Original Paper

The Roles of p38 MAPK and ERK1/2 in Coplanar Polychlorinated Biphenyls-Induced Apoptosis of Human Extravillous Cytotrophoblast-Derived Transformed Cells

Zhu Liu^a Hong-jie Ruan^b Ping-qing Gu^b Wen-yan Ding^b Xiao-hui Luo^b
Rong Huang^b Wei Zhao^b Ling-juan Gao^b

^aClinical Laboratory, Huangdao District of Traditional Chinese Medicine, Qingdao, ^bState Key Laboratory of Reproductive Medicine, Department of Clinical Laboratory, Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, Nanjing, China

Key Words

Apoptosis • Coplanar polychlorinated biphenyls (Co-PCBs) • Receptor for the globular heads of the human C1q (gC1qR) • p38 MAPK/ERK

Abstract

Background/Aims: The purpose of this study was to investigate the relationships among exposure to coplanar polychlorinated biphenyls (Co-PCBs), the expression of gC1qR and the underlying intracellular apoptotic signaling pathways of human extravillous cytotrophoblast (EVCT)-derived transformed cells (HTR-8/SVneo and HPT-8). **Methods:** Apoptosis in HTR-8/SVneo and HPT-8 cells was assessed using flow cytometric analysis. gC1qR expression was examined in the HTR-8/SVneo and HPT-8 cells using real-time qPCR and western blot analyses. The phosphorylations of p38 mitogen-activated protein kinase (p38 MAPK) (Thr180/Tyr182) and extracellular signal-regulated kinase (ERK) 1/2 (Thr202/Thr204) were detected using western blot analyses. **Results:** The HTR-8/SVneo and HPT-8 cells treated with Co-PCBs exhibited significantly increased gC1qR expression, p38 MAPK/ERK activation and an up-regulation of cellular apoptosis. These effects were abrogated by the application of gC1qR small interfering RNA (siRNA). Furthermore, apoptosis in HTR-8/SVneo and HPT-8 cells was observed upon treatment with Co-PCBs, and these effects were reversed by the p38 MAPK pathway inhibitor SB203580 or the ERK1/2 pathway inhibitor PD098059. **Conclusion:** These data support a mechanism wherein gC1qR plays a crucial p38 MAPK/ERK signaling pathway-dependent role in Co-PCBs-induced apoptosis of human EVCT-derived transformed cells.

Copyright © 2015 S. Karger AG, Basel

Z. Liu and H.-j. Ruan contributed equally to this work.

Ling-juan Gao

Clinical Laboratory, Nanjing Maternity and Child Health Care Hospital, Tianfei Alley,
Nanjing Mochou Road, 210004, Nanjing, (P.R. China)
Tel. +86 25 52226790, Fax +86 25 84460507, E-Mail gaolingjuan@njmu.edu.cn

Introduction

Coplanar polychlorinated biphenyls (Co-PCBs) are the most widespread and persistent endocrine-disrupting chemicals that affect female reproductive health. Environmental Co-PCBs exhibit estrogenic properties and exert effects that mimic endogenous mechanisms [1-2]. PCBs have been associated with decreased litter size and increased risks of spontaneous abortion, stillbirth, and foetal resorption in mice, rats, rhesus monkeys, guinea pig and rabbits [3-4]. In humans, maternal exposure to Co-PCBs has been associated with increased rates of spontaneous abortion and stillbirth [5]. In previous experiments, our findings have also revealed that Co-PCBs, such as 3, 3', 4'-tetrachlorobiphenyl [IUPAC number PCB-77], 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB-126), and 3, 3', 4, 4', 5, 5'-hexachlorobiphenyl (PCB-169), are closely associated with spontaneous abortion [6]. Moreover, Co-PCBs have toxic and biological effects on human trophoblasts [7]. However, the mechanisms involved in PCB-induced cell cytotoxicity in trophoblasts have not been completely elucidated.

