Identification of STAT-1 as a Molecular Target of IGFBP-3 in the Process of Chondrogenesis*

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The chondrogenesis process requires the ordered proliferation and differentiation of chondrocytes. Insulin-like growth factor-binding protein (IGFBP)-3, well characterized as the carrier of insulin-like growth factor (IGF), has been reported to have intrinsic bioactivity that is independent of IGF binding. The mechanisms involved in this IGF-independent action are still unclear. Using the RCJ3.1C5.18 chondrogenic cells, which in culture progresses from undifferentiated to terminally differentiated chondrocytes, we have shown previously that IGFBP-3 has an IGF-independent, antiproliferative effect in undifferentiated and early differentiated but not in terminally differentiated chondrocytes. In the present study, cDNA microarray analysis was used to screen for genes: 1) that were regulated by IGFBP-3 in early but not in terminally differentiated chondrocytes; 2) that were regulated specifically by IGFBP-3, but not by IGF-I; and 3) whose regulation was abolished by coinubcation of IGFBP-3 with IGF-I. Signal transducer and activator of transcription (STAT)-1 was the gene that, fulfilling the screening criteria, exhibited the greatest up-regulation by IGFBP-3 (>40-fold). STAT-1 gene up-regulation was confirmed by Northern analysis of cells treated with IGFBP-3 or transfected with an IGFBP-3 expression vector. Remarkably, similar results were obtained when cells were transfected with an IGFBP-3 mutant unable to bind IGFs, definitively demonstrating the IGF-independent action of IGFBP-3. Consistent with the up-regulation of STAT-1 mRNA, IGFBP-3 also increased STAT-1 protein expression. Furthermore, both IGFBP-3 and the IGFBP-3 mutant induced STAT-1 phosphorylation and its nuclear localization. An antisense STAT-1 oligonucleotide abolished the IGF-independent cell apoptosis induced by IGFBP-3. We have demonstrated that STAT-1 is a major intracellular signaling and transcriptional target of the IGF-independent apoptotic effect of IGFBP-3 in chondrogenesis.

Long bone growth is initiated by chondrogenesis, a strictly regulated process that requires proliferation and differentiation of chondrocytes at the growth plate. Control of chondrogenesis is a complex and poorly understood phenomenon. Coordination of several factors and signals is required to achieve adequate skeletal growth. The remarkable degree of growth failure observed in animals carrying null mutations of the genes encoding the insulin-like growth factors (IGFs) and the type I IGF receptor has clearly indicated the fundamental role of IGFs in the growth process (1, 2). Six IGF-binding proteins (IGFBPs), referred to as IGFBP-1–6, have been characterized as IGF carriers (3, 4). The multifunctional nature of some of the IGFBPs, and in particular IGFBP-3, has been characterized over the past few years, including an intrinsic bioactivity of IGFBP-3 that is independent of IGF binding (5). IGFBP-3, through this IGF-independent action, has been shown to control cell proliferation and to induce or enhance apoptosis (6–10). In addition to binding IGFs, IGFBP-3 has been demonstrated to bind to an array of cellular factors in different cell compartments. IGFBP-3 binds to the extracellular matrix and to cell membrane receptors and is transported in the nucleus by the importin β subunit, where it binds to the retinoid X receptor-α (11–17). Although extensively described, the IGF-independent concept remains controversial. Specifically, although multiple IGF-independent binding sites have been reported, the signaling pathways and transcriptional targets related to these interactions are still poorly understood. Additionally, in some experimental systems, the reported IGF-independent actions of IGFBP-3 may have alternative explanations.

Using the RCJ3.1C5.18 chondrogenic cell line as an established model to study chondrogenesis in vitro, we have reported a novel IGF-independent role for IGFBP-3 in this process (18). RCJ3.1C5.18 cells are especially suitable for this purpose. Over the 2 weeks of culture, they undergo a reproducible, time-dependent progression from chondroprogenitors to hypertrophic chondrocytes (19, 20). Furthermore, RCJ3.1C5.18 cells do not express IGFs or IGFBP-3; therefore the action of these peptides can be studied without interference from endogenous molecules (18). Using RCJ3.1C5.18 cells, we have reported that IGFBP-3 has an IGF-independent antiproliferative effect in undifferentiated and early differentiated chondrocytes but not in terminally differentiated chondrocytes (18).

