Inhibition of Apoptosis by ZVAD-fmk and DNA Fragmentation Assay

Where indicated, the tripeptide inhibitor Z-Val-Ala-Asp (O-Me)-fluoromethylketone (ZVAD-fmk, Alexis Biochemicals) was added to culture medium at a final concentration of 50 μM to inhibit the ced-3/ICE-family of proteases (caspases) and block apoptosis. For DNA ladder analysis, cell pellets were treated with a lysis solution (Gentra Systems), followed by treatment with RNase A at 37°C for 1 h. Next, a protein precipitation solution (Gentra Systems) was added to the cell lysate, followed by vortexing for 25 s and by centrifugation at 10,000*g* for 10 min. The superior phase was collected, and the genomic DNA was precipitated with isopropanol. Aliquots of 20 μg of genomic DNA were size-fractionated through a 1.5% agarose gel by electrophoresis. The DNA was visualized with ethidium bromide.

RESULTS

Expression of Sense or Anti-Sense Transcripts of Bcl-2 Is Accompanied by Alterations in Cell Morphology

We previously reported that Bcl-2 expression was down-regulated in primary human chondrocytes and IRC cells in response to apoptotic signals such as retinoic acid or serum withdrawal, while the expression of Bax was unchanged [Feng et al., 1998]. These studies were accompanied by additional work using stable IRC cell lines expressing either sense or anti-sense transcripts of Bcl-2. These lines have now been maintained under G-418 selection for multiple passages.We report that the level of Bcl-2 expression has remained constant for the various clonal lines (Fig. 1). Parental IRC cells or a line with an integrated CMV promoter vector express abundant Bcl-2 protein (Fig. 1, lanes 1 and 2). The level of endogenous Bcl-2 expression in four independent lines with an integrated construct expressing anti-sense Bcl-2 is generally reduced compared with the parental cells (Fig. 1, lanes 3–6). Conversely, in four independent cell lines with an integrated construct expressing full-length Bcl-2, there is abundant protein (Fig. 1, lanes 7–10). We chose the cell lines with the most dramatic differences in endogenous Bcl-2 expression levels for further analysis as described in this report.

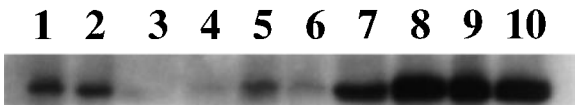

Fig. 1. The level of Bcl-2 protein detected by Western blotting is stable with extended passage in various IRC lines expressing either sense or anti-sense transcripts of Bcl-2. **Lane 1**, total cellular protein isolated from parental IRC cells; **lane 2**, total cellular protein isolated from an IRC line with an integrated plasmid containing the CMV promoter without an insert; **lanes 3–6**, total cellular protein isolated from four separate clonal IRC lines with a stably integrated plasmid that contained the CMV promoter driving the production of anti-sense Bcl-2; **lanes 7–10**, total cellular protein isolated from four4 separate clonal IRC lines with a stably integrated plasmid that contained the CMV promoter driving the production of sense transcripts of Bcl-2.

We have observed consistent differences in the morphology of the cells expressing the anti-sense Bcl-2 transcripts as compared with the lines expressing the sense transcript or the parental cells (Fig. 2). Parental IRC cells cultured in medium containing 10% serum grew as well-attached cells with polygonal morphology (Fig. 2A). These cells formed colonies when plated at low cell density and elaborated an abundant refractile extracellular matrix that stained strongly with Alcian Blue at low pH, indicative of the accumulation of the cartilage proteoglycan aggrecan (data not shown). IRC cells expressing anti-sense Bcl-2 were fibroblastic in appearance and did not form discrete colonies (Fig. 2B). Small, round detached cells undergoing apoptosis were observed even in cultures maintained in medium containing 10% serum. By contrast, IRC cells expressing the Bcl-2 sense transcript more closely resembled the parental cells, existing as well attached polygonal cells elaborating an extracellular matrix (Fig. 2C). In addition, these cells consistently formed colonies larger in size than what was observed with the parental cells. The various phenotypes shown in Figure 2 were observed with multiple clonal isolates for each of the sense and anti-sense cell lines.

