Award Number: DAMD17-97-1-7204

TITLE: Role of IGFBP-3/IGFBP-3 Receptor Interaction in Normal and Malignant Mammary Growth: A Potential Diagnostic Parameter and New Strategy for Endocrine Therapy

PRINCIPAL INVESTIGATOR: Youngman Oh, Ph.D.

CONTRACTING ORGANIZATION: Oregon Health Sciences University
Portland, Oregon 97201-3098

REPORT DATE: September 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Role of IGFBP-3/IGFBP-3 Receptor Interaction in Normal and Malignant Mammary Growth: A Potential Diagnostic Parameter and New Strategy for Endocrine Therapy

6. AUTHOR(S)
Youngman Oh, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oregon Health Sciences University
Portland, Oregon 97201-3098

E-MAIL: ohy@ohsu.edu

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. ABSTRACT: The proposal of my grant is to investigate the biological significance and mechanism of insulin-like growth factor binding protein-3 (IGFBP-3) as well as identification and characterization of the IGFBP-3 receptor in human breast cancer cells. As a third year task, we have successfully characterized binding specificity of the IGFBP-3 receptor to IGFBP-3. It revealed that only IGFBP-3 and its fragment (aa88-148) bind the IGFBP-3 receptor with high affinity, whereas IGFBPs -2, -4, -5 and -6 did not interact with the IGFBP-3 receptor, demonstrating specificity of the IGFBP-3 receptor. We have also identified the IGFBP-3/IGFBP-3 receptor-mediated signal transduction pathway in human breast cancer cells. Current studies demonstrated that the IGFBP-3/IGFBP-3 receptor axis causes cell cycle arrest in G1 phase and induces apoptosis. The underlying mechanisms are ablation of MAPK signaling cascades and increase of caspase activity, respectively. Further through investigation is currently in the process in my laboratory. As characterization of the structure-functional analysis of IGFBP-3 and the IGFBP-3 receptor, we identified differential effects of IGFBP-3 and those IGFBP-3 proteolytic fragments on ligand binding, cell surface association and IGF-I receptor signaling. Current findings under this grant support will provide pivotal evidence for clinical significance and potential application of the IGFBP-3/IGFBP-3 receptor axis in the prevention and/or treatment of human neoplasia, in particular, breast cancer.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Front Cover</td>
<td>1</td>
</tr>
<tr>
<td>Report Documentation Page</td>
<td>2</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>4</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>11</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>12</td>
</tr>
<tr>
<td>Conclusions</td>
<td>12</td>
</tr>
<tr>
<td>Appendices</td>
<td>13</td>
</tr>
</tbody>
</table>
I. INTRODUCTION.

The insulin-like growth factor binding proteins (IGFBPs) 1-6 bind IGF-I and IGF-II with high affinity and serve to transport the IGFs, prolong their half-lives, and modulate their proliferative and anabolic effects on target cells. The molecular mechanisms involved in the interaction of the IGFBPs with the IGFs and their receptors remain unclear, but these molecules appear, at least, to regulate the availability of free IGFs for interaction with IGF receptors. Recent studies from our laboratory and others demonstrated that some IGFBPs have ability to exert IGF-independent actions.

In this project, I proposed investigation of the characterization of the IGFBP-3-specific receptor, the elucidation of the pertinent signal transduction pathways and analysis of structure-function relationships in the IGFBP-3 in the context of growth control in human breast cancer.

II. BODY.

I. characterization of the IGFBP-3 receptor in human breast cancer cells (Tasks 1-7).

In our continuing investigation of the biological importance of IGFBP-3, we are characterizing specificity of the IGFBP-3 receptor binding to IGFBP-3 and involvement of the IGFBP-3 receptor on the IGFBP-3-induced growth inhibition.

As IGFBP-3 has been previously reported to specifically bind to the surface of breast cancer cells and subsequently exhibit growth suppressing activity, I further determined whether the IGFBP-3 receptor might participate in this process. Hs578T and MCF-7 human breast cancer cells were transiently transfected with a construct encoding IGFBP-3 receptor FLAG-tagged at the C-terminus (4-33F) or with vector alone. These cells were then subjected to a monolayer binding assay using 125I-labelled IGFBP-3. The overexpression of 4-33 resulted in a 30-60% increase in IGFBP-3 binding to the cell surface relative to cells expressing endogenous levels of 4-33 (Figure 1). This result was greatly magnified when the same assay was done using SF9 insect cells either uninfected or infected with virus harboring the 4-33F cDNA, as the infected cells overexpress 4-33F to a much greater degree compared to control cells. In these cells the increase in IGFBP-3 cell surface binding was nearly 3.5 fold over control. The increased IGFBP-3 binding was competed in a dose-dependent manner with the addition of cold IGFBP-3, and was unaffected by the presence of the FLAG-tag at the C-terminus of the 4-33 protein. Further, fragments of IGFBP-3 containing the putative binding region for 4-33 (amino acids 88-148) were able to successfully compete labelled full-length IGFBP-3 binding, but an N-terminal fragment comprised
of amino acids 1-97 was not. Additionally, other IGF binding proteins were unable to compete IGFBP-3 binding in this assay, demonstrating IGFBP-3 specificity.

A) IGFBP-3 Cell Surface Binding Assay

B) Competitive Binding Results

<table>
<thead>
<tr>
<th>IGFBP-3 fragments</th>
<th>Competitive binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-3 4-33</td>
<td>-</td>
</tr>
<tr>
<td>IGFBP-3 38-148</td>
<td>+</td>
</tr>
<tr>
<td>IGFBP-3 98-266</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other binding proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-2</td>
</tr>
<tr>
<td>IGFBP-4</td>
</tr>
<tr>
<td>IGFBP-5</td>
</tr>
<tr>
<td>IGFBP-6</td>
</tr>
</tbody>
</table>

Figure 1. A) Increased cell surface binding of IGFBP-3 to cells overexpressing 4-33. B) Competitive inhibition of IGFBP-3 cell surface binding with unlabelled IGFBP-3. Only fragments containing amino acids 88-148 were able to successfully compete full-length IGFBP-3 binding. Other IGF binding proteins were unable to compete.

The IGFBP-3 receptor involvement in IGFBP-3 biological function: cell cycle arrest and apoptosis.

To facilitate the study of the biological actions of 4-33 and IGFBP-3, we have generated a stably-transfected inducible IGFBP-3 MCF-7 breast cancer cell line using the ecdysone-inducible system (see Appendix 1). One of the sublines, designated MCF-7:BP-3 #3 (colony #3), inducibly produced IGFBP-3 at levels comparable to the endogenous levels produced by Hs578T cells. The induction of IGFBP-3 in these cells caused inhibition of growth and DNA synthesis as measured by incorporation of [3H]thymidine, to a similar degree as has been described for treatment of MCF-7 cells with exogenous IGFBP-3. Induced expression of IGFBP-3 in these cells further leads to cell cycle arrest at G1. We observed an increase in the percentage of cell in G1 from 72.1% to 78.1%, with a concurrent decrease in cells in S and G2/M phases (data not shown, see Appendix 1). Further, our data indicate that IGFBP-3 induces apoptosis in this cell system (Figure 2).
Figure 2. A) IGFBP-3-induced cell cycle arrest. B) IGFBP-3 induction of apoptosis, as measured by Annexin V binding and assays for caspase activity. * = p<0.05.

Studies of 4-33 in breast cancer cells which produce IGFBP-3 demonstrated that transient overexpression of 4-33 resulted in a significant increase in cell detachment / death over time, compared to little or no effect in cells which do not produce IGFBP-3 (data not shown). IGFBP-3-expressing Hs578T cells displayed fewer cells per field following transient overexpression of 4-33 compared to transfection with vector alone, while no such effect was seen in IGFBP-3-nonexpressing MCF-7 cells. We further investigated the effect of 4-33 and IGFBP-3 on cell proliferation as indicated by incorporation of [3H]thymidine during DNA synthesis. We compared wild type MCF-7 cells with the MCF-7:BP-3 #3 subline, with and without incubation with ponasterone A. As expected, induction of IGFBP-3 by ponasterone A resulted in an inhibition of DNA synthesis to an average of 55% of control levels (Figure 3). With the additional overexpression of 4-33 in these cells, DNA synthesis was further inhibited down to an average of 35% of control levels. Overexpression of 4-33 had no significant effect on DNA synthesis in the absence of IGFBP-3 (either wild type MCF-7 or uninduced MCF-7:BP-3 #3 cells), and ponasterone A had no inhibitory effect in wild type MCF-7 cells.

Figure 3. Growth inhibition of breast cancer cells by IGFBP-3 and 4-33. 4-33 enhances the IGFBP-3 growth-inhibitory effect on breast cancer cells as measured by thymidine incorporation. * = p<0.05.

2. Identification of the IGFBP-3/IGFBP-3 receptor-induced signal transduction pathway (Tasks 8 and 9)

As reported last year, we have successfully generated inducible IGFBP-3 stably transfected human breast cancer cell line, MCF-7:IGFBP-3 #3 (see Appendix 1). Dose-dependent inducible production of IGFBP-3 protein was detected in the induced stably-transfected cells, compared to undetectable levels in control parental and uninduced stably-transfected cells. Induction of IGFBP-3 in these cells showed dose-dependent inhibition of DNA synthesis as assessed by [3H]-thymidine incorporation assays. This inhibitory effect was abolished by co-treatment with Y60L-
IGF-I, an IGF analog which has significantly reduced affinity for the IGF receptor but retains high affinity for IGFBP-3, demonstrating specificity and IGF-independence. In addition, flow cytometry analysis showed that induced expression of IGFBP-3 led to an arrest of the cell cycle in G1-S phase. Induction of IGFBP-3 resulted in a significant decrease in the mRNA and protein levels of cyclin D, but not cyclin E, as well as concomitant decreases in the levels of cdk4, total-Rb, and phosphorylated-Rb, consistent with and presenting a possible mechanism for IGFBP-3-induced cell cycle arrest. Moreover, IGFBP-3 inhibited oncogenic Ras-induced phosphorylation of MAPKs, presenting the evidence for cross-talk of IGFBP-3 signaling with MAPK signal transduction pathway. IGFBP-3-expressing cells also displayed increased Annexin V binding compared to controls, exhibiting the IGFBP-3-induced apoptosis. Further studies demonstrated that IGFBP-3 caused an increase in caspase activities, suggesting a potential mechanism for the IGFBP-3-induced apoptosis. Taken together, present study shows that cellular production of IGFBP-3 leads to cell cycle arrest and induction of apoptosis, thereby inhibiting cell proliferation in these MCF-7 human breast cancer cells and suggesting that IGFBP-3 functions as a negative regulator of breast cancer cell growth, independent of the IGF axis.

