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PCBs Enhance Collagen I Expression from Human Peritoneal Fibroblasts

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Abstract

Objective—To test the effect of four polychlorinated biphenyl congeners (PCB-77, PCB-105, PCB 153 and PCB 180) on expression of three adhesion markers, TGF- β 1, VEGF, and type I collagen in normal human peritoneal and adhesion fibroblasts

Design—Cell culture study

Settings—University Research Laboratory

Patients—Primary cultures of normal peritoneal and adhesion fibroblasts were established from three patients.

Interventions—Fibroblasts were treated with PCB-77, PCB-105, PCB-153 or PCB-180, 20 ppm for 24 hours. Total RNA was extracted from each treatment and subjected to real-time RT/PCR.

Main Outcome and Measures—mRNA levels of type I collagen, VEGF and TGF- β 1.

Results—Normal human peritoneal fibroblasts expressed type I collagen, VEGF and TGF- β 1. Exposure of normal human fibroblasts to PCB-77, PCB-105, PCB-153 or PCB-180 did not affect mRNA levels of β -actin, the house keeping gene used to normalized RNA levels for the real-time RT/PCR, nor did it affect cell viability as assessed by trypan blue exclusion. PCB treatments, compared to control, resulted in no significant change for TGF- β 1 or VEGF mRNA levels, in normal peritoneal and adhesion fibroblasts. In marked contrast, type I collagen mRNA levels were markedly increased in response to the brief 24 hrs exposure to each PCB, treatment each ($P < 0.0001$) in both cell types.

Conclusion—The finding that PCB-77, PCB-105, PCB-153 or PCB-180 increased the expression of type I collagen in human normal peritoneal and adhesion fibroblasts is the first demonstration of involvement of organochlorines in the pathogenesis of tissue fibrosis. This may implicate organochlorine exposure as an etiologic factor in a wide variety of previously unlinked human ailments characterized by fibrosis.

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CAPSULE

Polychlorinated biphenyls (PCBs) enhance expression of collagen I, but not transforming growth factor beta 1 or vascular endothelial growth factor from human normal and adhesion peritoneal fibroblasts.

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Keywords

PCB-153; PCB-180; PCB-77; PCB-105; organochlorine; adhesions; fibrosis; collagen

INTRODUCTION

Post-operative adhesions have been demonstrated to develop in the vast majority of patients undergoing intraperitoneal surgical procedures (1,2). While the likelihood of developing postoperative adhesions is related to the pre-existing pathology, the extent of the surgical procedure, and the skill and expertise of the surgeon, these factors cannot totally explain which patients will not develop adhesions, or the severity and extent of adhesions that occur. Thus, it has generally been considered that some individuals are “predisposed” to adhesions, although it is unclear what these predisposing factors are.

Similarly, it is unclear why some individuals, but not others, develop conditions characterized by fibrosis at sites throughout the body. These include keloids, retroperitoneal fibrosis, etc. In part, some of these disorders are considered to have an immunologic basis.

In view of these observations, and the recent release of the Vallombrosa Consensus Statement on Environmental Contaminants and Human Fertility Compromise (3) (from a diverse group of scientists, patient advocacy groups, and national society representatives), which highlighted the potential role of environmental contaminants on growth and development, we wondered whether polychlorinated biphenyls (PCBs) could contribute to tissue fibrosis in men and women. As a first step to assess this question we designed the current study to examine the effect of four different PCBs on expression of three markers of adhesions, collagen I (the most common form of extracellular matrix in the body), transforming growth factor beta-1 (TGF- β 1; a molecular which regulates many components of the inflammatory responses), and vascular endothelial growth factor (VEGF; a molecule responsible for angiogenesis) from human normal peritoneal and adhesion fibroblasts. PCBs 153 and 180 were chosen because they are usually reported to be the two most common PCBs measured from human serum and tissue samples, and are usually present in the greatest amounts. PCBs 77 and PCB-105, were chosen because they are considered to be dioxin-like in terms of their mechanism of action and toxicity (4).

