RAPID COMMUNICATION

Prolactin inhibits the apoptosis of chondrocytes induced by serum starvation

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Abstract

The apoptosis of chondrocytes plays an important role in endochondral bone formation and in cartilage degradation during aging and disease. Prolactin (PRL) is produced in chondrocytes and is known to promote the survival of various cell types. Here we show that articular chondrocytes from rat postpubescent and adult cartilage express the long form of the PRL receptor as revealed by immunohistochemistry of cartilage sections and by RT-PCR and Western blot analyses of the isolated chondrocytes. Furthermore, we demonstrate that PRL inhibits the apoptosis of these same chondrocytes cultured in low-serum. Chondrocyte apoptosis was measured by hypodiploid DNA content determined by flow cytometry and by DNA fragmentation evaluated by the ELISA and the TUNEL methods. The anti-apoptotic effect of PRL was dose-dependent and was prevented by heat inactivation. These data demonstrate that PRL can act as a survival factor for chondrocytes and that it has potential preventive and therapeutic value in arthropathies characterized by cartilage degradation.

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Introduction

Chondrocytes are the only cells residing in cartilage and are responsible for the formation, maintenance, and turnover of a variety of extracellular matrix proteins. Indeed, chondrocytes are essential for the function of the tissue; their death is an important feature of cartilage replacement during bone formation (Poole 1991), but in aging and disease it can lead to cartilage degradation (Aigner & McKenna 2002). Various lines of evidence suggest that apoptosis is the main type of death in chondrocytes. Apoptotic chondrocytes have been detected during endochondral ossification (Hatori *et al.* 1995, Zenmyo *et al.* 1996), in aging cartilage (Adams & Horton 1998), and in arthropathies characterized by cartilage destruction, such as rheumatoid arthritis (Kim & Song 1999) and osteoarthritis (Kouri *et al.* 1997, Goggs *et al.* 2003). In osteoarthritis, the major age-associated joint disease, increased chondrocyte apoptosis has been correlated with the severity of cartilage damage (Hashimoto *et al.* 1998). Morphological and functional differences suggest that the mechanisms of chondrocyte apoptosis differ from those in other tissues (Roach *et al.* 2004, Pérez *et al.* 2005), but these mechanisms are unclear and little is known about the regulatory factors responsible for their control.

Prolactin (PRL) acts both as a circulating hormone and as a cytokine in a vast array of physiological functions that range from reproduction and osmoregulation to immunomodulation and angiogenesis (Bole-Feysot *et al.* 1998, Corbacho *et al.* 2002). In addition, PRL may serve as a regulatory factor for joint tissues. PRL can act directly on osteoblasts during development to regulate bone formation (Clément-Lacroix *et al.* 1999, Coss *et al.* 2000) and can activate synovial cell functions in rheumatoid arthritis (Nagafuchi *et al.* 1999). Furthermore, synovial fluid contains PRL (Ogueta *et al.* 2002) that may derive from plasma and/or may be produced locally. Indeed, PRL is expressed by synovial cells (Nagafuchi *et al.* 1999), chondrocytes (Macotela *et al.* 2006), and bone marrow-derived mesenchymal stem cells undergoing

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chondrogenic differentiation (Ogueta *et al*. 2002). In the latter, PRL may contribute to the acquisition of the chondrocytic phenotype as it stimulates the synthesis of proteoglycans and type II collagen (Ogueta *et al*. 2002). In support of chondrocytes being cellular targets of PRL, the PRL receptor has been detected in chondrocytes from neonatal rats (Coss *et al*. 2000), although its functional role remains to be determined. Because PRL is known to be a survival factor for various cell types (Ploszaj *et al*. 1998, Buckley 2001, Tessier *et al*. 2001, Ruffion *et al*. 2003, Perks *et al*. 2004, Asai-Sato *et al*. 2005) and chondrocyte survival is essential for cartilage function, the aim of the present work was to investigate whether the PRL receptor is expressed in articular chondrocytes from normal postpubescent and adult cartilage and can mediate an action of PRL on cell survival.