**Cell cycle arrest.** I examined the effect of the IGFBP-3 receptor on these specific proteins known to be involved in cell cycle progression and the apoptotic process. Hs578T and MCF-7 cells, and the IGFBP-3-constitutively expressing MCF-7.BP-3 #1 cell line were either left untreated, treated with an apoptosis inducer (sodium butyrate, NaB), or transfected with vector alone or the IGFBP-3 receptor. At 24 hours post-transfection cell lysates were harvested, assayed for protein content, and equal amounts of protein per sample were immunoblotted. Examination of cell cycle proteins cyclin D1, cyclin E, and p21/Waf1 revealed a specific decrease in the level of cyclin D1 protein in the IGFBP-3 receptor-transfected cells in the presence of IGFBP-3. Control transfected cells, and the IGFBP-3 receptor-transfected cells in the absence of IGFBP-3 had no effect on cyclin D1 levels. Cyclin E and p21/Waf1 were unaffected by these treatments, while proper induction of p21/Waf1 was seen with NaB treatment. Additionally, when the IGFBP-3 receptor-transfected cells were examined by immunofluorescence with antibodies against Cyclin D1, Rb and the IGFBP-3 receptor, a significant reduction in both Cyclin D1 and Rb immunodetectable protein levels occurred in 4-33-transfected cells, but not in neighboring untransfected cells (Figure 4).
untreated: + - - - + - - - + - - 
10 mM NaB: - + - - - + - - - + - -
CS2+: - - + - - - + - - - + - -
CS/BP-3R: - - - + - - - + - - + 

Cyclin D1

Cyclin E

p21/Waf1

Hs578T (BP-4+), MCF-7 (BP-3+), MCF-7:BP-3 #1 (BP-3+)

<table>
<thead>
<tr>
<th>vector</th>
<th>1-43F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>4-33</td>
</tr>
<tr>
<td>Rb</td>
<td>4-33</td>
</tr>
</tbody>
</table>

merged

merged

Figure 4. Effect of the IGFBP-3 receptor (4-33) and IGFBP-3 overexpression on cell cycle proteins in breast cancer cells. A) Specific decrease in the level of Cyclin D1 in cells overexpressing 4-33. The levels of Cyclin E and p21/Waf1 were unaffected. B) Immunofluorescence data showing specific reduction of immunodetectable levels of Cyclin D1 and Rb proteins in cells overexpressing 4-33, but not in neighboring untransfected cells.

A possible mechanism for this effect may be perturbation of p44/42 mitogen-activated protein kinase (MAPK) signaling pathways. Our data indicate that levels of phosphorylated MAPK, Cyclin D1, and phosphorylated retinoblastoma (Rb) proteins are significantly reduced upon induction of IGFBP-3 expression in MCF-7:BP-3 #3 cells (Figure 5).

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PonA (15 μM)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Cyclin

Phospho-Rb

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>6</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>PonA (15 μM)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Cyclin

Phospho-Rb

p44/42

Figure 5. Western immunoblot and immunofluorescence data showing a significant decrease in the levels of phospho-p44/42 MAPK, Cyclin D1, and phospho-Rb proteins with induction of IGFBP-3 expression.
**Apoptosis:** We further investigated whether the IGFBP-3 receptor plays a role in IGFBP-3-induced apoptosis. Hs578T, MCF-7, and induced MCF-7:BP-3 #3 cells were either untreated or transfected with vector or 4-33 and the cell cycle profile was analyzed by propidium iodide staining of DNA content followed by flow cytometry detection (Figure 6A). In each case, the IGFBP-3 receptor-transfected cells displayed an increase in the sub-G1 population and a concurrent decrease in the S/G2/M population compared to control cells. A peak in the sub-G1 range can be indicative of cells undergoing apoptosis. We investigated this further using an Annexin V assay, which is used to identify cells early in the apoptotic process. By incubating suspended cells with FITC-labelled Annexin V, coupled with concurrent propidium iodide staining without permeabilization of the plasma membrane, it is possible to discriminate between cells in early apoptosis and those in late apoptosis or necrosis using a two-color flow cytometric analysis. Hs578T cells were transfected with vector alone or the IGFBP-3 receptor, and subsequently harvested and assayed at 14, 24, 36, and 48 hours post-transfection. As shown in Figure 6B, at each time point, the population of early apoptotic cells was significantly increased in the cells transfected with the IGFBP-3 receptor, compared to control-transfected cells.

**Figure 6.** Induction of apoptosis by IGFBP-3 and the IGFBP-3 receptor (4-33). A) Cell cycle analysis of breast cancer cells either untransfected or transfected with vector or 4-33. B) Annexin V binding assay data from a time course of Hs578T cells transfected with vector or 4-33. Annexin V binding is an indicator of the early stages of apoptosis.

9
As IGFBP-3 has been shown to potentiate caspase activity, we examined this phenomenon as a potential mechanism for IGFBP-3/IGFBP-3 receptor biological function. Caspases are a family of evolutionarily related cysteine-dependent proteases, with an universal specificity for Asp in the P1 position, that play a prominent role during the progression of apoptosis. Activation of caspases and subsequent cleavage of critical cellular substrates are implicated in many of the morphological and biochemical changes associated with apoptotic cell death. Using an assay which detects activity of a broad range of caspases by incubating cell lysates with a mixture of purified fluorogenic peptide caspase substrates and measuring subsequent reaction kinetics, we demonstrated a measurable and reproducible increase in caspase activity (described as pmol substrate cleaved / min / mg protein) in Hs578T cells with transient overexpression of 4-33 compared to control-transfected cells (Figure 7).

![Caspase activity: Hs578T](image)

**Figure 7.** Potentiation of caspase activity in Hs578T cells transiently transfected with 4-33F compared to controls. * = p<0.05.

3. Characterization of structure-function aspects of IGFBP-3 action in human breast cancer. (Tasks 10-13)

Our previous report demonstrated that we have generated proteolytic fragments derived from plasmin-digested recombinant human IGFBP-3, synthetic fragments generated using the baculovirus expression system, and IGFBP-3 fragments in normal human urine. With each of these reagents we demonstrated retention of IGF binding of an N-terminal IGFBP-3 fragment, albeit with significantly reduced affinity as compared to the intact molecule. In addition, we demonstrated that these N-terminal fragments can bind specifically to insulin, and inhibit insulin receptor autophosphorylation. As further investigation, we identified differential effects of IGFBP-3 and those IGFBP-3 proteolytic fragments on ligand binding, cell surface association and
IGF-I receptor signaling (see Appendix #2). We demonstrated that IGFBP-3 showed a dose-dependent inhibition of autophosphorylation of the beta-subunit of IGF-I receptor (IGFIR). The (1-97)NH2-terminal fragment inhibited IGFIR autophosphorylation at high concentrations and this effect appears largely due to sequestration of IGF-I. In contrast, no inhibition of IGF-I induced IGFIR autophosphorylation was detectable with the (98-264) and (184-264)COOH-terminal fragments, despite their ability to bind IGF. However, unlike the (1-97)NH2-terminal fragment, the COOH-terminal fragments of IGFBP-3 retained their ability to associate with the cell surface and this binding was competed by heparin, similar to intact IGFBP-3 (Appendix #2). In addition, we are in the progress to synthesize IGFBP-3 mutants which show no binding affinity to IGFs, but retain full affinity for the IGFBP-3 receptor.

These preliminary data support the hypothesis that 4-33 is a functional receptor for IGFBP-3 in the breast cancer system, and that the interaction of IGFBP-3 with 4-33 may be an important mechanism in the IGF-independent, growth-inhibitory actions of IGFBP-3. These studies firstly demonstrated the underlying mechanism for the IGF/IGFBP-3 receptor-induced biological function; the IGFBP-3/IGFBP-3 receptor axis arrests cell cycle progression through ablation of the MAPK signaling cascades, and induces apoptosis via potentiating caspase activities. Current findings under this grant support will provide pivotal evidence for clinical significance and potential application of the IGFBP-3/IGFBP-3 receptor axis in the prevention and/or treatment of human neoplasia, in particular, breast cancer.

III. KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of the binding specificity of the IGFBP-3 receptor (4-33) to IGFBP-3 and other binding proteins.
- Characterization of the IGFBP-3-induced biological function in human breast cancer cells.
- Identification of the potential mechanisms for the IGFBP-3/IGFBP-3 receptor-mediated cell cycle arrest and induction of apoptosis.
- Characterization of IGFBP-3 proteolytic fragments on ligand binding, cell surface association and IGF-I receptor signaling.
IV. REPORTABLE OUTCOMES


V. CONCLUSIONS:

In summary, my laboratory has demonstrated IGFBP-3 / IGFBP-3 receptor interactions in the human breast cancer cell system, identified and initially characterized an IGFBP-3 interacting protein from breast cancer cells, and generated a polyclonal antibody against this interacting protein, and generated inducible IGFBP-3 stably transfected cell lines. Now, we have successfully characterized binding specificity of the IGFBP-3 receptor to IGFBP-3; Only IGFBP-3 and its fragment (aa88-148) bind the IGFBP-3 receptor with high affinity, whereas IGFBPs, -2, 4-, -5 and -6 did not interact with the IGFBP-3 receptor, demonstrating specificity of the IGFBP-3 receptor. We have also identified the IGFBP-3/IGFBP-3 receptor-mediated signal transduction pathway in human breast cancer cells. Current studies demonstrated that the IGFBP-3/IGFBP-3 receptor axis causes cell cycle arrest in G1 phase and induces apoptosis. The underlying mechanisms are ablation of MAPK signaling cascades and increase of caspase activity, respectively. Further through investigation is currently in the process in my laboratory. As characterization of the structure-functional analysis of IGFBP-3 and the IGFBP-3 receptor, we demonstrated that these N-terminal fragments can bind specifically to insulin, and inhibit insulin receptor autophosphorylation. As further investigation, we identified differential effects of IGFBP-3 and those IGFBP-3 proteolytic fragments on ligand binding, cell surface association and IGF-I receptor signaling. As laid out in the Statement of Work, We have completed Technical Objective 1, Tasks 1-7 in this year. We have accomplished Technical Objective 2, Tasks 8-9 and published 2 papers. Technical Objective 3, Tasks 10-11 are finished and published work. Tasks 11-12 are currently underway. In order to finish those Tasks, one-year extension was requested and proved by the U.S. Army Medical Research Committee
VI. APPENDICES


DIFFERENTIAL EFFECTS OF INSULIN-LIKE GROWTH FACTOR (IGF)-BINDING PROTEIN-3 AND ITS PROTEOLYTIC FRAGMENTS ON LIGAND BINDING, CELL SURFACE ASSOCIATION, AND IGF-I RECEPTOR SIGNALING

CAYATHRI R. DEVII, DOO-HYUN YANG, RON G. ROSENFELD, AND YOUNGMIN OH

Department of Pediatrics, School of Medicine, Oregon Health Science University, Portland, Oregon 97201-3082

ABSTRACT

Insulin-like growth factors (IGF)-binding protein-3 (IGFBP-3), the predominant IGF carrier protein in circulation, is posttranslationally modified in vivo by IGFBP-3 proteolytic cleavage into multiple fragments. Based on the cloned and predicted structures of an IGF-protease in the IGFBP-3 protein, FLAG-epitope tagged IGF-BP3, N-terminal (1-47), intermediate fragment (48-148), and COOH-terminal fragments (149-256) were generated in a recombinant and coexpressed system and assayed by Western ligand blot and affinity cross-linking assays, for their ability to bind IGF and insulin. The N-terminal and COOH-terminal fragments bound both IGF and insulin specifically (albeit with significantly reduced affinity) for IGF than for insulin, when compared with intact IGFBP-3. The effect of IGFBP-3 and the fragments on IGF-I receptor (IGFIR) signaling pathways was studied by testing IGF-I induced receptor autophosphorylation in IGFIR-overexpressing NIH-3T3 cells. IGFIR-IR showed a dose-dependent inhibition of autophosphorylation of the subunit of IGFIR. The IGF-I intermediate fragment inhibited IGFIR autophosphorylation at high concentrations, and this effect was largely attributable to sequestration of IGF-I. In contrast, no inhibition of IGF-I induced IGFIR autophosphorylation was detectable with the N-terminal and COOH-terminal fragments, despite their ability to bind IGF. Moreover, stimulation of the IGF-I receptor, as measured in cultured cells with intact IGFBP-3, was reduced by IGFBP-3, as well as by both IGF-independent and IGF receptor-independent mechanisms, through binding to specic IGF-I receptor sites (6W).