In the present study, we evaluate the mechanisms involved in the IGF-independent action of IGFBP-3 in chondrogenesis.

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¶ The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; STAT, signal transducer and activator of transcription; WIB, Western immunoblot; FGFR, fibroblast growth factor receptor; IFN, interferon.
In particular, our study was designed to identify IGFBP-3 target genes and intracellular signaling pathways involved in the pro-apoptotic, IGF-independent action of IGFBP-3 during the process of chondrogenesis.

EXPERIMENTAL PROCEDURES

Chemical Reagents—Recombinant nonglycosylated human IGFBP-3 expressed in *Escherichia coli* was generously supplied by Celtrix Pharmaceuticals Inc. (Santa Clara, CA). Human recombinant IGF-I was purchased from GroPep Pty. Ltd. (Adelaide, Australia). Fetal bovine serum, H9251-minimum essential medium and sodium pyruvate were purchased from Invitrogen. Dexamethasone and H9252-glycerophosphate were obtained from Sigma. Ascorbic acid was obtained from Wako Pure Biochemicals Industries, Ltd. (Osaka, Japan). Anti-phosphorylated signal transducer and activator of transcription (STAT)-1 (Tyr701), anti-STAT-1, and anti-p38 mitogen-activated protein kinase polyclonal antibodies were obtained from Cell Signaling Technology (Beverly, MA).

Cell Culture—RCJ3.1C5.18 cells, generously donated by Dr. Jane E. Aubin (University of Toronto), were grown in H9251-minimum essential medium supplemented with 15% heat-inactivated fetal bovine serum, 10 H11002 M dexamethasone, and 2 mM sodium pyruvate. The cells were plated at a density of 6 H11003 10^4 cells/well in six-well dishes. After reaching confluence (4 days), fresh growth medium supplemented with 50 g/ml of ascorbic acid and 10 mM H9252-glycerophosphate was added. The differentiating cells were fed again with supplemented medium at days 7 and 10 of culture. We have previously shown that RCJ3.1C5.18 cells grown in this manner maintain their differentiated chondrocytic phenotype, sequentially acquire at 7 days culture markers of early chondrocytic differentiation (type II collagen and proteoglycan synthesis), and progressively acquire at 10 and 14 days culture markers of terminal differentiation (type X collagen and alkaline phosphatase activity) (20).

Plasmid Constructs and DNA Transfections—The GGG-IGFBP-3 mutant cDNA was generated by site-directed mutagenesis at residues Ile56, Leu80, and Leu81 to Gly56, Gly80, and Gly81, as described previously (21). Binding studies (including BIAcore analysis) showed that the GGG-IGFBP-3 mutant protein, generated in *E. coli* and baculovirus expression systems, had abolished affinity for IGFs (21). For transfection, hIGFBP-3 and GGG-IGFBP-3 mutant cDNAs were subcloned into the pCMV6 vector as described previously (21). The cells were seeded in six-well dishes and 24 h later were transfected with 4 g of expression vector plasmid using Mirus Transit LT-1, as described by the manufacturer (PanVera, Madison, WI). IGFBP-3 and GGG-IGFBP-3 levels were measured in conditioned media of transfected cells. The media were concentrated 7–10-fold using Centricon 3 columns (Amicon, Boston, MA), and IGFBP-3 and GGG-IGFBP-3 concentrations were measured using a commercial immunoradiometric assay (IRMA) kit for hIGFBP-3 (Diagnostic System Laboratories, Inc., Webster, TX). The anti-IGFBP-3 antibodies employed in the IRMA kit recognized the GGG-IGFBP-3 mutant with the affinity similar to wild-type IGFBP-3 (21).