METHODS

Source and Culture of Human Fibroblasts

Fibroblasts were isolated from adhesions and normal peritoneum as previously described (5–7). Briefly, at the initiation of the surgery following entry into the abdominal cavity, normal parietal peritoneal tissues from the anterior abdominal wall lateral to the midline incision and adhesion tissue were excised from three patients who were undergoing laparotomy for pelvic pain. The sampled normal peritoneum was at a minimum at least 3 inches from any adhesions. The patients did not have active pelvic or abdominal infection and were not pregnant and did not have endometriosis. All patients gave informed written consent to tissue collection, which was conducted under a protocol approved by the Wayne State University institutional review board.

Harvested normal peritoneal and adhesion tissue samples were immediately placed in standard media (DMEM medium containing 10% fetal bovine serum, 2% penicillin and streptomycin). Tissues were cut into small pieces in a sterile culture dish and transferred to another fresh T-25 flask with 3 mL of Dispase solution (2.4 U/ml, GIBCO BRL, Life Technologies, Inc., Grand

Island, NY). The flasks were incubated overnight at 37°C in an environ-shaker (Lab-Line Instruments, Inc., Melrose Park IL).

Samples were then centrifuged for 5 minutes at 1400 X *g*, transferred into a fresh T-25 flask with pre-warmed DMEM medium, and put in a 37°C incubator (95% air and 5% CO₂). Outgrowth of fibroblasts generally took 2 weeks. When confluence was reached, the cells were transferred to 90-mm tissue culture dishes and cultured in standard media with 10% FBS. Thereafter, the confluent dishes were subcultured by trypsinization (1:3 split ratio). Studies were conducted using the third to fifth cell passage to maintain comparability.

Treatment of Cells

Primary cultures of fibroblasts isolated from normal peritoneal tissues and adhesions were cultured at the same density in dishes with DMEM media containing 10% FBS at 37°C at 5% CO₂, 95% air incubator. Cells were treated with PCB-77, PCB-105, PCB 153, or PCB-180 (20 ppm) under normal (20% O₂) conditions for 24 hours. All experiments were performed in triplicate. We obtained the PCBs from the Michigan Department of Community Health Analytic Chemistry laboratory. The concentration was chosen based on the upper limit of human *in vivo* levels.

Real Time RT/PCR for Type I collagen, VEGF, and TGF-β1

Total RNA was isolated from normal peritoneal and adhesion fibroblasts with the use of the monophasic solution of phenol and GITC/Trizol method as previously described (8,9).

Preparation of Complimentary DNA (cDNA)—A 20 µl reaction volume, including 1µg total RNA, 1µl oligo(dT) (500 µg/ml) (Invitrogen, CA) and 1µl 10mM dNTP Mix (Invitrogen, CA) was heated to 65° C for 5 minutes and then quick chilled on ice. A master mixture containing 4 µl 5X First Strand Buffer, 2 µl 0.1M DTT, 1 µl RNaseOut Recombinant Ribonuclease Inhibitor (40units/µl) (Invitrogen, CA) was added, and incubated at 42° C for 2 minutes. 1µl (200 units) of SuperScript II (GIBCO BRL) was added to each reaction and incubated for 50 minutes at 42° C, to inactivate the enzyme by heating at 70° C for 15 minutes.

Optimal oligonucleotide primers for real-time RT-PCR amplification of reverse-transcribed cDNA were selected with the aid of software program Oligo 4.0 (National Bioscience, Inc., Plymouth, MN). Sequences of the oligonucleotides used for amplification of VEGF, and β-actin mRNA are shown in Table I.

The following human oligonucleotide primers, which amplify variable portions of the protein coding regions, were used:

Locus	Sense (5'→3')	Antisense (5'→3')	bp
β-actin	CTA TGA CTT AGT TGC GTT ACA C	GCA TTA CAT AAT TTA CAC GAA AGC	288
TGF-β1	AAC AAT TCC TGG CGA TAC	AAG CAA TAG TTG GTG TCC	101
VEGF	TTG CTT GCC ATT CCC CAC TTG	TCT TTC CTC TTT CTG CTG GTT TCC	332
Type I Collagen	TGT CTT ATG GCT ATG ATG AG	ATC CAA ACC ACT GAA ACC	335

Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Analysis—Quantitative RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen) and a Cepheid 1.2f Detection System. RT-PCR was performed in a 25 µl total reaction volume including 12.5 µl of 2X QuantiTect SYBR Green RT-PCR master mix, 3ul of cDNA template, and 0.2 µM each of target specific primers designed to amplify a part of each gene. To quantify each target transcript, a standard curve was constructed with serial dilutions of standard plasmid (Invitrogen). Amplification was performed as follows: 95° C for 10 minutes, and 40 cycles of 95° C for 15 seconds and different annealing temperature (55–60° C). After PCR, a melting curve analysis was performed to demonstrate the specificity of

the PCR product as a single peak. A control, containing all the reaction components except for the template, was included in all experiments. The amount of each mRNA (collagen I, VEGF, TGF- β 1) was then normalized to a housekeeping gene, β -actin. The PCR reaction conditions for VEGF, TGF- β 1 and collagen I were programmed for each primer as follows: An initial cycle was performed at 94° C for 5 minutes, followed by 35 cycles of denaturation at 94° C for 1 minute, annealing at 60° C for 1 minute for VEGF and collagen I and 56° C for 1 minute for TGF- β 1, and then a final cycle at 72° C for 7 minutes to allow completion of product synthesis.

Statistical analysis

Factorial ANOVA was conducted on each dependent measure using SPSS version 10.0 for Windows (SPSS, Chicago, IL). Data are expressed as mean $\pm$ SEM. Results were considered significant when $p < 0.05$. The table demonstrates raw data of mRNA copies per microgram of RNA, expressed for each marker in normal peritoneal and adhesion treated and untreated fibroblasts.

RESULTS

Culturing of the human peritoneal and adhesion fibroblasts in the presence of PCB-77, PCB-105, PCB-153 or PCB-180 had no effect on mRNA levels of β -actin, the housekeeping gene used to normalize RNA levels for the real-time RT/PCR, nor did it affect cell viability as assessed by the trypan blue exclusion method. As previously reported, collagen I mRNA expression was significantly lower in normal peritoneal fibroblasts (4521 ± 287) as compared to adhesion fibroblasts (6700 ± 281 ; $p < 0.001$). Treatment of the normal fibroblasts with PCB-180 resulted in a seven fold increase in collagen I mRNA expression ($p < 0.001$); similar marked increases were seen with PCB-77, PCB-105, and PCB-153 (Table 1). In fact, the PCB stimulated levels exceeded the collagen I mRNA levels seen in the untreated adhesion fibroblasts. Similarly, in the adhesion fibroblasts, treatment with PCB-180 also resulted in a marked increase in collagen I expression ($p < 0.0001$). A similar effect was manifested when the adhesion fibroblasts were treated with PCB-77, PCB-105, and PCB-153 (Table 1; each $p < 0.0001$).

Also as previously reported, mRNA expression of TGF- β 1 and VEGF was higher in adhesion fibroblasts than normal peritoneal fibroblasts from the same patients. In contrast to the effects of PCBs on collagen I expression, these compounds had no appreciable effect on mRNA expression of TGF- β 1 or VEGF from either the normal or adhesion fibroblasts (Table 1; NS).

DISCUSSION

Polychlorinated biphenyls were manufactured by the chemical industry and include a total of 209 congeners which are formed by addition of chlorine atoms to one or more positions on two connected benzene rings. PCBs have physical and chemical characteristics such that they can persist for long periods of time, are lipophilic, and bioaccumulate with ascent up the food chain. The manufacture of PCBs was halted in industrialized countries since the 1970s and 1980s due to their adverse effects on reproductive outcomes in wildlife (10). However, because of the large quantities of these contaminants remaining in nature, they are still hazardous to the health of animals and humans. PCBs have been implicated in a variety of adverse health conditions including cancer of the breast and prostate gland, reproductive failure, immunologic disorders, endocrine disorders such as thyroid disease, metabolic derangements, and developmental disorders both in utero and in early childhood (11,12).

