Interleukin-11 inhibits expression of insulin-like growth factor binding protein-5 mRNA in decidualizing human endometrial stromal cells

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Introduction

Differentiation of endometrial stromal cells into decidual cells is essential for successful embryo implantation. Interleukin (IL)-11 signalling is critical for normal decidualization in the mouse. The expression of IL-11 and its receptors during the menstrual cycle, and the effect of exogenous IL-11 on the decidualization of human endometrial stromal cells in vitro, suggests a role for this cytokine in human decidualization. As the downstream target genes of IL-11 are also likely to be critical mediators of this process, this study aimed to identify genes regulated by IL-11 in decidualizing human endometrial stromal cells in vitro. Stromal cells isolated from endometrial biopsies were decidualized with 17β estradiol (E) and medroxyprogesterone acetate (EP) in the presence or absence of exogenous IL-11, and total RNA used for cDNA microarray analysis and real-time RT–PCR. Microarray analysis revealed 16 up-regulated and 11 down-regulated cDNAs in EP + IL-11-treated compared with EP-treated cells. The most down-regulated gene was insulin-like growth factor binding protein-5 (IGFBP-5) (3.6-fold). Using real-time RT–PCR, IL-11 was confirmed to decrease IGFBP-5 transcript abundance 102-fold (P < 0.016; n = 6). No difference in IGFBP-5 immunostaining intensity was detected in stromal cells decidualized in the presence or absence of IL-11, and there was no effect of exogenous IGFBP-5 on the progression of steroid-induced in vitro decidualization. Interactions between IL-11 and its target genes, including IGFBP-5, may contribute to the regulation of decidualization and/or mediate communication between the decidua and invading trophoblast at implantation.

Key words: decidualization/endometrium/IGFBP-5/interleukin-11/microarray

Materials and methods

Experimental subjects

Endometrial biopsies (n = 19; experiments 1–14) were obtained between days 7 and 26 of the menstrual cycle from women with regular menstrual cycles and no known endometrial dysfunction. Approval was obtained from the Human Ethics Committee at Monash Medical Centre, Melbourne, Australia. Informed consent was obtained from all women participating in the study and all procedures were conducted in accordance with the guidelines in The Declaration of Helsinki. Histological dating of the menstrual cycle was performed by an

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experienced gynaecological pathologist, according to the method of Noyes et al. (1975), and samples were identified as early proliferative (n = 1), mid-proliferative (n = 4), late proliferative (n = 5), early secretory (n = 1), mid-secretory (n = 6) or late secretory (n = 2). Samples for culture were collected into Dulbecco’s modified Eagle’s medium (DMEM; Thermo Electron Corporation, Melbourne, Australia).

Endometrial stromal cell culture

Stromal cells were isolated from endometrial biopsies, as described previously (Zhang and Salamonson, 1997; Dimitriadis et al., 2002). Briefly, tissue was finely minced with scissors and digested in 45 IU/ml bacterial collagenase type III (Worthington Biochemical Corporation, Freehold, NJ, USA) and 3.5 μg/ml deoxyribonuclease (DNase; Boehringer Mannheim) in sterile phosphate-buffered saline (PBS). After 40 min of agitation at 37°C, the digested tissue was filtered sequentially through 45 and 10 μm nylon filters to remove large epithelial cells. The cell suspension was then centrifuged, and the pellet resuspended in 1:1 mixture of DMEM/Ham’s F-12 (F12; Thermo Electron Corporation) containing 10% charcoal-stripped fetal calf serum (CS-FCS). Cells were seeded onto 10 cm diameter tissue culture dishes in 2 ml DMEM/10% CS-FCS, 2 mM l-glutamine (Thermo Electron Corporation) and 20 μl of 10 μM dithiothreitol (DTT), 10 μg/ml Gentamycin 10 × 10 μM linoleic acid (Sigma), 10 μg/ml bovine serum albumin (BSA; 0.1%; Sigma), 10 μg/ml monomeric antinuclear (Dimitriadis et al., 2002). Cells were kept under a light microscope and made up to the required volume in DMEM/10% CS-FCS. Cells were allowed to adhere for 2–4 days with medium changes every 2 days. Stromal cells prepared using the above method in this laboratory have been previously shown to be >97% pure as assessed by immunostaining for cytokeratin, vimentin and leukocyte common antigen (Dimitriadis et al., 2002). Experiments 7, 8, 9 and 13 were conducted on pools of isolated endometrial stromal cells from 2 or 3 women in the same menstrual phase. All other experiments used individual biopsies. Once confluent, the cells were washed twice with sterile PBS and subjected to 48 h in serum-free medium to suppress endogenous IL-11 production (Dimitriadis et al., 2002). On experimental day 0, fresh serum-free medium containing TSL: transferrin (10 μg/ml; Sigma), sodium selenite (25 ng/ml; Sigma),inositol acid (10 μM; Sigma) and bovine serum albumin (BSA; 0.1%; ICN Biomedicals, Aurora, OH, USA) was added to each well (Dimitriadis et al., 2002), and duplicate or triplicate wells treated with 17β estradiol alone (10–8 M in absolute ethanol; Sigma), Ei, E plus medroxyprogesterone acetate (10–7 M in absolute ethanol; Sigma; EP) or with either recombinant human IL-11 (100 ng/ml; gift from Dr. Lorraine Cobb, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) or neutralizing anti-human IL-11 polyclonal antibody (5 μg/ml; R&D Systems Inc., Minneapolis, MN, USA; AB) to completely neutralize endogenous IL-11. Medium was collected and stored at −20°C, and treatments were replaced every 2–3 days. At the completion of culture (days 10–12), cells were washed twice in sterile PBS and harvested for subsequent RNA analyses.