Materials and Methods

Chondrocyte isolation

Articular chondrocytes were isolated from femoral epiphyseal cartilage of male, postpubescent, Wistar rats (8 weeks old, 130–150 g body weight (bw)) as described (Shakibaei *et al*. 1997) with modifications (Macotela *et al*. 2006). The cells were either stored immediately at –80 °C for subsequent total RNA extraction, or processed for immunoprecipitation/Western blot analysis or cell culture experiments.

RT-PCR

Total RNA was extracted and quantified, and 5 μg were reverse transcribed in a 25-μl reaction using Moloney murine leukemia virus reverse transcriptase (Promega). Two-microliter aliquots were used for cDNA amplification by PCR using oligonucleotides specific for all forms of the PRL receptor (sense primer A: CCC CAA CTC CTG CTT CTT TAG, and antisense primer B: TAT TTT TGG CCC AGG AAC TA), or oligonucleotides specific for the short form (sense primer C: ATC CTG GGA CAG ATG GAG GAC, and antisense primer D: TGG CTG AGG CTG ACA AAA GAG), or for the long PRL receptor isoform (sense primer C, and antisense primer E: AGA CAG TGG GGC TTT TCT CCT). In all cases, amplification was for 40 cycles and for 30 s at 94 °C, 45 s at 56 °C, and 45 s at 72 °C for primers A-B; and 30 s at 94 °C, 45 s at 56 °C, and 60 s at 72 °C for primers C-D and C-E. The products resulting from reactions with primers A-B, C-D, and C-E were 582 bp, 1017 bp and 1344 bp respectively (Fig. 1A).

Immunoprecipitation/Western blot

Freshly isolated chondrocytes (2×10^6) were resuspended in lysis buffer (0·5% Nonidet P-40, 0·1% SDS, 50 mM Tris, 150 mM NaCl, 1 μg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride, pH 7) and immunoprecipitated with anti-rat PRL receptor U-5 monoclonal antibody (U-5 MAb, 5 μg) using the previously reported technique (Corbacho *et al*. 2000). U-5 MAb is directed against the extracellular region of the PRL receptor (Okamura *et al*. 1989) and was a gift from P A Kelly from INSERM U-584, Paris, France. Immunoprecipitates were subjected to SDS-PAGE on an 8% acrylamide gel under reducing conditions, then blotted and probed with a 1:500 dilution of U-5 MAb. The antigen–antibody complex was detected using the alkaline phosphatase second antibody kit (Bio-Rad Laboratories).

Immunohistochemistry

Bones from the knee joint of adult rats (250 g bw) were dissected, fixed, decalcified, and dehydrated for paraffin embedding. After deparaffination and rehydration, longitudinal 6-μm paraffin sections were blocked with 2% bovine serum albumin, 1% normal goat serum, and 0·3% Triton-X in phosphate-buffered saline (pH 7·4) for 1 h. Sections were then incubated overnight with a 1:100 dilution of U-5 or U-6 anti-rat PRL receptor MAbs. The U-6 MAb is also directed against the extracellular region of the PRL receptor (Okamura *et al*. 1989) and was provided by P A Kelly. Finally, after incubation with biotin-conjugated secondary antibody for 1 h, sections were developed using the avidin–biotin complex detection kit (Vector Laboratories, Burlingame, CA, USA).

Chondrocyte culture

Chondrocytes were seeded (2×10^5 cells/cm^2) on wells or on 1% fibronectin-coated glass coverslips and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 0·5% fetal bovine serum (FBS) and 1% penicillin/streptomycin for 48 h at 37 °C in the presence or absence of rat PRL (biological grade; National Hormone Pituitary Program, Torrance, CA, USA). Cells cultured in 10% FBS-DMEM served as negative control for apoptosis.

