Bioinformatic Analysis of Benzo- α -Pyrene-Induced Damage to the Human Placental Insulin-Like Growth Factor-I Gene

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Abstract

Introduction: Intrauterine growth restriction (IUGR) has been associated with exposure to polycyclic aromatic hydrocarbons (PAHs) which are released in the combustion of oil, fuel, gas, garbage, and tobacco. Pregnant women exposed to PAHs are at risk of the effects of these environmental toxins; for example, benzo- α -pyrene (B α P) is able to enter the blood stream and could contribute to IUGR or other developmental abnormalities via effects on the placental cells. Since IUGR has been associated with decreased cord blood concentrations of immunoreactive insulin-like growth factor I (ir-IGF-I) and IUGR has been associated with disordered development and fetal programming, we tested the effects of B α P on human placental trophoblast cells in culture. **Experimental:** IGF-I expression and activation was studied using an immortalized human placental trophoblast cell line (HTR-8). The cells were treated with vehicle control or 1 μ mol/L B α P, or 5 μ mol/L B α P for 12 hours. RNA was extracted and the exons of IGF-I were amplified using reverse transcriptase-polymerase chain reaction (RT-PCR). The ir-IGF-I expression levels were compared using gel electrophoresis. The PCR products were sequenced, and levels of mutation were measured with comparative sequence analysis. A computational protein analysis (computer simulation) was performed in order to assess the potential impact of B α P-associated mutation on IGF-I protein function. **Results:** The IGF-I expression decreased considerably in B α P-treated cells relative to untreated controls ($P < .05$), also in a dose-dependent manner. Comparative sequence analysis indicated that the level of B α P exposure correlated with the percentage of base pair mutations in IGF-I nucleotide sequences for both treatment groups ($P < .05$). Shifts were observed in the open reading frame, indicating a possible change in the IGF-I start codon. Protein folding simulation analysis indicated that the base pair changes induced by B α P weakened IGF-I-IGF binding protein (IGFBP) interaction. **Conclusions:** In concordance with the previous findings, exposure of human placental trophoblast cells to B α P exposure results in reduction of IGF-I expression and base pair mutations. The direct action of B α P on the placenta indicates that it may not be necessary for B α P to access other maternal tissues in order for gene abnormalities to occur. Given that PAHs are known to work through aryl hydrocarbon hydrolase (AHH), these results are likely due to the presence of AHH in HTR cells. Computational modeling of B α P actions on IGF-I, substrate–ligand binding, supports the biological premise of this work and underlines the need to determine actual biological effects rather than equating immune to bioactivity of IGF-I.

Keywords

bioinformatics, benzo- α -pyrene (B α P), intrauterine growth restriction (IUGR), fetal growth restriction (FGR), polycyclic aromatic hydrocarbons, IGF-I

Introduction

Insulin-like growth factor-I (IGF-I; somatomedin) is produced by the placenta and known to play a distinct role in cell metabolism, development, cell division, and survival, in each case regulating cell mass and organ growth.¹ In several species, IGF-I and other members of the IGF family have been shown to regulate uterine, placental, and fetal development,^{2,3} and circulating levels of IGF-I in the maternal and fetal compartments have been shown to relate directly to the growth of the fetus and placenta.^{4,5} Likewise, mutations and polymorphisms in the IGF

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family and in IGF receptors and cofactors have been implicated in intrauterine growth restriction (IUGR) and a number of other clinical syndromes.^{2,6-10,23-29}

In humans, the IGF-1 gene comprises a single-copy 85 kb sequence located on chromosome 12q22-q23 that contains 6 exons and 3 introns (NC_000012: c101398454-101313806). When processed, these messenger RNA (mRNA) products lead to a single circulating bioactive 70 amino acid form of IGF. The action of IGF-1 requires binding with cell surface IGF-1 receptors (IGF-1Rs) that are present in most tissues from embryogenesis to birth.²¹ The binding of IGF-1 to IGF-1R requires noncovalent IGF-1 binding to IGF binding proteins (IGFBPs), of which 7 human varieties have been characterized²²; essentially, all IGF-1 circulates bound to IGFBPs. The IGF-1 sites that bind to the IGF-1 receptors and those that bind to IGFBP binding domains are overlapping but not identical, resulting in unequal competition between IGF-1R and IGFBP for binding to IGF-1, even with minimal protein abnormality. This is a delicately balanced system and malfunction of its components has numerous downstream effects²²; in particular, IGF-1R genetic mutations, abnormally low IGF-1R concentrations, downregulation of IGF-1 signaling, and improper functioning of IGFBPs have all been associated with improper fetal growth and the clinical syndrome IUGR.^{7,23-29}