Total RNA isolation

Total RNA was extracted from cells using an RNeasy™ MiniKit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Genomic DNA was removed from samples using RNase-free DNase I (DNase-free™ kit; Ambion, Austin, TX, USA) for 30 min at 37°C. RNA concentration and quality was assessed by spectrophotometer and agarose gel electrophoresis. To concentrate the RNA for microarray analysis, samples were reprecipitated using linear acrylamide (Ambion), according to the manufacturer’s instructions, and resuspended in RNase-free water to approximately 500 ng/μl. An estimated 250 ng total RNA was loaded onto an RNA Labchip (Agilent Technologies, Palo Alto, CA, USA), for RNA concentration and quality analysis using an Agilent 2100 Bioanalyzer (Agilent Technologies). The resulting electrophoreograms confirmed that each sample was free from degradation and genomic DNA contamination.

Human 15K cDNA microarray hybridization

Custom glass cDNA microarrays were produced at the Department of Pathology, University of Cambridge, United Kingdom (http://www.path.cam.ac.uk/). The arrays contained approximately 15 000 cDNAs spotted in duplicate over two glass slides. Total RNA samples from EP-treated or EP + IL-11-treated cells from the same patient (n = 6; experiments 1–6) were prepared for microarray analysis using the following protocol: 1 μg total RNA per sample, using the SMART™ PCR cDNA Synthesis Kit (BD Biosciences Clontech, Palo Alto, CA, USA). Total RNA was incubated with 10 pmol 3′-SMART CDS primer IIA and 10 pmol template-switching primer (Petalidis et al., 2003) in a volume of 5 μl at 72°C for 2 min, then quenched on ice for 2 min. The following reagents were added to each reaction: 2 × 5′-first-strand buffer, 1 μl 20 mM diithiothreitol (DTT), 1 μl 10 mM deoxyribonucleoside 5′-triphosphates (dNTPs; Sigma) and 1 μl PowerScript reverse transcriptase (BD Biosciences Clontech) and incubated at 42°C for 1 h. A 2 μl aliquot of the first-strand cDNA was then used as the template for second-strand amplification. Each amplification reaction consisted of 2 μl first-strand cDNA, 75 μl distilled water (dH2O), 10 μl GeneAmpl® 10 × PCR buffer II (Applied Biosystems, Foster City, CA, USA), 5 μl 50 mM MgCl2, 2 μl 10 mM dNTPs, 4 μl 5′ PCR primer IIA and 2 μl AmpliTaq DNA polymerase (Applied Biosystems). Thermocycling consisted of 95°C for 1 min, then 16 cycles of 95°C for 5 s, 65°C for 5 s and 68°C for 6 min.

Fluorescent-labelled target cDNA was prepared by mixing 21 μl of amplified cDNA with 20 μl 2.5 × random primer reaction buffer (BioPrime DNA Labelling System, Invitrogen, Carlsbad, CA, USA) and incubating at 95°C for 5 min. The following reagents were then added on ice: 5 μl low-C dNTP mix (5 mM dATP, 5 mM dGTP, 5 mM dTTP, 2 mM dCTP, 2 μl Cy3 or Cy5-dCTP (Amersham Pharmacia Biociences) and 40 U Klenow polymerase I (Invitrogen) and incubated at 37°C for 2 h in the dark. The reactions were terminated by the addition of 5 μl stop buffer (Invitrogen). Cy3- and Cy5-labelled cDNA targets were purified separately on AutoSeq G-50 columns (Amersham Pharmacia Biociences), then pooled and combined with 10 μl human Cot-1 DNA (Gibco-BRL Life Technologies, Paisley, UK), 12 μl 3 M NaOAc (pH 5.2) and 305 μl 100% ethanol and pelleted by centrifugation for 10 min. The pellet containing Cy3 (EP) and Cy5 (EP + IL-11)-labelled cDNA from a single patient was resuspended in 50 μl hybridization buffer (40% deionized formamide, 5 × saline sodium citrate (SSC), 5 × Denhardt’s solution, 1.65 mM sodium pyrophosphate, 50 mM Tris–HCl, pH 7.4, 0.1% sodium dodecyl sulphate (SDS)), with the addition of 40 ng/μl human Cot-1 DNA, 150 ng/μl Poly A+ [poly(A)+40–60; Amersham Pharmacia Biociences] and 75 ng/μl yeast tRNA (Sigma). This mixture was heat denatured at 95°C for 5 min, incubated at 50°C for 5 min and centrifuged for 5 min before application to an array. Microarrays were prehybridized with hybridization buffer at 50°C for 1 h, washed in 2 × SSC and dH2O, dipped in isopropanol and air-dried. Hybridization with target cDNA was then performed under a Teflon-edged coverslip (Lifter Slips; Erie Scientific, Portland, NH, USA) at 50°C in a preheated humidified chamber for 16 h. Hybridizations were repeated using the alternate dye combinations to account for any differential fluorescent dye incorporation. Post-hybridization washing was as follows: twice in 2 × SSC, twice in 0.1 × SSC/0.1% SDS and twice in 0.1 × SSC each for 5 min at room temperature in the dark. Finally, slides were rinsed in two changes of dH2O and dipped in isopropanol before drying by centrifugation for 2 min. Slides were scanned using an Axon GenePix 4000B microarray reader (Axon Instruments, Union City, CA, USA) and GenePix Pro 4.0 software (Axon Instruments) to generate pairs of 16-bit TIFF files. Following manual quality control for hybridization artefacts, red (Cy5) and green (Cy3) median foreground and background fluorescence intensity measurements for each spotted DNA sequence were extracted for export to the data analysis software Acuity 3.1 (Axon Instruments).