In this study, we have examined the effect of PCB-77, PCB-105, PCB-153, and PCB-180 on markers of the adhesion phenotype including expression of collagen I, TGF- β 1, and VEGF

mRNA from human normal peritoneal and adhesion fibroblasts from the same patients. PCBs 153 and 180 were chosen for our initial analysis of the effect of organochlorines because they have consistently been among the most frequently identified in human serum and tissues from diverse parts of the world including the United States, Canada, Italy, Denmark, Sweden, Brazil, United Kingdom, Belgium, Germany, Greece, Thailand and Russia. Additionally, the levels of these two PCBs are among or are, the highest measured, and thus their body burden would be the highest and is evident in men and women of different ages, in pregnant women, and in neonates. Thus if PCBs were to effect fibrosis (including adhesion development), it would be likely (although not definitive, as different PCBs have different structures and postulated mechanisms of action) that these two congeners would have an effect on the markers of the adhesion phenotype. Two additional PCB congeners, PCB 77 and PCB 105 were also utilized to examine whether effects would be elicited with congeners with dioxin-like properties (4), which is especially important given their association with the severity of dioxin-induced endometriosis in primates (13).

Current understanding of the mechanism(s) leading to adhesion development includes tissue injury resulting in collection of blood and serosanguinous fluid from the sites of injury, which congeal to form a fibrinous proteinaceous mass between adjacent tissue surfaces. These sites of tissue injury are relatively hypoxic and deprived of nutrients as a result of the predisposing pathologic condition, or the surgical procedure performed. Teleologically, the body tries to create an adhesion to resupply oxygen and nutrients to these tissues.

Fibroblasts migrate to the site of tissue injury, and if the proteinaceous mass is present, migrate into the mass. This process is accentuated by the production of inflammatory cytokines and growth factors. Within the proteinaceous mass the fibroblast produce extracellular matrix material including collagen, leading to the development of an adhesion bridging to the previously injured tissue. To facilitate resupply of oxygen and nutrients to the tissue, angiogenic factors such as VEGF are produced, leading to angiogenesis and blood vessel formation.

In this study, we identified a consistent, marked stimulation of collagen I mRNA expression by either PCB-77, PCB-105, PCB-180 or PCB-153, to a level even exceeding that observed in adhesion fibroblasts. Surprisingly, it occurred in the absence of a significant effect on expression of TGF- β 1 and VEGF, which are commonly elevated as part of the process of adhesion development. Thus the mechanism for the collagen I elevation was not a generalized inflammatory response, as that would have been associated with elevation of these other markers as well. While at this time the mechanism for such a selective effect is unknown, it may relate to enhanced reactive oxygen species (ROS) production or depletion of free radical scavengers such as glutathione, as previously suggested by studies examining the effects of PCB 180 in rats (14).

To the best of our knowledge, this is the first report to examine, and to demonstrate, that PCBs may contribute to intraperitoneal fibrosis, as well as to adhesion development. Our observations are consistent with previously reported effects of PCBs in the liver of mice (15) and the thyroid of the harbour porpoise (16), as well as recent reports which have linked dioxin (another organochlorine) to development of endometriosis in monkeys and humans. While future studies will need to further extend these studies to other organochlorine compounds, additional components of the adhesion phenotype, as well as to clinical epidemiologic studies, it appears possible that PCBs may in part contribute to fibrogenic processes.

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TABLE 1

Statistical Summary Table of Collagen I, TGF- β 1, and VEGF following 24h exposure to PCB-077, PCB-105, PCB-153 or PCB-180 (Means and Standard Errors) $p < 0.0001$ vs. $p < 0.0001$

	Control Treatment	PCB-077 Mean +/- SEM	PCB-105 Mean +/- SEM	PCB-153 Mean +/- SEM	PCB-180 Mean +/- SEM
Collagen I					
Normal Fibroblasts	4521 +/- 287	21602 +/- 2128*	28572 +/- 258*	27680 +/- 416*	28621 +/- 402*
Adhesion Fibroblasts	6700 +/- 281	16885 +/- 634*	22032 +/- 246*	22187 +/- 271*	22635 +/- 594*
TGF-β1					
Normal Fibroblasts	3002 +/- 148	2606 +/- 221	2430 +/- 82	2495 +/- 31	2549 +/- 122
Adhesion Fibroblasts	10643 +/- 905	10565 +/- 262	10386 +/- 1036	10638 +/- 1022	10773 +/- 456
VEGF					
Normal Fibroblasts	678 +/- 82	626 +/- 28	703 +/- 22	774 +/- 65	769 +/- 131
Adhesion Fibroblasts	1515 +/- 62	1521 +/- 75	1469 +/- 75	1424 +/- 125	1050 +/- 127