The insulin-like growth factors (IGF) I and II play an active role in cell proliferation and survival in association with distinct and specific IGF-binding proteins designated as IGFPs I and II. The IGF binding protein-3 (IGFBP-3) is the major IGF binding protein in adult serum, binds both IGFs with high affinity and specificity, and serves as a carrier of IGFs, prolonging their half lives, as well as modulating their proliferative and anabolic effects on target cells by regulating IGF bioavailability. Exogenous IGFBP-3 has also been demonstrated to specifically inhibit the proliferation of various cells, including fibroblasts, epithelial cells, and vascular smooth muscle cells, and it thus has a direct inhibitory effect on a variety of tissues (3). Decreased cell growth was observed when human IGFBP-3 complementary DNA (cDNA) was transfected into human glioblastoma cells (4) and rat fibroblast cells derived from neurons or embryonic rat cells (5). The mechanism of this inhibition seems to be both IGF-independent and IGF receptor-dependent mechanisms. Through binding to specific IGF-I receptor sites (6W).

IGFBP-3 may be posttranslationally modified by IGF binding (IGFBP-3) protease(s) present in both biological fluids and culture media (plasma, growth-stimulatory agent, fetal serum, urine, serum, and urine) (9) and those whose activity has been demonstrated only in vitro is that of streptomycin 3, thymulin (10). Serum IGF binding protein-3 has been found in the plasma of diabetics. (12), renal (13), pregnancy (14), and malignancy (15, 16), and following traumatic conditions or invasive procedures, such as surgery. Cleavage sites in IGFBP-3 have been located at the beginning of the mid-domain (residues 99-660), particularly residue 97, which is in the cleavage site for P-Na, plasma, human serum, and serum yields a fragment of approximately 16 kDa or 20 kDa (glycosylated IGFBP-3) (10). However, only I terminal fragments, containing a highly basic heparin binding domain, have only been detected in vivo by plasmin digestion of intact IGFBP-3 and those fragments seem to inhibit degradation of other binding proteins (17). It is recognized that IGF binding also occurs in the mucosal layer outside of the bloodstream (18, 19), and that, under certain enzymatic processes, it can be further inhibited by sequestration of IGF. Both intact IGFBP-3 and IGFBP-3 proteolytic fragments have been shown to be capable of blocking the mitogenic effects of IGF (20). Whether these actions primarily represent IGF-independent or IGF-dependent remains to be determined.
Our laboratories have demonstrated that the NH2-terminal recombinant (fragment 1-193) of IGF-BP3 (1, 87) and (1-97) retains the ability to bind IGF, albeit with substantially reduced affinity. Additionally, these fragments specifically bind insulin and stimulate insulin binding to its receptor (7, 22). Based on these studies, it has been hypothesized that the neuropeptide Y and COOH-terminal sequence, as well as the appropriate conformation generated by disulfide bonds in the six classical IGF-BPs, are all required for high affinity binding of IGFs. A recent study has indicated that a natural C-terminal fragment of human IGF-BP3 retained partial IGF-binding activity (23), and a COOH-terminal, 15-kDa IGF-BP5 fragment (isolated from human plasma) showed similar results (24). However, there is limited information on the binding characteristics of the IGF-BP3-COOH-terminal domain and the neuropeptide Y biological effects of prolyl-hydroxyproline-fragments containing either the NH2- or COOH-terminal residues.

In this study, we demonstrate the ability of COOH-terminal fragments of IGF-BP3 to bind IGF-1. The (1-97) IGF-BP3 fragment and the (1-193)NH2-terminal fragment are both characterized, respectively, by their ability to bind insulin with low affinity, but with higher affinity when the case for intact IGF-BP3. Additionally, we have examined the effect of intact IGF-BP3 and the IGF-BP3 fragments on IGF-I-stimulated autophosphorylation of the IGF-I receptor (IGF-IR). 

Materials and Methods

Antibodies and reagents

IGF-I and IGF-II were purchased from EMD Biosciences (San Diego, CA). Body, cysc, and tubulin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal antibody (1E5) specific for IGF-I receptor (IGF-IR) was purchased from Amersham International PLC. The antibody was produced by mice and was shown to cross-react to insulin, insulin-like growth factor (IGF) receptor, and IGF-IR. The antibody was generated by Dr. D. B. Drucker (Department of Endocrinology and Metabolism, Oregon Health Sciences University). Recombinant human IGF-I and IGF-II were purchased from Biorad Laboratories, Inc. (Richmond, CA).

Cell culture

NIH 3T3 cells were maintained in the human IGF-BP3 were kindly gifted from Dr. C. P. Robinson, Department of Pediatrics, Oregon Health Sciences University. The cells were cultured in DMEM with 10% FCS and 95% CO2 at 37°C. Culture medium consisted of DMEM with 15% FCS and 95% CO2 at 37°C.

Generation and purification of recombinant IGF-BP3-3 proteolytic fragments

(1-193) IGF-BP3 and (88-193) IGF-BP3-3 FLAG epitope tagged fragments were generated and purified as described above. The constructs were expressed in E. coli in the presence of ammonium sulfate and purified by affinity chromatography. The purified proteins were analyzed by SDS-PAGE and Western blotting. The purified proteins were then subjected to SDSPAGE and Western blotting.

Affinity cross-linking

Insulin (1 mg/mL) or the NH2- and COOH-terminal fragments were incubated with (1-77) or (1-193) human IGF-BP3 (100 mg/mL), and the insulin or IGF-I was then added to the cells. The insulin was added to the cells, and the cells were then subjected to Western blotting. The insulin or IGF-I was added to the cells, and the cells were then subjected to Western blotting.

Western blot analysis

Western blot analysis was performed as described by Harlow et al., with minor modifications. Briefly, samples of IGF-I (1-77), (1-97), and (1-193) IGF-BP3-3 fragments, and (1-77) IGF-BP3-3 fragments, were resolved by SDS-PAGE (12%) and transferred to nitrocellulose membranes. The membranes were then probed with a monoclonal antibody (1E5) specific for IGF-IR and visualized by enhanced chemiluminescence (ECL).
THE EFFECT OF IGFBP-3 FRAGMENTS ON IGFII SIGNALING

of 100 μg/ml heparin (Sigma), in binding buffer, was added to the cells. In a similar experiment, cells were treated with heparin (100 μg/ml) for 1 h, before addition of the peptides as listed above. The transfections were carried out at 15°C for 3 h. The cells were washed with PBS and cross-linked with EDC, as described above. The iodinated cell lysates were then run on a 15% SDS-PAGE gel and immunoblotted with anti-IGFBP-3 monoclonal antibody and detected with enhanced chemiluminescence.

ECL-induced IRF4 autophosphorylation assay

Consistent with previous use of recombinant IL-10 plasmids, NIH-3T3 cells were exposed for 3 min to 10 ng of TGFα, which had been preincubated with or without intact IGFBP-3 (10 ng) or (36-265)IGFBP-3 (10 ng) for 2 h at 4°C. The reaction was stopped by subtilisin digestion (1%) and then processed for 5 min at 4°C as described elsewhere. The samples were separated by SDS-PAGE (7.5%) under reducing conditions and visualized by immunoblot analysis. For immunoblot analysis, the filters were blocked in Tris-buffered saline (TBS) with 5% nonfat dry milk for 1 h at room temperature and then incubated with anti-IGFBP-3 monoclonal antibody (1 μg/ml) diluted by TBS + 0.1% Triton X-100 (TBT) for 1 h at room temperature. The filters were then washed in TBS and exposed in a 3:6:1 solution of Nafion or ECL-reagent (Medical Pharm Inc) for 1 h at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system.

Results

Expression of the IGFBP-3 recombinant fragments

Based on the overexpression and predicted YAP recognition sites in IGFBP-3 and the recognition sites for other known IGFBP-3 proteases, such as metalloproteases and plasminogen activators, IGFBP-3 and four different recombinant fragments were generated in a baculovirus and/or Escherichia coli expression system. Each peptide was coupled to a FLAG-epitope tag at the carboxyterminus, as shown in Fig. 1. A. The purified proteins were immunoblotted with anti-FLAG M2 or anti-IGFBP-3 monoclonal antibody for estimation of their molecular weights. Intact IGFBP-3 and all the fragments were detectable by anti-FLAG M2 antibody under reducing (Fig. 1A) and non-reducing conditions. Diminished forms of the proteins were identified in anti-FLAG XL immunoprecipitates run under non-reducing conditions (data not shown). Small differences in the Mr for intact IGFBP-3 (1-97), IGFBP-3 (1-49), and IGFBP-3 (1-265) suggest that the variable intermediate species of IGFBP-3, which is purely based on the avid affinity of the protein, may have arisen because of N-linked glycosylation. There are three potential N-glycosylation sites (Fig. 1A); Asn93, Asn95, and Asn99, in IGFBP-3 (90-126). The anti-FLAG XL antibody detected intact IGFBP-3 and (90-126)IGFBP-3, under both reducing (Fig. 1A) and non-reducing conditions (data not shown). The fragment 1-97 was detectable only under non-reducing conditions. The peptide and (90-126) fragments were not detected effectively with this antibody.

Analysis of IGF binding to the IGFBP-3 proteolytic fragments

To determine whether the regions encompassed by the proteolytic fragments contained a functional IGF-binding or cross-linking site, the proteins were incubated with [125I]IGF I and then affinity cross-linked with DBS and analyzed by SDS-PAGE. The data in Fig. 2A demonstrate that 125I-IGFI can be cross-linked to the (1-97)IGFBP-3 and (90-265)IGFBP-3 fragments in a dose-dependent manner. Significant IGF cross-linking was observed at 0.25 μM concentrations of (1-97)IGFBP-3, which was completely saturated by 100 nM concentrations. In the case of (90-265)IGFBP-3, a dose-dependent increase in IGF binding with increasing protein concentrations with saturation of binding occurred at 0.5 μM concentration range. The expected sizes of the individual proteolytic fragment coupled to [125I]IGF I were detected, shown as 25 and 41 kDa bands, respectively. A faint band at 39 kDa is potentially a dimethylated form of 1-97 fragment cross-linked to IGF I.