Human 15K cDNA microarray data analysis

Normalization and identification of differentially expressed genes were performed on log-transformed data using the analysis tools provided in Acuity 3.1. Loess slide normalization was applied to each set of hybridization replicates in turn, to normalize the red and green channels relative to one another. Quality control criteria were then applied to remove data arising from spots of poor quality, resulting in a data set where each spot fulfilled the following criteria: flags = 0 (removing spots that had been ‘flagged’ during manual quality control as containing artefacts), F635%Sat < 2 (removing spots with more than 2% pixel saturation in the foreground red channel), F532%Sat < 2 (removing spots with more than 2% pixel saturation in the foreground green channel) and Rgn R2 > 0.6 (removing spots with low uniformity). Spots that did not fulfill these criteria in arrays from at least five of six patients were excluded. Differentially
expressed genes were then defined as those that were more than 2-fold up- or down-regulated in at least four out of six patients. The coefficient of variation across patients for each gene was assessed as a measure of statistical significance.

Real-time quantitative RT–PCR
To validate the microarray data for IGFBP-5, we performed real-time quantitative RT–PCR on the same total RNA samples used for microarray analysis (n = 6; experiments 1–6). Total RNA (500 ng per sample) was reverse transcribed in triplicate at 46°C for 1.5 h in 20 μl reaction mixture using 100 ng random hexanucleotide primers (Roche, Mannheim, Germany) and 6 IU AMV reverse transcriptase (Roche) in the presence of cdNA synthesis buffer (Roche), 1 mM dNTPs (Roche), 10 mM DTT (Roche) and 10 IU ribonuclease inhibitor (RNasin; Promega, Annandale, Australia). The resulting cdNA mixtures were heated at 95°C for 5 min before storage at −20°C in small volumes to avoid freeze-thawing. Negative controls were performed by omission of reverse transcriptase.

For real-time quantification of IGFBP-5 mRNA transcript levels, PCR was carried out using a Roche LightCycler (Roche, Indianapolis, IN, USA). Before LightCycler analysis, standard cdNA for IGFBP-5 was generated using a PCR expression block cycler (Thermo Hybaid Instruments, Franklin, MA, USA). A 1 μl aliquot of RT product was amplified in a total volume of 40 μl using 4 μl of 10 × PCR buffer (100 mM Tris–HCl, 15 mM MgCl₂, 500 mM KCl, PH 8.3; Roche), 62.5 μM dNTPs, 10 pmol specific sense (5’-CGGGTATTGCTCAACGAA-3’) and antisense (5’-TCTTGGGGAGTGATCTCCT-3’) primers (PrimerBank ID 10834982at; Wang and Seed, 2003; Sigma Genosys, Castle Hill, New South Wales, Australia) and 2.5 IU Taq DNA polymerase (Roche). The block cycler PCR amplification consisted of a hot start at 95°C for 5 min followed by 40 cycles of denaturation at 94°C for 50 s, annealing at 61°C for 40 s and extension at 72°C for 40 s. The optimal annealing temperature was determined by testing a 12-step thermal gradient, either side of the expected annealing temperature of 5°C lower than the lowest primer melting temperature (Tₘ), as estimated by Net Primer software (http://www.premierbiosoft.com/netprimer). The final extension was performed at 72°C for 10 min. The PCR product was electrophoresed on a 1.5% agarose gel containing 200 ng/ml ethidium bromide. The single amplified product band was excised from the gel and purified using the UltraClean GelSpin DNA purification kit (Mo Bio Laboratories, Solana Beach, CA, USA). The cdNA concentration was measured using a spectrophotometer, and an IGFBP-5 standard curve was generated using serial 1:10 dilutions of this standard cdDNA with sterile water. Samples were diluted 1:5 before LightCycler analysis.

The cdNA templates (triplicate RT reactions for each of six patients; 4 μl) were added to sterile LightCycler capillaries (Roche) to a total volume of 20 μl containing SYBR Green L, dNTPs, Taq DNA polymerase and reaction buffer (LightCycler FastStart DNA Master SYBR Green I Kit; Roche), supplemented with 5 pmol of the above sense and antisense primers and an optimized concentration of 3 mM MgCl₂. An initial denaturing step was performed for 10 min at 95°C, before 45 cycles of 95°C for 15 s, 61°C for 5 s and 72°C for 10 s. Fluorescence was monitored continuously during cycling at the end of each elongation phase. At the end of the program, melting curve analysis confirmed the specificity of the 116 bp reaction product (melting temperature, 87.7°C).

