Antiproliferative Effects of Insulin-like Growth Factor-binding Protein-3 in Mesenchymal Chondrogenic Cell Line RCJ3.1C5.18

RELATIONSHIP TO DIFFERENTIATION STAGE*

Chondrogenesis results from a complex equilibrium between chondrocyte proliferation and differentiation. Insulin-like growth factors (IGFs) have a crucial role in chondrogenesis, but their mechanisms of action are not well defined. IGF-binding protein-3 (IGFBP-3) is the major carrier for circulating IGFs in postnatal life, and has been shown to have IGF-independent effects on proliferation of several cancer cell lines. In this study, we have evaluated the IGF-independent and -dependent effects of IGFBP-3 on chondrocyte proliferation and the relationship of these effects with chondrocyte differentiation stage. We used the RCJ3.1C5.18 nontransformed mesenchymal chondrogenic cell line, which, over 2 weeks of culture, progresses through the differentiation pathway exhibited by chondrocytes in the growth plate. We demonstrated that IGFBP-3 inhibited, in a dose-dependent manner (1–30 nM), the proliferation of chondroprogenitors and early differentiated chondrocytes, stimulated by des-(1–3)-IGF-I and longR1-IGF-I (IGF-I analogs with reduced affinity for IGFBP-3), and by insulin and IGF-I. In terminally differentiated chondrocytes, IGFBP-3 retained the ability to inhibit cell proliferation stimulated by IGF-I, but had no effect on cell growth stimulated by insulin, or des-(1–3)-IGF-I or longR1-IGF-I. By monolayer affinity cross-linking, we demonstrated a specific IGFBP-3-associated cell-membrane protein of ~20 kDa. We determined that IGFBP-3 has an antiproliferative effect on chondrocytes and, that this effect is related to the differentiation process. In chondroprogenitors and early differentiated chondrocytes, antiproliferative effect of IGFBP-3 is mainly IGF-independent, whereas, following terminal differentiation this effect is IGF-dependent.

Skeletal growth is a complex phenomenon involving numerous regulatory mechanisms that modulate the dynamic equilibrium of the growth plate. Mesenchymally derived growth plate chondrocytes proliferate and terminally differentiate to produce a cartilage template for linear growth. Several growth factors are involved in this process, but how this tightly ordered equilibrium is controlled is still not well understood. Insulin-like growth factors (IGFs)1 appear to play a central role in chondrogenesis, but studies directed at clarifying the precise mechanisms of IGF action have led to contradictory results and theories (1). The somatomedin hypothesis postulates that IGF-I produced in extraskeletal tissues is transported to the growth plate, where it acts as an endocrine factor. The dual effector theory advocates that IGF-I is produced in the growth plate under the stimulation of growth hormone (GH) and acts as a paracrine/autocrine factor; GH may also act independently, by promoting the differentiation of resting chondrocytes to chondrocytes (1). However, to date, clinical and experimental evidence does not definitively support either theory. Moreover, the regulatory action of IGF-binding proteins (IGFBPs) in chondrogenesis requires careful evaluations.

IGFBPs are part of an IGFBP/IGFBP-related-protein superfamily, of which six proteins (termed IGFBP-1 to -6) bind IGFs with high affinity (3). IGFBP-3 is the major circulating IGFBP present during postnatal life (2). IGFBP-3 is GH-dependent, prolongs IGF half-life, and carries IGF to target tissues. In addition, a direct effect of IGFBP-3 on cell proliferation that is independent of IGF binding has been demonstrated in several cell lines, and this effect may involve association with cell-membrane proteins (2, 4, 5). Based upon an increasing understanding of the IGFBP system, we have recently proposed an IGFBP regulatory hypothesis as part of an integrated model of the GH-IGF-IGFBP-3 effects in the growth process (1). The proposed model accounts for the effects of circulating IGFBPs, but the possibility of a direct, IGF-independent effect of IGFBP-3 on the growth plate has yet to be addressed.