For estimation of the affinity of IGF I binding, the proteolytic fragments were affinity cross-linked with [125I]IGF I in the presence of increasing amounts of unlabeled IGF I or...
THE EFFECT OF IGFBP-3 FRAGMENTS ON IGF-I SIGNALING

Analysis of insulin binding to IGFBP-3 fragments

The observations that insulin could compete for IGF-I binding to the fragments led us to assess their insulin binding activity. Both (1-97)IGFBP-3 and (98-264)IGFBP-3 showed a strong binding to insulin, in contrast with that observed with IGFBP-3 at similar concentrations (Fig. 4c). Unlabeled insulin was able to displace insulin binding to both the NH2 and COOH-terminal fragments (Fig. 4d), although even higher concentrations of unlabeled insulin could not completely displace insulin binding to the (98-264)IGFBP-3, suggesting a slow dissociation rate. Insulin concentrations required to achieve 50% inhibition of insulin binding to IGFBP-3 showed an IC50 value ranging between 0.3-0.4 µM, whereas nearly 1 was found in the case of insulin binding to the (98-264) fragment (Fig. 4c). In summary, the (1-97)IGFBP-3 showed significantly higher affinity for insulin, relative to the (98-264)IGFBP-3 fragment.

In the Western blot analysis with phosphorylated insulin, both the NH2- and COOH-terminal domains showed no binding to the (1-97)IGFBP-3, similar to the Western blot (data not shown).

IGFBP-3 and 1-97 fragments inhibit IGF-I interaction with the IGFIR

In conclusion, whether the ability of intact IGFBP-3 and the amino- and carboxy-terminal fragments to bind IGFBP-3 to the IGFIR lead to phosphorylation of the IRS-1 molecule in NIH 3T3 cells expressing the IGFIR (NTH-3T3-IGFIR). The data in Fig. 5A showed that 125I-IGF-I specifically cross-linked with the IGFIR using a 250-300-kDa band under non-reducing conditions (320 µg/ml IGF-I binding to IGFIR, 30 µg/ml IGFIR in NTH-3T3 cells). Cross-linking of intact IGF-BP-3 and the (1-97)NH2-terminal fragment was completely inhibited by IGF-I, while the (98-264)IGFBP-3 was partially displaced by 100 µM unlabeled IGF-I. Further, the (1-97)IGFBP-3 complex formation was completely inhibited by preincubation with the 125I-IGF-I with unlabeled IGF-I (IC50 = 30 µM) and about 50% inhibited by preincubation with 250 µM concentration of (1-97)NH2-terminal fragment. The cross-linked band was not inhibited, however, by preincubation of the 125I-IGF-I with 100 µM (98-264)IGFBP-3 or the (1-97)IGFBP-3 fragment.

The same set of samples were analyzed on a microplate and probed with 250,000 cpm 125I-IGF-I and D91 (see Experimental Procedures). Results from Fig. 5B show that the carboxyterminal fragments (98-264), (51-264), and intact IGFBP-3 moieties associated with the cell surface in the presence of IGF-I. (1-97)IGF-BP-3, however, showed no cell-associated band. The carboxy-terminal fragments have the ability to associate with the cell surface.

Because, compared with the NH2-terminal fragment, the (98-264)COOH-terminal IGFBP-3 fragment failed to inhibit binding of IGF-I to the IGFIR, we wanted to study the ability of the fragments to associate with the cell surface in the absence of IGF-I. Monolayer cross-linking was carried out with the FLVG epitope tagged intact IGFBP-3 (1-97) or (98-264) fragments in NH3T3IGFIR cells, and the cell-associated proteins were detected by immunostaining the cell layers with anti-IGF-BP-3 monoclonal antibody. The (98-264) carboxyterminal fragment and intact IGFBP-3 molecules associated with the cell surface (1-97)IGF-BP-3, however, showed no cell-associated band (Fig. 6, lanes 2 and 5). Further, there was no detectable shift in molecular weights of the cross-linked proteins when compared with control (Fig. 1B) cross-linked protein preparations. To test whether the ability of intact IGFBP-3 and the carboxy-terminal fragment to bind to the cell surface was via the heparin-binding domain, cells were preincubated with heparin (100 µg/ml) and then treated with the permeant, alternatively, the permeant was preincubated with heparin and then added to the cells followed by monolayer cross-linking in both cases (Fig. 6). Similar results were observed in all types of experiments, i.e., heparin blocked the cell surface association of intact IGFBP-3 (Fig. 6, lanes 3 and 4) and the (98-264)IGFBP-3 fragment (Fig. 6, lanes 6 and 7).

Inhibition of IGF-I signaling

Because intact IGFBP-3 and its fragments have the ability to bind IGFBPs and thereby disrupt its interaction with the IGFIR, we analyzed the potential biological manifestation of this interaction on IGF-I signaling. This was carried out by testing the effect of IGFBP-3 and its fragments on IGF-I-induced 2A5-mAb autophosphorylation in NIH 3T3-L1 cells. Control experiments with IGF-I revealed that 5 min treatment with 7-14 µM of peptide showed maximal intensity autophosphorylation of the 95-kDa band of the b-subunit of IGF-I in NIH 3T3-L1 cells (Fig. 7A). IGFBP-3 inhibited IGF-I stimulated autophosphorylation of the IGF-I receptor b-subunit in a dose-dependent manner (Fig. 7B).

Quantification of the inhibition of the phosphorylation subunit of IGF-I was carried out by densitometric analysis of the specific 95-kDa band and the 116-kDa non-specific band in each gel. The ratio of the two bands intensities was used to normalize and calculate the percentage of maximal IGF-I-stimulated IGF-I autophosphorylation detected in the presence of IGF-I and the IGFBP-3 fragments (Fig. 7C). IGFBP-3 caused 50% inhibition of the IGF-I-induced autophosphorylation at 5-20 ng concentrations and by 15-20 ng 125I-IGFBP-3 concentrations, complete inhibition of IGF-I autophosphorylation was observed. In contrast, the (1-97)IGFBP-3 fragments inhibited complex autophosphorylation only at higher concentrations (41-79% inhibition at 100-250 ng concentrations). The (98-264)IGFBP-3 and (98-264)IGFBP-3 fragments, however, did not show any significant inhibition of IGF-I autophosphorylation even at 750 ng concentrations (Fig. 7, B and C).

Discussion

We report herein that IGFBP-3 fragments are capable of binding IGF-I and IGF-II, although with lower affinity than that seen with intact IGFBP-3. Further, the fragments have
Fig. 5. Monolayer affinity cross-linking with 125I-IGF-I. A. 125I-IGF-I was preincubated at 4°C in the presence or absence of unlabeled IGF-I (100 ng), IGFBP-3 (50 ng), or fragment (25 ng) and then these treatments were added to semiconfluent monolayers of NIH 3T3 IGFIR cells for 3 h at 4°C. After washing, the cells were fixed, and cell extracts were run on a 5% SDS PAGE gel. The arrow indicates the IGFIR complex. B. A set of the same cell extracts were run on a 5% SDS PAGE gel under reduced conditions and immunoblotted with MS and FGS antibodies.

The ability to bind insulin with higher affinity than observed with intact IGFIR-3. The principal conclusion is that the high affinity binding of IGF-I by IGFIR-3 requires proper tertiary configuration of the Nhe- and COOH-terminal domains. This observation is further supported by the recent concept of an IGFBP-3 superfamily (30, 31). Over the course of evolution, the classical IGFBLPs, which have well-conserved Nhe- and COOH-terminal domains, evolved into high-affinity IGF-binding proteins (2). In contrast, the IGRs and their low-affinity IGFBLPs only share the conserved Nhe-terminal domain (32). This structural difference, combined with the present data, strongly implicates the importance of the IGFIR COOH-terminal domain in conferring high-affinity IGF binding.

The concept that interaction between Nhe- and COOH-terminal domains is essential for high-affinity IGF binding was initially considered based on observations that proteolysis of IGFBLPs in biological fluids results in fragments that have diminished or no binding affinities for IGF-I (21). The in vitro generation of recombinant fragments or fragments isolated by limited proteolysis supports the in vivo data. A 16-kDa fragment corresponding to the Nhe terminus and a small portion of the carboxyl-terminal region, generated by proteolytically modifying IGRBP-3, specifically cross-linked to both IGF-I and II, although with a 20-fold lower affinity than intact IGRBP-3 (24, 33, 34). Similarly, a carboxyl-terminal 23-kDa IGFBP-3 fragment from osteoclast-like cells demonstrated decreased IGF binding affinity (36). Deletion mutants of the carboxyl-terminal domains of IGRBP-3 and IGRBP-1 have resulted in a decrease in IGF affinity, thereby demonstrating the importance of the highly conserved COOH-terminal fragment in the carboxyl-terminal region (38, 39). The present study
THE INHIBITORY FRAGMENTS ON IGFIR SIGNALING

Fig. 5. Effect of IGFIR on cell surface association of IGFBP-3 and its fragments. Control NTR-IGFBP-3(186-264) cells were treated with either peptides (lane 3), untrated cells (lane 4), IGFIR (lane 5), (98-264)IGFBP-3 (lane 6), (110-264)IGFBP-3 (lane 7), (110-264)IGFBP-3 (lane 8), or with peptides (peptides with IGFBP-3 (lane 9), IGFIR (lane 10), IGFIR (IGFBP-3 (lane 11), or with peptides) (peptides with IGFBP-3 (lane 12), IGFIR (lane 13), IGFIR (IGFBP-3 (lane 14), or with peptides). All the treatments were carried out for 15 min at 37°C. After treatment, the cells were cross-linked, and cell lysates were run on a 4% SDS PAGE and immunoblotted with anti-IGFBP-3 polyclonal antibody. The arrow indicates the cell-surface-associated species.

We have shown that IGFBP-3 causes a dose-dependent inhibition of IGF-I-induced IGFIR autophosphorylation in NDF 3T3 cells overexpressing the IGFIR. This inhibition occurs at all 1:1 mol ratios of IGFBP-3 to IGF-I, suggesting an IGF-dependent mechanism of modulation of receptor signaling. The (11-97)NH2-terminal fragment in IGFIR, in addition to binding IGF-I through its IGF receptor, also binds IGFBP-3 at a site distinct from the cognate site on IGFBP-3. This site on IGFBP-3 is likely to be the IGFIR fragments' ability to bind IGFBP-3 and IGF-I receptor, and the site on IGFBP-3 is likely to be the IGFIR fragments' ability to bind IGFBP-3 and IGF-I receptor, respectively.