During the reproductive cytotoxicity injury, complement activation is increasingly recognised as a major contributor. During complement activation, C1q can itself function as a potent extracellular signal for a wide range of cells, resulting in the induction of ligand-specific biologic responses. Decidual stroma is known to overexpress and to release the globular heads receptor of C1q (gC1qR). gC1qR is a 34-kDa glycosylated phosphoprotein that has been implicated in cell signaling and was initially identified as a protein of the mitochondrial matrix [8]. There is evidence that gC1qR mediates many biological responses, including inflammation, infection and immune regulation [9]. Examples of such responses include growth perturbations, morphological abnormalities and the initiation of apoptosis [10].

gC1qR has been reported to be an endogenous substrate of mitogen-activated protein kinase (MAPK) and, upon mitogenic stimulation, gC1qR translocates to the nucleus in a MAPK-dependent manner in cervical cancer cells [11]. The MAPK signaling pathway plays an important role in cellular proliferation, differentiation, migration and apoptosis [12-13]. There are three mainly MAPK pathway: ERK1/2 pathway, which responds to growth- and differentiation-factor, and the p38 and JNK pathway, which respond to stress conditions. In recent decades, some cytotoxicity molecules have been reported to induce cellular apoptosis, and p38 MAPK and ERK1/2 signaling pathway were deemed stress responsive and thus involved in the process of apoptosis [13-14]. However, it has been not clear whether MAPK signaling pathway is involved in the apoptotic process induced by Co-PCBs on human trophoblasts.

The aim of present study was to comprehensively identify the cytotoxicity generated by Co-PCBs in human trophoblasts, the constitutive expression of gC1qR and the underlying intracellular signaling pathways. Our results provide evidence for an important role for the p38 MAPK/ERK signaling pathways in Co-PCBs-induced apoptosis in human extravillous cytotrophoblast (EVCT)-derived transformed cells.

Materials and Methods

Reagents

PCBs congeners were obtained from Ultra Scientific (North Kingstown, RI). Dimethyl sulfoxide (DMSO) was purchased from Fisher BioReagents (Thermo Fisher Scientific, Pittsburgh, PA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). The Phototope-HRP Western Blot Detection System, including an anti-mouse/rabbit IgG, an HRP-linked antibody, a biotinylated protein ladder, 20X LumiGLO Reagent and 20X peroxide, was purchased from Cell Signaling Technology (Beverly, MA, USA). The Annexin V-FITC/Propidium Iodide (PI) Flow Cytometry Assay Kit was purchased from Invitrogen (Carlsbad, CA, USA). SB203580 (a p38 MAPK pathway inhibitor), PD098059 (an ERK1/2 pathway inhibitor) and antibodies directed against gC1qR (catalogue number: sc-271201) and β -actin (catalogue number: sc-

130301) were purchased from Santa Cruz (Santa Cruz, CA, USA). Phosphorylated p38 MAPK (*p-p38 MAPK) (Thr180/Tyr182) and p38 MAPK (catalogue number: #8203), phosphorylated ERK (*p-ERK) (Thr202/Tyr204) and ERK (catalogue number: #8201) were purchased from Cell Signaling Technology. gC1qR small interfering RNA (siRNA) and negative siRNA (i.e., siRNA directed toward an unrelated gene and used as a negative control) were synthesised by Wuhan Genesil Biotechnology Co., Ltd. (Wuhan, China). Cell culture supplies were purchased from Life Technologies (Gaithersburg, MD, USA). Unless otherwise specified, all other reagents were of analytical grade.