A STAT-1 morpholino antisense oligonucleotide (GeneTools LLC, Philomath, OR) was designed based upon the published rat STAT-1 cDNA sequence (GenBank® accession number AF205604: 5’-GCT-GAAGCTGACACCTGACAT-3’), and corresponded to the first 25

![Fig. 1. STAT-1 is an IGFBP-3 target gene in early differentiated cells as assessed by cDNA microarray analysis. The columns represent samples obtained from cells at early (7 days of culture) or late (14 days of culture) stage of differentiation treated under serum-free conditions with IGF-I, IGF-I plus IGFBP-3, IGFBP-3, or untreated (Control). A, spot analysis. Red indicates gene expression above the median; green indicates gene expression equal to or below (lighter) the median. B, sample over control ratios are represented.](http://www.jbc.org/)

![Fig. 2. Exogenous IGFBP-3 increases STAT-1 mRNA in chondroprogenitors and early differentiated chondrocytes. Total RNA was obtained from cells cultured for 4, 7, and 14 days (4D, 7D, and 14D) incubated in serum-free medium for 24 h without (Control) or with IGFBP-3. Northern analysis was performed using a STAT-1 cDNA probe that corresponds to the STAT-1 cDNA fragment present in the microarrays. The 18 S rRNA bands are shown to demonstrate equal RNA loading. This is a representative gel that has been repeated at least three times.](http://www.jbc.org/)
nucleotides of the STAT-1 open reading frame. The cells were seeded in six-well dishes and 24 h later were treated with or without the morpholino STAT-1 antisense oligo, using the special delivery morpholino system as described by the manufacturer (GeneTools LLC). Twenty-four hours after antisense treatment, the cells were transfected with 4 μg of expression vector plasmid (IGFBP-3, GGG-IGFBP-3, or empty vector), using Mirus Transit LT-1 as described. Twenty-four hours after transfection, the cells were subjected to a quantitative apoptosis assay, and total RNA was obtained and subjected to Northern blot analysis for STAT-1. Total cell extracts were also obtained and subjected to Western immunoblot (WIB) analysis for phosphorylated STAT-1. Each experiment was performed twice in triplicate.

Microarrays—13,824 unique mouse genes available from Research Genetics (Huntsville, AL) were spotted in duplicate on superaldehyde-coated glass slides. Detailed descriptions of printing, processing, and data analysis procedures are available from medir.ohsu.edu—geneview. Ten μg of total RNA were used from indirect biotinylated probe synthesis. Each source RNA was used to screen two independent arrays. Data normalization and analysis were performed using trimmed mean approaches as described by Eisen et al. (23). Detailed methods are available on the web page web.

Total RNA was obtained from early differentiated (7 days of culture) and terminally differentiated chondrocytes (14 days of culture) using RNeasy columns as described by the manufacturer (Qiagen, Inc., Santa Clarita, CA). The cells were treated with IGFBP-3 (1 μg/ml), IGF-I (100 ng/ml), or IGFBP-3 (1 μg/ml) plus IGF-I (100 ng/ml) or left untreated (control) for 24 h in serum-free conditions. This experiment was performed in triplicate. Ten μg of total RNA were hybridized with DNA microarrays.

Northern Blot Analysis—The STAT-1 cDNA spotted on the microarrays (GenBank™ accession number AI449540) was generated by PCR, verified by DNA sequencing matched through BLAST analysis (www.ncbi.nlm.nih/BLAST/) to GenBankTM data base, and used as a probe. Total RNA was obtained from cells cultured for 4, 7, or 14 days and then incubated for 24 h in serum-free medium with or without 1 μg/ml of recombinant IGFBP-3. Total RNA was also obtained at different time point after transfection from cells transfected with expression vector plasmid (IGFBP-3, GGG-IGFBP-3, or empty vector) or left untransfected. Total RNA was extracted from cultured cells, as described by the manufacturer, using RNeasy columns (Qiagen) and quantified by spectrophotometric analysis. Eight to ten μg of RNA were subjected to Northern blot analysis as described previously (18, 24). The STAT-1 probe was labeled by random priming with [α-32P]dCTP, and hybridization was performed in Rapid-hyb buffer (Amersham Biosciences). Washed filters were autoradiographed, and densitometric analysis was done with a GS700 Imaging Densitometer (Bio-Rad). 18 S RNA was used as an internal control for RNA loading.