The importance of IGF-1 in placental development has been demonstrated in studies of IGF-1 null mice; such mice are typically stunted and die in the early neonatal period, but they may be rescued by exogenous IGF-1 administration.^{8,11} In humans IGF-1 mutations, acquired polymorphisms, and base pair deletions are associated with clinical IUGR¹²⁻¹⁸ and low concentrations of the protein in the maternal compartment have been reported in cases of IUGR.^{2,7,19,20} Despite these observations, the causes of gene mutation and protein dysfunction have not yet been characterized and no evidence has appeared testing the relationship of immunoassay levels of IGF-1 to bioassay results in IUGR or that treatment with IGF1 can forestall IUGR. This is an important gap in knowledge since IUGR has been linked to serious post-natal complication including the fetal origin of adult disease.³⁰⁻⁵¹

There is mounting evidence demonstrating that expression of IGF-1 is subject to exposure of environmental factors. For example, many recent studies have demonstrated the existence of causal and dose-related relationships between maternal cigarette smoke exposure to pregnancy disorders, abnormal fetal development, and adverse outcomes on adult health.^{3,51-57} It has also been observed that maternal exposure to cigarette smoke corresponds to the appearance of DNA damage in offspring,^{55,58,59} some of which has been linked specifically to benzo- α -pyrene (B α P), a polyaromatic hydrocarbon (PAH) also found in automobile exhaust, wood smoke, and charred food.⁶⁰ When inhaled, B α P is quickly absorbed into the bloodstream and has been shown to act at the gene level by intercalating between base pairs, disrupting the helical structure of the DNA, and predisposing it to further mutation.⁶¹⁻⁶⁴ Although a great deal is known about the capacity of B α P to have mutagenic and carcinogenic impacts on exposed tissues, the hypothesis that this PAH is linked to IUGR has not yet been tested.

In an effort to address the effects of PAHs on the results of placental production of IGF1, we treated immortalized human placental trophoblast cells with 2 doses of B α P and compared the outcome with IGF-1 expression by untreated controls. The RNA of the treated cells was used to show effects of B α P on the IGF gene and computer simulation to model the induced mutations on IGF-1 protein folding and on IGFBP binding.

Materials and Methods

Cells

The human placenta is an extremely complex and plastic organ. Its tissues grow and are eclipsed in rapid succession. It has a different structure from most animal placentas and cannot be mimicked by the use of animal material. Therefore, in this initial study of the effects of B α P, we tested a well-characterized and stable human cell line from a fixed period of gestation rather than primary cultures, whole tissues, or cells/tissues from other species. The human placental trophoblast cell lines (HTR-8) are immortalized human first trimester extravillous trophoblast cells,⁶⁵ a gift provided by Dr Seth Guller (Yale University).

Cell Treatment and RNA Isolation/Analysis

Early-pass (<10) cells were grown to subconfluency (~70%) in Dulbecco modified eagle medium with 15% bovine serum, 75 cm² canted-neck, vented cap (BD/Falcon, Franklin Lakes, New Jersey). Following 24 hours without fetal bovine serum (starvation), the cells were either left untreated (control) or treated with 1 or 5 μ mol/L B α P (Sigma, St. Louis, Missouri) for 48 hours. The treatment media was changed daily.

Total mRNA extraction was done using the TRIzol method, as described by the manufacturer (Invitrogen Life Technologies, Grand Island, New York). The treated cells were lysed directly in the culture dish by adding 1 mL TRIzol reagent per 10 cm² area of culture dish. The contents of the dish were then passed through a small-bore Pasteur pipette to disrupt the remaining cell membranes and to homogenize the lysate before transferring it to pointed polypropylene tubes (Eppendorf, Hauppauge, New York).