HTR-8/SVneo and HPT-8 Cell Lines and Cells Culture

The human extravillous cytotrophoblast (EVCT)-derived transformed cell lines (HTR-8/SVneo and HPT-8) were obtained from Hangzhou Hibio Bio-tech Co., Ltd. (Hangzhou, Zhejiang, China). The first trimester EVCT cell line was termed HTR-8 and was immortalised by SV40 tag; the HTR-8/SVneo cells retained all of the phenotypic and functional characteristics of the parental mortal HTR-8 cells. The in situ expression markers of EVCT included cytokeratins 18 and 8, human placental lactogen (hPL), human chorionic gonadotropin (hCG), human leukocyte antigen G (HLA-G) and type IV collagenase. Another immortalised primary cell clone (HPT-8) exhibited cytokeratin 7, cytokeratin 18, vimentin, cluster of differentiation antigen 9, epidermal growth factor receptor, stromal cell-derived factor 1 and placental alkaline phosphatase. These cells secreted prolactin, estradiol, progesterone and hCG and were positive for HLA-G, which is a marker of extravillous trophoblasts [15]. Thus, in this experiment, the HTR-8/SVneo and HPT-8 cells were chosen as ideal in vitro models to study the biology of normal trophoblasts. The HTR-8/SVneo and HPT-8 cells were grown in Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% foetal bovine serum and maintained in 5% CO₂ at 37 °C. Four dose groups were prepared: (I) control-vehicle (DMSO); (II) low dose-PCB 77 (40 µM) + PCB 126 (40 µM) + PCB 169 (40 µM); (III) medium dose-PCB 77 (80 µM) + PCB 126 (80 µM) + PCB 169 (80 µM); (IV) high dose-PCB 77 (120 µM) + PCB 126 (120 µM) + PCB 169 (120 µM) (diluted in DMSO). The p38 MAPK inhibitor SB203580 and ERK1/2 inhibitor PD098059 were dissolved in DMSO and diluted to a final working concentration of 20 µM and 30 µM respectively. After 1-h pretreatment with SB203580 or PD098059, HTR-8/SVneo and HPT-8 cells were treated with 80 µmol/L Co-PCBs for 24 h, SB203580 and PD098059 are maintained in the medium during the 24h Co-PCBs treatment. Cell lysates were prepared for further experiment.

Observation of Apoptosis morphology

After an initial incubation to approximately 80% confluence, the HTR-8/SVneo and HPT-8 cells were treated with different concentrations of Co-PCBs and cultured for 24 h. After washing three times with PBS, the cells were immersed immediately for 2 h in phosphate-buffered 2.5% glutaraldehyde. After an overnight wash in a 0.1 M sodium phosphate buffer, the cells were postfixed in 1% OsO₄ in a 0.1 M phosphate buffer (pH 7.4) for 1 h and stained with 1% uranyl acetate. Afterwards, the cells were dehydrated and flat-embedded in Durcupan (Fluka Chemic AG, Sweden). For electron microscopy (EM), ultrathin sections (60–70 nm) were stained with lead citrate, examined at 3700X and 12500X magnification and photographed using a Zeiss 10⁹ electron microscope.

Immunohistochemical Analysis

Immunohistochemistry for gC1qR and p38 MAPK/ERK in human villous tissues of abortion tissues was performed according to the manufacturer's instructions. In brief, paraffin-embedded sections of villous tissues were deparaffinized, and then heated for 3 minutes to retrieve antigens in a pressure pot. Then the sections were incubated with mouse anti-human gC1qR monoclonal antibody at 1: 200 dilution or rabbit anti-human p38 MAPK/ERK polyclonal antibody at 1: 150 overnight at 4°C. The slides were then incubated with peroxidised goat anti-rabbit/mouse secondary antibody for 30 min at 37°C, and 3,3'-diaminobenzidine (DAB) staining was used to evaluate the chromogenic reaction. All incubation steps were performed in humidified chambers.

gC1qR siRNA-expressing Plasmid Construction and Transfection

The gC1qR siRNA was designed to target the 408-426 nucleotide portion of the human gC1qR mRNA; the forward sequence was 5'-AAC AAC AGC AUC CCA CCA ACA UU-3'. pGenesil-1 was used as the vector

backbone to construct a gC1qR siRNA-expressing plasmid. The BamHI and HindIII restriction site overhangs were located near the 5' ends of the two oligonucleotides and the siRNA was synthesised, annealed and ligated into the BamHI and HindIII restriction sites in the pGenesil-1 expression vector. An unrelated gene was used as a negative control. Transient transfection of siRNA into HTR-8/SVneo and HPT-8 cells was conducted using Lipofectamine 2000 according to the manufacturer's instructions. Briefly, 3.0 µg siRNA and 8.0 µl of the transfection medium were incubated for 25 min, then the mixture was added to the cells. Culture medium was then removed at 6 h after transfection and replaced by minimum essential medium (MEM) supplemented with 10% FCS for 54 h. Green fluorescence protein was directly observed using a fluorescence microscope to evaluate the ratio of transfection.