The abundance of 18S rRNA was measured in the same samples as above. Samples were diluted 1:50 before LightCycler analysis using 5 pmol sense (5’-CGGCTACACATCCAAGGAA-3’) and antisense (5’-CGGGGCTACGCGGCT-3’) primers and 4 mM MgCl₂. An initial denaturing step was performed for 10 min at 95°C, before 35 cycles of 95°C for 15 s, 60°C for 5 s and 72°C for 10 s. Melting curve analysis confirmed the specificity of the 187 bp product reaction (melting temperature, 86.8°C).

Statistical analysis of real-time RT–PCR data
Duplicate RT reactions for each sample, the standard curve and a no RT negative control were analysed in the same run, and each run was repeated. A mean value for the absolute IGFBP-5 mRNA concentration in each sample was calculated relative to the standard curve using the fit points function of the LightCycler software, with the mean concentration of 18S rRNA for each sample used to control for RNA input. Following normalization, absolute amounts of IGFBP-5 mRNA in EP-treated and EP + IL-11-treated cells were tested for normality and statistically analysed using the Wilcoxon matched pairs test function of GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). A one-tailed P value of less than 0.05 was considered a significant difference.

IGFBP-5 immunocytochemistry
To confirm differential expression of IGFBP-5 at the protein level, cells treated with EP or EP + IL-11 were prepared for immunocytochemistry on day 12 (n = 4; experiments 7–10). Following the collection of culture medium, cells were washed in sterile PBS and dislodged from the plate by incubation in 0.12% trypsin (JRH Biosciences, Lenexa, KS, USA) for 5 min at 37°C. Small volumes (20 μl) of the resulting cell suspensions were centrifuged at 30% power for 10 min onto SuperFrost® glass slides (Menzel-Gläser, Braunschweig, Germany) using a Centrifuge (Hettich, Bach, Germany), fixed in 90% ethanol for 10 min, air-dried and stored at room temperature until use. Antigen retrieval was carried out by microwaving on high (1200 W) in 0.01 M sodium citrate (BDH Laboratory Supplies) for 5 min, followed by cooling in running dH₂O for 5 min. Endogenous peroxidase activity was quenched by incubating cells in 3% hydrogen peroxide (BDH Laboratory Supplies) for 10 min.

The primary antibody was rabbit anti-human IGFBP-5 (Upstate Biotechnology, Lake Placid, NY, USA; 06-110), and the negative control was prepared by preadsorbing the anti-body with a 5-fold excess of rhIGFBP-5 (GroPep, Adelaide, Australia) at 4°C for 72 h. Before the application to the cytosplns, both primary anti-body and negative control were preincubated at 1:50 dilution in non-immune block containing 10% normal swine serum, 2% normal human serum in Tris-buffered saline (TBS)/0.1% Tween 20 (Bio-Rad Laboratories) for 30 min at room temperature. Cytosplns were also incubated in non-immune block for 30 min, before the application of the primary anti-body or negative for 2 h at room temperature. The secondary anti-body was biotinylated swine anti-rabbit IgG (DakoCytomation, Glostrup, Denmark), diluted 1:200 in non-immune block. Secondary anti-body binding was detected using the Vectastain ABC Elite/HRP Kit (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer’s instructions. Between incubation steps, cytosplns were washed in TBS for 5 min, TBS/0.1% Tween for 5 min, then TBS for 5 min. Protein localization was visualized as brown staining using the liquid DAB–plus substrate chromogen system (DakoCytomation), with Harris hematoxylin counterstain.

IGFBP-5 immunoblotting
An enzyme-linked immunosorbent assay (ELISA) has not yet been developed for human IGFBP-5 (Yang and Chaum, 2003), therefore dot and western blotting were used to semi-quantify secreted and cytoplasmic IGFBP-5 in conditioned media and cell lysates.

Following collection of culture medium on day 12 of decidualization, cells treated with EP or EP + IL-11 (n = 4; experiments 7–10) were washed in sterile PBS and homogenized in ice-cold lysis buffer [50 mM Trizma base (Sigma), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF] with 1:500 dilution of protease inhibitor cocktail set III (Calbiochem, San Diego, CA, USA). Homogenates were incubated at 4°C on an orbital shaker for 15 min to fully lyse the cells. Lysates and media were centrifuged at 4°C at 14 000 × g for 15 min, and the supernatants assayed for total protein content using a BCA protein assay kit (Pierce, Rockford, IL, USA).

For dot blotting, serial dilutions of rhIGFBP-5 standard (GroPep), 15 μg total protein from each culture medium sample and 3 μg total protein from each cell lysate sample were applied directly to a nitrocellulose membrane and allowed to air dry. For western blotting, 100 ng rhIGFBP-5 standard and 15 μg total protein from each cell lysate sample were separated by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane.