The cDNA Synthesis and Reverse Transcriptase-PCR Analysis

The Enhanced Avian First Strand Synthesis Kit (Sigma-Aldrich, St. Louis, Missouri) was used to create placental complementary DNA (cDNA). The following was added to a polymerase chain reaction (PCR) tube: 5 μ g RNA template (extracted from human placental cell line, see above), 1 μ L deoxynucleotide mix, 1 μ L 3' antisense-specific primer, and double distilled water (ddH₂O) quantity sufficient to bring total volume to 10 μ L. Sample tubes were placed in the thermal cycler at 70°C for 10 minutes. The tubes were then placed on ice, centrifuged, and the following components were added: 2 μ L 10 $\times$ buffer for enhanced avian reverse transcriptase (eAMV-RT), 1 μ L eAMV-RT, 1 μ L RNAase inhibitor, and 6

μL ddH₂O. The reaction tubes were incubated at 25°C for 15 minutes. The tubes were placed in the thermal cycler at 50°C for 50 minutes. The samples were then used for PCR as follows: the following was added to a sterile PCR tube for each sample (control, 1 $\mu\text{mol/L}$, 5 $\mu\text{mol/L}$ of B α P), 12.5 μL Reaction Ready Hot Start Sweet PCR master mix (PA-007; Super-Array BioScience, Frederick, Maryland), 9.5 μL ddH₂O, 1 μL cDNA template, and 1 μL primer.

Exons 1 to 4 of IGF-1 were amplified using the following specific PCR primer pairs:

	Forward Primer:	Reverse Primer:
Exon 1	5'-GCTAAATCTCAC TGTCAGTCTGCTAAATT-3'	5'-GAATTCCTCCCAAT GACAACAAAGAG-3'
Exon 2	5'-CCTGATTAATG ACAGTCGGTG-3'	5'-CCAGATAC GGGCACTCATC-3'
Exon 3	5'-GCATTTCAACA TGAGGCGACTCTG-3'	5'-GGATCCCACCCA GGTGGGCTTAC-3'
Exon 4	5'-GCTCATTCAAA GGGACAACATGGG-3'	5'-TGCTCCTCTCTCAT CATCCTTGCC-3'

After amplification, the PCR products were separated using gel electrophoresis, eluted from the gel, purified, and sequenced by the Big Dye Terminator Chemistry (Applied Biosystems Inc, California). Normalization of results was carried out using simultaneously amplified placental glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the standard for control of gene expression. The density of the GAPDH band was analyzed compared to that of total IGF-1 as well as its individual exon. The analysis of band density was done using NIH-Image and Image-J software (Scion, Frederick, Maryland). All the above studies were performed in triplicate or greater, $N \geq 3$.

RNA Quality and Quantity

Following PCR, these were assessed using gel electrophoresis (Bio-Rad) on denaturing gel (40 mL 10 $\times$ MOPS, 360 mL ddH₂O, and 70 mL 37% formaldehyde) at 50 V for about 2 hours or until the dark blue marker dye had migrated about three-fourth across the length of the gel. The gel was then viewed under an ultraviolet light transilluminator and images were computationally registered then digital gel photos were analyzed using the NIH Image software, Scion Image for Windows (Scion, Frederick, Maryland).

Bioinformatics Analyses of IGF-1 Gene Changes

ClustalW multiple alignment. Nucleotide sequences generated using PCR were compared to sequences of the respective gene region to the authentic source (GenBank: NC α 000012: c101398454-101313806). The GenBank sequences were considered to be the standard (control) for this study as the sequences were amplified from normal control untreated HTR-8 placental cells. Multiple alignments of the sequences were produced using online software, ClustalW, from the

European Bioinformatics Institute (www.ebi.ac.uk/Tools/msa/clustalw2/). The alignments were then further analyzed using online software, GeneDoc, from the National Resource for Biomedical Supercomputing (www.nrbsc.org/gfx/genedoc/ebinet.htm)⁶⁹ to generate an alignment with similarity shading to show the degree of similarity and dissimilarity of the treated sequences to the reference sequence.

Open reading frame analysis. The open reading frame (ORF) tool provided by National Center for Biotechnology Information (NCBI) was used to translate exon 2 of the control and B α P-treated cells' sequences of IGF-1 (codes for amino acids 1-27 of the bioactive form of IGF-1) and all potential results were searched in Basic Local Alignment Search Tool (BLASTp) for similarity within the nonredundant protein database for humans.

Proteinsort secondary structure modeling. The CGC Wisconsin package (Proteinsort application) was used to model the secondary structure of the reference primary structure of IGF-1 and those determined by the ORF analysis (confirmed by BLASTp) which generated values for the hydrophilicity (H), surface probability (S), and chain flexibility (F) of the protein. A Spearman rank coefficient correlation was performed to compare non-Gaussian distributed scores.