Interestingly, the (98-264) fragment, the (1-97)IGFBP-3 fragment, and the (1-97)IGFBP-3 fragment, failed to show any inhibition of IGFBP-3 binding, despite its ability to bind IGF-I, as revealed by in vitro binding analysis. The COOH-terminal fragments (110-264) and (110-264) also failed to compete for 125I-IGF-I binding and cross linking to the IGFIR, which, as shown with intact IGFBP-3 and the (1-97)NH2-terminal (IGFBP-3) fragment, suggests that the inability of the fragments containing the COOH-terminal domain of IGFBP-3 to inhibit IGF-I binding to the IGFIR could be attributable to the following mechanisms: 1) the COOH-terminal fragment binds to IGF-I, and the entire complex is still capable of binding to anti-autophosphorylating the IGFBP-3, implying that the binding site on IGFBP-3 for the complex and the COOH-terminal region of IGFBP-3 are different; and 2) the COOH-terminal domain of IGFBP-3 possesses an extracellular matrix (ECM) binding region, and it is possible that in the cellular environment, the fragments containing the COOH-terminal domains are more prone to associate with the cell surface and are not available to sequester IGF-I, especially given their low affinity for IGF-I. To test these hypotheses, the ability of the FLAG epitope tagged fragments to associate with the cell surface was examined in the presence of IGF-I, with subsequent cross-linking and by analysis of cell lysates on immunoblot probed with anti-IGFBP-3 or αM-antibody antibodies. Our data indicate that the COOH-terminal fragments (98-264) and (110-264) have the ability to associate with the cell surface in the presence of IGF-I, unlike the NH2-terminal fragment (1-97)IGFBP-3, which showed no cell-surface association. Further, there was no shift in molecular weight of the cell-surface associated bands, and heparin blocked the binding of both intact and the (98-264)IGFBP-3 fragment to the cell surface, ruling out the possibility of interaction of the fragments with the cell surface and thereby supporting the second hypothesis. This, in agreement with an earlier study (40), which reported that an IGFIR 2 deletion fragment, lacking the 184-264 region, failed to show any cell-surface association. These two putative heparin-biding motifs in IGFBP-3, located at amino acids 148-153 and 219-226 in the central and carboxy-terminal regions, respectively, and the carboxy-terminal motif has been shown to have a fold higher affinity for heparin (34). Recently, Broman et al., 1996 (42), have identified two heparin-binding motifs (K3-X-X-X-G) within the ECM binding region (201-218) in the carboxy-terminal region of IGFBP-3, which allow escape of the insulin-like growth factor I (IGF-I) in affinity for human IGF-I. This region is highly conserved in IGFBP-5, 3, and 4, and is known to contain the heparin-binding domain. The authors have suggested that the IGF-I and ECM-binding sites partially overlap, and heparin binding to the basic amino acids might obfuscate with IGF-I interaction in vivo.

Previous studies with mini-receptor constructs and with isolated domains or proteolysis fragments of the IGF-I (43) urokinase receptor (44), GH receptor (45), and IGF-I, to name a few, have confirmed the involvement of two or more ligand contact regions. Similarly, in the case of IGFBP-3, it seems that the IGF-I and insulin binding domains are bipartite and possibly overlapping. In our biological system, the stoichiometry of IGF-I binding to IGF-I seems to be 3:1. We propose that both NH2- and COOH-terminal domains have residues that are capable of binding IGF-I and insulin with low affinity. However, there is simultaneous interaction of the two unpaired half-site, so intact IGFBP-3, which creates a high-affinity IGF-I binding site on the molecule. Simultaneously, this interaction leads to a markedly reduced ability.
THE EFFECT OF IGFBP-3 FRAGMENTS ON IGFII SIGNALLING

A

<table>
<thead>
<tr>
<th>IGF-I, nm</th>
<th>0</th>
<th>1</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>1</td>
<td>7</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>MW (kDa)</td>
<td>-116</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

[Diagram showing IGFBP-3 and IGF-I interactions]

C

[Graphs showing IGFBP-3 and IGF-I binding]

Acknowledgments

We are grateful to Danna Germain and Elizabeth Wilson for technical support and to Dr. Victor Shao and Stephen M. Topp for helpful discussions.

IGFBP-3 and IGF-I antibodies were separated by SDS-PAGE under reducing conditions and visualized by immunoblot analysis using chemiluminescence detection. The arrows indicate the 86 kDa fragment of IGFBP-3 and the symbols represent the bands corresponding to the fragments of IGF-I and IGFBP-3.
THE EFFECT OF LGF-S FRAGMENTS ON URTICARIA

References

Cellular Expression of Insulin-like Growth Factor Binding Protein-3 Arrests the Cell Cycle and Induces Apoptosis in MCF-7 Human Breast Cancer Cells

Ho-Seong Kim, Angela R Ingermann, Junko Tsubaki, Stephen MTwigg and Youngman Oh

Department of Pediatrics, Oregon Health Sciences University, Portland, Oregon

Running Title: IGFBP-3 arrests cell cycle and induces apoptosis
Address correspondence to: Youngman Oh, Ph.D., Associate Professor
Department of Pediatrics
Oregon Health Sciences University
3181 S.W. Sam Jackson Park Road, NRC-5
Portland, OR 97201-3098
Tel: 503-494-1930
Fax: 503-494-0428
Email: ohy@ohsu.edu
Summary:
The IGFBPs are classically known to bind IGFs and modulate the IGF signaling system, however, emerging data have suggested that IGFBPs may play more active, IGF-independent roles in growth regulation in various cell systems. In support of this hypothesis, IGFBPs, in particular IGFBP-3, have been recently shown to potently inhibit proliferation of various cell types in an IGF-independent manner. However, the specific mechanism for the IGF-independent action of IGFBP-3 is not yet clearly understood. In the present study, we have demonstrated a novel, IGF-independent role for IGFBP-3; cell cycle arrest and induction of apoptosis in MCF-7 human breast cancer cells. MCF-7 cells, which do not produce IGF peptides, were stably transfected with an IGFBP-3 cDNA construct using the ecdysone-inducible expression system. Dose-dependent inducible production of IGFBP-3 protein was detected in the induced stably-transfected cells, compared to undetectable levels in control parental and uninduced stably-transfected cells. Induction of IGFBP-3 in these cells showed dose-dependent inhibition of DNA synthesis as assessed by [³H]-thymidine incorporation assays. This inhibitory effect was abolished by co-treatment with Y60L-IGF-I, an IGF analog which has significantly reduced affinity for the IGF receptor but retains high affinity for IGFBP-3, demonstrating specificity and IGF-independence. In addition, flow cytometry analysis showed that induced expression of IGFBP-3 led to an arrest of the cell cycle in G1-S phase. Induction of IGFBP-3 resulted in a significant decrease in the mRNA and protein levels of cyclin D, but not cyclin E, as well as concomitant decreases in the levels of cdk4, total-Rb, and phosphorylated-Rb, consistent with and presenting a possible mechanism for IGFBP-3-induced cell cycle arrest. Moreover, IGFBP-3 inhibited oncogenic Ras-induced phosphorylation of MAPKs, presenting the evidence for cross-talk of IGFBP-3 signaling with MAPK signal transduction pathway. IGFBP-3-expressing cells also displayed increased Annexin V binding compared to controls, exhibiting the IGFBP-3-induced apoptosis. Further studies demonstrated that IGFBP-3 caused an increase in caspase activities, suggesting a potential mechanism for the IGFBP-3-induced apoptosis. Taken together, present study shows that cellular production of IGFBP-3 leads to cell cycle arrest and induction of apoptosis, thereby inhibiting cell proliferation in these MCF-7 human breast cancer cells and suggesting that IGFBP-3 functions as a negative regulator of breast cancer cell growth, independent of the IGF axis.

Key Words
IGFBP-3, inducible stable cell line, cell cycle arrest, apoptosis, breast cancer, cyclin D1, retinoblastoma protein, caspase
Introduction:

The insulin-like growth factor binding proteins (IGFBPs) are components of the IGF signaling system, and their superfamily is comprised of six high affinity species (IGFBPs 1-6) and several low affinity binders (IGFBP-related proteins (IGFBP-rPs)) (1-5). The classical role of the IGFBPs involves IGF binding and modulation of IGF signaling, however, recent data suggest that some IGFBPs may play more active, IGF-independent roles in growth regulation in various cell systems (6-19). In particular, IGFBP-3 has been shown to potentially inhibit proliferation of various cell types in an IGF-independent manner. This concept of IGF-independent action of IGFBP-3 is supported by demonstrations that (1) exogenous IGFBP-3 binds to specific proteins on cell surface and this interaction is strongly correlated with the ability of IGFBP-3 to inhibit cell growth (8, 9); (2) overexpression of a transfected human IGFBP-3 cDNA inhibits cell proliferation (10-12); (3) IGFBP-3 mediates transforming growth factor-b (TGF-b)- (13), retinoic acid- (14), antiestrogen- (15), vitamin D analogs- (16), and tumor necrosis factor-a (TNF-a)- (17) induced growth inhibition; (4) regulation of IGFBP-3 gene expression plays a role in signaling by p53, a potent tumor-suppressor protein (18); and (5) IGFBP-3 fragments inhibit the stimulation of DNA synthesis induced either by IGF-I or insulin (19). Recently, several reports have demonstrated that IGFBP-3 induces apoptosis in PC-3 prostate cancer (20) and MCF-7 breast cancer cells (21), and increases ceramide-induced apoptosis in Hs578T breast cancer cell line (22). However, the specific mechanism for the IGF-independent action of IGFBP-3 is yet to be elucidated. Moreover, applications of the purified IGFBP-3 from biological fluids and recombinant species have showed limitation for the biological studies due to concerns about the purity, bioactivity and post-translational modification of IGFBP-3. In this study, we have investigated a novel, IGF-independent role for IGFBP-3; cell cycle arrest and induction of apoptosis in MCF-7 human breast cancer cells by inducible cellular expression of IGFBP-3.

We hypothesized that IGFBP-3 inhibits cell growth in an IGF-independent manner and were interested to determine whether its growth-inhibitory effects involve regulation of the cell cycle arrest and/or induction of apoptosis. To address these questions, we generated a subline of MCF-7 cells (which do not produce IGF peptides) stably transfected with an inducible IGFBP-3 cDNA construct using the ecdysone-inducible expression system. This controlled system was used to look carefully at the effects of induced IGFBP-3 expression on the cell cycle and apoptosis. We then investigated possible mechanisms of growth inhibition and the signal transduction pathways involved in inhibitory actions of IGFBP-3.
**Experimental Procedures**

**Materials**

Cells were purchased from American Type Culture Collection (Rockville, MA). Tissue culture reagents and plastics were purchased from Mediatech (Herndon, VA), Becton Dickinson (Franklin Lakes, NJ) and Nunc (Naperville, IL). Monoclonal antibodies against cyclin D1 were purchased from NeoMarkers (Fremont, CA), cyclin D3 from Calbiochem (Cambridge, MA), cyclins A, E and poly(ADP-ribose) polymerase (PARP) from Santa Cruz Biotechnology (Santa Cruz, CA), cyclin-dependent kinase (cdk) 4 from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against retinoblastoma protein (Rb), phospho-Rb, p44/42 mitogen-activated protein kinase (MAPK), and phospho-p44/42 MAPK were purchased from New England Biolabs/Cell Signaling Technology (Beverly, MA). Monoclonal antibody against IGFBP-3 and a radioimmunoassay kit for IGFBP-3 were generously provided by Diagnostic Systems Laboratories (Webster, TX). Recombinant human IGF-I analog, Y60L-IGF-I was the generous gift of Protigen Inc. (Mountainview, CA). DEVD-AMC and LEHD-AMC fluorogenic caspase substrate peptides were purchased from Biomol (Plymouth Meeting, PA). The ecdysone-inducible expression system was from Invitrogen (Carlsbad, CA). Human cyclin D1 cDNA was purchased from American Type Culture Collection (Rockville, MA). A constitutively active Ras (RasV12) cDNA expression construct was the kind gift of Dr. Philip Stork, Vollum Institute, OHSU, (Portland, OR). DNA preparations were made using kits from Qiagen (Chatsworth, CA).