DNA labeling technique for flow cytometric analysis

At the end of the incubation, chondrocytes were trypsinized and sedimented. Cell pellets were fixed in 80% ethanol for 60 min at 4 °C, washed twice, incubated with RNAse (300 μg/ml) for 20 min at 4 °C and stained with propidium iodide (50 μg/ml) for 15 min at 4 °C in the dark. Propidium iodide fluorescence of nuclei was measured by flow cytometry on a fluorescence-activated cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA) with a 560 nm dichromatic mirror and a 600 nm band

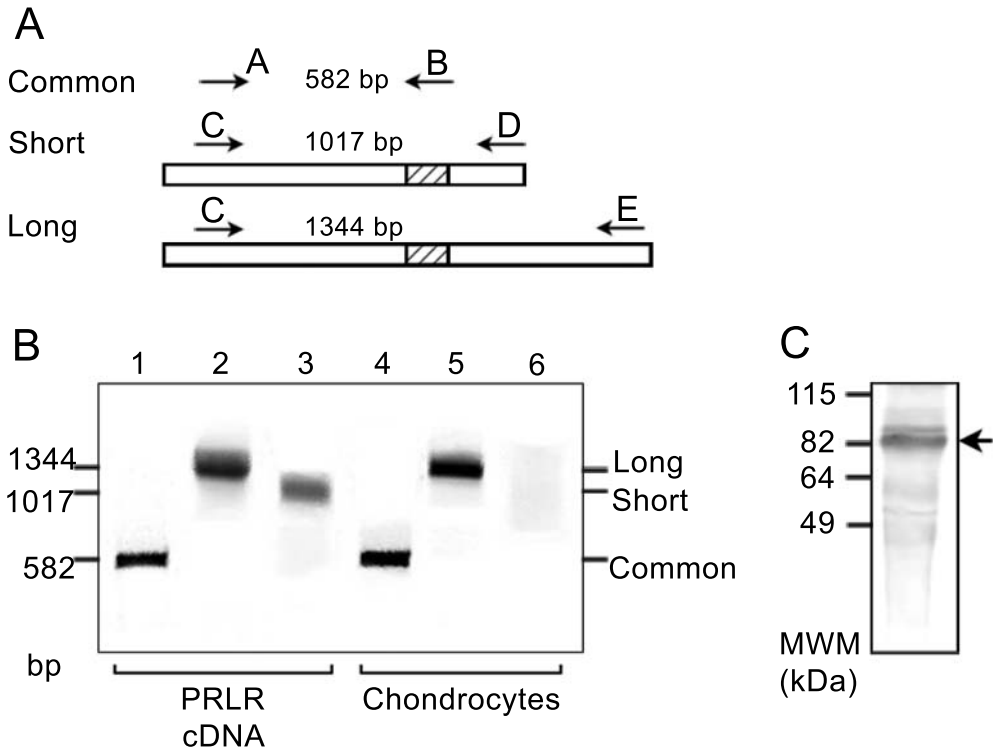

Figure 1 Expression of the long PRL receptor mRNA and protein in chondrocytes isolated from postpubescent rat articular cartilage. (A) Schematic representation of cDNAs encoding short and long forms of PRL receptor in the rat. The positions of oligonucleotide probes (arrows) complementary to sequences encoding the extracellular region (primer A and primer C) or the membrane domain (hatched area, primer B) common to all forms, or the cytoplasmic domain specific for the short form (primer D), or for the long form (primer E) are shown. Predicted sizes of PCR products for each primer combination are in basepairs (bp). (B) PCR products from reverse transcribed total RNA from chondrocytes (lanes 4, 5, and 6), or from rat PRL receptor cDNA (PRLR cDNA; lanes 1, 2, and 3) amplified with the three combinations of PRL receptor primers shown in (A). (C) Immunoprecipitation/Western blot analysis of PRL receptors in chondrocytes. Chondrocytes (2×10^6) were lysed, immunoprecipitated with U-5 anti-rat PRL MAb and blotted with the same U-5 MAb. The apparent molecular weights of markers (MWM) and an immunoreactive protein of 85 kDa (arrow) are indicated.

pass filter. For each sample, 10^4 cells were analyzed and percentage values denoted the proportion of propidium iodide positive cells.