Loss of Bcl-2 Expression Is Correlated With Down-regulation of Aggrecan Expression

IRC cells have a highly differentiated phenotype and express the major cartilage matrix proteins, such as collagen II, collagen XI, link protein, and aggrecan [Horton et al., 1988; Oxford et al., 1994]. We determined the level of aggrecan mRNA expression in multiple clonal IRC lines expressing either anti-sense or sense Bcl-2 mRNA when cultured in medium contain-

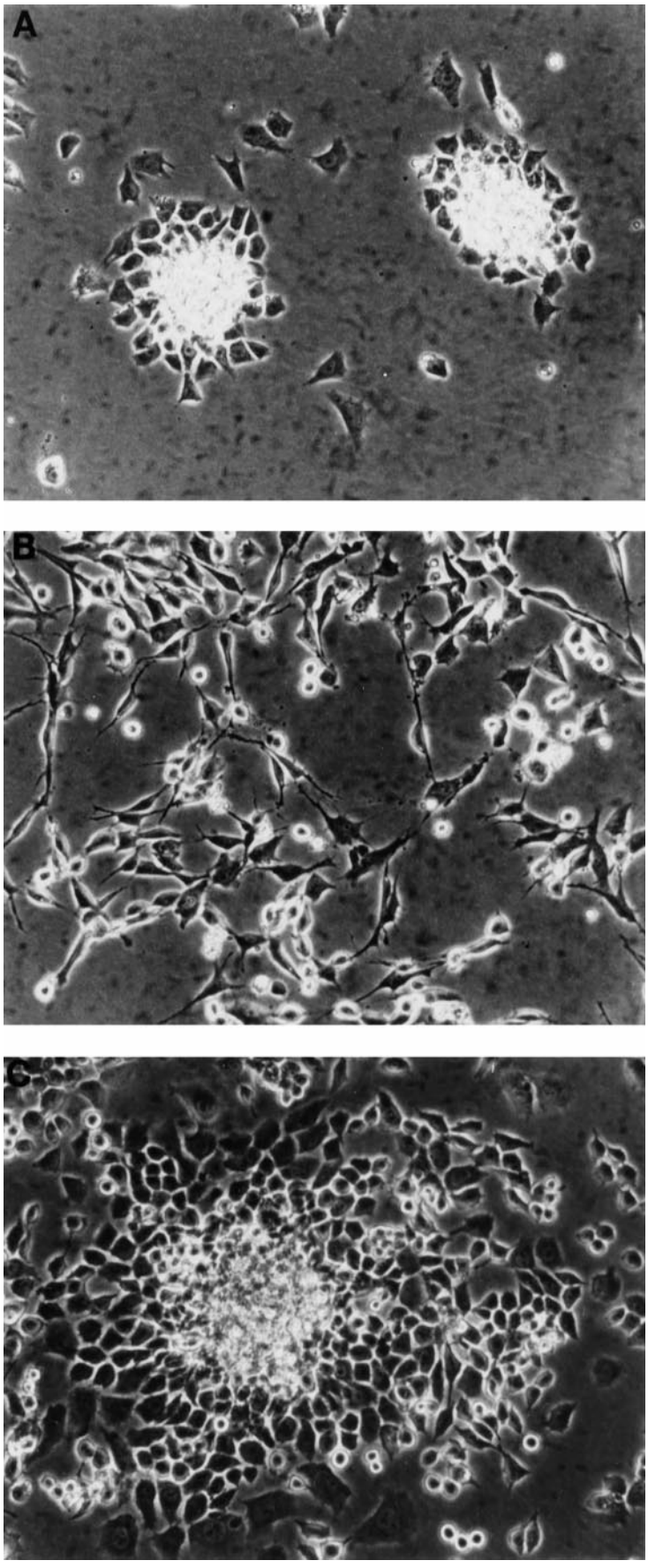

Fig. 2. The expression level of Bcl-2 is correlated with differences in the morphology of IRC cells. Phase-contrast photomicrographs of **(A)** parental IRC cells; **(B)** a clonal IRC line expressing antisense Bcl-2; and **(C)** a clonal IRC line expressing sense Bcl-2. The cultures were all seeded at the same cell density (3×10^6 cells/100-mm dish) and maintained in medium with 10% serum for 5 days. The final magnification for all three images is $\times$400

ing 10% serum. Northern blotting showed that overexpression of anti-sense Bcl-2 mRNA with the resulting loss of Bcl-2 protein expression lead to down-regulation of aggrecan expression in three separate clonal isolates (Fig. 3, lanes 3–5). The overexpression of sense BCl-2 did not significantly alter the expression of aggrecan in separate clonal isolates cultured in medium containing 10% serum (Fig. 3, lanes 6–8), as compared with the parental IRC cells (Fig. 3, lane 1) or cells transfected with a control plasmid (Fig. 3, lane 2). These results suggest that a minimum level of Bcl-2 expression may be required to maintain aggrecan expression.