**Generation of an MCF-7-derived Inducible IGFBP-3 Cell Line**

A cDNA encoding human IGFBP-3 was cloned into the pIND expression vector. This construct was cotransfected with pVgRXR (encoding a hybrid ecdysone / retinoid X receptor) into MCF-7 cells using FuGene 6 transfection reagent (Roche, Indianapolis, IN). Cells were split 48 h later to low density into selective medium containing G418 (800 μg/ml) and zeocin (100 μg/ml). After 14 days, isolated foci of selection-resistant cells were subcultured and expanded. To test for the proper inducible expression of IGFBP-3, each clone was cultured in the presence of the inducer ponasterone A (2-15 μM). Conditioned media (CM) were collected to test for ponasterone-inducible expression of IGFBP-3 by western immunoblot. Hybrid receptor only-transfected (MCF-7:EcR) cells were used as a negative control.

**Cell Cultures**

All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4.5 g/liter glucose, 110 mg/liter sodium pyruvate, and 10% fetal bovine serum. Stably-transfected MCF-7:EcR cells were maintained in selective medium containing 100 μg/ml zeocin. Stably-transfected MCF-7:BP-3 cells were maintained in selective medium containing 800 μg/ml G418 and 100 μg/ml zeocin. For studies involving the induction of IGFBP-
3 expression, cells were seeded and cultured until 60-70% confluent, then switched to serum-free media with or without 15 μM ponasterone A for 72 h, unless otherwise indicated in the text. CM were collected and centrifuged at 1000 X g for 10 min to remove cell debris.

[^3H] Thymidine Incorporation Assay

Cells were seeded and cultured in 24-well dishes. After three days of IGFBP-3 induction, a 4 h pulse of 0.1 μCi of[^3H] thymidine (25 Ci/mM; NEN, Boston, MA) in a volume of 25 μl was added to each well. Cells were incubated, and the rate of DNA synthesis was estimated by measuring the trichloroacetic acid-precipitable radioactivity, as described previously (23).

Flow Cytometry: Cell Cycle and Apoptosis Assays

Cells were seeded and cultured in 6-well dishes, then induced to express IGFBP-3. Cells were harvested, pelleted at 1000 rpm for 5 min and washed three times with phosphate-buffered saline (PBS). For cell cycle analysis, each sample was resuspended in propidium staining solution (50 mg/ml propidium iodide, 100 U/ml RNase A, 0.1% Triton X-100, 0.1% sodium azide in PBS) and incubated for 30 min in the dark. Analysis of apoptotic cells was performed using FITC-conjugated Annexin V (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer’s directions. Data were collected on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with an argon laser. The data were analyzed using Cell Quest software (Becton Dickinson).

Western Immunoblotting

Cell lysates were prepared as described previously with minor modifications (24). In brief, confluent cells were washed with cold PBS, then scraped from plates in the presence of cold RIPA lysis buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% Na DOC, 0.1% SDS, containing a protease inhibitor cocktail (Roche, Indianapolis, IN). Cell lysates were rocked for 15 min at 4°C, then centrifuged to remove cell debris. The aliquots were stored at -70°C until use. Conditioned media samples were fractionated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions, while cell lysate samples were fractionated under reducing conditions. Fractionated proteins were electrotransferred onto Hybond-ECL nitrocellulose (Amersham Pharmacia, Arlington, VA). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST), and incubated with primary antibodies diluted in TBST for 2 h at room temperature or overnight at 4°C. Membranes were washed in TBST, then incubated with horseradish peroxidase-conjugated secondary antibodies (Southern Biotechnology Associates, Birmingham, AL), diluted 1:7000, for 1 h at room temperature. Immunoreactive proteins were detected using Renaissance Western Blot Chemiluminescence reagents (NEN, Boston, MA).
**Northern Blot Analysis**

Total RNAs from monolayer cultures of IGFBP-3-transfected stable cell line cultured with or without ponasterone A were isolated using the RNeasy RNA isolation kit (Qiagen, Chatsworth, CA), and quantitated by absorbance at 260 nm. Five μg of total RNA were electrophoresed on 1% formaldehyde gels and transferred to GeneScreen Plus nylon membranes (NEN). Membranes were UV crosslinked and stained in 0.02% methylene blue / 0.3 M NaOAc, pH 5.5 to verify equivalent loading and transfer. 32P-labelled cDNA probes were prepared using the Prime It kit (Stratagene, Cedar Creek, TX). Membranes were hybridized in ULTRAHyb buffer (Ambion, Austin, TX) overnight at 42°C, and washed in 0.1 X SSC as described (3).

**Densitometric and Statistical Analysis**

Densitometric measurement of immunoblots were performed using a Bio-Rad GS-670 Imaging densitometer (Bio-Rad, Melville, NY). All experiments were conducted at least three times. The data were analyzed with Student’s t test, using the Microsoft Excel 98 software package.

**Caspase Assay**

Cells were seeded in 96-well plates until 90% confluent, then incubated in triplicate with or without ponasterone A for the times indicated. Cells were lysed in 30 μl per well of ice-cold lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 0.1% Triton X-100, 1 mM DTT, 0.1 mM EDTA). 20 μl of each lysate was used in assays for caspase activity using a combination of two fluorogenic peptide substrates, DEVD-AMC and LEHD-AMC, which together cover specificity for a wide range of caspases. Lysates were distributed into a 96-well black plate and diluted in 190 μl of assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol). Serial dilutions of free 7-amino-4-methylcoumarin (AMC, Sigma, St. Louis, MO) diluted in assay buffer were included to generate a reference standard curve for determination of the amount of AMC released in each reaction. The plate was pre-incubated for 10 minutes at 37°C, then the reactions were started with the addition of DEVD-AMC and LEHD-AMC to each well to a final concentration of 40 μM each. Reaction kinetics were monitored for up to 16 hours at 37°C in a Bio-Rad Fluoromark fluorometer, with plate readings taken every 10 minutes at excitation/emission of 390nm/460nm. Of the remaining cell lysate, 5 ml were assayed for protein content. Data were analyzed from the linear portion of the reactions using Microplate Manager software (Bio-Rad), and final results were adjusted for protein content.
**Immunocytochemistry**

Cells were seeded in 8-chamber slides and cultured until 70% confluent, then incubated with or without ponasterone A for 48 or 72 hours. Cells were then rinsed twice in PBS, fixed in 4% paraformaldehyde, then rinsed again in PBS. For some antibodies, cells were additionally incubated in ice-cold methanol for 2 minutes on ice. Slides were blocked in 5% normal goat serum / PBS / 0.1% Triton for 1-2 hours at RT, then incubated with primary antibodies diluted in blocking solution at 4°C overnight. Slides were rinsed 3 times 5 minutes in PBS and incubated with secondary antibodies diluted in blocking solution for 1 hour at RT. Slides were rinsed as before, and cells were covered in 50% glycerol before coverslipping. Data were collected on a Nikon (Melville, NY) Diaphot 300 inverted fluorescent microscope equipped with a 1.3 megapixel CCD camera (Princeton Instruments, Trenton, NJ) using IPLab software (Scanalytics, Fairfax, VA).
Results

Induced Expression of IGFBP-3 in the MCF-7-derived Ssable Cell Lines

For these studies we developed a subline of MCF-7 human breast cancer cells which expresses IGFBP-3 when cultured in medium containing an inducing compound, ponasterone A. The parental MCF-7 cells do not express detectable levels of IGFBP-3, and also do not express IGF peptides (25), making this cell line an ideal choice. A total of 16 selection-resistant clones were generated, and these were tested for inducible expression of IGFBP-3 by western immunoblotting of conditioned media (CM) samples. Figure 1 shows the panel of IGFBP-3 protein production from these 16 clones. IGFBP-3 was expressed in clones #2, #3, and #6 in an inducible manner, while constitutively expressed in clones #1 and #16. Expression of IGFBP-3 could be detected from 24 h at the concentrations of ponasterone A ranging from 1 to 15 µM without affecting the cell viability (data not shown). Clone #3 (MCF-7:IGFBP-3 #3) was used for further experiments. Quantitative analysis of IGFBP-3 protein levels in CM of induced MCF-7:IGFBP-3 #3 cells using a radioimmunoassay indicated that maximal levels of IGFBP-3, ranging from approximately 100-150 ng/ml, occurs on day 3 at the concentration of 15 µM ponasterone A.

IGF-independent Inhibition of DNA synthesis by IGFBP-3

Induced expression of IGFBP-3 by ponasterone A resulted in an inhibition of DNA synthesis compared to IGFBP-3-uninduced cells (MCF-7:IGFBP-3 #3 without ponasterone A), shown in Fig. 2A. This inhibitory effect of IGFBP-3 was dose-dependent, with 45% inhibition at a concentration of 10 µM ponasterone A (p<0.001). Meanwhile, an inhibition of DNA synthesis in pVgRXR-transfected control cells (MCF-7:EcR) was not prominent. This effect of IGFBP-3 does not result from blocking the mitogenic actions of IGFs by preventing their binding to IGF receptors, because MCF-7 cells, which do not produce IGF peptides (25), were cultured in SFM in our system to exclude the effects of IGFs. Moreover, this IGFBP-3-induced inhibitory effect was abolished by co-treatment with Y60L-IGF-I, an IGF analog with a leucine for tyrosine substitution at amino acid position 60, has a 100-fold reduced affinity for IGF receptors but full affinity for IGFBP-3, demonstrating the specificity of the IGF/IGF receptor-independent action of IGFBP-3 (Fig. 2B).

IGFBP-3 Arrests the Cell Cycle and Regulates Cell Cycle-related Proteins

To identify the mechanism for the growth-inhibitory effect of IGFBP-3, we performed flow cytometry analysis. MCF-7:IGFBP-3 #3 and EcR cells cultured in SFM with or without ponasterone A were used to analyze the cell cycle profile by propidium iodide staining of DNA content followed by flow cytometry detection. The treatment of MCF-7:IGFBP-3 #3 with 15 µM ponasterone A caused a decrease in the percentage of cells in the S phase, from 18.4% in the absence of ponasterone A to 13.8% and an accumulation of cells in the G1 phase from 72.1% to 78.1% (Fig. 3). There was no
change in the cell cycle distribution in EcR cells treated with ponasterone A (data not shown). These results suggest that induced expression of IGFBP-3 leads to a cell cycle arrest in the G1/S phase. We further examined whether IGFBP-3 affects the levels of cell cycle regulatory proteins, such as cyclin D1, cyclin D3, cyclin A, cdk4, Rb, and phospho-Rb, which are known as key cell cycle regulatory proteins for progression through G1 phase of the cell cycle in breast epithelial cells (27, 28). Firstly, we examined steady-stable cyclin D1 mRNA level by northern blotting using MCF-7:IGFBP-3 #3 cells cultured in SFM with or without ponasterone A (Fig. 4A). Both cyclin D1 mRNA species, approximately 4.5 and 1.5 kb in size, were observed in MCF-7:IGFBP-3 #3 cells. A significant decline in 4.5 kb cyclin D1 mRNA levels were observed from 24 h after addition of ponasterone A. In contrast, the 1.5 kb mRNA species was not affected by addition of ponasterone A. The expression of IGFBP-3 mRNA was observed from 12 h, before the decrease in expression of cyclin D1 mRNA. Hybridization with β-actin showed equal loading of the gel. Further, immunoblot analysis revealed a concomitant decline in the levels of cyclin D1 protein (Fig. 4B). Addition of ponasterone A resulted in a reduction in cyclin D1 in MCF-7:IGFBP-3 #3 cells, but not in EcR cells. Decreased levels of cyclin D1 in MCF-7:IGFBP-3 #3 cells with ponasterone A was reversed by co-treatment of Y60L-IGF-I (Fig. 4C), suggesting the IGFBP-3 either directly or indirectly is involved in regulating cyclin D1 expression in an IGF/IGF receptor-independent manner.

