Expression of IGFBP-3 by Human Retinal Endothelial Cell Cultures: IGFBP-3 Involvement in Growth Inhibition and Apoptosis

Polyxenie E. Spoerri, Sergio Caballero, Sylvia H. Wilson, Lynn C. Shaw, and Maria B. Grant

PURPOSE. Growth hormone (GH), insulin-like growth factor (IGF), and somatostatin (SST) modulate each other’s actions. SST analogues have been successfully used to treat proliferative diabetic retinopathy (PDR) that is unresponsive to laser therapy and to retard the progression of severe nonproliferative retinopathy to PDR. In this study, the endogenous expression of IGF-binding protein (IGFBP)-3 was examined in human retinal endothelial cells (HRECs), the direct effects of IGFBP-3 on HRECs were evaluated, and the possible involvement of IGFBP-3 in mediating the growth inhibitory effects of SST receptor (SSTR) agonists in HRECs was assessed.

METHODS. The cellular localization of IGFBP-3 was examined with anti-IGFBP-3 and fluorescein-conjugated goat anti-rabbit IgG. HRECs were exposed to varying concentrations of human recombinant IGFBP-3, and growth inhibition was evaluated by thiazolyl blue (MTT) conversion. Apoptosis was examined using fluorochrome-annexin V staining. Conditioned media (CM) from SSTR2 agonist (L779976)–treated or SSTR3 agonist (L796778)–treated HRECs were analyzed by ELISA for changes in expression of IGFBP-3.

RESULTS. HREC immunostaining showed cell surface and cytoplasmic IGFBP-3. Exogenous IGFBP-3 induced a dose-dependent inhibition of HREC proliferation and staining with fluorochrome-annexin V showed numerous apoptotic HRECs. HRECs exposed to the SSTR2 or SSTR3 agonists expressed IGFBP-3 in a concentration-dependent manner.


Numerous studies support a role for growth hormone (GH) and insulin-like growth factor (IGF) in proliferative diabetic retinopathy (PDR).1–3 IGF-I is the mediator of the mitogenic effects of GH and has direct effects on the abnormal growth of retinal vasculature.4,5 Somatostatin (SST), a hypothalamic inhibitory hormone,6 blocks GH release from the anterior pituitary and also decreases the expression of IGF-I.7 GH, SST, and IGF-I modulate each other’s actions in vivo.

IGF-I is found in the blood in association with IGF-binding proteins (IGFBPs).8 IGFBPs may either inhibit or potentiate the bioavailability of IGF through several mechanisms.9 Six IGFBPs with high affinity to IGF have been identified, as well as four other molecules with low affinity (IGFBP-related peptides).10 The first five IGFBPs preferentially bind IGF-I over IGF-II. IGFBP-3, the primary carrier of IGF in circulation, forms a 150-kDa complex with IGF-I that modulates the amount of bioavailable free IGF and inhibits its transfer from circulation to tissue sites of action.11

By competitively binding IGFs and preventing their binding to the type I IGF receptor (IGF-IR), all IGFBPs can demonstrate growth-inhibitory effects. Aside from its effects on IGF-I/IGF-IR interactions, IGFBP-3 can stimulate apoptosis and inhibit cell proliferation in a variety of cell types directly and independently of binding IGF-I or -II.12,13 IGF-I–knockout fibroblasts, when transfected with a vector containing the IGFBP-3 gene, demonstrate growth inhibition that correlates with the magnitude of expressed IGFBP-3.14 Human epithelial keratinocytes synthesize IGFBP-3, which inhibits IGF-I’s ability to stimulate mitosis.15 IGFBP-3 has been shown to mediate apoptosis in prostate cancer cells independent of binding IGF-I and removing IGF-I for receptor activation.15 Furthermore, a rhIGFBP-3 fragment inhibited IGF-I–stimulated DNA synthesis in chick embryo fibroblasts.16 Moreover, IGFBP-3 has been shown to participate actively in apoptotic pathways triggered by p53,17 Wilms tumor gene (WT-1),18 cytokines,18,19 and retinoic acid.20,21