Serum Withdrawal Is Associated With Decreased Expression of Aggrecan That Is Prevented by Overexpression of Bcl-2

We previously reported that, in both primary chondrocytes and IRC cells exposed to serum withdrawal, the expression of Bcl-2 is down-regulated and the cells undergo apoptosis. Thus, the cell lines with constitutive overexpression of Bcl-2 exposed to serum withdrawal provide a good model with which to explore a potential causal relationship between Bcl-2 and aggrecan expression. An IRC line containing an integrated vector plasmid minus an insert expressed abundant aggrecan mRNA when cultured in medium containing 10% serum (Fig. 4, lane 2). However, when this control IRC line

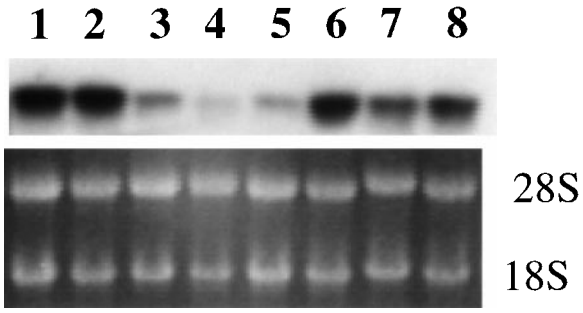

Fig. 3. Expression of anti-sense Bcl-2 results in the down-regulation of aggrecan mRNA in multiple clonal lines of IRC cells. The various IRC lines were maintained for several days in monolayer culture in medium containing 10% serum and harvested for RNA isolation. After size-fractionation by agarose gel electrophoresis, the RNA was transferred to a filter and probed with a cDNA for aggrecan as described in the material and methods. **Lane 1**, parental IRC cells; **lane 2**, IRC line containing an integrated vector with the CMV promoter only; **lanes 3–5**, three different clonal isolated of IRC cells expressing anti-sense Bcl-2 transcripts; **lanes 6–8**, three different clonal isolates of IRC cells expressing the sense transcript of Bcl-2. The anti-sense and sense cDNA of Bcl-2 is driven by the CMV promoter.

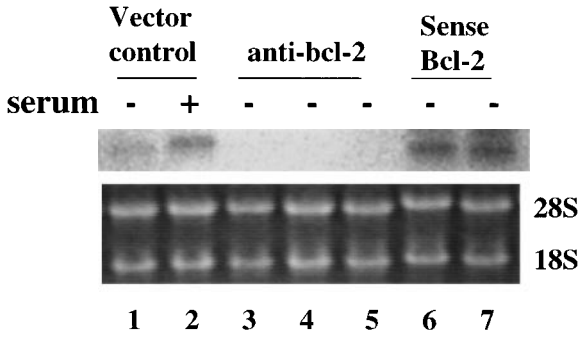

Fig. 4. Serum withdrawal is associated with decreased expression of aggrecan that is prevented by overexpression of Bcl-2. The various IRC lines were maintained in medium with or without 10% serum for 48 h and then processed and analyzed for aggrecan expression as described in Fig. 3 and under Material and Methods. **Lane 1**, IRC line containing an integrated vector with the CMV promoter only cultured in serum-free medium; **lane 2**, The CMV-control line cultured in medium containing 10% serum; **lanes 3–5**, three different clonal isolated of IRC cells expressing anti-sense Bcl-2 transcripts cultured in serum-free medium; **lanes 6**, **7**, two different clonal isolates of IRC cells expressing the sense transcript of Bcl-2. The anti-sense and sense cDNA of Bcl-2 is driven by the CMV promoter.