Membranes were blocked with 5% skim milk in TBS/0.05% Tween (TBS-T–MILK) for 1.5 h at room temperature, then incubated for 30 min at room temperature with rabbit anti-human IGFBP-5 (Upstate Biotechnology) at a dilution of 1:2000. After washing with TBS-T, horseradish peroxidase-conjugated goat anti-rabbit IgG (DakoCytomation) was applied at 1:15 000 for 30 min at room temperature. Following washing, IGFBP-5 (dot or 31 kDa band) was visualized by applying ECL plus western blotting detection reagent (Amersham Pharmacia Biosciences) for 2 min then exposing to Hyperfilm ECL (Amersham Pharmacia Biosciences) for 6 min.
IGFBP-5 functional studies
To determine the effect of exogenous IGFBP-5 on hormone-induced decidualization, endometrial stromal cells \((n = 4; \text{experiments 11–14})\) were plated at \(0.25 \times 10^6\) cells per well in a 24-well plate and grown to confluence in DMEM/F12 with 10% CS-FCS for 2–4 days. Once confluent (experimental day 0), the medium was removed and replaced with DMEM/F12 containing 2% CS-FCS. Recombinant human IGFBP-5 was reconstituted in 10 mM hydrochloric acid at a concentration of 200 \(\mu\)g/ml, and rhIGFBP-3 (gift from Dr. Kate Hale, Prince Henry’s Institute of Medical Research, Melbourne, Australia) was reconstituted in 10 mM acetic acid and 0.05% BSA at a concentration of 1470 \(\mu\)g/ml. Both proteins were diluted in DMEM/F12 + 2% CS-FCS to 50 \(\mu\)g/ml and stored at \(-80^\circ\)C in small aliquots. Before use, the 50 \(\mu\)g/ml IGFBP-5 stock was serially diluted, and the same volume of each dilution added to the appropriate wells. Wells were treated in triplicate with E alone or EP + 0, 1, 10, 100, 300 or 1000 ng/ml rhIGFBP-5 or 1000 ng/ml rhIGFBP-3. Cells were first treated with IGFBP-5 or -3 and incubated at 37°C for 15 min, followed by the addition of hormones. Medium was collected, and treatments were replaced every 48–72 h. On day 12, medium was collected and stored at \(-20^\circ\)C until use. Cells were washed in sterile PBS and dislodged from the plate by incubation in 0.12% trypsin for 5 min at 37°C. Triplicate wells were pooled, and the cells in a 10 \(\mu\)l sample counted using a hemocytometer under a light microscope.

PRL ELISA
Immunoreactive PRL produced by cultured endometrial stromal cells treated with E, EP, EP + IL-11 or EP + AB was measured quantitatively using a PRL ELISA kit (Bioclone Australia, Marrickville, New South Wales, Australia), according to the manufacturer’s instructions. Total protein content was assessed using a BCA protein assay kit (Pierce). Conditioned media collected from cell cultures were thawed and concentrated 5- to 15-fold using a vacuum dryer before assay in duplicate, with results subsequently corrected for concentration factor. The sensitivity of the PRL assay was 10 mIU/l. Following a positive test for normality, absolute values (m IU/\(\mu\)g total protein) for experiments 1–6 (treated with E, EP, EP + IL-11 or EP + AB) were analysed using the repeated measures one-way ANOVA and Bonferroni’s multiple comparison post hoc test functions of GraphPad Prism 4.0. Absolute values for experiments 11–14 (treated with E, EP or EP + increasing concentrations of IGFBP-5 or -3) were analysed using the repeated measures one-way ANOVA and Tukey’s multiple comparison post hoc test functions of GraphPad Prism 4.0. Different post hoc tests were used as Bonferroni’s test is recommended for less than five groups and Tukey’s for more than five groups. In both analyses, a two-tailed \(P\) value of less than 0.05 was considered a significant difference.

Results
Morphological changes and PRL production by human endometrial stromal cells treated with E, EP, EP + IL-11 or EP + AB
Isolated human endometrial stromal cells treated for 12 days with E alone did not display the morphological changes characteristic of decidualization, maintaining a spindle-shaped fibroblast-like appearance (Figure 1A). In contrast, stromal cells treated with EP over the same time course took on a more rounded, polygonal shape with a larger nucleus and more cytoplasm (Figure 1B). With the addition of IL-11, cells became even more enlarged, indicating enhanced decidualization (Figure 1C). Cells treated with EP + AB resembled most closely those treated with EP (Figure 1D).

Measurement of PRL secretion from human endometrial stromal cells treated with E, EP, EP + IL-11 or EP + AB revealed a statistically significant increase in PRL production with the addition of progesterone (EP compared with E) and with exogenous IL-11 (EP + IL-11 compared with EP; Figure 2). The addition of AB resulted in PRL secretion that was not significantly different from EP alone (Figure 2), confirming that endogenous IL-11 production was effectively suppressed in serum-free conditions.

Differential gene expression in response to IL-11
To identify genes regulated by IL-11 during in vitro decidualization, endometrial stromal cells were decidualized with EP or EP + IL-11.
**Validation of IGFBP-5 mRNA expression by real-time RT–PCR**

To confirm that IGFBP-5 mRNA was lower in abundance when endometrial stromal cells were decidualized in the presence of exogenous IL-11, real-time quantitative RT–PCR was carried out using the same RNA samples used for microarray analysis (Figure 3). Although the microarray data showed a 3.6-fold lower amount of IGFBP-5 in IL-11-treated cells, real-time RT–PCR revealed a consistent and much greater down-regulation of 102 ± 17-fold (mean ± SEM; \( P = 0.016 \)). Among six individual cell preparations derived from patients at different stages of the menstrual cycle, the fold change in IGFBP-5 expression ranged from 3 to 469.