Studies directed at the evaluation of IGFBPs in chondrogenesis have produced contradictory findings, primarily due to the lack of a good in vitro model. In most studies, primary cultures of chondrocytes have been used. Under these conditions, however, the chondrocytes undergo a process that has been termed dedifferentiation (6), which is characterized by a change in shape, attachment, and a loss of cartilage-specific markers. Despite these limitations, receptors for IGFs and GH have been identified in chondrocytes (7–11). Some studies have reported

1 The abbreviations used are: IGF, insulin-like growth factor; GH, growth hormone; IGFBP, IGF-binding protein; des-IGF-I, des-(1–3)-IGF-I; LR1-IGF-I, longR1-IGF-I; WLB, Western ligand blotting; WIB, Western immunoblotting; FGFR-3, fibroblast growth factor receptor-3; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DSS, disuccinimidyl suberate.
that, in primary cultures of articular chondrocytes, IGFBP-5 is the major IGFBP (12), whereas others have reported that IGFBP-2 is the most abundant IGFBP in cultured chondrocytes (13).

In the current study, we have employed a nontransformed clonal chondrogenic cell line, RCJ3.1C5.18. The RCJ3.1C5.18 cell line is a mesenchymal stem cell system that, without requiring biochemical or oncogenic transformation, spontaneously and sequentially undergoes chondrocyte differentiation (14-16). We have previously reported that RCJ3.1C5.18 cells sequentially acquire, over 2 weeks of culture, markers for chondrocytic differentiation and terminal differentiation (Table I (14). Although our data are based upon an in vitro situation the morphology, the histochemical markers and the temporal sequential acquisition of the chondrocytic phenotype in this cell system is identical to the chondrogenesis process that occurs in vivo. This makes our system ideal and unique for studying chondrocyte cellular and molecular regulation, and suggests that our findings are relevant to the in vivo process.

Using the RCJ3.1C5.18 in vitro model for chondrogenesis, this study is aimed at: 1) evaluating the biological effects of IGFBP-3 on chondrocyte proliferation; 2) characterizing the relationship of this effect with differentiation stage; and 3) examining the IGF-independent effect of IGFBP-3 on chondrocyte proliferation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant IGF-I, des-[1–3]-IGF-I (des-IGF-I), and longR (LR-IGF-I) IGF-I were purchased from GroPep Pty. Ltd. (Adelaide, Australia). Des-[1–3]-IGF- and LR-IGF-I exhibit 30–100-fold reduced affinity for IGFBP-3, but unaltered affinity for the type I IGF receptor compared with IGFs (17, 18). IGF-I and IGF-II were generously supplied by Celtrix Pharmaceuticals Inc. (Santa Clara, CA). Recombinant human IGFBP-2 was purchased from Austral Biologicals (San Ramon, CA). Recombinant human IGFBP-5 was purchased from GroPep. Human recombinant insulin was purchased from Sigma. A rabbit polyclonal antibody against human IGFBP-3, αIGFBP-3g1, was generated and characterized in our laboratory (19). A polyclonal antibody against human IGFBP-2 that weakly recognizes IGFBP-3, called αHEC 1, was generated and characterized in our laboratory (20). In rats, αHEC 1 has been characterized to be specific for IGFBP-2 (12). A polyclonal antibody against rat IGF-I was previously produced and characterized in our laboratory (12, 21). IGFBP-3* was iodinated by a modification of the chloramine-T technique (5). Iodinated glycosylated IGFBP-3 was generously provided by DiagnoTech, Inc. (Adelaide, Australia). Des-(1–3)-IGF- and LR 3-IGF-I exhibit 30–100-fold reduced affinity for IGFBP-3, but unaltered affinity for the type I IGF receptor compared with IGFs (17, 18). IGF-I and IGF-II were generously supplied by Celtrix Pharmaceuticals Inc. (Santa Clara, CA). Recombinant human IGFBP-2 was purchased from Austral Biologicals (San Ramon, CA).

**Cell Culture**—RCJ3.1C5.18 cells, generously donated by Dr. Jane E. Aubin (University of Toronto, Toronto, Ontario, Canada), were grown in α-minimal essential medium supplemented with 15% heat-inactivated fetal bovine serum, 10 μM dexamethasone, and 2 mM sodium pyruvate. Cells were plated at a density of 6 × 10^4 cells/well in six-well dishes. After reaching confluence (4 days), fresh growth medium supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate was added. Differentiating cells were fed again with supplemented medium at days 7 and 10 of culture. Cultures were monitored over a total period of 14 days. We have previously shown that RCJ3.1C5.18 cells grown in this manner maintain their differentiated chondrocytic phenotype (14). As shown in Table I, cells sequentially acquire at 7 days of culture markers of chondrocytic differentiation (type II collagen and proteoglycans synthesis) and progressively acquire at 10 and 14 days of culture markers of terminal differentiation (type X collagen and alkaline phosphatase activity).