TUNEL assay

Chondrocytes were fixed with fresh 4% paraformaldehyde for 1 h at room temperature and permeabilized by treatment with 0·1% Triton X-100 in 0·1% sodium citrate buffer (pH 7·4) for 2 min at 4 °C. Following permeabilization, apoptotic cells were visualized by the TUNEL method using a detection kit (Roche Diagnostics) and fluorescence microscopy.

Cell death ELISA

Fragmented nucleosomal DNA was measured using the ELISA kit from Boehringer Mannheim, according to the manufacturer's instructions. After incubation, medium containing floating cells was harvested, and the cells on the plate were trypsinized briefly. Floating and trypsinized cells were combined, sedimented, counted, lysed in the lysis buffer of the kit and transferred to a microtiter plate to quantitate nucleosomes. The results are expressed as optical density units per 5×10^4 cells relative to those of control cells (incubated with 10% FBS).

Statistical analyses

All results were replicated in three or more independent experiments. Data are presented as the mean ± S.E.M. As appropriate, Student's unpaired *t*-test or one-way ANOVA followed by Tukey's test to compare individual means was used for statistical comparisons. The significance level was set at 5%.

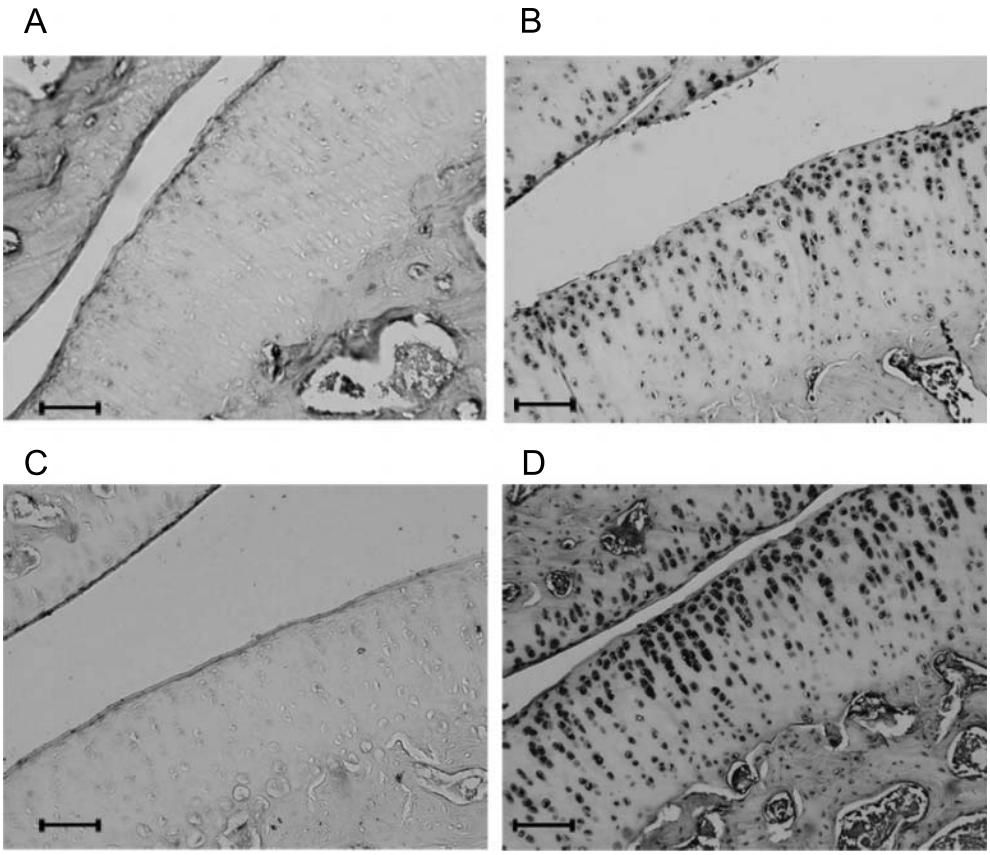

Figure 2 Immunohistochemical detection of PRL receptors in articular chondrocytes. Sections of femoral heads from adult rats immunolabeled with the U-5 (B) or the U-6 (D) anti-rat PRL MAb are shown. Both show MAbs labeled cells throughout the cartilage. No positive reaction was detected in the absence of primary antibodies (A) or by using control mouse IgG_1 (R&D Systems, Minneapolis, MN, USA) as primary antibodies (C). Scale bar=100 μm.

Results

The long form of the PRL receptor is expressed in chondrocytes

In order to study whether PRL can act directly on chondrocytes to regulate their survival, we first examined the expression of PRL receptors in articular chondrocytes isolated from normal, postpubescent rat cartilage. The PRL receptor mRNA was investigated by RT-PCR (Fig. 1). Amplification using primers common to all forms of the PRL receptor or specific for the long form of the PRL receptor yielded products having the expected