was cultured for 48 h in medium without serum, the level of aggrecan expression was dramatically reduced (Fig. 4, lane 1). As expected, in three separate clonal isolates of IRC cells expressing anti-sense transcripts of Bcl-2, there was negligible aggrecan mRNA when the cells were cultured for 48 h in medium without serum (Fig. 4, lanes 3–5). However, in two separate clonal isolates of IRC cells containing a plasmid vector in which Bcl-2 cDNA was driven by a CMV promoter, the level of aggrecan mRNA remained high, even when the cells were cultured for 48 h in medium lacking serum (Fig. 4, lanes 6 and 7). These results are consistent with a model in which Bcl-2 is involved in a signal cascade that is initiated by external trophic factors and functions directly or indirectly to regulate aggrecan expression.

Serum Withdrawal and Expression of Anti-Sense Bcl-2 Down-regulate Aggrecan Expression Independent of Apoptosis

Because both serum withdrawal and the expression of anti-sense Bcl-2 mRNA induce apoptosis in IRC cell, it may be argued that down-regulation of aggrecan expression by either of these signals results from the induction of apoptosis, and not the level of Bcl-2 per se. In order to distinguish between these two possibilities, we used the tripeptide inhibitor of the ced3/ICE family of proteases, ZVAD-fmk, which can inter-

rupt the apoptosis signaling cascade downstream of Bcl-2 in many cell systems [Jacobsen et al., 1996]. Agarose gel electrophoresis of DNA isolated from the various cell lines showed that 50 mM ZVAD-fmk completely inhibited full apoptosis of IRC cells induced either by serum withdrawal (Fig. 5, cf. lanes 3 and 4) or by anti-sense Bcl-2 (Fig. 5, cf. lanes 5 and 6) during the time period studied (48 h in culture). As we had previously determined that our cDNA probe for aggrecan detected the expected transcript for aggrecan, we next used dot-blot analysis to determine whether aggrecan was still down-regulated in IRC cells under these conditions. Control IRC cells cultured for 48 h in medium containing 10% serum expressed abundant aggrecan mRNA transcripts (Fig. 6, lane 1). The control cell line cultured for 48 h in medium without serum but in the presence of

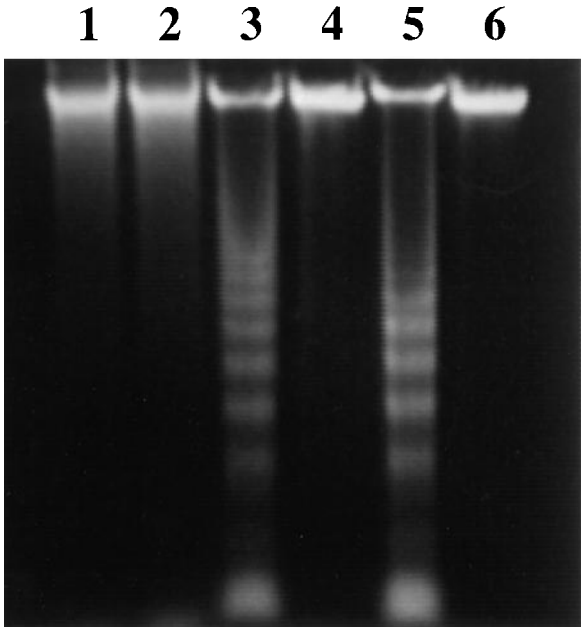

Fig. 5. ZVAD-fmk inhibits apoptosis of IRC cells exposed to serum withdrawal and in cells overexpressing anti-sense Bcl-2. Genomic DNA was isolated from the various IRC lines after 48 h in culture in medium with or without serum as indicated. The DNA was size-fractionated on an agarose gel and the DNA visualized with ethidium bromide. **Lane 1**, parental IRC cells cultured in medium containing 10% serum; **lane 2**, IRC line containing an integrated vector containing the CMV promoter only cultured in medium containing 10% serum; **lane 3**, CMV promoter control IRC line cultured for 48 h in serum-free medium; **lane 4**, CMV promoter control IRC line cultured for 48 h in serum-free medium + 50 mM ZVAD-fmk; **lane 5**, IRC line expressing anti-sense Bcl-2 cultured for 48 h in medium containing 10% serum; **lane 6**, IRC line expressing anti-sense Bcl-2 cultured for 48 h in medium containing 10% serum + 50 mM ZVAD-fmk.