**IGFBP-5 protein expression by immunocytochemistry**

As changes in mRNA levels are unlikely to have functional consequences without a concomitant change in protein expression, criteria described in Materials and Methods, there were 11 genes and 1 expressed sequence tag (EST) up-regulated and 10 genes down-regulated in the presence of exogenous IL-11. It is striking that many of these genes are known to be associated with the extracellular matrix (ECM); PRG2, CHI3L1, CHI3L2, LGALS3BP, and DPT. The most up-regulated gene was IL-1\( \beta \), which is thought to play a role in implantation in the mouse (Simon et al., 1994), primate (Strakova et al., 2000) and human (Frank et al., 1995; Mizuno et al., 1999). The most down-regulated gene was IGFBP-5, showing a mean 3.6-fold difference in expression between EP-treated and EP + IL-11-treated cells. As a role for IGFBP-5 in decidualization has not been previously described, we selected IGFBP-5 for further examination.

**Table 1.** Genes up- or down-regulated by exogenous IL-11 (+, up-regulated; –, down-regulated)

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The Department of Pathology ID is the unique identifier given to each cDNA on the microarray, and the GenBank Accession number is specific to that cDNA sequence. The UniGene cluster to which that sequence belongs and the common gene name are also given.
immunocytochemistry to detect IGFBP-5 protein was carried out on cytospin; cells with strong positive staining for IGFBP-5 and those that were completely negative. Positive staining was observed at the cell surface and within vesicles in the cytoplasm, with some nuclear staining evident (Figure 4A and B). No staining was observed in the preadsorption control (n = 2), where the primary anti-body was replaced with anti-body preadsorbed with a 5-fold excess of IGFBP-5 peptide (Figure 4C).

**IGFBP-5 protein expression by immunoblotting**

Dot blotting showed no detectable IGFBP-5 in culture media from EP – IL-11-treated or EP + IL-11-treated cells (data not shown). Similarly, although a specific 31 kDa band was detectable in the IGFBP-5 standard by western blotting, there was no IGFBP-5 detectable in lysates from cells treated with EP or EP + IL-11 (data not shown). Extended exposure of the membrane to film for 2 h was still unable to reveal an IGFBP-5 signal in the cell lysates.

**Effect of exogenous IGFBP-5 on decidualization of endometrial stromal cells**

To determine whether the down-regulation of IGFBP-5 mRNA during decidualization with EP + IL-11 has functional importance for this process, increasing concentrations of exogenous IGFBP-5 were added to endometrial stromal cells as they were decidualized with EP. Cells were decidualized in the presence of 2% CS-FCS, rather than in the absence of serum, to more closely mimic physiological conditions. By comparing PRL secretion from E- and EP-treated cells, it was evident that stromal cells from each of the four patient biopsies successfully decidualized, to a variable degree (Figure 5). Only one of the four cell

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**Figure 3.** Real-time RT–PCR for insulin-like growth factor binding protein-5 (IGFBP-5) (n = 6; experiments 1–6). Absolute amounts of IGFBP-5 mRNA in endometrial stromal cells decidualized with estradiol + medroxyprogesterone acetate (EP) or EP + interleukin-11 (IL-11) from each of six patients were divided by the corresponding 18S rRNA amount. Values in fg/μl are represented on a log_{10} scale, and corresponding values from the same patient are joined by a solid line. Each experiment is represented by a different symbol, as in figure 2. *P = 0.0156 (Wilcoxon matched pairs test).

**Figure 4.** Representative micrographs of immunocytochemistry for insulin-like growth factor binding protein-5 (IGFBP-5) (experiment 10), following treatment for 12 days with estradiol + medroxyprogesterone acetate (EP) (A) or EP + interleukin-11 (IL-11) (B). Positive staining appears brown with blue counterstain. (A) EP-treated, arrow indicates nuclear staining (increased magnification inset); (B) EP + IL-11-treated, arrow indicates cytoplasmic vesicle staining, arrow head indicates cell surface staining (increased magnification inset) and (C) preadsorption negative control. Scale bar, 25 μm.
IL-11 inhibits IGFBP-5 during decidualization

preparations showed significantly lower secretion of PRL when treated with exogenous IGFBP-5 (Figure 5B). Suppression of PRL secretion compared with EP treatment was seen at 1, 10 and 1000 ng/ml, with an apparent bell-shaped curve in response to increasing IGFBP-5 concentrations. As a control for IGFBP-5-specific effects, 1000 ng/ml IGFBP-3 was added to triplicate wells for two cell preparations (Figure 5C and D). An inhibitory effect of IGFBP-3 on decidualization was seen in one of these experiments (Figure 5D). Differences in PRL secretion were not a function of altered cell proliferation, as none of the treatments significantly affected cell number measured at day 12 (data not shown). Overall, there were no reproducible effects on PRL secretion of adding exogenous IGFBP-5 to decidualizing endometrial stromal cells.