lengths of 582 bp or 1344 bp respectively (Fig. 1B, lanes 4 and 5). These products were similar to the control PCR bands amplified from the rat PRL receptor cDNA (lanes 1 and 2). No product was amplified from chondrocytes when primers specific for the short form of the PRL receptor were used (lane 6). To determine whether the PRL receptor mRNA was translated into protein, PRL receptors were immunoprecipitated from chondrocyte lysates and analyzed by immunoblot (Fig. 1C). The anti-rat PRL receptor U-5 MAb precipitated a major protein of 85 kDa, the expected size for the long form of the PRL receptor (Okamura *et al*. 1989), and several minor proteins above and below this size. Some of the latter are close to the size estimated for the intermediate (65 kDa) and the short (40 kDa) PRL receptor variants, but additional work is needed to clarify their nature, inasmuch as no short PRL receptor mRNA was detected. In addition, the presence of PRL receptors was examined by immunohistochemistry in sections of femoral heads from adult rats (Fig. 2). Chondrocytes throughout femoral articular cartilage stained positively for PRL receptors as revealed by the anti-rat PRL receptor U-5 and U-6 MAbs (Fig. 2B, D), and no positive labeling was evident in their absence (Fig. 2A) nor with control IgGs of the same isotype (Fig. 2C).

PRL inhibits apoptosis of articular chondrocytes induced by serum deprivation

Next, we determined whether primary chondrocytes isolated from rat postpubescent articular cartilage could be

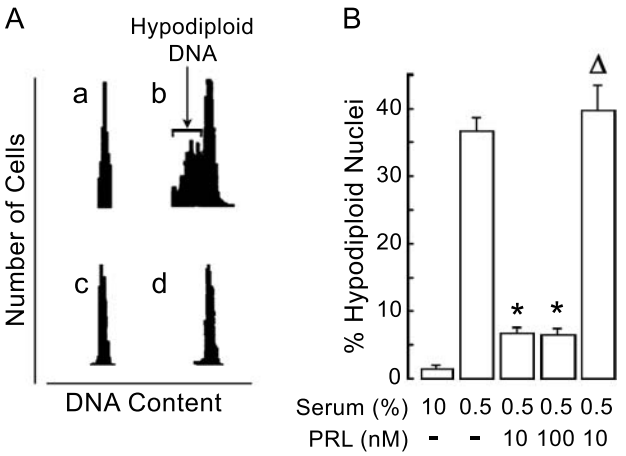

Figure 3 PRL inhibits low-serum-induced apoptosis of articular chondrocytes. (A) Representative experiment in which chondrocytes were cultured for 48 h in medium containing 10% serum (a) or 0·5% serum, either alone (b) or in combination with 10 nM (c) or 100 nM (d) PRL. The cells were then stained with propidium iodine and analyzed by flow cytometry. Cells containing hypodiploid DNA (apoptotic cells) are indicated. (B) Percentage of cells with hypodiploid DNA from three independent experiments performed as in (A). PRL was used before and after (Δ) heat inactivation for 30 min at 90 °C. Values are means ± S.E.M. **P*<0·05 vs 0·5% serum in the absence of PRL.