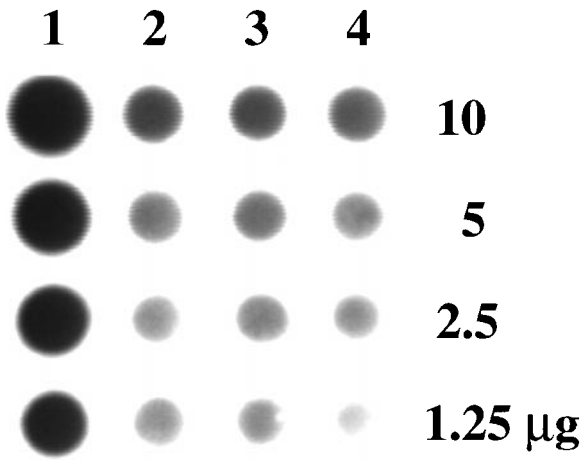

Fig. 6. Aggrecan mRNA is down-regulated in IRC cells with reduced Bcl-2 expression in conditions where full apoptosis is blocked by ZVAD-fmk. **Lane 1**, Control IRC line cultured for 48 h in medium containing 10% serum; **lane 2**, Control IRC line cultured for 48 h in serum-free medium + 50 mM ZVAD-fmk; **lane 3**, IRC line expressing anti-sense Bcl-2 cultured for 48 h in medium containing 10% serum; **lane 4**, IRC line expressing anti-sense Bcl-2 cultured for 48 h in medium containing 10% serum + 50 mM ZVAD-fmk.

50 mM ZVAD-fmk displayed a dramatic reduction in the steady-state level of aggrecan mRNA transcripts (Fig. 6, lane 2), although these cells were prevented from undergoing apoptosis (Fig. 5, lane 4). A clonal line expressing anti-sense-Bcl-2 cultured in medium containing 10% serum showed decreased aggrecan expression (Fig. 6, lane 3), which was not prevented when the cells were cultured in the presence of 50 mM ZVAD-fmk (Fig. 6, lane 4).

DISCUSSION

We report on an extension of previous work in our laboratory, and of other investigators, examining the mechanistic relationship between apoptosis, the regulation of differentiated phenotype, and the expression of Bcl-2, using a chondrocyte cell model. First, we demonstrate that the expression of anti-sense Bcl-2 in a chondrocyte cell line results in a switch from the more polygonal morphology associated with the differentiated phenotype to a fibroblastic morphology. In addition, we report that inhibition of the endogenous expression of Bcl-2 in these cells either by the expression of anti-sense mRNA or by serum withdrawal results in a loss of expression of aggrecan, a proteoglycan specific for differentiated chondrocytes and that overexpression of Bcl-2 will block the loss of aggrecan expression by IRC cells exposed to

serum withdrawal. Most significantly, aggrecan expression is down-regulated in response to decreased Bcl-2, even if downstream events of apoptosis are blocked with the caspase inhibitor ZVAD-fmk. These results support a model in which Bcl-2 plays a novel role in maintaining the expression of the matrix protein aggrecan in chondrocytes, independent of its role in preventing apoptosis. This work has significance not only for the regulation of chondrocyte gene expression but for the more general role of Bcl-2 as a regulator of differentiation-specific gene expression as well.