Real-time Quantitative Polymerase Chain Reaction (Real-time qPCR)

According to the manufacturer's instructions, total RNA was isolated from the cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). gC1qR mRNA expression was detected with forward primer 5'-AAT CAC ACG GTA GAC ACT GAA ATG CC-3' and reverse primer 5'-CAT CAT CCC ATC TAA AAT GTC CCC TG-3', which were used with the FAM/TAMRA-labelled probe 5'-TGC TCC AGT TCA ACC AAC GTC CTT CTC-3'. β-actin was quantified using forward primer 5'-TCA CCC ACA CTG TGC CCA TCT ATG A-3' and reverse primer 5'-CAT CGG AAC CGC TCA TTG CCG ATA G-3' with the FAM/TAMRA-labelled probe 5'-ACG CGC TCC CCC ATG CCA TCC TGC GT-3'. The following thermal cycling conditions was used via an ABI PRISM 7300 sequence detection system: 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All of the reactions were performed in 50 µL reaction volumes in triplicate. Standard curves were generated for gC1qR and β-actin. The actin gene was used as an internal control in all of the PCR experiments. Relative amounts of gC1qR mRNA were normalised to actin mRNA using the following formula: $2^{-\Delta\Delta CT} = 2^{-(CT_{gC1qR} - CT_{actin})_{Time x} + (CT_{gC1qR} - CT_{actin})_{Time 0}}$.

Western Blot Analysis

Following different treatments, the HTR-8/SVneo and HPT-8 cells were prepared on ice in lysis buffer (10 mM Tris-HCl, pH 7.8, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 100 mM NaCl, 10 mM EDTA) supplemented with protease inhibitors for 30 min. The supernatants were obtained by centrifugation at 15,000 × g at 4 °C for 20 min. The protein samples (50 µg) were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The transferred membranes were probed with specific primary monoclonal rabbit anti-phospho-p38 MAP kinase (Thr180/Tyr182), anti-phospho-ERK1/2 (Thr202/Tyr204), anti-p38 MAP kinase, anti-ERK and monoclonal mouse anti-actin antibodies overnight at 4 °C followed by horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualised using an enhanced chemiluminescence (ECL) Western Detection System and quantified by densitometry using Quantity One (Bio-Rad, USA).

Apoptotic Cell Detection

The HTR-8/SVneo and HPT-8 cells were exposed to Co-PCBs and/or different signaling pathway inhibitors for 24 h. The cells were washed and resuspended in binding buffer (2.5 mM CaCl₂, 10 mM HEPES, pH 7.4, and 140 mM NaCl). The cells were incubated in the dark with 5 µL each of Annexin V-FITC and propidium iodide for 15 min. Binding buffer was then added to each tube, and the samples were analysed using a Beckman Coulter Epics XL flow cytometer. Annexin-V-FITC (-)/PI (-) staining indicated live cells, Annexin-V-FITC (+)/PI (-) staining indicated cells that were in the early stages of apoptosis, and Annexin-V-FITC (+)/PI (+) staining indicated cells that were in the late stages of apoptosis or necrosis. The apoptosis ratio was calculated as the percentage of apoptotic cells among the treated sample compared to that among the control.

Statistical Analyses

The results are presented as the mean ± the standard deviation (SD) and analysed using student's *t* test when comparing two groups. To compare more than two groups, we used one-way ANOVA and *post hoc* Turkey tests, and p-values below 0.05 were considered to be significant and denoted as follows: ****p* < 0.001; ***p* < 0.01; **p* < 0.05.