Molecular dynamic similarity assessment of IGFBP-1. The crystal structure of the human IGFBP-5 was downloaded from Protein Data Bank (PDB; www.PDB.org) and used as a template to model by homology the human IGFBP-1. The sequence of human IGFBP-1 was retrieved in FASTA format from Swiss-Prot (<http://expasy.org/sprot/>), using the Sequence Retrieval System (<http://www.expasy.org/srs5/>) as the search and retrieval tool. The template for homology modeling of human IGFBP-1 was searched using BLASTp from the NCBI Website (<http://blast.ncbi.nlm.nih.gov/>) with the human IGFBP-1 sequence as query against the PDB using default parameters. The target human IGFBP-5 (PDB code: 1H59) template sequences were aligned using ClustalW (<http://www.ebi.ac.uk/clustalw/>) with default parameters. Comparative modeling of human IGFBP-1 was carried out using the alignment interface of Swiss-Model (<http://swissmodel.expasy.org/SWISS-MODEL.html>). The alignment in ClustalW format was used as the input for Swiss-Model. The Swiss-Model server returned the homology model for a length of 44 amino acid residues, which was evaluated using the SWISS-MODEL/Structure Assessment server, an online Ramachandran Plot Assessment Server. The 3-dimensional (3D) model structure of human IGFBP-1 was validated through PROCHECK from the European Bioinformatics Institute (EBI) Website (<http://www.ebi.ac.uk/thornton-srv/software/PROCHECK>) and Whatif programs (<http://swift.cmbi.ru.nl/whatif/>). The former uses Ramachandran plots to analyze the α -and- α angle distributions for the refined model and to analyze the amino acid backbone conformation. Ramachandran plots of the amino acid residues in the allowed region and overall G-factor were considered using this program.

Figure 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) of insulin-like growth factor I (IGF-I) expressed in placental trophoblast cells. A, Results of RT-PCR demonstrate a decrease in density with increasing dosage of B α P in the lane for IGF-I, indicating an inverse relationship between benzo- α -pyrene (B α P) exposure and expression of IGF-I. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) lane depicted below was used as a control to normalize the intensity levels between the groups. B, The area under each curve represents the expression level of each band in the IGF-I lane, above, relative to that of the corresponding GAPDH band. Although the level of IGF-I is comparable to that of GAPDH for the control band, dose 1 and dose 2 differ significantly from one another and from the control.

Simulation of the Human IGF-I/IGFBP-1 Complex Formation

The IGFBP-1 model obtained was superimposed to the IGFBP-5 structure of the IGF-1/IGFBP-5 complex (pdb code: 1h59) by electronically “lifting out” the IGFBP-5 and replacing it with the IGFBP-1 model and studied as follows: first, the geometry of the IGF-1/IGFBP-1 complex model was optimized in vacuo

Figure 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of exons 1 to 4 of insulin-like growth factor I (IGF-I) gene expression levels in control versus benzo- α -pyrene (B α P)-treated cells. Compared to control cells, control (left), B α P dose 1-, and B α P dose 2-treated cells exhibit decreased expression, respectively.

Figure 3. Percentage similarity to wild-type insulin-like growth factor I (IGF-I) of control placental cells and groups treated with doses 1 and 2 of benzo- α -pyrene (B α P).

using the “Steepest descent” algorithm to the RMS gradient of 0.001 kcal/($\text{\AA}$ mol) followed by the “Conjugate gradient” algorithm to the same RMS gradient. Molecular minimizations and molecular dynamics (MD) simulations were conducted using the Insight II program (Accelrys, San Diego, California) with the consistent valence force field. The Discover3 module of the Insight II (Accelrys) program, a molecular modeling program, was used to optimize the model by energy minimization through its simulation and visualization tool.