**Analysis of IGFBPs in Conditioned Media and Cell Lysates**—Cell lysates were obtained from cells cultured for 4, 7, 10, and 14 days, and conditioned media were obtained from cells cultured similarly but incubated for 24 h in serum-free media. For preparation of cell lysates, cells were solubilized for 30 min at 4 °C in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol) containing a mixture of protease inhibitors (Boehringer Mannheim, Mannheim, Germany) including 1 mM phenylmethylsulfonyl fluoride. Cell lysates were cleared by centrifugation and protein concentrations were determined by the Lowry assay (Bio-Rad). Conditioned media (100 μl), cell lysates (10 μg of proteins), or rat serum (2 μl) were subjected to Western ligand blot analysis (WLB); a mixture of 125I-IGF-I and 125I-IGF-II (1.5 × 10^6 cpm of each) was used as described previously (19, 22). In the current study, we have employed a nontransformed clonal chondrogenic cell line, RCJ3.1C5.18. The RCJ3.1C5.18 cell line is a mesenchymal stem cell system that, without requiring biochemical or oncogenic transformation, spontaneously and sequentially undergoes chondrocyte differentiation (14–16). We have previously reported that RCJ3.1C5.18 cells sequentially acquire, over 2 weeks of culture, markers for chondrocytic differentiation and terminal differentiation (Table I (14). Although our data are based upon an in vitro situation the morphology, the histochemical markers and the temporal sequential acquisition of the chondrocytic phenotype in this cell system is identical to the chondrogenesis process that occurs in vivo. This makes our system ideal and unique for studying chondrocyte cellular and molecular regulation, and suggests that our findings are relevant to the in vivo process.

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**IGFBP-3 Effect on Chondrogenesis**

| Culture time point | Differentiation stage morphology | Histochemical markers | Table I: Chondrocyte differentiation markers sequentially acquired by RCJ3.1C5.18 cells over 2 weeks of culture (14) |
|--------------------|---------------------------------|----------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| 4 days             | Undifferentiated mesenchymal cells; chondroprogenitors | Type II collagen     |
| 7 days             | Early differentiated cells; few chondrocyte nodules     | Proteoglycan synthesis |
| 10 days            | Differentiated cells; numerous chondrocyte nodules      | Type XI collagen     |
| 14 days            | Terminally differentiated cells; numerous chondrocyte nodules; mineralization | Type II collagen     |
|                    |                                                                | Proteoglycan synthesis |
|                    |                                                                | Type X collagen       |
|                    |                                                                | Alkaline phosphatase activity |

Cells were plated at a density of 6 × 10^4 cells/well in six-well dishes. After reaching confluence (4 days), fresh growth medium supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate was added. Differentiating cells were fed again with supplemented medium at days 7 and 10 of culture. Cultures were monitored over a total period of 14 days. We have previously shown that RCJ3.1C5.18 cells grown in this manner maintain their differentiated chondrocytic phenotype (14). As shown in Table I, cells sequentially acquire at 7 days of culture markers of chondrocytic differentiation (type II collagen and proteoglycans synthesis) and progressively acquire at 10 and 14 days of culture markers of terminal differentiation (type X collagen and alkaline phosphatase activity).
IGFBP-3 Effect on Chondrogenesis

RESULTS

Analysis of IGFBPs in Conditioned Media and in Cell Lysates—To determine the IGFBPs produced by RCJ3.1C5.18 cells, conditioned media and cell lysates were obtained after 4, 7, 10, and 14 days of culture and subjected to WLB analysis (lanes 1–10). For conditioned media, cells were incubated for 24 h in serum-free medium. Lanes 1–4, cell lysates obtained at the indicated culture time point; lanes 5–8, the corresponding conditioned media; lane 10, normal rat serum (NRS). Cell lysate (CL, lane 11) was subjected to immunoprecipitation using preimmune rabbit serum (PI), lanes 13 and 17), or a HEC polyclonal antibody against IGFBP-2 (aBP-2, lanes 14 and 15), or a polyclonal antibody against IGFBP-4 (aBP-4, lane 18) and then to WLB analysis.