Somatostatin receptors (SSTRs) are an attractive therapeutic target for regression of neovascularization. SSTR stimulation blocks angiogenesis in several model systems,22–24 including a mouse model of oxygen-induced retinopathy.3 Somatostatin analogues have achieved success in the clinical arena by retarding the progression of advanced retinopathy.25,26 However, signal transduction pathways used by SSTRs to reduce retinal endothelial cell viability have not been elucidated. In other systems, SSTRs signal through two main pathways, resulting in apoptosis and arrest of the cell cycle.27 One pathway involves coupling through pertussis-toxin-sensitive G proteins of the Gαq/11 class, leading to inhibition of production of cyclic AMP (cAMP).28 This inhibition may be the mechanism of action for the cytostatic and apoptotic effects of SST in cells where proliferation is promoted by cAMP. The second pathway leads to increased activation of the tyrosine phosphatases SHP-129 and -2,30 which inhibit signaling through tyrosine kinase receptors. In a previous study from this laboratory, the inhibitory effect of SSTR agonists in HRECs was active only after 48 to 72 hours of exposure,31 suggesting the possible expression or secretion of a new protein in response to exposure to SSTR agonists.

In this study, we asked whether agonist activation of SSTRs could lead to secretion of IGFBP-3, which can facilitate both an autocrine and paracrine growth rate regulation of endothelial...
cells. To evaluate the role of IGFBP-3 in cultured HRECs, the surface and intracellular localization of IGFBP-3 was examined by immunocytochemistry. We also examined the direct effect of exogenously administered IGFBP-3 on HREC proliferation and apoptosis. Our results suggest that IGFBP-3 may be a determining factor in the vascular complications associated with retinopathies.

**MATERIALS AND METHODS**

**Cell Culture**

Human eyes were obtained from the National Disease Research Interchange within 36 hours of death (n = 4 donors). The research protocol adhered to the tenets of the Declaration of Helsinki for research involving human tissue. Human retinal endothelial cells were prepared and maintained as previously described, and cells in passages 3 and 4 were used in the present study. The identity of HRECs was validated by demonstrating endothelial cell incorporation of fluorescence-labeled acetylated LDL and by fluorescence-activated cell-sorting analysis, as previously described.

**IGFBP-3 Localization by Immunostaining**

Cells were grown to 90% confluence on 2% gelatin-coated eight-well chamber slides and were serum deprived, as in our previous study.

The 48- to 72-hour serum-deprived cultures were washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 20 minutes at room temperature. Fixed cell monolayers were washed in 0.05 M Tris-buffered saline (TBS), permeabilized using 0.2% Triton X-100 in TBS, for 30 minutes at room temperature followed by incubation in TBS + Triton X-100 containing 10% normal goat serum for 30 minutes at room temperature. The cells were then incubated in TBS containing rabbit anti-IGFBP-3 (Upstate Biotechnology, Lake Placid, NY) for 1 hour at 37°C. To test for immunospecificity, the antibody was incubated with 25 μg/mL recombinant IGFBP-3 (catalog no. 12-131; Upstate Biotechnology) at 37°C for 30 minutes and then added to the cells as just described. After washing with TBS, the secondary antibody, fluorescein–conjugated goat anti-rabbit IgG (Sigma, St Louis, MO), in TBS was applied. The cells were incubated for 1 hour at 37°C, washed with TBS, covered in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) under a coverslip, and examined and photographed with a fluorescence microscope (Axiovirt; Carl Zeiss, Thornwood, NY).

**Effect of IGFBP-3 on Proliferation of HRECs**

The effects on proliferation of HRECs were measured by a modified thiazolyl blue (MTT) assay, based on the ability of live cells to take up thiazolyl blue and convert it into dark blue formazan. In brief, 6 x 10^4 cells per well (96-well microtiter plates) were incubated with 100 μL of culture medium overnight. The cells were then treated with 0, 10, 50, 100, 500, or 1000 ng/mL human recombinant IGFBP-3 in serum-free medium for 48 hours. MTT solution (0.5 mg/mL, final concentration; Sigma, St. Louis, MO) was added to each well, the cells were further incubated at 37°C for 2 hours, and the medium was aspirated and replaced with isopropanol. MTT conversion was measured with a microplate spectrophotometer at a wavelength of 595 nm. Each condition was assayed in triplicate with cells from at least three donors.

**Effect of IGFBP-3 on HREC Apoptosis**

HRECs were grown to 70% confluence (10^4 cells/cm²) in eight-well chamber slides coated with 2% gelatin. The medium was replaced with serum-free medium containing 1 μg/mL of human recombinant IGFBP-3 and incubated for 48 hours. The cells were washed one time with ice-cold PBS and incubated in biotin-conjugated annexin V (500 ng/mL; Molecular Probes, Eugene, OR) for 15 minutes at room temperature. After a wash with 1 x annexin-binding buffer (ABB; Molecular Probes), the cells were incubated in ABB containing propidium iodide (PI; 100 μg/mL) plus an avidin-conjugated fluorochrome (20 μg/mL; Alexa Fluor 488; Molecular Probes) for 30 minutes in the dark at room temperature and then washed one time with ABB. Three slides from each of three separate donors were examined with a fluorescence microscope (Axiovirt 135; Carl Zeiss).