The energy-minimized model of IGF-1/IGFBP-1 complex was placed in the center of a rectangular box soaked with water molecules. All calculations were performed with NVT and periodic boundary conditions.⁷⁸ An atom-based distance cutoff was applied at 8 $\text{\AA}$ for both nonbonded electrostatic and Van der Waals interactions. All MD simulations were performed at room temperature (298.15 K) and at pH 7. The solvated system was initially energy minimized by conjugated gradient method for 5000 iterations with the protein backbones constrained. To further optimize the arrangement of the solvent molecules around the modeled IGF-1–IGFBP-1 complex and to alleviate high-energy regions, solvent molecules were allowed to relax by running a short 150 ps MD simulation with

Figure 4. Multiple sequence alignment for exon 2 of insulin-like growth factor I (IGF-I) gene displaying areas of similarity and dissimilarity with the wild-type IGF-I sequence. Box shading shows areas of similarity of IGF-I sequence between the wild-type, control, and treated cells. Sites shaded black were consistent with wild-type IGF-I (unmutated) in all experimental groups. Dark gray shading indicates bases that did not mutate in the wild-type sequence, control, or dose 1-treated sequence, and unshaded sites indicate mutated bases in the dose 2-treated sequence. Light gray shading indicates bases that did not mutate in the wild-type sequence or the control but did exhibit mutations in both dose 1- and dose 2-treated sequences.

Figure 5. A. Summary of artificially induced and naturally occurring polymorphisms at the amino acid level in the bioactive form of insulin-like growth factor I (IGF-I). These are displayed in relation to the side chains of the molecule and the respective effect on binding to receptors and binding proteins is displayed.

A)

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>gi|11024682|ref|NP_000609.1| insulin-like growth factor 1 (somatomedin
C) [Homo sapiens]
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(A)

GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA

(B)

GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA

(C)

GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA

T C T

A - RESIDUES WHICH WILL RESULT IN ALTERED BINDING WHEN MUTATED
 B - IN VIVO AMINO ACID CHANGE REPORTED
 C - REPORTED SNP SITES (coding: 2 synonymous; 1 non-synonymous)

B)

Figure 6. This figure shows (A) the translated amino acid sequence of insulin-like growth factor 1 (IGF-1) with the polymorphic amino acids highlighted and (B) the 3-dimensional (3D) model of IGF-1, made with Cn3D, highlighting in yellow the polymorphic regions of the protein which correspond to the highlighted amino acids in panel A. For references to color, please see the online version.

position restraints applied to all the protein atoms. Subsequently, protein alone was minimized with 5000 steps followed by the minimization of the entire system for an additional 5000 iterations. These systems were energy minimized with the method described above until the maximum derivative was lower than $0.001 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$. Finally, approximately 1 ns MD simulations were performed for the solvated model IGF-1/IGFBP-1 complex. During the MD simulations, the IGF-1 backbone atoms were fixed. The MD simulation time step was set as 2 fs and the trajectories were saved every 1 ps for further analyses using the ANALYSIS module of the Insight II program.

Results

Gel Electrophoresis of IGF-1 Exon RNA

Expression of IGF-1 was reduced in a dose-dependent manner ($P < .05$; Figure 1). Moreover, there was differential expression of IGF-1 exons in BxP-treated placental cells compared to the untreated control cells ($P < .05$; Figure 1). Normalization against GAPDH demonstrated that treatment of placental trophoblast cell lines with both doses of BxP (ie, 1 and 5 $\mu\text{mol/L}$) strongly reduces expression of IGF-1 exons' RNA in comparison to IGF-1 exon expression by untreated controls ($P < .01$). There is clear difference between expression in 1 $\mu\text{mol/L}$

Table 1. Level of Positive Identity (%) and E Values for Homologies Found Between Experimental and Database Sequences of IGF-1 When Comparing BLASTp to the nr Human Database, Which Demonstrates That Treatment With B α P Affected the Similarity Level Between Wild-Type “Normal” IGF-1 and That Produced by Treated Placental Cells.

Treatment	Query Length (aa)	Identities (%)	Positives (%)	Gaps (%)	E Value
Control/untreated	55	76	83	1	1.00E-14
Dose 1: 1 μ mol/L B α P	58	62	68	13	1.00E-09
Dose 2: 5 μ mol/L B α P	Blasted against BLASTp as amino acid sequences and as nucleotide sequence returned using BLASTn (there were no hits)				

Abbreviations: BAP, benzo- α -pyrene; IGF-1, insulin-like growth factor 1.

L treated groups and 5 μ mol/L treated groups (Figures 1, 2, and 3). Using the gene-walking technique (PCR amplification of individual exons followed by exonic sequencing), there was reduced expression of IGF-1 exons 1 to 4 in both the control and the B α P-treated placental cells. This difference appeared to be dose related in all exons (Figure 2).