Western Blot Analysis—Total cell extracts were obtained at different time points after transfection from cells transfected with expression vector plasmid (IGFBP-3, GGG-IGFBP-3, or empty vector) or from untransfected cells. For preparation of total cell extracts, the 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, and 10% glycerol) containing a mixture of protease inhibitors (Roche Molecular Biochemicals) including 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium vandate. Total cell extracts were cleared by centrifugation, and the protein concentrations were determined (Bio-Rad). The total cell extracts (100 μg of protein) were subjected to WIB with specific primary antibodies, using ECL (PerkinElmer Life Sciences), as described previously (18, 25). The membranes were rehybridized with an anti-p38 antibody as an internal control for the protein amount loaded. Densitometric analysis was done with a GS700 Imaging Densitometer (Bio-Rad).

Immunocytochemistry—The cells were seeded in six-well dishes and 24 h later were transfected with 4 μg of expression vector plasmid (IGFBP-3, GGG-IGFBP-3, or empty vector). Twenty-four hours after transfection, the cells were fixed in 100% methanol for 10 min at −20 °C, washed with TBST buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Triton X-100), incubated for 1 h at room temperature with biotinylated secondary antibody (Vector Laboratories Inc., Burlingame, CA), washed, incubated for 30 min at room temperature with 0.6% hydrogen peroxide, washed, incubated 1 h at room temperature with ABC reagents (VectorStain ABC kit; Vector Laboratories Inc.), washed, and incubated with DAB reagent (0.01% hydrogen peroxide, 0.2 mg/ml diaminobenzidine tetrahydrochloride in phosphate-buffered saline). The reaction was monitored under a microscope and was stopped with water.

Measurement of Apoptosis—A cell death detection enzyme-linked immunosorbent assay kit was used to measure cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) generated in the early phase of apoptosis (Roche Molecular Biochemicals). The assay is based on a quantitative sandwich enzyme immunoassay, using antibodies directed against DNA and histones. This allows the specific determination of mono- and oligo-nucleosomes, which are released into the cytoplasm of apoptotic cells. The cells were seeded in six-well dishes and 24 h later were treated with or without the STAT-1 antisense oligo. Twenty-four hours after antisense treatment, the cells were transfected with 4 μg of expression vector plasmid (IGFBP-3, GGG-IGFBP-3, or empty vector). Twenty-four hours after transfection, the cell lysates were subjected to Western blot analysis for phosphorylated STAT-1. Each experiment was performed twice in triplicate.

Fig. 3. Transfection of chondroprogenitors with IGFBP-3 or GGG-IGFBP-3 expression vectors induces STAT-1 mRNA. Total RNA was obtained from chondroprogenitor cells transfected with IGFBP-3 or the GGG-IGFBP-3 mutant expression vectors, transfected with the control vector containing no cDNA insert (Empty vector), or left untransfected (Untransf). Northern analysis was performed as described in legend to Fig. 2. This is a representative gel that has been repeated at least three times.

Fig. 4. Effect of IGFBP-3 on total STAT-1 by WIB analysis. The cell lysates were obtained from chondroprogenitors transfected with IGFBP-3 (lanes 3, 6, and 9) or with empty vector (lanes 1, 4, and 7) or left untransfected (lanes 2, 5, and 8). The cell lysates were obtained, respectively, 6, 12, and 24 h after transfection and subjected to WIB analysis for total STAT-1. The 91-kDa (STAT-1α) and 84-kDa (STAT-1β) bands, representing alternatively spliced products of STAT-1 gene, are marked. The membranes were rehybridized with an anti-p38 antibody to demonstrate equal protein loading. This is a representative gel that has been repeated at least three times.
were prepared and subjected in duplicate to the cell death detection assay.