**Induction of IGFBP-3 by SSTR Agonists**

HRECs were grown in 96-well gelatin-coated tissue culture dishes (10^4 cells/well) and were serum deprived for 24 hours before treatment with SSTR agonists (a gift from Jim Schlafer, Merck Pharmaceuticals, Rahway, NJ). HRECs were treated with the SSTR agonists L779976 (type 2 selective at <1 nM; activates type 3 receptor at >100 nM) or L796778 (type 3 selective) diluted in serum-free medium to 0.1, 0.3, 1, 3, 10, 30, 100, and 300 μM. The cells were incubated for 72 hours, and the conditioned medium (CM) was collected for analysis by ELISA (Diagnostic Systems Laboratories, Inc., Webster, TX) for the quantitative measurement of IGFBP-3. Cells incubated in either serum-free medium or normal growth and maintenance medium were used for negative and positive controls, respectively. Each condition was assayed in triplicate with cells from at least three separate donors.

**RESULTS**

**IGFBP-3 Localization by Immunostaining**

HREC monolayers that reacted with an antibody specific for human IGFBP-3 showed that most cells had diffuse cytoplasmic and/or surface IGFBP-3 (Figs. 1A, 1B). This staining was spe-
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**Figure 2.** IGFBP-3 induced a concentration-dependent decrease in cell proliferation as measured by conversion of the tetrazolium salt, MTT. Data represent mean ± SD of triplicate conditions using cells from three separate donors after 48 hours exposure to the IGFBP-3. *P < 0.01 compared to untreated cells.

specific for IGFBP-3, as there was no staining when the antibody was preadsorbed with human recombinant IGFBP-3, nor was there staining when nonimmune serum was used instead of the IGFBP-3–specific antibody (not shown).

**Effect of IGFBP-3 on Proliferation of HRECs**

IGFBP-3 inhibited HREC proliferation and survival in a concentration-dependent manner, as measured by MTT conversion. A statistically significant inhibitory effect (*P < 0.01*) was observed at IGFBP-3 concentrations exceeding 50 ng/mL (Fig. 2).

**Effect of IGFBP-3 on HREC Apoptosis**

HRECs treated with recombinant IGFBP-3 (1 μg/mL) for 48 hours showed numerous apoptotic green fluorescent cells that stained with the fluorochrome and annexin V (Fig. 3A). Control HRECs showed very few green fluorescent cells (Fig. 3B). A few necrotic cells were present in treated and untreated HRECs, distinguished by red nuclei as a result of PI’s binding tightly to the nucleic acids in the cells. Live cells showed little or no fluorescence (Fig. 3).

**Induction of IGFBP-3 by SSTR Agonists**

HRECs exposed to SSTR agonists secreted IGFBP-3 in a concentration-dependent manner, as measured by ELISA (Fig. 4). L779976 induced some IGFBP-3 at concentrations at which it activates SSTR2 (<1 nM), with maximum induction of expression occurring at concentrations that activate SSTR3 (>100 nM). The amount of IGFBP-3 induced by L796778 (SSTR3 selective) was also dependent on agonist concentration and reached maximum levels at an agonist concentration of 30 nM. The data were normalized to untreated cells (in serum-free medium) and are expressed as multiples of increase. The actual concentrations of IGFBP-3 ranged from approximately 1.5 to approximately 50 ng/mL.

**DISCUSSION**

Our data demonstrated production of IGFBP-3 in HRECs. The immunocytochemical findings showed diffuse cellular staining corresponding to cytoplasmic IGFBP-3. Serum IGFBP-3 could have been released from the 150-kDa complex by its association with endothelial cell surface proteoglycans.1,2 However, HRECs were serum deprived long enough to allow for the decay of any IGFBP-3 that could have originated from serum in the culture medium.

IGFBP-3 has been shown to be translocated into the nucleus, compatible with having a nuclear localization sequence in the midregion.35,36 This suggests that nuclear IGFBP-3 may control gene expression and play a role in IGF-independent actions in some cell types. However, unlike immunocytochemical studies of dividing human keratinocytes,37 we did not detect intranuclear IGFBP-3.