Results

Co-PCBs Induce Apoptosis in EVCT-Derived Transformed Cells

To quantitatively evaluate whether Co-PCBs triggered apoptotic death, the rates of apoptosis of the HTR-8/SVneo and HPT-8 cells were assessed by flow cytometry following treatment with different concentrations of Co-PCBs (0, 40, 80, and 120 $\mu\text{mol/L}$) for 24 h in vitro. As shown in Fig. 1A, the numbers of apoptotic cells (the early and the late apoptotic cells were distributed in the Q1_LR and Q1_UR regions) observed following treatment

Fig. 1. Co-PCBs induce apoptosis in EVCT-derived transformed cell lines. A: HTR-8/Vneo and HPT-8 cells (2×10^6) were exposed to Co-PCBs at concentrations of 40, 80, and 120 $\mu\text{mol/L}$ and compared to DMSO-exposed cells (0 $\mu\text{mol/L}$ Co-PCBs). After 24 h of treatment, the cells were subjected to flow cytometric analysis to detect apoptotic death. The cells were double-stained with annexin V-FITC and PI. The early and the late apoptotic cells were distributed in the Q1_LR and Q1_UR regions, respectively. The necrotic cells were located in the Q1_UL region. The results are expressed as the means $\pm$ SD of three separate experiments (N = 3). ***p < 0.001, **p < 0.01, *p < 0.05 versus DMSO group. B: Electron microscopic observations of the HTR-8/SVneo and HPT-8 cells. No morphologic changes were observed in the cells treated with DMSO. The morphological changes observed in the Co-PCB-treated cells included blebbing, cell shrinkage, nuclear fragmentation (The white arrows). During the chromatin condensation phase, electron-dense nuclear material was aggregated peripherally under the nuclear membrane, and apoptotic bodies consisting of cytoplasm with tightly packed organelles were present in cells treated with different concentrations of Co-PCBs (40, 80, and 120 $\mu\text{mol/L}$ PCBs; 3700X). The results are detected via three separate experiments (N = 3).

with Co-PCBs at concentrations of 0, 40, 80, and 120 $\mu\text{mol/L}$ were 10.1 ± 0.03 , 19.4 ± 0.05 , 35.8 ± 0.06 , and 47.9 ± 0.08 , respectively. These data indicated the Co-PCBs concentration-dependently induced moderate to strong levels of apoptosis. Although treatment with 120 $\mu\text{mol/L}$ Co-PCBs induced the highest rate of apoptosis, the concentration of 80 $\mu\text{mol/L}$ Co-PCBs was used in subsequent experiments.

The cytotoxicity of Co-PCBs in HTR-8/SVneo and HPT-8 cells was also confirmed with morphological studies. Electron microscopy observations of the Co-PCB-treated cells (40, 80, and 120 $\mu\text{mol/L}$) at 24 h (Fig. 1B) revealed characteristic pathological subcellular changes and death. These changes included blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. During the chromatin condensation phase, electron-dense nuclear material was aggregated peripherally under

Fig. 2. The effect of Co-PCBs on gC1qR expression levels in EVCT-derived transformed cells lines. HTR-8/SVneo and HPT-8 cells (2×10^6) were exposed to Co-PCBs (0, 40, 80, and 120 $\mu\text{mol/L}$) for 24 h. A: The relative gC1qR gene expression levels are demonstrated. The gC1qR expression levels were analysed by real-time PCR. $\Delta\Delta\Delta p < 0.001$, $\Delta\Delta p < 0.01$, $\Delta p < 0.05$ versus DMSO group; $*p < 0.05$ versus PCBs (40 $\mu\text{mol/L}$); $\Delta p < 0.05$ versus PCBs (80 $\mu\text{mol/L}$). B: The gC1qR protein levels were measured in HTR-8/SVneo and HPT-8 cells using western blot analysis. The graph depicts the relative gC1qR protein levels normalised to β -actin. The results are expressed as the means $\pm$ SD of three separate experiments ($N = 3$). $\Delta\Delta\Delta p < 0.001$, $\Delta\Delta p < 0.01$, $\Delta p < 0.05$ versus DMSO group; $*p < 0.05$ versus PCBs (40 $\mu\text{mol/L}$); $\Delta p < 0.05$ versus PCBs (80 $\mu\text{mol/L}$).

the nuclear membrane, and apoptotic bodies consisting of cytoplasm with tightly packed organelles were present. The PCB-treated cells exhibited concentration-dependent chromatin condensation and nuclear fragmentation. However, in the DMSO (0 $\mu\text{mol/L}$ PCBs) treated-groups, the morphologies of the HTR-8/SVneo and HPT-8 cells exhibited no obvious apoptotic features.