Further analysis of various cell cycle-regulated proteins was performed in MCF-7:IGFBP-3 #3 cells cultured in SFM with or without ponasterone A at 0, 24, 48, 72 h. As shown in Fig. 5A, the levels of cyclin D1, cdk4, total Rb, and phosphorylated Rb were decreased from day 1 and levels of cyclin A from day 3, while the levels of cyclins D3 and E were unchanged. These results suggest that IGFBP-3 specifically decreases the levels of cyclin D1, cdk4, and total and phosphorylated Rb in these cells, presenting a possible mechanism for IGFBP-3-induced cell cycle arrest in G1/S phase. Ultimately, the decreased levels of Rb and phosphorylated Rb may serve to directly suppress exit from G1 phase. Additionally, as shown in Fig 5B, immunocytochemistry experiments revealed a similar pattern of decreased protein levels. Immunodetectable levels of Cyclin D1 and phosphorylated Rb proteins were significantly reduced in cells induced to express IGFBP-3 compared to controls.

Effect of IGFBP-3 on the MAPK signaling pathway

Previous studies indicated that mitogen-activated protein kinase (MAPK) cascades modulate the expression of cyclin D1 (29), thus we examined the effect of IGFBP-3 on MAPK cascade proteins. Fig. 6A shows that the levels of phosphorylated, but not total p44/42 MAPK was decreased in MCF-7:IGFBP-3 #3 cells after induction of IGFBP-3 with ponasterone A. Further, immunofluorescent microscopy studies demonstrated that induced expression of IGFBP-3 results in significant decrease as well as disturbed subcellular localization of phosphorylated p44/42 MAPK (Fig. 6B). This suggests that the IGFBP-3-mediated decrease in cyclin D1 results, at least in part, of modulation of p44/42
MAPK activity.

To further investigate the cross-talk between IGFBP-3 signaling and the MAPK signaling cascades, we transiently transfected constitutively active Ras (RasV12) into our cell system. As shown in Fig. 7, overexpression of oncogenic Ras resulted in stimulation of DNA synthesis as well as activation of p44/42 MAPK. Induction of IGFBP-3 expression caused a significant inhibition of both oncogenic Ras-induced DNA synthesis and p44/42 MAPK phosphorylation. Taken together, these data suggest that IGFBP-3 antagonizes Ras-MAPK signaling cascades.

IGFBP-3 induces apoptosis

At the same time, we determined whether IGFBP-3 could induce apoptosis using the annexin V binding assay, which is used to identify cells in the early stages of the apoptotic process (26). Fig. 8A shows that induced expression of IGFBP-3 caused an increase in the percentage of cells in the apoptosis, from 1.5% in the absence of ponasterone A to 36%, suggesting that IGFBP-3 induces apoptosis in this cell system. Additional indication that IGFBP-3 induces apoptosis came from results of assays for caspase activity. The data in Fig 8B demonstrate that induction of IGFBP-3 expression causes an increase caspase activity in these cells as measured by incubating cell lysates with a combination of the purified fluorogenic caspase substrate peptides DEVD-AMC and LEHD-AMC. Caspase activity in uninduced cells lysates was detected at an average of 7.8 pmol AMC released/min/mg to an average of 10 pmol/min/mg with induced IGFBP-3 expression (p<0.05) at 48 hrs. In addition, IGFBP-3 increases caspase activity in a dose-dependent manner. The topoisomerase II inhibitor etoposide, and taxol, which reversibly binds to tubulin, were used as control apoptosis inducers. To present further evidence of capase activation by IGFBP-3, we examined cleavage of one caspase substrate, poly(ADP-ribose) polymerase (PARP) by immunoblotting. This nuclear enzyme is proteolytically cleaved by activated caspases during apoptosis (30). As shown in Fig. 8C, the induced expression of IGFBP-3 resulted in an increase of the 85 kDa carboxy terminal fragment of PARP, confirming that IGFBP-3 induces apoptosis at least, in part, through activation of caspases in MCF-7 breast cancer cell system.
Discussion

A growing accumulation of data has demonstrated that some IGFBPs, including IGFBPs -1, -3, -5, and presumably IGFBP-rPs, have their own IGF-independent biological actions (3, 5, 8-11). In particular, the IGF-independent effects of IGFBP-3 have been reported in various cell systems by treatment of recombinant IGFBP-3 exogenously or use of stable transfection systems. However, its action requires relatively high concentrations of recombinant IGFBP-3, ranging 500-1500 ng/ml to achieve biological effects of IGFBP-3 (8, 11, 20, 21). At present study, we utilize the ecdysone-inducible expression system in MCF-7 human breast cancer cells, and thereby examining the biological effects of endogenous IGFBP-3 expressed under controlled induction. As demonstrated, human endogenous IGFBP-3 was induced ranging 100-150 ng/ml in our cell system, of which concentrations appear to be comparable to those obtained in conditioned media after treatment of various reagents, such as TGF-b, RA, TNF-a and entiesterogen, for investigation of biological function of induced IGFBP-3 in various cell systems. Our results demonstrate that this lower concentration of endogenous IGFBP-3 is sufficient to inhibit DNA synthesis and induces apoptosis, despite relatively high concentrations (300-1000 ng/ml) of recombinant IGFBP-3 which were required to obtain similar biological effects of IGFBP-3 in MCF-7 cells (21). It is tempting to speculate that difference in the sensitivity of IGFBP-3 on biological function may result from the difference of IGFBP-3 preparations, that is a natural vs. recombinant form, and endogenous secretory protein from the cell or exogenous form added to the cultures. In addition, post-translational modifications, such as glycosylation and phosphorylation, may affect the sensitivity. On the other hand, sensitivity to IGFBP-3 may determine whether the cell types express the oncogenes or other molecules which influence the signaling pathways in the development of IGFBP-3 insensitivity. Martin and Baxter (31) reported that resistance to IGFBP-3 is induced in normal mammary epithelial cells transfected with oncogenic ras, thereby activating the MAPK/ERK pathway. In contrast, MCF-10A normal human mammary epithelial cells, which require 10-100 ng/ml human plasma-derived IGFBP-3 to achieve a similar level of inhibition to that seen with 500-1500 ng/ml in the transformed cells, are considerably more sensitive to IGFBP-3 than breast cancer cells. Increased activity of oncogenic Ras-dependent signaling pathways is implicated in the development of IGFBP-3 insensitivity (31). It is of note that MCF-7 cells do not express oncogenic Ras, which may explain why such low concentrations of IGFBP-3 are sufficient to exert its biological effects.

The IGF-independent effect of IGFBP-3 has been extensively investigated in variety of cell systems, however, the mechanisms by which these actions are exerted are not fully elucidated. Recent studies have proposed that IGFBP-3 functions as an apoptosis-inducing agent and that this action is mediated through a p53- and IGF-independent pathway in PC-3 prostate cancer cells (20), whereas IGFBP-3 has no direct inhibitory effect on Hs578T breast cancer cells but could accentuate apoptosis induced by ceramide (22). On the other hand, a vitamin D3 analog (Ro 24-5531) inhibits cell growth and increases the IGFBP-3 mRNA and protein levels in human osteosarcoma cell line, and the
inhibition in cell growth is accompanied by a decrease in the expression of p34cdc2, a protein critically involved in cell cycle regulation. These studies have provided circumstantial evidence that IGFBP-3 involves cell growth arrest (32). Our present studies focus on identification of the potential mechanism for IGFBP-3-induced growth inhibition, and demonstrate for the first time that IGFBP-3 induces cell cycle arrest in G1 phase by regulating expression of cell cycle-regulatory proteins, in particular cyclin D1, as well as inducing apoptosis by modulating proapoptotic caspase activities.

Since IGFBP-3 prevents cell cycle progression at G1 phase, we determined whether IGFBP-3 affects cell cycle-regulated proteins in MCF-7:IGFBP-3 #3 cells. Components responsible for the coordinated progression through the cell cycle include the cyclin-dependent kinases (cdks), regulatory cyclin subunit, and cdk inhibitors (33-35). Once extracellular signals activate the synthesis of the regulatory cyclin subunit, appropriate sites on the catalytic subunit must be phosphorylated by the cdk-activating kinase (CAK, also known cdk7) to phosphorylate the product of the Rb gene, resulting in the derepression of E2F/DP-dependent transcription and passage through S phase of the cell cycle (36, 37). D-type cyclins (cyclins D1, D2, and D3), in conjunction with their catalytic partners, cdk4 and cdk6, have been known to execute their critical functions during mid-to-late G1 phase, as cells cross a G1 restriction point (33). Overexpression of cyclin D1 can shorten the G1 cell cycle phase, decrease cell size, reduce requirements for growth factors (38-40). Microinjection of antisense constructs or antibodies to cyclin D1 into normal fibroblasts can prevent them from entering S phase (38, 41). In contrast, cyclin E is expressed later in G1 phase and its expression is periodic and maximal at the G1-S transition (42). In our study, we have found that induction of IGFBP-3 leads to inhibition of expression of cyclin D1 mRNA followed by a reduction in protein levels, and concomitant decrease of cdk4, total Rb, and phospho-Rb proteins, indicating a possible mechanism of cell cycle arrest in G1/S phase. In contrast, cyclin D3, cyclin A, and cyclin E do not show any change, suggesting that cyclin D1 is a major player in the regulation of G1-S phase progression by IGFBP-3 in MCF-7 cells. Cyclin D1 is important for neoplastic transformation as well as cell cycle progression. When cyclin D1 is cotransfected with other oncogenes, such as activated Ha-ras or adenovirus E1A into human fibroblast, malignant transformation of cells has been reported (43, 44). Overexpression of cyclin D1 in the mammary gland of transgenic mice induces mammary carcinoma (45). Moreover, dysregulated cyclin D1 expression have been observed in human neoplasia, including breast cancer (46, 47). These results suggest that IGFBP-3, which is able to modulate cyclin D1 expression, has a potential role in a strategy for anti-cancer therapy.

The expression of cyclin D1 is known to be regulated by transcriptional, translational, and posttranscriptional processes (48, 49). Multiple signaling pathways seem to be involved in the regulation of cyclin D1 expression at a transcriptional level. Previous studies have shown that cyclin D1 expression is regulated by the p42/p44 MAPK, p38 MAPK, and Jun kinases (JNKs) (29). Moreover, direct induction of cyclin D1 can be achieved by serum, growth factors, cytokines, Rb, oncogenic Ras, and Src kinase (29, 50-53). Ectopic expression of E2F1 inhibits the cyclin D1
protein at the transcriptional level, suggesting a negative feedback for cells already in S phase (54). Decreased expression of cyclin D1 by IGFBP-3 shown in this study may be, at least in part, associated with the decreased level of p42/p44 MAPK activity. Our results demonstrated that induced expression of IGFBP-3 results in inhibition of not only basal level of phosphorylation of p42/p44 MAPK but also oncogenic-Ras-induced phosphorylation of p42/p44 MAPK, indicating that IGFBP-3 appears to interact with the Ras-MAPK signaling cascades, presumably on a downstream effector of Ras and thereby regulating cyclin D1 expression and subsequent cell cycle progression. More proximal events of IGFBP-3-induced antagonism of the MAPK signaling pathway will be the subject of future studies in our laboratory.