IGFBP-3 induced a concentration-dependent decrease in proliferation of HRECs as reported for numerous cell systems. IGFBP-3 inhibits cell proliferation by inducing apoptosis in HRECs as well as promoting cell cycle arrest. Our findings provide further evidence that IGFBP-3 induced apoptosis in HRECs, as shown by the bright green fluorescent annexin V staining, specific for apoptotic cells, after exposure to IGFBP-3. Annexin V is a protein that binds in a calcium-dependent manner to phosphatidyl serine (PS).38,39 PS in apoptotic cells is located at the outer leaflet of the plasma membrane. A few necrotic cells were present in both control and treated cultures and were easily distinguished from apoptotic cells by red fluorescence. Live cells in the background showed a low level of fluoresce.38,39

**Figure 3.** HRECs treated with human recombinant IGFBP-3 (1 μg/mL) for 48 hours and stained with annexin V and a fluorochrome as well as PI. (A) Numerous apoptotic cells showed bright green fluorescence, and a few necrotic cell nuclei took up the red fluorescent PI. Live cells in the background showed a low level of fluorescence. (B) Control nontreated cells showed very few apoptotic cells. A few necrotic cell nuclei were present in both control and treated cultures.
independent of IGF-IR.46 Such fragments enhance the apoptotic effect of the ceramide analogue C2 on Hs578T breast carcinoma cells.41

IGF-I-independent effects of IGFBP-3 may be mediated by a specific receptor or by other cell surface proteins that bind to IGFBP-3. IGFBP-3 has been shown to bind to breast cancer cell surface proteins of 20-, 26-, and 50-kDa, resulting in inhibition of cell growth.42 The midregion of IGFBP-3 has been identified as the receptor-binding domain.43 In addition, the type V transforming growth factor (TGF)-β receptor has been shown to bind IGFBP-3, suggesting that IGFBP-3 mediates its action in part through this receptor.44 IGFBP-3 has also been shown to interact with numerous molecules that may regulate its action on cells, including procarboxypeptidase A,45 transferrin,46 fibrinogen and fibrin47, and heparin.48 Despite all these reports, a specific, IGFBP-3 receptor has not yet been confirmed.

Our findings suggest that IGFBP-3 may also mediate in part the growth-inhibitory effect of SSTR agonists in HRECs. Previous studies have shown that both SST and the SST analogue octreotide diminishes the proliferative activity of cultured mouse endothelial cells.49 The inhibitory capacity of SST appears to be mediated by the presence of SSTRs.45 At least five different subtypes of specific, high-affinity SSTRs (types 1 to 5) have been cloned in humans, mice and rats.46,49,50 Previous studies from this laboratory have shown that HRECs have SSTRs, and exposure to octreotide decreases proliferation of HRECs in response to IGF-I and basic fibroblast growth factor (bFGF).51

Induction of protein tyrosine phosphatase by SST plays a key role in mediating growth arrest through SSTR2 or apoptosis through SSTR3. SSTR2 induces cell cycle arrest by a phosphotyrosine phosphatase (PTP)-dependent modulation of mitogen-activated protein kinase (MAPK), associated with induction of retinoblastoma tumor suppressor protein (Rb) and p21 (cyclic-dependent kinase inhibitor). In contrast, SSTR3 triggers PTP-dependent apoptosis accompanied by activation of p53 and increases expression of p53-regulated genes that regulate apoptosis, such as Bax.49-53 Activation of p53 has also been shown to upregulate IGFBP-3 and participate in apoptotic pathways triggered by p53.15,54

In this study, we observe that activation of both SSTR2 and -3 increased expression of IGFBP-3. The levels of IGFBP-3 observed after activation of SSTR were sufficient to have physiological consequences, including initiation of apoptosis in HRECs. Our data support the theory that SSTR agonists, as antiangiogenic therapeutic agents for treatment of PDR, may mediate their growth-inhibiting effect on HRECs, in part by increasing expression of IGFBP-3. Furthermore, in the present study HRECs clearly expressed IGFBP-3 in vitro. Exogenous IGFBP-3 in HRECs induced growth inhibition and apoptosis consistent with other studies of different cell types. This report is the first to demonstrate IGFBP-3 growth inhibition and apoptosis in retinal endothelial cells. Thus, endogenous expression of IGFBP-3 may be a key regulator of endothelial cell behavior in both disease and health.

References