ClustalW multiple alignment and ORF Analyses. The ClustalW multiple alignment comparison of IGF-1 sequences obtained from treated cells' RNA versus that from nontreated ones. A clear correlation was found between the number of mutated nucleotides and the amount of the treatment dose of B α P (Figure 3). A clear change in the IGF-1 nucleotide sequences at the exonic levels was also noticed when treated with B α P (Figure 4).

Observed sequence changes were random and categorized into different classes:

Base substitutions: base substitutions were relatively common among the exposed samples. The bases adenine (A), cytosine (C), thymine (T), and guanine (G) were substituted with another noncomplementary base, indicating a loss of PCR replication fidelity of bases.

Unknown bases: unknown base insertions were numerous in the B α P-exposed samples and they are depicted as N in the nucleotide sequences.

Inserted/deleted bases: deleterious genotoxic process of base insertion and deletion was observed for B α P-exposed samples. In this process, one or more bases were incorporated/removed within the ORFs and in other regions of the gene. Such base incorporation/deletion within the ORF will produce altered amino acid codons and frame shifts. The mechanism(s) of base changes was not apparent in B α P-exposed samples.

The patterns of changes in the nucleic acid bases within the ORFs of the PCR-amplified IGF-1 gene exons are illustrated in Figure 4. Chemical modifications of the nucleotide bases (N) by B α P apparently disabled the fidelity of the polymerase function and resulted in generation of an unknown/absent

product in the PCR procedure. The nature of such chemical modification of bases is not known; however, random sequence changes from C to A and T; T to G, A, and C; A to G, T, and C; and finally, G to T and C characterized the ORF sequences of IGF-1 gene in B α P-treated placental samples.

The outcome of gene changes (both artificially induced and naturally occurring) at the amino acid level was summarized in the bioactive form of IGF-1 and compared to the normal IGF-1 amino acid sequences that have been assigned roles in binding to IGFBP (Figure 5). The analysis included the change in relation to the side chains of the molecule and the respective effect on binding to receptors and binding proteins. It is clear from this analysis that there is a correlation between the nucleotide/amino acid site of change and the ability of IGF-1 to interact with its ligand (IGFRBP) and the receptor. These data were followed by the data we obtained from the current study and show the translated amino acid sequence of IGF-1 with the polymorphic amino acids highlighted and B: the 3D model of IGF-1, made with Cn3D, highlighting in yellow the polymorphic regions of the protein which correspond to the highlighted amino acids in panel A (Figure 6).

The ORF analysis is summarized in Table 1. There was a maximum of 1 hit for the control and 1 μ mol/L B α P-treated sequences, and no hits for the sequence treated with 5 μ mol/L of B α P. Using the conventional cutoff of 75% positive identity to declare homology between protein sequences, only the control was confirmed to be homologous to IGF-1. However, the E values for the results returned for all 3 sequences demonstrate that the similarity calculated by BLASTp did not occur by chance (the E values are all $< e^{-10^4}$ which is the level at which significant homology is assumed). This analysis demonstrates that while B α P did not mutate IGF-1 beyond recognition, nucleotide changes result in enough sequence differences to alter the protein structure so that the tertiary-folded structure is no longer homologous to wild-type/normal human IGF-1.

Protein modeling and homology assessment. The results of the Proteinsort secondary structure model analysis of reference and experimental IGF-1 sequences are shown in Table 2. Although data show that control sequences are compliant with the normal secondary structure of IGF-1 at a reasonable level of statistical significance,⁶⁶ those proteins isolated from B α P-treated lines do not comply with normal IGF-1's structure (or correlate with a low enough statistical significance to be considered uncorrelated). In addition to the loss of homology at the level of their primary sequence, detailed above, the observation of decreased homology supports the theory that altering of IGF-1 caused by treatment with B α P results in altered biological function.

The amino acid sequence alignment using our modeled human IGFBP-1 based on the standard IGFBP-5 revealed approximately 52% sequence identity (Figure 7) and Procheck analysis revealed that a total of 94.0% of the residues of these sequences were located in the “most favored” regions of the Ramachandran plot, while 6% of the residues were found in “additionally allowed” regions, implying that the backbone conformation of IGFBP-1 was consistent. Subsequent tests

Table 2. Summary of Spearman Rank Correlation Coefficients (ρ) for Hydrophobicity, Surface Probability, and Chain Flexibility Predictions of the Secondary Structure of IGF-I When Compared to the Reference Amino Acid Sequence Predictions.