Beyond arrest of the cell cycle, our data also indicate that cellular expression of IGFBP-3 promotes apoptosis in MCF-7 cells. Apoptosis is a major multi-faceted form of cell death, that has been implicated as playing a role in several human diseases, including cancer. There are a series of events involved in the commitment and execution of apoptotic cell death, several of which have been well characterized. Among these are changes in the plasma membrane, with the enzymatically-driven translocation or “flipping” of phosphatidylserine (PS) to the extracellular surface. The result of this process can be detected utilizing the binding properties of Annexin V, which binds preferentially to PS and other negatively charged phospholipids. Our results show a clear and significant increase in annexin V binding in cells induced for IGFBP-3 expression relative to controls. Another indicator of the apoptotic process is caspase activity. Caspases are a family of evolutionarily related cysteine-dependent proteases, with an universal specificity for Asp in the P1 position, that play a prominent role during the progression of apoptosis. Activation of caspases and subsequent cleavage of critical cellular substrates are implicated in many of the morphological and biochemical changes associated with apoptotic cell death. Using an assay which detects activity of a broad range of caspases, we demonstrate a measurable and reproducible increase in caspase activity in IGFBP-3-induced cells relative to control uninduced cells, and further the increase in caspase activity was dose-dependent with regard to IGFBP-3. Furthermore, increased cleavage of the caspase substrate poly(ADP-ribose) polymerase (PARP) was observed after induction of IGFBP-3 expression. The nuclear enzyme PARP is proteolytically cleaved by activated caspases, primarily caspases 3 and 7 during apoptosis, but can also be cleaved in vitro by a wide range of caspases (30). It is of note that MCF-7 cells do not express caspase 3 due to a functional deletion of the gene (55), suggesting that the IGFBP-3-induced activation of caspase activity may be mediated primarily through caspase 7 and others. Nevertheless, these three lines of evidence indicate that induction/promotion of apoptosis is a major effect of cellular expression of IGFBP-3 in these cells.

Our previous studies have demonstrated that IGFBP-3 inhibits cell growth in an IGF-independent manner through an IGFBP-3 receptor in Hs578T breast cancer cells (8, 9, 13). In addition, we have sequenced and characterized a novel gene/protein which specifically interacts with IGFBP-3, designated IGFBP-3 receptor (BP3-R) (unpublished data). When we transfecBP-3R into IGFBP-3-induced cells, DNA synthesis was further inhibited (by an aver-
age of 65%) compared to control IGFBP-3-induced cells (an average of 45%), suggesting that IGFBP-3 and BP-3R appear to cooperatively suppress DNA synthesis and cell growth, to an extent greater than that seen with IGFBP-3 alone (unpublished data). Furthermore, BP-3 R alone without induction of IGFBP-3 results in no significant changes in DNA synthesis and cell growth in the same cell system, suggesting necessity of interaction between IGFBP-3 and BP-3R for IGFBP-3-induced biological function.

We thus concluded that cellular expression of IGFBP-3 inhibits DNA synthesis and cell growth through the cell cycle arrest in G1 phase and induction of apoptosis at physiological concentrations in an IGF-independent manner in MCF-7 breast cancer cells. Regardless of the underlying mechanisms, the present study demonstrates that IGFBP-3 decreases the levels of cyclin D1 protein, followed by cdk4, Rb, and phospho-Rb, indicating a possible mechanism of cell cycle arrest. Although we cannot exclude a possible additional posttranscriptional and translational regulation of cyclin D1 by IGFBP-3, our results suggest that IGFBP-3 decreases the cyclin D1 expression at the level of transcription, in part, through the decline in p42/p44 MAPK expression. This novel cell cycle regulatory and apoptosis-inducing aspect of IGFBP-3 have clinical significance in the prevention and/or treatment of human neoplasia, particularly in conjunction with IGFBP-3 receptor.
References

35. Reed, S. I. (1997) Cancer Surv. 29, 7-23
Fig. 1. Panel of IGFBP-3 expression in 16 clones tested for induction with ponasterone A. MCF-7 cells were stably transfected using the ecdyson-inducible system. Transfected cells were selected in G418- and Zeocin-containing medium. Incubation of the cells with Ponasterone A induces the expression of IGFBP-3. Clones 1 and 16 constitutively expressed IGFBP-3; clones 2, 3, and 6 expressed IGFBP-3 in an inducible manner.

Fig. 2. Inhibitory effect of IGFBP-3 on DNA synthesis in inducible stably transfected MCF-7 cells. A) Cells were treated with ponasterone A at concentrations of 0-10 μM for 72 h in SFM prior to assessing DNA synthesis by [3H]-thymidine incorporation. Significant decreases in DNA synthesis compared with noninducible control (receptor only) transfected cells were seen. B) Cells were treated with Y60L-IGF-I (100 ng/ml), an IGF-I analog with significantly reduced affinity for the IGF receptor but high affinity for IGFBPs, in the presence or absence of ponasterone A (10 μM) as indicated for 72 h prior to assay for [3H]-thymidine incorporation. The inhibitory effect of IGFBP-3 was abolished by Y60L-IGF-I, demonstrating IGFBP-3 specificity and IGF-independency. * = p<0.05, ** = p<0.001.

Fig. 3. Cell cycle arrest in the IGFBP-3-induced cells. Asynchronous MCF-7:IGFBP-3 #3 cells were seeded with or without ponasterone A in SFM for 72 h. The percentages of cells in the various phases of the cell cycle were determined by propidium iodide staining for DNA content and subsequent flow cytometry. These data show that induced expression of IGFBP-3 resulted in arrest of the cell cycle in G1 phase.

Fig. 4. Induced IGFBP-3 expression causes a reduction in Cyclin D1 at mRNA and protein levels. A) Northern blot analysis of a time course of cyclin D1 and IGFBP-3 expression in induced and uninduced cells. With the induction of IGFBP-3, expression of the 4.5 kb cyclin D1 mRNA species is decreased. Expression of the 1.5 kb species is not affected. B-actin was used as a control. B) Western blot analysis of Cyclin D1 protein in the MCF-7:IGFBP-3 #3 and MCF-7:EcR cells after treatment with increasing concentrations of ponasterone A for 72 h. Induction of IGFBP-3 results in a significant decrease in the level of Cyclin D1. C) Co-treatment with Y60L-IGF-I reverses the decreased level of cyclin D1 showed in IGFBP-3-induced cells, demonstrating specificity of IGFBP-3.

Fig 5. Cell cycle proteins affected by induction of IGFBP-3 expression. A) Western blot analysis of various cell cycle-related proteins in the MCF-7:IGFBP-3 #3 cells cultured in the presence or absence of ponasterone A at indicated time. The expression of cyclin D1, cdk4, total Rb, and phospho-Rb starts to decline from day 1 in the IGFBP-3-induced cells, presenting a possible direct mechanism for IGFBP-3-induced cell cycle arrest. A decrease of cyclin A expression is evident after day 3. B) Immunofluorescent staining of cells showing the decrease in Cyclin D1 and phosphorylated Rb detectable levels with the induction of IGFBP-3.
Fig. 6. IGFBP-3 induction causes a decrease in active MAPK. A) Western blot analysis of p44/42 mitogen-activated protein kinase (MAPK) and phosphorylated p44/42 MAPK in the MCF-7:IGFBP-3 #3 cells cultured the presence or absence of ponasterone A at indicated time. The phosphorylation of MAPKs declines with induction of IGFBP-3. B) Immunofluorescent staining of phospho-p44/42 MAPK in control uninduced and IGFBP-3-induced cells. A decrease in detectable levels of phospho-MAPK is evident, as well as perturbed subcellular localization.

Fig. 7. IGFBP-3 antagonizes Ras-induced MAPK signaling. MCF-7:IGFBP-3 #3 cells were transfected with a constitutively active Ras (RasV12) construct, which increased A) DNA synthesis and B) phosphorylation of p44/42 MAPK. Induction of IGFBP-3 expression abrogated both of these RasV12-induced effects.

Fig. 8. Induction of apoptosis in the MCF-7:IGFBP-3 #3 cells. A) Asynchronous MCF-7:IGFBP-3 #3 cells were seeded with or without ponasterone A for 72 h. Cells were incubated with Annexin V, then binding of Annexin V was determined by flow cytometry. IGFBP-3-induced cells showed significantly increased binding of Annexin V, an indicator of cells undergoing apoptosis. B) Asynchronous MCF-7:IGFBP-3 #3 cells were cultured with or without ponasterone A, and cell lysates were assayed for caspase activity. Induction of IGFBP-3 caused a dose-dependent increase in caspase activity compared to control uninduced levels. C) Detection of PARP cleavage to the p85 protein species as detected by western immunoblot. An increase in the p85 species was seen in lysates from IGFBP-3-induced cells compared to uninduced controls. * = p<0.05.
<table>
<thead>
<tr>
<th>Clone No.</th>
<th>0</th>
<th>2</th>
<th>10 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[image]</td>
<td>[image]</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>[image]</td>
<td>[image]</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>[image]</td>
<td>[image]</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>[image]</td>
<td>[image]</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>[image]</td>
<td>[image]</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>[image]</td>
<td>[image]</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>[image]</td>
<td>[image]</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>[image]</td>
<td>[image]</td>
<td>16</td>
</tr>
</tbody>
</table>
MCF-7: IGFBP-3 #3

<table>
<thead>
<tr>
<th></th>
<th>- Pon A</th>
<th>+ Pon A</th>
</tr>
</thead>
<tbody>
<tr>
<td>% G_0/G_1</td>
<td>72.1</td>
<td>78.1</td>
</tr>
<tr>
<td>% S</td>
<td>18.4</td>
<td>13.8</td>
</tr>
<tr>
<td>% G_2/M</td>
<td>9.5</td>
<td>8.1</td>
</tr>
</tbody>
</table>
A

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pon A</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Cyclin D1

4.5 kb

1.5 kb

IGFBP-3

β-actin

B

<table>
<thead>
<tr>
<th>Pon A (µM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7: EcR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7: IGFBP-3 #3</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

Cyclin D1

C

<table>
<thead>
<tr>
<th>Pon A</th>
<th>-</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y60L-IGF-I</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

MCF-7:IGFBP-3 #3

Cyclin D1
A

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>6</th>
<th>18</th>
<th>36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pon A (15 μM)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Phospho-p44/42 MAPK

- p44/42 MAPK

B

<table>
<thead>
<tr>
<th></th>
<th>IGFBP-3</th>
<th>Hoechst</th>
<th>phospho-MAPK</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Pon A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Pon A</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
MCF-7:IGFBP-3 #3

Thymidine incorporation (% of control)

- Pon A
+ Pon A

pcDNA  RasV12

Phospho-p44/42 MAPK