Different concentrations of Co-PCBs Cause gC1qR Expression in EVCT-derived Transformed Cells

Our previous study demonstrated that gC1qR plays an important role in the induction of apoptosis. Thus, to investigate the relationship between Co-PCBs and gC1qR expression in EVCT-derived transformed cells lines, HTR-8/SVneo and HPT-8 cells were treated with different concentrations of Co-PCBs (0, 40, 80, and 120 $\mu\text{mol/L}$). The results of real-time PCR and western blot analyses demonstrated that the gC1qR expression levels were significantly increased in the Co-PCBs (40, 80, 120 $\mu\text{mol/L}$) group compared to the DMSO-treated groups (Fig. 2A-B). Moreover, gC1qR gene expression in the Co-PCBs 80 $\mu\text{mol/L}$ group was notably higher than that in the Co-PCBs 40 $\mu\text{mol/L}$ group, and the expression level of the Co-PCBs 120 $\mu\text{mol/L}$ group was significantly higher than that of the Co-PCBs 80 $\mu\text{mol/L}$ group. Quantification of the data revealed showed that the gC1qR expression level increased in a concentration-dependent manner following Co-PCBs treatment. These findings suggest that Co-PCBs induce gC1qR gene expression.

The effect of Co-PCBs on gC1qR expression and the p38 MAPK and ERK1/2 signaling pathway in gC1qR-silenced EVCT-derived transformed cells lines

In this experiment, our results indicated that the gC1qR expression and the p38 MAPK, ERK1/2 signaling pathway were significantly increased in spontaneous abortion villous tissues comparing with induced abortion villous tissues (Fig. 3A). Next, we further explored the effect of Co-PCBs on gC1qR expression and the p38 MAPK, ERK1/2 signaling pathway in gC1qR-silenced EVCT-derived transformed cells lines by transfecting HTR-8/SVneo and HPT-8 cells with 100 ng empty vector, 100 ng gC1qR siRNA or 100 ng negative siRNA. After 72 h, the cells were treated with 80 $\mu\text{mol/L}$ Co-PCBs for 24 h, and gC1qR gene and protein expression levels were analysed by real-time PCR and western blot analysis, respectively (Fig. 3B). These findings demonstrated that the gC1qR mRNA and protein expression levels were significantly decreased in the PCBs and gC1qR siRNA groups compared to the PCBs and empty vector group. However, there was no difference between the PCBs + empty vector group and the PCBs + negative siRNA groups ($p > 0.05$). In contrast, the gC1qR mRNA and protein expression levels in the PCBs + negative siRNA group were significantly higher than those of the PCBs + gC1qR siRNA groups.

To demonstrate phosphorylation of p38 and ERK1/2 is part of the cellular response to Co-PCBs exposure, cells were treated with plain medium or Co-PCBs (80 μM), and after 24 h, the level of phospho-p38 MAPK and phosphor-ERK1/2 were examined in the HTR-8/SVneo and HPT-8 cells by western blot analyses. The results indicated that Co-PCBs increased the level of phosphorylation of p38 MAPK (Thr180/Tyr182) and the phosphorylation of ERK1/2 (Thr202/Thr204) compared with the plain medium group (Fig. 3C).

To explore the related signaling mechanisms, phospho-p38 MAPK and phosphor-ERK1/2 levels were examined in the HTR-8/SVneo and HPT-8 cells by western blot analyses. As shown in Fig. 3D, the phosphorylation of p38 MAPK (Thr180/Tyr182) and the phosphorylation of ERK1/2 (Thr202/Thr204) were rapidly decreased in the PCBs + gC1qR siRNA group compared to the PCBs + empty vector group. There were no differences in phospho-p38 MAPK or phosphor-ERK1/2 protein expressions between the PCBs + empty vector group and the PCBs + negative siRNA group ($p > 0.05$). However, the phosphorylated p38 MAPK and ERK1/2 protein levels were significantly higher in the PCBs + negative siRNA group than in the PCBs + gC1qR siRNA group. These data demonstrate that Co-PCB treatment activated the p38 MAPK and ERK1/2 signaling pathways.