Previously we created cell lines in which either sense or anti-sense transcripts of Bcl-2 were expressed in a constitutive fashion from the CMV promoter [Feng et al., 1998]. With extended passage of these cells, it became apparent that the morphology of different clonal isolates for each cell line showed consistent morphological characteristics. When it was confirmed that the various cell lines continued to express the expected level of Bcl-2 protein after multiple passages, we concluded that the morphological differences were related, either directly or indirectly, to Bcl-2 expression. Our findings are similar to those reported for human mammary epithelial cells infected with a retrovirus carrying a Bcl-2 expression vector [Lu et al., 1995]. Specifically, these investigators reported that overexpression of Bcl-2 promoted epithelial to mesenchymal conversion of cells in monolayer culture and promoted branching morphogenesis when the cells were placed in collagen gels. It was concluded that some of the morphological affects were secondary to an increased survival advantage of the Bcl-2 expressing clones but, also, that Bcl-2 facilitated differentiation in the mammary gland in addition to regulating cell death. We do not know the precise relationship between the expression of Bcl-2 and the regulation of cell morphology. It has been established that chondrocytes, like many other cell types, require trophic factors in either an autocrine or paracrine fashion in order to survive [Ishizaki et al., 1994]. It has also been shown that chondrocytes maintained at high cell density have a more stable phenotype as compared with cells cultured at low density [Watt, 1988]. One possibility is that overexpression of Bcl-2 confers a survival advantage to the chondrocyte, so that even in the presence of serum, the cells expressing high levels of this protein may accumulate more of the factors required for maintenance of the phenotype. However, the fact that we observed the morphologic differences even in cells cultured at clonal densities would suggest that the effect might be mediated on an individual cell basis. As discussed below, decreased Bcl-2 levels result in loss of aggrecan expression. Other conditions in which aggrecan is down-regulated, such as with retinoic acid treatment, also produce a fibroblastic phenotype in chondrocytes [Horton et al., 1987]. It is possible that the change in the morphology is secondary to the loss of normal extracellular matrix expression. It will be important to determine the effects of culturing the cells in the presence of exogenously added matrix proteins to establish whether this will prevent or delay the morphological changes.

We previously reported that the treatment of IRC cells with retinoic acid suppressed Bcl-2 expression induced apoptosis and inhibited the production of aggrecan [Feng et al., 1988]. It is well established that IRC cells, as well as primary chondrocytes isolated from a variety of sources, respond to retinoids by switching off the expression of chondrocyte-specific genes [Horton et al., 1987, 1988]. The fact that overexpression of Bcl-2 did not block this down-regulation of aggrecan is consistent with the fact that retinoids act through cytosolic receptors which bind directly to specific sequences in the promoters and enhancers of target genes and regulate transcription [Meier, 1997].

We report that aggrecan expression was suppressed in IRC cells subjected to serum-withdrawal and in IRC lines expressing anti-sense Bcl-2. This finding is consistent with studies showing that aggrecan is down-regulated in the hypertrophic chondrocytes that are undergoing apoptosis in the growth plate [Mundlos et al., 1991; Chen et al., 1995]. Several studies address possible mechanisms that are operating to control both differentiation and apoptosis. For example, neuroblastoma cell lines treated with genistein (a specific inhibitor of protein tyrosine kinase) undergo differentiation and display increased apoptosis accompanied by down-regulation of N-*myc* expression [Brown et al., 1998]. This finding suggests that the regulation of both apoptosis and differentiation in this cell type may be linked through the *myc* proto-oncogene. The constitutive expression of human inducible heat shock protein 70 in HL-60 cells promotes the differentiation of the cells as evidenced by biochemical markers and pre-

vents the apoptosis that is normally associated with terminal differentiation [Kwak et al., 1998]. Furthermore, it has recently been reported that HL-60 cells overexpressing Bcl-2 still displayed biochemical differentiation in response to retinoids, although the cells did not undergo the apoptosis that is normally observed with terminal differentiation in parental cells [Ueno et al., 1998]. Finally, it was reported that the stable expression of wild-type p53 in a poorly differentiated pancreatic carcinoma cell line caused upregulation of the p21/WAF1gene and induced apoptosis in the majority of the cells; however, a subpopulation (30%) of the cells survived and acquired a more differentiated phenotype [Lang et al., 1998]. These studies suggest that differentiation and apoptosis, although related, have unique regulatory pathways.