Treatment	ρ H	Correlation	ρ S	Correlation	ρ F	Correlation
Control	0.477491	$P < .01$	0.593469	$P < .001$	0.654204	$P < .005$
Dose 1	-0.21013	None	0.121318	None	-0.46559	None
Dose 2	0.238462	$P < .2$	0.248311	$P < .2$	0.328893	$P < .025$

Abbreviation: IGF-I, insulin-like growth factor I.

IGFBP-5	ALAEGQSCG <u>Y</u> TERCAQGLRCLPRQDEEK <u>P</u> LHALLHGRGVCLNE
	AL G +CGV T RCA+GL C E++PLHAL G+G C+ E
IGFBP-1	ALPLGAACG <u>V</u> ATARCARGLSICALPGEQQ <u>P</u> LHALTRGQGACVQE

Figure 7. Sequence alignment of insulin-like growth factor-binding protein I (IGFBP-I) with IGFBP-5 receptor. The key residues involved in the interaction with IGF-I are displayed in bold and underlined.

Figure 8. Plots of molecular dynamics (MD)-simulated internuclear distances, and the root-mean-square deviation (RMSD) for atomic positions of the insulin-like growth factor-binding protein I (IGFBP-I) versus simulation time for IGF-I binding with IGFBP-I. Trace D1 and D2 represent the bidentate H bond distances between the 2 oxygens of the IGF-I^{Glu3} carboxyl group and the N-H backbone of IGFBP-I^{Leu94} residue.

performed on a number of stereochemical parameters, including peptide planarity, bad nonbonded interactions, main chain hydrogen bonding energy, and standard deviations of 1 angle (ie, the first torsion angle of the side chain) also indicated reliable protein structure.

The Whatif quality report showed a comparison of our IGFBP-1 sequence to high-quality experimental structures deposited in the online PDB and resulted in a z score of -0.54 . This indicated that the model was reliable, given that a z score ≥ -5.0 indicates poor packing.

MD simulation of IGF-I/IGFBP complexing. Comparison of the simulated IGF-I/IGFBP-1 complex structure with the x-ray structure of IGF-I/IGFBP-5 (1H59.pdb) revealed no major structural differences between the IGFBP-1 and the IGFBP-5 in a bound state with IGF-I. Most of the key residues of IGFBP-1 involved in the interaction with IGF-I were conserved in IGFBP (Figure 7).

Inspection of the trajectory of the MD simulation revealed that the positions of IGFBP-1 backbone atoms remained stable

after approximately 500 ps of the MD simulation. The final results of the MD simulation of the IGF-I/IGFBP-1 complex are shown in Figure 9, which reveals that the carboxyl group of IGF-I^{Glu3} residue and the N-H backbone atom of the IGFBP-1^{Leu94} residue occupy the positions suitable for the formation of the favorable hydrogen bonds seen in Figure 8.

In addition to these important hydrogen bonds, Figure 9 also reveals several favorable interactions between the IGFBP-1 and the IGF-1 protein. As observed in the IGF-1/IGFBP-1 binding, the IGFBP-1^{Leu94} side chain is also lodged in the hydrophobic pocket involving Leu5, L54, and L57 of IGF-1. Moreover, the IGFBP-1^{V73} residue is stabilized by forming a tight dipole-quadrupole interaction between the methyl group of the Val73 side chain and the phenyl ring of IGF-1^{F16} residue, as reflected by the distances D3 (Figure 8).

Figure 9 displays stick model image of the residues of the side chain of IGF-1 that are involved in the polymorphism seen during this experiment. Since the Glu3 and Phe16 residues of IGF-1 are involved in the interaction with the IGFBP-1 receptor, mutation of these residues should considerably decrease the binding mode of IGFBP-1 to IGF-1. Mutation of other residues may also decrease the affinity of IGF-1 to its receptor because of the induced conformational changes.