Statistics—The data are presented as the means ± S.D. Statistical differences between the means were assessed by one-way analysis of variance, followed by the Student-Newman-Keuls test for pairwise comparisons. The statistical significance was set at \( p < 0.05 \).

RESULTS

STAT-1 Expression Is Regulated by IGFBP-3 during Chondrogenesis

Identification of Differential STAT-1 Gene Expression Using Microarray Analysis—We have previously reported that IGFBP-3 has IGF-I-independent antiproliferative action in undifferentiated and early differentiated chondrocytes but not in terminally differentiated chondrocytes (18). Because of this selective biological action of IGFBP-3, microarray gene profiling analyses were carried out to select genes: 1) that were regulated by IGFBP-3 in early but not in terminally differentiated cells; 2) whose regulation was abolished by coinfection of IGFBP-3 with IGF-I; and 3) that were regulated by IGFBP-3 but not by IGF-I. The STAT-1 fulfilled all the screening criteria that we used for the microarray analysis; among the genes that met our criteria, STAT-1 showed the highest regulation by IGFBP-3. Consistent with the increase in STAT-1 mRNA, IGFBP-3 increased total STAT-1 protein compared with untreated control cells (Fig. 2). Effects similar to those observed in cells treated with exogenously added IGFBP-3 were seen in undifferentiated chondroprogenitors transfected with expression vectors encoding wild-type IGFBP-3 or the IGFBP-3 mutant with abolished affinity for IGFs (GGG-IGFBP-3). Thus, both IGFBP-3 and GGG-IGFBP-3 produced a marked increase in STAT-1 mRNA (Fig. 3) compared with untransfected cells or cells transfected with the empty vector. IGFBP-3 and GGG-IGFBP-3 had a prolonged effect on STAT-1 gene expression (up to 24 h after transfection).

Fig. 5. Effect of GGG-IGFBP-3 mutant on STAT-1 total by WIB analysis. The cell lysates were obtained from chondroprogenitors transfected with GGG-IGFBP-3 (lane 4) or IGFBP-3 (lane 3) or with empty vector (lane 2) or left untransfected (lane 1). The cell lysates were obtained 12 h after transfection and subjected to WIB analysis as described in the Fig. 4 legend. This is a representative gel that has been repeated at least three times.

Fig. 6. IGFBP-3 increases STAT-1 phosphorylation by WIB analysis. The cell lysates were obtained from chondroprogenitors transfected with IGFBP-3 (lanes 3, 6, 9, 12, and 15) or with empty vector (lanes 2, 5, 8, 11, and 14) or left untransfected (lanes 1, 4, 7, 10, and 13). The cell lysates were obtained, respectively, 6, 12, 24, and 48 h after transfection and subjected to WIB analysis for phosphorylated STAT-1. This is a representative gel that has been repeated at least three times.

STAT-1 Northern Blot and Western Blot Analyses—To confirm the results of the microarray analysis, we performed Northern blot analysis for STAT-1. As shown in Fig. 2, in undifferentiated (4 days of culture) and early differentiated (7 days of culture) cells, IGFBP-3 treatment resulted in a marked increase in STAT-1 mRNA. In terminally differentiated cells (14 days of culture), IGFBP-3 did not affect STAT-1 gene expression (Fig. 2). Effects similar to those observed in cells treated with exogenously added IGFBP-3 were seen in undifferentiated chondroprogenitors transfected with expression vectors encoding wild-type IGFBP-3 or the IGFBP-3 mutant with abolished affinity for IGFs (GGG-IGFBP-3). Thus, both IGFBP-3 and GGG-IGFBP-3 increased STAT-1 gene expression (Fig. 3) compared with untransfected cells or cells transfected with the empty vector. IGFBP-3 and GGG-IGFBP-3 had a prolonged effect on STAT-1 gene expression (up to 24 h after transfection).

Fig. 7. GGG-IGFBP-3 mutant induces STAT-1 phosphorylation by WIB analysis. The cell lysates were obtained from chondroprogenitors transfected with GGG-IGFBP-3 (lane 4) or IGFBP-3 (lane 3) or with empty vector (lane 2) or left untransfected (lane 1). The cell lysates were obtained 12 h after transfection and subjected to WIB analysis as described in the Fig. 4 legend. This is a representative gel that has been repeated at least three times.