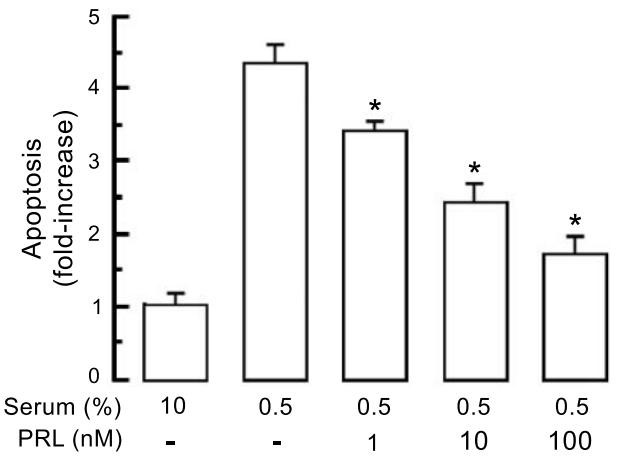

Figure 5 PRL inhibits low-serum-induced apoptosis of articular chondrocytes. Chondrocytes were cultured for 48 h in medium containing 10% serum or 0·5% serum, alone or in combination with different concentrations of PRL. Apoptosis was quantified using an ELISA for nucleosome detection. Results are presented as means ± S.E.M. of three independent experiments normalized to the amount of apoptosis observed in control chondrocytes cultured in 10% serum. **P*<0·05 vs 0·5% serum in the absence of PRL.

induced to undergo apoptosis by the classic signal of serum deprivation. Rat articular chondrocytes were incubated with 0·5% serum, and the amount of apoptosis was measured by propidium iodide staining and fluorescence-activated cell sorting analysis (Fig. 3). This method of flow cytometry quantitates the percentage of cells with hypodiploid DNA occurring because of DNA fragmentation. Incubation in 0·5% serum resulted in a peak of apoptotic cells with DNA hypoploidy that was absent when cells were cultured in 10% serum. The number of apoptotic cells induced by low-serum corresponded to approximately 35% of total chondrocytes. Coincubation with 10 or 100 nM PRL prevented the appearance of hypodiploid cells induced by low-serum. The two PRL concentrations were equally effective, and their protective action was abolished by heat denaturation (Fig. 3B).

PRL protection against apoptosis was confirmed by the detection of DNA strand breaks in cells using the TUNEL method (Fig. 4). Incubation with 0·5% serum increased the number of TUNEL-positive chondrocytes by sixfold compared with cells cultured in 10% serum. The increase in TUNEL-positive chondrocytes was prevented by PRL in a dose-dependent manner (Fig. 4B).

Finally, chondrocyte apoptosis was determined using an ELISA that measures DNA nucleosomal fragments (Fig. 5). Low-serum resulted in a fourfold increase in the amount of apoptosis, and this increase was inhibited by increasing concentrations of PRL.