Discussion

Treatment of human endometrial stromal cells with exogenous IL-11 during progesterone-induced decidualization alters their gene expression, with the most down-regulated gene being IGFBP-5. By microarray analysis, IGFBP-5 mRNA was 3.6-fold lower in abundance in EP + IL-11-treated compared with EP-treated cells. This was confirmed by quantitative real-time RT–PCR, showing a mean 102-fold lower abundance of IGFBP-5 in IL-11-treated cells. This study demonstrates for the first time that IGFBP-5 mRNA is inhibited by IL-11 during in vitro decidualization, which may have functional importance in the maternal response to implantation.

IGFBPs (IGFBP-1 to -6) bind IGF-I and -II with high affinity and can either amplify or attenuate IGF effects on target cells (reviewed in Rosenfeld et al., 1990; Cohen et al., 1991). IGFBPs can also exert IGF-independent effects (reviewed in Jones and Clemmons, 1995; Clemmons, 1997). When bound to the cell surface, IGFBP-5 can enhance IGF activity by concentrating IGF in close proximity to its receptors (Jones et al., 1993). IGF-independent actions of IGFBP-5 may be mediated via a distinct IGFBP-5 receptor and signal transduction pathway (Abrass et al., 1997; Andress, 1998; Kuemmerle and Zhou, 2002). IGFBP-5 also has a nuclear targeting sequence and is translocated into the nucleus of actively dividing cells in an IGF-independent manner (Schedlich et al., 1998). Once inside the nucleus, IGFBP-5 would be capable of modulating the transcription rate of target genes (Mohan and Baylink, 2002). IGFBP-5 is also thought to induce apoptosis by sequestering IGF-I and inhibiting its pro-survival function (Tonner et al., 1997).

Regulation of IGFBP-5 is likely to be complex, and both transcriptional and posttranscriptional mechanisms have been demonstrated (reviewed in Mohan and Baylink, 2002). Previously identified regulators of IGFBP-5 include growth hormone, parathyroid hormone, glucocorticoid, vitamin D3, IGFs, BMPs, TGFβ and Interleukins, and IL-11 can now be added to this list.

There is evidence that IGFBPs are involved in the maternal regulation of implantation (reviewed in Simmen et al., 1995). Normal embryonic and placental development are dependent on the IGF system in mice (Baker et al., 1993; Liu et al., 1993; Powell-Braxton et al., 1993). Placental phenotype has not been examined in either the IGFBP-5 null mouse (Schuller et al., 1999) or the IGFBP-5 transgenic mouse (Salih et al., 2004), but the transgenics have significantly reduced litter sizes at 10 days post coitum (dpc) due to a maternal defect. Reductions in litter size directly correlate with increased maternal IGFBP-5 expression levels. This supports the functional significance
of reduced IGFBP-5 levels in the decidua during early pregnancy, but remains to be tested.

During early pregnancy in the rat, IGFBP-5 mRNA is expressed by non-decidualized stromal cells near the luminal epithelium and at the antimesometrial boundary between the secondary decidual zone and the non-decidualized endometrium (Cerro and Pintar, 1997). As pregnancy proceeds, IGFBP-5 mRNA expression is undetectable in decidualized cells near the embryo (Cerro and Pintar, 1997), consistent with the decreased IGFBP-5 mRNA expression in IL-11-enhanced human decidualized cells in this study.

In the human endometrium, cyclic changes in steroid hormones have been shown to regulate both mRNA and protein for IGFBPs (Bell et al., 1988; Giudice et al., 1991b). IGFBP-1, -2 and -3 mRNAs are more abundant in the progesterone-dominant secretory phase compared with the estrogen-dominant proliferative phase of the cycle (Bell et al., 1988; Giudice et al., 1991b). In culture, human endometrial stromal cells secrete abundant IGFBP-1 and also IGFBP-2 when decidualized with progesterone (Bell et al., 1991; Giudice et al., 1991a). In contrast, IGFBP-5 mRNA is expressed diffusely in the stroma and is more abundant in the proliferative compared with the secretory phase (Zhou et al., 1994). However, a different investigation of the cyclic expression pattern of IGFBP-5 mRNA did not detect hormonal regulation in the human endometrium (Rutanen et al., 1994).

During human pregnancy, decidualized stromal cells of the maternal decidua basalis and parietalis express all six of the IGFBPs in variable abundance (Han et al., 1996). IGFBP-1, -2, -4 and -6 mRNAs are expressed in most decidual cells, whereas IGFBP-3 and -5 mRNAs are only expressed by a subset of decidual cells, consistent with the IGFBP-5 immunostaining pattern in this study. Also consistent with the data presented here, women in their first trimester of pregnancy have significantly lower levels of serum IGFBP-5 than non-pregnant women (Baxter et al., 2002), and a lower proportion of this is complexed with both IGF and acid-labile subunit (ALS), resulting in greater access of IGFs to the tissues.