One point in the apoptotic pathway that might be linked to the regulation of differentiation-specific genes is the activation of specific caspases that occurs down-stream to the action of the Bcl-2 family of proteins. The caspase family comprises several related cysteine proteases [Cohen, 1997] that mediate many of the terminal events in apoptosis, including DNA fragmentation, and chromatin condensation [Allen et al., 1998]. Certain known substrates of caspases may not directly be involved in apoptosis, suggesting that specific caspases could be involved in "cross-talk" with other cellular pathways that might influence the expression of specific genes. With this model in mind, we hypothesized that the inhibition of the apoptotic process down-stream of Bcl-2 with a general caspase inhibitor would block the down-regulation of aggrecan induced by expression of anti-sense Bcl-2 or serum withdrawal. Surprisingly, in multiple experiments, we observed the same degree of suppression of aggrecan expression with either serum withdrawal or anti-sense Bcl-2 in the presence of ZVAD-fmk, even though full apoptosis was inhibited as evidenced by the lack of DNA fragmentation. This finding has led us to conclude that the regulation of aggrecan is linked to the expression of Bcl-2 more directly, rather than through its inhibition of apoptosis. Although novel, this interpretation is in concert with an emerging concept that Bcl-2 plays a role in cellular function beyond its direct control of apoptosis. First, it has been reported that Bcl-2 is expressed in certain neuronal populations beyond the developmental time period where extensive neuronal cell death is occurring suggesting an alternative role for this protein [Merry et al., 1994]. More recent work has demonstrated neurons deficient in Bcl-2 extended axons more slowly as compared with wild-type cells, although there were no significant differences in cell survival between the two cell types [Hilton et al., 1997]. A separate group reported a related finding in which they demonstrated that decreased expression of Bcl-2 played a key role in the loss of ability of mammalian CNS neurons to regenerate axons past a certain developmental period [Chen et al., 1987]. Most significant was their finding that ZVAD-fmk could block apoptosis and promote cell survival of the older neurons but did not replace the need for Bcl-2 to stimulate regeneration. The precise mechanism by which Bcl-2 might control differentiated properties of cells is unknown but probably involves the control of transcription of specific genes. Numerous studies that suggest that Bcl-2 can regulate signaling pathways and transcription factors that regulate a wide variety of target genes not necessarily involved in apoptosis. For example, overexpression of Bcl-2 in fibroblasts inhibits the activation of c-jun N-terminal kinase (JNK) by interleukin-1β (IL-1β) (a nonapoptotic signal) independent of the rac1 GTPase [Lee et al., 1998]. Two separate studies have reported that Bcl-2 down-regulates the activity of the transcription factor NF-κB [Lin et al., 1995; Grimm et al., 1996], while another study has found that Bcl-2 activates NF-κB in neonatal ventricular myocytes through the degradation of the cytoplasm inhibitor IκB-α [de Moissac et al., 1998]. Additional studies are needed to explore the possible link between the regulation of these and other pathways by Bcl-2 and the control of differentiation-specific gene expression in chondrocytes and other cell types.

A final point of discussion is the potential importance of a regulatory pathway controlling chondrocyte matrix gene expression that involves Bcl-2. The regulation of chondrocyte differentiation and the expression of specific cartilage matrix proteins is critical both for proper development of the skeletal system and for the normal function of diarthrodial joints in the adult. Chondrocytes appear to rely on external signals such as PTHrP or other growth factors to maintain the expression of Bcl-2 that, in turn, controls the viability and differentiated state of the cell. Several transgenic models have

confirmed that perturbations of PTHrP signaling (either too much or too little) result in severe skeletal abnormalities [Karaplis et al., 1994; Lanske et al., 1996; Weir et al., 1996]. Mice with reduced Bcl-2 expression resulting from targeted gene disruption also display abnormal skeletal development [Nakayama et al., 1994]. The expression patterns of specific cartilage matrix proteins have not been examined in these models. It is significant, however, that mice bearing an autosomal recessive mutation in the aggrecan gene display a phenotype similar to the Bcl-2 knockout animals, namely dwarfism and early postnatal mortality [Watanabe et al., 1997].

In addition to the control of skeletal development, the expression of Bcl-2 could play a significant role in the biology of the articular chondrocyte in mature organisms. There is a significant increase in the incidence of cartilage degeneration as a function of age in animals and humans. In addition, chondrocytes isolated from the cartilage of old animals are less responsive to growth factors in terms of the expression of cartilage matrix proteins [Martin et al., 1997]. We recently reported that the incidence of apoptosis increases in the articular cartilage of mice and rats as they age [Adams et al., 1998], although the expression of Bcl-2 as a function of aging as not been determined in the articular cartilage. It is possible that declining expression of Bcl-2 in certain subpopulations of chondrocytes in the articular cartilage as a function of age both render these cells more sensitive to the induction of apoptosis and diminish the capacity of these cells to repair the cartilage by retarding the expression of matrix proteins. We are currently testing this hypothesis by monitoring the level of Bcl-2 expression in the articular chondrocytes of young and old animals by in situ hybridization and immunostaining.

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