$[^{3}H]$Thymidine (0.8 μCi/ml) in serum-free medium for 18 h. Incubations were terminated by washing with ice-cold PBS. Incorporation of $[^{3}H]$Thymidine into DNA was determined as uptake of radioactivity in trichloroacetic acid-precipitable material, as described previously (29). Cells at days 4, 7, 10, and 14 of culture were incubated in serum-free medium for 24 h; cell monolayers were then trypsinized and cells counted in a hemocytometer.

Cell-surface IGFBP-3 Binding—Affinity cross-linking of IGFBP-3 to cell monolayers was performed as described by Massagué et al. (30) with some modifications. Equilibrated cells were incubated with $^{125}$I-IGFBP-3 ligand-receptor complexes were cross-linked using 27 mM disuccinimidyl suberate (DSS) for 15 min at 4°C. Cross-linking reaction mixture was aspirated and quenched by addition of 10 mM Tris-containing detachment buffer. The excess of Tris quenched the unreacted DSS in solution; samples were processed as rapidly as possible to rapidly achieve the complete quenching of residual DSS internalized into the cell compartments by adding SDS-sample buffer. Cells were detached, lysed, and cell debris removed by centrifugation. Supernatants were subjected to immunoprecipitation using $\alpha$-IGFBP-3g1 antibody as reported previously (19, 23). Immunoprecipitates were dialyzed against SDS-sample buffer, boiled, centrifuged, and supernatants electrophoresed in SDS-PAGE under reducing and non-reducing conditions. Gels were dried and exposed to film.

Statistical Analysis—Data are presented as means ± S.D. The statistical differences between means were assessed by unpaired Student’s $t$ test or analysis of variance. Statistical significance was set at $p < 0.05$.

IGFBP-5. Northern blotting analysis was carried out, and IGFBP-5 mRNA was identified in RCJ3.1C5.18 cells (data not shown).

The 43–39-kDa doublet that corresponds to intact IGFBP-3 in rat serum (Fig. 1, lane 10) was not detected, even when cells were treated with increasing doses (1–15 nM) of IGF-I. To determine if the absence of intact IGFBP-3 was the result of specific conditioned media protease activity, conditioned media was subjected to protease assay. No IGFBP-3 proteolysis was detectable in conditioned media of chondrocytes at any time point of the culture (data not shown).

Northern Blotting Analysis for IGFBP-3 and Ribonuclease Protection Assay for IGFs—We determined that no IGFBP-3 mRNA was present in RCJ3.1C5.18 cells at any stage of differentiation by Northern blotting analysis (Fig. 2, lanes 2–4). RNase protection assays revealed that no IGF-I or IGF-II mRNA was expressed in RCJ3.1C5.18 cells at any stage of differentiation (Fig. 3, lanes 4–7 and lanes 8–12). The fact that no IGFBP-3, IGF-I, and IGF-II mRNAs were present in RCJ3.1C5.18 chondrogenic cells at any time point of the cell culture makes this an ideal system for studying the effects of IGFBP-3 and IGFs without interference from endogenous peptides.