IGFBP-3 induces phosphorylated STAT-1 nuclear localization by immunocytochemistry. The cells were subjected to immunocytochemistry for phosphorylated STAT-1 24 h after transfection with either IGFBP-3 or empty vector or left untransfected.

STAT-1 Phosphorylation and Nuclear Localization Induced by IGFBP-3 in Chondroprogenitors

To investigate the IGF-independent effect of IGFBP-3 on STAT-1 activation, we performed WIB of phosphorylated STAT-1. In chondroprogenitors, IGFBP-3 transfection resulted in an increase in STAT-1 phosphorylation, compared with untransfected cells or cells transfected with empty vector (Fig. 6). The effect of IGFBP-3 on STAT-1 phosphorylation was observed up to 24 h after transfection (Fig. 6). Similarly to
IGFBP-3, the GGG-IGFBP-3 mutant increased STAT-1 phosphorylation (Fig. 7), with a similar prolonged effect.

To determine whether phosphorylated STAT-1 translocated to the nucleus, immunocytochemical analysis of phosphorylated STAT-1 cellular localization was performed. As shown in Fig. 8, transfection with IGFBP-3 resulted in the nuclear localization of phosphorylated STAT-1, whereas only a faint signal for phosphorylated STAT-1 was seen in the cytoplasm of untransfected cells or cells transfected with the empty vector (Fig. 8).

**STAT-1 Expression and Phosphorylation Have a Functional Role in IGFBP-3-induced Apoptosis in Chondroprogenitors**

It has been previously reported that STAT-1 is a key signaling molecule that mediates the apoptotic activity of fibroblast growth factor receptor 3 (FGFR-3) (27–31). Therefore, we decided to investigate whether STAT-1 has a functional role in the IGFBP-3-induced pro-apoptotic effect in chondroprogenitors. We used a STAT-1 antisense oligonucleotide to inhibit the endogenous expression of STAT-1. As shown in Fig. 9, IGFBP-3 induced apoptosis in chondroprogenitors. Similar results were obtained if chondroprogenitors were transfected with GGG-IGFBP-3 mutant, therefore demonstrating that IGFBP-3 has an IGF-independent pro-apoptotic action in chondroprogenitors. If cells were treated 24 h before transfection with STAT-1 antisense, the level of apoptosis induced by either IGFBP-3 or GGG-mutant was significantly reduced to the level observed in cells treated with the empty vector or in untransfected cells (control). The STAT-1 antisense reduced the level of STAT-1 mRNA (Fig. 10), STAT-1 total protein (Fig. 11), and phosphorylated STAT-1 (Fig. 12) induced by IGFBP-3 or GGG-IGFBP-3, demonstrating that the STAT-1 antisense indeed inhibited endogenous STAT-1 mRNA expression and activation. The STAT-1 antisense had no effect on apoptosis when compared with controls (untransfected cells or cells transfected with empty vector) that did not receive antisense. The antisense delivery reagents were not toxic to cells (data not shown).

**DISCUSSION**

In the present study, we identified STAT-1 as a major transcriptional gene target and intracellular signaling factor in the IGF-independent pro-apoptotic effect of IGFBP-3 in chondrogenesis. We used cDNA microarray analysis to identify genes that were regulated by the IGF-independent action of IGFBP-3. We have previously reported that IGFBP-3 has an IGF-independent antiproliferative action in early but not in terminally differentiated RCJ3.1C5.18 chondrogenic cells (18). We used this evidence to develop specific criteria for profiling IGFBP-3-related genes by cDNA microarray analysis. We determined that STAT-1 was the most prominent gene up-regulated by IGFBP-3 in early but not in terminally differentiated cells. STAT-1 expression was not affected by IGF-I by itself, although IGF-I abolished the STAT-1 up-regulation induced by IGFBP-3. Northern analysis confirmed that STAT-1 was a target gene for the IGF-independent action of IGFBP-3 in undifferentiated and early differentiated chondrocytes. Using the GGG-IGFBP-3 mutant with abolished affinity for IGFs, we have conclusively demonstrated that IGFBP-3-induced regulation of STAT-1 expression occurs via an IGF-independent mechanism. We have also demonstrated that IGFBP-3 induces STAT-1 phosphorylation and its nuclear translocation. Furthermore, we have demonstrated that the inhibition of STAT-1 transcription by STAT-1 antisense significantly reduces the IGF-independent pro-apoptotic effect of IGFBP-3 in undifferentiated chondrocytes. Taken together, these findings indicate that STAT-1 has a functional role in the pro-apoptotic biological action of IGFBP-3 in the process of chondrogenesis.