Discussion

In this study, DNA sequences of trophoblast cells treated with B α P were observed to exhibit apparent dose-related degrees of mutation within specific exons of IGF-1. This finding is of critical importance given the established changeover in splicing during development and the accepted relationship of IGF-1 mutation to IUGR.²⁰ Our studies further substantiate and dilate on the possibility that B α P-induced damage to IGF-1 may be important in the pathophysiology of this serious clinical syndrome. The decrease in IGF-1 expression with increasing exposure to B α P supports previous reports of the IGF-1-IUGR-smoking connection.⁶⁰ Our *in silico* studies regarding the molecular mechanisms behind the B α P-IGF-1-IUGR relationship are of special interest. Bioinformatics analyses and

Figure 9. Ribbon view of the final structure of the molecular dynamics (MD)-simulated IGF-1/IGFBP-1 complex. The residues involved in the insulin-like growth factor 1 (IGF-1) polymorphism are displayed in stick mode. Residues of IGF binding protein 1 (IGFBP-1) involved in the interaction with IGF-1 are displayed in ball mode.

protein modeling performed observed B α P-induced IGF-1 base changes that could be analyzed as altering the structure of the IGF-1 protein. They also revealed possible differences in the binding of post-B α P IGF1 to IGF-binding proteins. These are all important findings that must be shown to occur in the placenta of women exposed to cigarette smoke.

The *in silico* analyses of normal versus B α P-mutated IGF-1 confirmed that IGFBP-1 homology of backbone conformation (Procheck), residue contact (Whatif), and protein folding stability (MD simulation) for the RNA of untreated cells were well within the established ranges of reliable protein structures and that our 3D model accurately portrayed the IGF-1/IGFBP-1 interface at the binding site, and was adequate for use in evaluation of the effects of B α P-induced IGF-1 mutation on the ability of this complex to form properly. Results indicated that altered structure of the IGF-1 protein should decrease its binding to IGFBP-1, and would decrease affinity of the IGF-1 receptor for the protein. This implies that although immunoreactive (ir)-IGF-1 may be found in serum samples from human participants (especially newborns), the isoform detected may be of compromised function. Because of the sensitivity of the epitope-based immunoassays, modification of certain amino acid sites could result in inaccurate estimates of biological activity through the use of these immunoassay methods. This is important because immunoassay and immunohistochemistry have been the only sources for quantitative measurements of IGF-1 protein expression and in the literature the term “IGF-1” has been synonymous with “immunoreactive-IGF-1.” This fusion of terms has implications for interpretation of these measurements; immunomethods are dependent on the identification of a unique sequence of amino acids (epitope) that can only be found in the putative protein. This means that the epitope used to determine the presence of the protein may not be relevant to biological action and deletion of the biologically

active portion of a protein may not be detected by immunoassay. Further, tertiary folding of the molecule may obscure the presence of the epitope to the antibody, disconnecting biological activity from measured ir-protein. Although Western blotting makes the appreciation of ir-protein more reliable, it does not resolve the bio-to-immune-activity conundrum. Since almost all the studies of the impact of IGF-1 expression levels on clinical pathologies have employed immunohistochemical assay and staining, there is a need for a more comprehensive, molecular understanding of the human placental IGF-1 in health and disease. Likewise, it is important to evaluate the biological activity of placental gene products given the known effects of abnormalities of the gene and its protein products.^{2,6-8} The proteomic and computational results in this study support the use of more rigorous methods to evaluate IGF-1 in normal and IUGR pregnancy and offspring. Given that there have been numerous unexplained reports of such phenomena our current findings could be a step toward a paradigm shift, in which critical protein functions are associated with structure rather than blood or serum concentration by epitope-driven assays.

Knowledge of the biological function of the IGF-1 molecule and its protein-protein interactions has recently been increased by site-directed mutagenesis studies that have altered the primary structure of the IGF-1 protein in a known fashion and allowed detailed study of the binding interactions of both the IGF-1 receptor and IGFBPs. These artificial polymorphisms at the amino acid level and the 1 known noncontiguous small nucleotide polymorphisms are summarized in Figures 6 and 7(A and B). These figures demonstrate that, in concordance with our findings, any genetic or etiological cause of a change in the amino acid sequence of IGF-1 would most likely produce a change in binding with the IGF-1R, IGFBP-1, or both. This would result in a reduction or failure of downstream signaling

from the IGF-1 system, a mechanism through which IUGR could occur.

In summary, this study showed that B α P exposure caused mutations of the IGF-1 gene in immortalized human trophoblast cells. The gene products include abnormal sequences that may adversely affect binding to IGF-1-specific binding proteins. Such effects could play a role in the etiology of IUGR. These effects could escape immunoreagent-based tests such as immunoassay.

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