Antiproliferative Action of IGFBP-3 in RCJ3.1C5.18 Chondrogenic Cells: IGF-independent and IGF-dependent Effects—We next assessed the IGF-dependent and IGF-independent antiproliferative actions of IGFBP-3 in RCJ3.1C5.18 chondrogenic cells and the relationship to cell differentiation stage. IGF-I, IGF-II, des-(1–3)-IGF-I, LR$^3$-IGF-I, and insulin, promoted, in a dose-dependent manner (1–15 nM), cell proliferation at all stages of differentiation, as determined by $[^{3}H]$thymidine incorporation; at 15 nM, IGFs and IGF analogs typically stimulated thymidine incorporation by 10-fold. IGFBP-3 inhibited DNA synthesis in progenitor (4 days of culture) and early differentiated (7 days) chondrocytes stimulated by insulin, des(1–3)-IGF-I, and LR$^3$-IGF-I by 80% (Fig. 4, A and B). In differentiated chondrocytes (10 days), 30 nM IGFBP-3 inhibited proliferation stimulated by insulin, des(1–3)-IGF-I, and LR$^3$-IGF-I by 40% (Fig. 4C). In terminally differentiated chondrocytes (14 days), IGFBP-3 had no effect on DNA synthesis stimulated by insulin, des(1–3)-IGF-I, and LR$^3$-IGF-I (Fig. 4D); IGFBP-3 continued, however, to inhibit cell proliferation stimulated by IGF-I by 80% at all stages of cell differentiation (Fig. 4, A–D).

During the early stages of chondrogenesis (days 4 and 7 of
cells treated with LR²-IGF-I at day 14 of culture, although IGFBP-3 continued to inhibit IGF-I-induced proliferation by 45%.

**Association of IGFBP-3 with Cell-surface Proteins**—We performed IGFBP-3 WIB analysis to detect the amount of exogenously added human recombinant IGFBP-3 (≈29 kDa) presents in conditioned media obtained from cells after 24 h incubation with IGFBP-3 alone or with IGFBP-3 plus IGF-I or des-(1–3)-IGF-I. The amount of IGFBP-3 was decreased when cells were incubated with IGFBP-3 alone or with des-(1–3)-IGF-I (Fig. 7, lanes 1, 2, 4, 5, 7, 8, 10, and 11) as compared with cells incubated with IGFBP-3 plus IGF-I (Fig. 7, lanes 3, 6, 9, and 12). This decrease in exogenously added IGFBP-3 detectable in the conditioned media was not due to inhibition of IGFBP-3 proteolysis in the presence of IGF-I, since no increase in IGFBP-3 fragments was detected by WIB analysis (Fig. 7) or by an IGFBP-3 protease assay (data not shown). These observations suggested the possibility that IGFBP-3 was associating with cell membranes, and that IGF-I, but not des-(1–3)-IGF-I, was promoting dissociation of IGFBP-3 from the cell surface into the conditioned media.

To investigate binding of IGFBP-3 to cell-surface proteins, ¹²⁵I-IGFBP-3 was cross-linked to RCJ3.1C5.18 cell monolayers, immunoprecipitated by IGFBP-3 antibody, and the communoprecipitated proteins analyzed by SDS-PAGE. As shown in Fig. 8, a prominent ~50-kDa radiolabeled band was identified in the immunoprecipitate (Fig. 8, lanes 3–5 and lanes 8–9); presumably an ~21-kDa protein cross-linked to the 29-kDa iodinated recombinant IGFBP-3. This ~50-kDa band was not present when ¹²⁵I-IGFBP-3 tracer was cross-linked in the absence of cells (Fig. 8, lanes 1 and 2). The appearance of the ~50-kDa band was inhibited by coinubation of cells with unlabeled (cold) IGF-I (Fig. 8, lanes 6 and 7) or with unlabeled IGFBP-3 (Fig. 8, lanes 11 and 12). A predominant ~50-kDa band was also detected when samples obtained by cross-linking ¹²⁵I-IGFBP-3 to cell monolayers and immunoprecipitation with IGFBP-3 antibody were electrophoresed in SDS-PAGE under reducing conditions, confirming that the cross-linked complex represents specific binding of ¹²⁵I-IGFBP-3 to the cell surface, rather than nonspecific adherence. The ~50-kDa ¹²⁵I-IGFBP-3 monolayer affinity cross-linked band was detectable at day 7 as well as at day 14 of cell culture.