The IGF-independent action of IGFBP-3 has to date remained an elusive concept. Several lines of evidence have indicated that IGFBP-3, by mechanisms not related to IGF binding, directly controls or potentiates the effects of other growth factors in controlling cell growth and apoptosis (6–10). However, because of the complexity of the IGF-IGFBP-3 system,
these studies were not totally conclusive proof of an IGF-independent action of IGFBP-3. Several IGFBP-3 binding sites have been found in different cell compartments; IGFBP-3 has been reported to bind to extracellular matrix, to cell membrane proteins, to nuclear membrane proteins, and to the nuclear retinoid X receptor-α (11–16). IGFBP-3 bioactivity could be ascribed to only some of these interactions, and functional and distinctive IGFBP-3 signaling pathways are virtually unknown. Fanayan et al. (32) were able to demonstrate that IGFBP-3 induces Smad2 and Smad3 phosphorylation in T47D breast cancer cells, but specific IGFBP-3 bioactivity could not be attributed to Smad phosphorylation; on the other hand, they found that IGFBP-3 was synergistic with TGF-β in inducing Smad phosphorylation and cell growth inhibition. Conover et al. (33) have reported that in bovine fibroblasts, cell-associated IGFBP-3 enhances IGF-induced cell growth through the phosphatidylinositol 3-kinase pathway; however, IGFBP-3 alone did not exhibit bioactivity. Our current demonstration of IGFBP-3-induced STAT-1 activation represents the first functional intracellular signaling pathway for the IGF-independent action of IGFBP-3.

STAT-1 as a Molecular Target of IGFBP-3

FIG. 11. STAT-1 antisense oligonucleotide reduces total STAT-1 protein by WIB analysis. Chondroprogenitors were treated with (lanes 1–4) or without (lanes 5–8) a STAT-1 antisense oligonucleotide and 24 h later were transfected with IGFBP-3 or GGG-IGFBP-3. Forty-eight after treatment with or without STAT-1 antisense, the cell lysates were obtained from chondroprogenitor cells transfected with IGFBP-3 (lanes 4 and 8), GGG-IGFBP-3 mutant (lanes 3 and 7), or empty vector (lanes 2 and 6) or left untransfected (lanes 1 and 5). The cell lysates obtained were submitted to WIB analysis for total STAT-1. Densitometric analysis of total STAT-1 levels are normalized for p38 levels. 