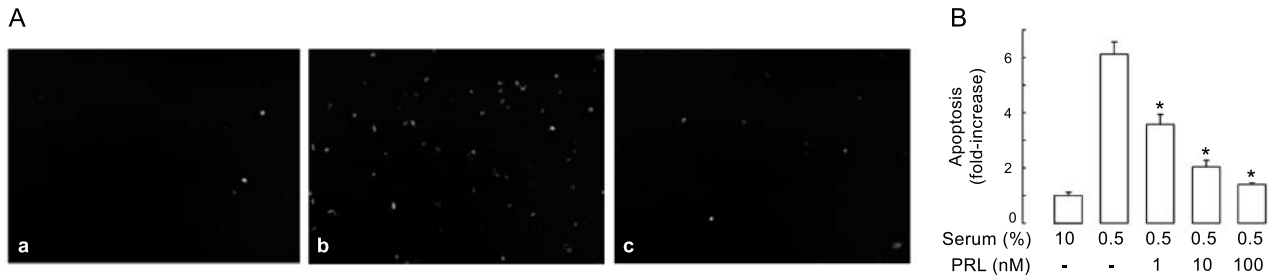

Figure 4 PRL inhibits low-serum-induced apoptosis of articular chondrocytes. (A) Representative experiment in which chondrocytes were cultured on coverslips for 48 h in medium containing 10% serum (a) or 0·5% serum, either alone (b) or in combination with 100 nM PRL (c). TUNEL-positive cells (apoptotic cells) are indicated by bright nuclear fluorescence. (B) Percentage of TUNEL-positive cells from three independent experiments performed as in (A). Six microscopic fields per coverslip were selected at random and visually scanned to record the total number of TUNEL-positive cells relative to those in control cells (incubated with 10% FBS). **P*<0·05 vs 0·5% serum in the absence of PRL.

Discussion

Our present study is the first to report that PRL can protect chondrocytes against apoptotic death. Apoptosis was effectively antagonized by PRL. The cellular response to low-serum is compatible with apoptosis, as characterized by flow cytometry quantification of cells with hypodiploid DNA, TUNEL staining, and ELISA measurement of DNA fragments. These three methods identify the degradation of DNA by cleavage between nucleosomes, a hallmark of apoptosis. Consistent with the protective effect of PRL, we also show that chondrocytes express the long form of the PRL receptor mRNA and that this message is translated into significant amounts of protein in the isolated cells and in chondrocytes *in situ*.

The anti-apoptotic effect of PRL was dose related in the TUNEL and ELISA assays. In the flow cytometry assay, the two PRL concentrations tested (10 and 100 nM) were equally potent. The reasons for the same potency are unclear, but might stem from errors in collecting the small amount of light associated with dead cells, which are difficult to spin down by ordinary low-speed centrifugations. The mechanisms underlying the anti-apoptotic effect of PRL in chondrocytes need to be investigated. The survival action of PRL has been associated with the upregulation of Bcl-2, a family of proteins in mouse mammary epithelial cells and in breast cancer cells (Ploszaj *et al.* 1998, Peirce & Chen 2004), and downregulation of Bcl-2 plays an important role in chondrocyte apoptosis induced by serum withdrawal (Feng *et al.* 1998).

The observation that articular chondrocytes from postpubertal rats undergo apoptosis in response to low-serum is in agreement with other *in vitro* studies using 0% serum and chick embryo and adult rat sterna chondrocytes (Ishizaki *et al.* 1994) or adult human and rabbit articular chondrocytes (Feng *et al.* 1998). These findings indicate that, similar to most vertebrate cells, chondrocytes require growth factor signaling for survival. Various hormones and growth factors promote the survival of cultured chondrocytes, including dexamethasone, insulin, basic fibroblast growth factor, transforming growth factor (TGF)-β and insulin-like growth factor-I (Quarto *et al.* 1992, Ishizaki *et al.* 1994, Gruber *et al.* 2000, Lo & Kim 2004). The present results add PRL to this list and raise the important question of whether PRL is one of the factors regulating the survival of articular chondrocytes *in vivo*.

Articular cartilage is the thin layer of smooth hyaline cartilage that covers the joint surfaces of a bone. Adult articular cartilage is thought to be a postmitotic tissue and because there is virtually no cell turnover, preservation of cell viability is essential for its function (Aigner & Kim 2002). Because cartilage is avascular, serum-borne survival factors must be able to diffuse through the tissue from the synovial fluid of the joint. However, due to the sparse distribution of chondrocytes encased within the extracellular cartilage matrix, an autocrine survival mechanism may be more efficient, and chondrocytes do produce survival-promoting factors (Ishizaki *et al.* 1994). In this regard, PRL is a component of human synovial fluid (Ogueta *et al.* 2002, Rovensky *et al.* 2005), and may derive from plasma, since most proteins with a molecular mass of less than 100 kDa readily transfer from one fluid space to the other (Perman 1980). In addition, PRL can be generated by chondrocytes. Isolated chondrocytes from rat articular cartilage express PRL mRNA and protein (Macotela *et al.* 2006), and bone marrow-derived mesenchymal progenitor cells express PRL mRNA during chondrogenic differentiation (Ogueta *et al.* 2002).