**DISCUSSION**

In this study, we have demonstrated that IGFBP-3 has an antiproliferative effect on RCJ3.1C5.18 cells, which, under *in vitro* conditions, progress through the differentiation steps observed in growth-plate chondrocytes *in vivo*. The mechanisms involved in this growth-inhibitory effect of IGFBP-3 are clearly related to the differentiation process. During the early stage of RCJ3.1C5.18 differentiation, IGFBP-3 action appears to be independent of its ability to bind IGF. This conclusion is supported by the ability of nanomolar concentrations of IGFBP-3 to inhibit stimulation of DNA synthesis and cell proliferation by insulin, des-(1–3)-IGF-I, LR²-IGF-I, and IGF-I. In terminally differentiated cells, on the other hand, the inhibitory effects of IGFBP-3 become exclusively IGF-dependent. This transition is marked by the failure of IGFBP-3 to inhibit des-(1–3)-IGF-I, LR²-IGF-I, and insulin-induced DNA synthesis at day 10 and day 14 of culture, despite continued inhibition of IGF-I stimulation.

To determine whether the IGF-independent effect of IGFBP-3 was specific, we used IGFBP-2, which has a similar affinity for IGF-I, and the IGF-I analog des-(1–3)-IGF-I (17), and IGFBP-5, which has been shown to have potential IGF-independent effects (31–33). IGFBP-2 had no effect on cell growth stimulated by des-IGF-I, but was capable of inhibiting...
IGF-I-stimulated cell proliferation. IGFBP-5 had no effect on insulin-stimulated DNA synthesis in early differentiated as well as in terminally differentiated chondrocytes. Although we cannot exclude that other IGFBPs or IGFBP-related proteins can have an IGF-independent effect on chondrogenesis, this effect seems to be peculiar to IGFBP-3 and is not demonstrated by other IGFBPs which have similar affinities for IGF and IGF analogs or have been shown to have IGF-independent effects. Interestingly, IGFBP-5, in contrast to IGFBP-3, inhibited IGF-I-induced DNA synthesis at the early stage of chondrogenesis, but had no effect or even a slight enhancement of IGF-I-stimulated proliferation in terminally differentiated chondrocytes.

**IGFBP-3 Effect on Chondrogenesis**

![Graphs showing the effects of IGFBP-3 on DNA synthesis in RCJ3.1C5.18 chondrogenic cells.](http://www.jbc.org/)

**FIG. 4.** IGF-independent and IGF-dependent effects of IGFBP-3 on DNA synthesis in RCJ3.1C5.18 chondrogenic cells: relationship to cell differentiation stage. Cells cultured for 4, 7, 10, and 14 days were changed to serum-free medium for 4 h and then incubated for an additional 18 h with [3H]thymidine without and with IGFBP-3 and, respectively, insulin, des-(1–3)-IGF-I, longR3-IGF-I, or IGF-I. Incorporation of [3H]thymidine into DNA was determined as uptake of radioactivity in trichloroacetic acid-precipitable material. A, 4 days of culture; B, 7 days of culture; C, 10 days of culture; D, 14 days of culture. Results are expressed as percentage of the control, which was given an arbitrary value of 100%.

**FIG. 5.** Dose-dependent IGFBP-3 effect on des-(1–3)-IGF-I-stimulated DNA synthesis. Cells cultured for 4, 7, 10, and 14 days were changed to serum-free medium for 4 h and then incubated for an additional 18 h with [3H]thymidine with IGF-I or des-(1–3)-IGF-I and increasing doses of IGFBP-3. Incorporation of [3H]thymidine into DNA was determined as uptake of radioactivity in trichloroacetic acid-precipitable material. A, 4 days of culture; B, 7 days of culture; C, 10 days of culture; D, 14 days of culture. Results are expressed as percentage of the control, which was given an arbitrary value of 100%.
Enhancement of IGF proliferative effect by IGFBP-5 has been demonstrated in other systems, like osteoblasts, and it has been speculated that inhibition of IGFBP-5 binding to extracellular matrix can facilitate the delivery of IGF to IGF-I receptor (31–33).

IGFBP-3 binds to a membrane-associated protein located on chondrocytes, and this binding can be totally ablated by IGF-I, presumably due to dissociation of IGFBP-3 from the cell membrane. Binding of $^{125}$I-IGFBP-3 can also be competed by excess of unlabeled IGFBP-3. This specific cell surface-associated protein has a mass of ~21 kDa (after subtracting 29 kDa of iodinated IGFBP-3).