Using differential display, IGFBP-5 was shown to be down-regulated in a premalignant human trophoblast cell line compared with a normal invasive trophoblast cell line and may therefore be a potential tumour suppressor (Lee et al., 2001). The premalignant cells displayed increased proliferation, migration and invasion, combined with loss of IGFBP-5. As IGFBP-5 can prevent IGF-I from binding to its receptor, these effects may be owing to unrestrained IGF-I activity. It could therefore be hypothesized that spatially and temporally reduced IGFBP-5 secretion by decidual cells at the fetal–maternal interface may allow trophoblast invasion to proceed. The involvement of IL-11 in this process would provide a mechanism by which fully decidualized cells could enhance the invasiveness of adjacent trophoblast.

The down-regulation of IGFBP-5 mRNA by IL-11 in decidualizing human endometrial stromal cells shown here is consistent with data from other microarray analyses. During in vitro decidualization of decidual fibroblasts from term human placenta, IGFBP-5 was downregulated 20-fold and was the most regulated mRNA in this system (Brar et al., 2001). IGFBP-5 mRNA was also down-regulated 4.7-fold in human endometrial stromal cells treated with progesterone for 3 days (Okada et al., 2003). However, neither study used an additional method of IGFBP-5 mRNA nor protein quantification to confirm the microarray expression data. Tierney et al. (2003) found that IGFBP-5 mRNA was down-regulated 80-fold in stromal cells decidualized under serum-free conditions with 8-bromo-cAMP for 48 h. Interestingly, these authors also showed that IL-11 was up-regulated 63-fold in decidualized cells at the same time point. Regulation of both IGFBP-5 and IL-11 was corroborated by semiquantitative RT–PCR.

The in vitro decidualization protocol used in these studies produced variable responses in cells prepared from different endometrial biopsies and probably reflects biological variation among women with respect to hormonal state, reproductive and other medical history, compounded by the differences between phases of the menstrual cycle at which the biopsies were collected. Reports obtained retrospectively indicated some abnormal pathology for two of six biopsies used for the microarray analysis. Tissue from the same woman as the biopsy used in experiment four showed ‘mildly disordered histology’ and that used in experiment five was designated ‘poorly developed endometrium, low grade chronic endometritis’. Interestingly, cultured cells from these biopsies showed the lowest PRL secretion in response to the decidualizing stimuli (Figure 2), and the lowest change in IGFBP-5 mRNA expression when treated with IL-11. In the future, gene expression analysis may therefore be a powerful tool in the diagnosis of endometrial abnormalities.

Despite the significant down-regulation of IGFBP-5 mRNA in cells treated with IL-11, immunocytochemistry did not reveal decreased IGFBP-5 protein secretion at this time. Immunoreactive IGFBP-5 protein is known to be produced in relatively low abundance in human endometrial cells (Lai et al., 1996). Confirming this, IGFBP-5 could not be detected in culture medium or cell lysates using either dot blot or western blot. Any change in protein secretion may have therefore been too subtle to detect by immunostaining. In addition, cells were removed from the culture plate using trypsin, which may have affected IGFBP-5 protein expression at the cell surface. There is evidence that IGFBP-5 mRNA stability and transcription rate are affected independently of or before changes in protein secretion (Lee et al., 2001) and that IGFBP-5 protein may be stored before release (De los Rios and Hill, 2000). The detection of IGFBP-5 in cytoplasmic vesicles (Figure 4) supports this. Once synthesized, the levels of IGFBP-5 protein may also be tightly regulated by degradative enzymes (Conover, 1996). The studies described here were performed on isolated endometrial stromal cells in vitro rather than in the context of whole decidual tissue with its associated ECM components. The effects of epithelial cells, leukocytes and ECM molecules on the response of decidualizing cells to exogenous IL-11 are unknown.

The addition of exogenous IGFBP-5 to decidualizing stromal cells did not reproducibly affect their PRL secretion, indicating that either exogenous IGFBP-5 has no functional role in decidualization or its effects are masked by the in vitro model system. The complex of IGF-I and rhIGFBP-5 is able to bind free ALS present in serum, forming a 150 kDa complex that is unable to enter cells (Twigg et al., 1998). The experiments described here to determine the effect of exogenous IGFBP-5 on in vitro decidualization were carried out in the presence of 2% serum to more closely mimic in vivo conditions, so it is possible that the IGFBP-5 was unable to access the decidualizing cells. However, this does not exclude a role for endogenous IGFBP-5 in decidualization.

During progesterone-induced decidualization of human endometrial stromal cells, IL-11 treatment consistently down-regulated the expression of IGFBP-5 mRNA. Although decreased IGFBP-5 protein production was not detected in this study by immunocytochemistry, significantly reduced mRNA levels are likely to have functional importance for the decidual cell. Owing to the low level of IGFBP-5 protein expression in decidual cells, more sensitive assays are now required to determine whether these mRNA changes are reflected at the protein level (Yang and Chaum, 2003). If so, IGFBP-5 may be involved in the proliferation and differentiation of endometrial stromal cells into decidual cells and/or in the communication between the decidua and invading trophoblast at implantation.

Acknowledgements

The authors thank Chelsea Stoikos and Dr Kate Hale for technical assistance, Judi Focking and the surgeons for sample collection and all the women who donated tissue. Microarrays were kindly provided by Dr Rob Furlong of the...


Submitted on August 14, 2005; revised on September 20, 2005; accepted on September 22, 2005