FIG. 12. STAT-1 antisense oligonucleotide reduces STAT-1 phosphorylation by WIB analysis. Chondroprogenitors were treated with (lanes 1, 3, 5, and 7) or without (lanes 2, 4, 6, and 8) a STAT-1 antisense oligonucleotide and 24 h later were transfected with IGFBP-3 or GGG-IGFBP-3. Forty-eight after treatment with or without STAT-1 antisense, the cell lysates were obtained from chondroprogenitor cells transfected with IGFBP-3 (lanes 7 and 8), GGG-IGFBP-3 mutant (lanes 3 and 4), or empty vector (lanes 1 and 2) or left untransfected (lanes 5 and 6). The cell lysates obtained were submitted to WIB analysis for phosphorylated STAT-1. Densitometric analysis of total STAT-1 levels was normalized for p38 levels.
rived from STAT-1 knockout mice have been found to be defective in FGF-mediated growth inhibition (28). Recently, Sahni et al. (30), in an in vivo study, reported further evidence that STAT-1 is a modulator of FGF inhibition of bone growth. They reported that the chondrodysplastic phenotype, similar to human achondroplasia, observed in transgenic mice overexpressing FGF-2 was corrected if the animals were crossed with STAT-1 knockout mice (30). In the absence of STAT-1 function, the increased apoptosis and reduced chondrocyte proliferation in mice overexpressing FGF-2 were restored to nearly normal levels (30). In particular, STAT-1 deficiency repressed the apoptosis induced by the FGF-2 transgene in chondroprogenitors and proliferative chondrocytes and had no effect on the apoptosis observed in hypertrophic chondrocytes (30). Our study is the first to report STAT-1 activation by the IGFBP-3 and to elucidate the role of STAT-1 in IGFBP-3-induced apoptosis in chondroprogenitors. IGFBP-3 actions seem to have several similarities with the pathways and the biological actions observed in mice overexpressing FGF-2 or mediated by FGRFR-3-activating mutations. IGFBP-3 induces STAT-1 activation that, in turn, mediates its pro-apoptotic action. IGFBP-3 has selective antiproliferative and pro-apoptotic actions in chondroprogenitors but not in hypertrophic chondrocytes. The mechanisms through which IGFBP-3 induces STAT-1 gene expression, as well as the apoptotic pathways involved in the STAT-1 activation by IGFBP-3, remain to be elucidated. We hypothesize that in chondrogenesis, STAT-1 activation by IGFBP-3 may be required for proper negative control to balance the actions of mitogenic pathways, so that the appropriate balance of chondrocyte proliferation and differentiation can be achieved.

STAT-1 gene and protein expression are increased by interferons (IFNs) via IFN-stimulated response elements in its promoter (35–40). It has been proposed that the prolonged elevated levels of STAT-1 protein and gene expression induced by IFNs can be attributed to homodimerization of phosphorylated STAT-1 or its heterodimerization with STAT-2, formation of the complexes GAF (transcription factor γ-activated factor) and ISGF3 (IFN-stimulated gene factor 3) with IRF-9 (IFN regulatory factor 9), that bind IFN-stimulated response elements and result in increased STAT-1 gene and protein expression and phosphorylation (36, 38–40). We speculate that IGFBP-3 might have a signaling pathway similar to type I IFNs, inducing STAT-1 phosphorylation as well as increased STAT-1 gene and protein expression. An alternative hypothesis is that IGFBP-3 might induce STAT-1 gene expression and phosphorylation by two independent cellular responses.

There are few studies directed at the evaluation of IGFBP-3 in chondrogenesis. IGFBP-3 (protein and gene expression) has been reported to be increased (up to 24-fold) in synovial fluid and articular chondrocytes from patients with rheumatoid arthritis and osteoarthritis. In these conditions IGF-I has been also reported increased (up to 3.5-fold), leading to the hypothesis that the net result in rheumatoid arthritis and osteoarthritis would be an increase of unbound IGFBP-3 that might determine decreased cell growth and apoptosis through its IGF-independent action (41, 42). The other observation regarding the role of IGFBP-3 in the growth process derives from the growth pattern of transgenic mice overexpressing IGFBP-3 (43). Transgenic mice overexpressing IGFBP-3 exhibit a reduction in growth pattern of transgenic mice overexpressing IGFBP-3 (43). In conclusion, we have demonstrated that STAT-1 functions as a downstream mediator of IGFBP-3 signaling, modulating the pro-apoptotic action of IGFBP-3 during the process of chondrogenesis. Our study provides new perspectives in understanding the IGF-independent role of IGFBP-3 in the growth process. Further studies, however, are needed to elucidate the molecular mechanisms through which IGFBP-3 induces STAT-1 phosphorylation and gene expression, as well as the downstream targets of STAT-1 that mediate IGFBP-3-induced apoptosis.

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Identification of STAT-1 as a Molecular Target of IGFBP-3 in the Process of Chondrogenesis

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