Chondrogenesis is a complex phenomenon that results from the ordered and sequential proliferation and differentiation of chondrocytes in the growth plate. The GH-IGF system appears to play a crucial role in chondrogenesis, but the relative contributions of GH and IGF to the process have not been resolved (1). In RCJ3.1C5.18 chondrocytes, IGFs and, interestingly, insulin, stimulate DNA synthesis and cell proliferation at nanomolar concentrations. The present study reports an IGFBP-independent antiproliferative effect of IGFBP-3 in the regulation of normal cell growth in a system that mimics the in vivo process of chondrogenesis. This finding suggests that this IGFBP-3 effect is not only important in the control of cancer and fibroblast cell line growth, as reported previously (4, 5, 34), but also in physiological conditions, such as chondrogenesis. We have demonstrated that IGFBP-3 has an IGFBP-independent antiproliferative effect on chondroprogenitors and early differentiated

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**Fig. 6.** Effect of IGFBP-3 on des-(1–3)-IGF-I-stimulated DNA synthesis. IGF-independent antiproliferative effect of IGFBP-3 is specific. Cells cultured for 4 (panel A) and 7 days (panel B) were changed to serum-free medium for 4 h and then incubated for an additional 18 h with $^{3}$H-thymidine and with the following peptides: des-(1–3)-IGF-I; des-(1–3)-IGF-I and IGFBP-3; des-(1–3)-IGF-I and IGFBP-2; IGF-I; IGF-I and IGFBP-3; IGF-I and IGFBP-2. Results are expressed as percentage of the control, which was given an arbitrary value of 100%.

**Fig. 7.** Western immunoblotting analysis for exogenously added human recombinant IGFBP-3. Cells cultured for 4 days (lanes 1–3), 7 days (lanes 4–6), 10 days (lanes 7–9), and 14 days (lanes 10–12) were treated with recombinant human IGFBP-3 with or without IGF-I or des-(1–3)-IGF-I in serum-free media for 24 h. Conditioned media were subjected to WLB analysis using $\alpha$-IGFBP-3g1 antibody. Lanes 1, 4, 7, and 10 cells incubated with IGFBP-3 alone; lanes 2, 5, 8, and 11, cells treated with IGFBP-3 and des-(1–3)-IGF-I; lanes 3, 6, 9, and 12, cells incubated with IGFBP-3 and IGF-I; lane 13, human recombinant IGFBP-3 (30 nM); lane 14, serum pooled from pregnant women (2 µl).

**Fig. 8.** Cell-surface IGFBP-3 binding. Affinity cross-linking of IGFBP-3 to cell monolayers. Cells were incubated with $^{125}$I-IGFBP-3 without (lanes 3, 4, 8, and 9) or with unlabeled (cold) IGF-I (lane 7) or IGFBP-3 (lanes 11 and 12). $^{125}$I-IGFBP-3 ligand-receptor complexes were cross-linked with disuccinimidyl suberate. Supernatant were subjected to immunoprecipitation using $\alpha$-IGFBP-3g1 antibody. Immunoprecipitates were subjected to SDS-PAGE analysis, and gels were dried and exposed to film. Lanes 1 and 2, tracer cross-linked in the absence of cells and subjected to immunoprecipitation. The arrow indicates cross-linked complex of $^{125}$I-IGFBP-3 and cell surface-associated protein.
IGFBP-3 receptor-like protein with a molecular weight similar to the specific cell membrane-associated protein identified in RCJ3.1C5.18 cells (5). It is of note that the current finding of a similar IGFBP-3 receptor, as well as an IGF-independent effect of IGFBP-3, is in a nontransformed cell system. Interestingly, the IGFBP-3 receptor was present in cells at every differentiation stage, whereas the IGF-independent antiproliferative effect was only evident during the early stages of differentiation.

Further studies are needed to determine whether different post-receptor signaling mechanisms or intranuclear localization processes for IGFBP-3 can explain this selective differentiation-dependent, growth inhibitory effect of IGFBP-3.

In conclusion, these studies demonstrate IGF-independent and IGF-dependent effects of IGFBP-3 on chondrocyte proliferation; the nature of these IGFBP-3 effects relates to the stage of cellular differentiation. These findings provide new insight into the biological actions of IGFBP-3, as well as the impact of cell differentiation, and characterize a novel, informative model for the investigation of the role of the IGF-IGFBP-3 system in the growth process.

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