Furthermore, chondrocytes express the PRL receptor. PRL receptors have been detected in chondrocytes from cartilage in the diaphysis of digits in newborn rats (Coss *et al.* 2000) and in bone marrow-derived mesenchymal stem cells undergoing chondrogenic differentiation (Ogueta *et al.* 2002). PRL receptors exist in several isoforms that differ primarily in the sequence and length of the cytoplasmic domain (Bole-Feysot *et al.* 1998). Of interest is the observation that upon chondrogenic differentiation, mesenchymal stem cells switch expression from the intermediate isoform to the long isoform of the PRL receptor (Ogueta *et al.* 2002). In agreement, we show that chondrocytes from postpubertal articular cartilage express the long form of the PRL receptor. Moreover, this receptor isoform is functional in chondrocytes, because PRL stimulates the synthesis of proteoglycans and type II collagen in the chondrocytic mesenchymal cells (Ogueta *et al.* 2002) and protects articular chondrocytes against low-serum-induced apoptosis (present results).

Although chondrocytes respond to PRL *in vitro*, the *in vivo* action of PRL on cartilage function remains to be established. Targeted disruption of the PRL receptor reduces bone development and growth, but this action appears to be independent of a defect in cartilage (Clément-Lacroix *et al.* 1999). Bone alterations were observed mostly in calvaria, where bone formation occurs by intramembranous ossification, and there was no skeletal alteration indicative of a defect of endochondral bone formation. Given that endochondral ossification requires chondrocyte apoptosis, the absence of an endochondral ossification phenotype in these mice would argue against a role for PRL in cartilage survival. While redundant mechanisms could compensate for the loss of PRL action, it is also possible that the anti-apoptotic effect of PRL does not occur in chondrocytes involved in bone development and growth. Articular chondrocytes and cells in prenatal and growth plate cartilage are known to have differences that reflect specific functions and long-term survival (Karsenty & Wagner 2002). For example, unlike other chondrocytes, articular chondrocytes are normally arrested before hypertropic differentiation leading to apoptosis, allowing cartilage on the articular surface to persist, which is essential for proper joint function.

Multifunctional proteins can exhibit differential effects on chondrocyte subpopulations. For example, TGF-β stimulates chondrogenesis of undifferentiated multipotent mesenchymal cells (Leonard *et al.* 1991), but it blocks hypertropic differentiation of articular chondrocytes, promoting the survival of articular cartilage (Serra *et al.* 1997). Evaluation of the effect of PRL receptor gene deletion on the aging skeleton or in degenerative joint diseases may provide strong support for the role of PRL as a mediator of articular cartilage survival.

Importantly, loss of adult articular cartilage results from diverse actions including age, loading, and trauma, and it is the major cause of joint dysfunction and disability in rheumatoid arthritis and osteoarthritis (Goggs *et al.* 2003). Investigations leading to the identification of chondrocyte apoptosis inhibitors should have a major impact on the prevention and treatment of a wide range of disabling rheumatological conditions. Of interest is the fact that PRL has been detected in the synovial fluid of patients with rheumatoid arthritis and osteoarthritis (Rovensky *et al.* 2005), and that it is expressed by synovial cells in rheumatoid arthritis (Nagafuchi *et al.* 1999). A better understanding of the effect of PRL on chondrocyte survival both *in vivo* and *in vitro* should help elucidate its probable contribution to cartilage function under both healthy and diseased states.

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