Fig. 3. The effects of Co-PCBs on gC1qR-silenced EVCT-derived transformed cells lines. **A:** The gC1qR and p38 MAPK/ERK levels in human villous tissue. **a:** The results of immunohistochemical staining. The positive results for gC1qR antigen in human villous tissues by immunohistochemistry ($\times 200$). 1: staining of monoclonal anti-gC1qR antibody in spontaneous abortion tissues; 2: staining of monoclonal anti-gC1qR antibody in induced abortion tissues. **b:** The results of immunohistochemical staining. The positive results for p38 MAPK antigen in human villous tissues by immunohistochemistry ($\times 400$). 1: staining of monoclonal anti-p38 MAPK antibody in spontaneous abortion tissues; 2: staining of monoclonal anti-p38 MAPK antibody in induced abortion tissues. **c:** The results of immunohistochemical staining. The positive results for ERK1/2 antigen in human villous tissues by immunohistochemistry ($\times 200$). 1: staining of monoclonal anti-ERK1/2 antibody in spontaneous abortion tissues; 2: staining of monoclonal anti-ERK1/2 antibody in induced abortion tissues. **B:** HTR-8/SVneo and HPT-8 cells (2×10^6) were treated with PCBs (80 $\mu\text{mol/L}$). After 24 h, the cells were transfected with 100 ng of empty vector, 100 ng of gC1qR siRNA or 100 ng of negative siRNA. The relative gC1qR gene expression levels in the HTR-8/SVneo and HPT-8 cells. **a:** The gC1qR mRNA expression levels were analysed with real-time PCR. ** $p < 0.01$, # $p > 0.05$ versus the PCBs and empty vector groups;

▲▲p < 0.01 versus the PCBs and gC1qR siRNA group. b: The gC1qR protein levels were measured in the HTR-8/SVneo and HPT-8 cells using western blot analyses. The graph depicts the relative gC1qR protein levels normalised to β -actin. The results are expressed as the means $\pm$ the SD of three separate experiments (N = 3). ***p < 0.001, #p > 0.05 versus PCBs and empty vector groups; ▲▲p < 0.01 versus the PCBs and gC1qR siRNA group. c: The p38 MAPK and ERK1/2 activations in the cultured EVCT-derived transformed cells lines. The HTR-8/SVneo and HPT-8 cells (2×10^6) were treated with plain medium or Co-PCBs (80 μ mol/L). After 24 h, cells were lysed and examined for p*-p38 and p*-ERK1/2 by western blot analyses. The p38 MAPK and ERK1/2 activities were determined as described. The graph represents the ratio of the phosphorylated p38 MAPK to total p38 MAPK, and the ratio of the phosphorylated ERK1/2 to total ERK1/2 respectively. The results are expressed as the means $\pm$ SD of three separate experiments (N = 3). ***p < 0.001 versus the plain medium group. d: The p38 MAPK and ERK1/2 activations in the cultured EVCT-derived transformed cells lines. The HTR-8/SVneo and HPT-8 cells (2×10^6) were lysed and examined for p*-p38 and p*-ERK1/2 by western blot analyses. The p38 MAPK and ERK1/2 activities were determined as described. The graph represents the ratio of the phosphorylated p38 MAPK to total p38 MAPK, and the ratio of the phosphorylated ERK1/2 to total ERK1/2 respectively. The results are expressed as the means $\pm$ SD of three separate experiments (N = 3). ***p < 0.001, #p > 0.05 versus the PCBs and empty vector group; ΔΔΔp < 0.001 versus PCBs and gC1qR siRNA groups.

Fig. 4. The effect of Co-PCBs combined with SB203580 or PD098059 on gC1qR expression and apoptosis in the EVCT-derived transformed cells lines. After 1-h pretreatment with 20 μ M SB203580 (a p38 MAPK pathway inhibitor) or 30 μ M PD098059 (a ERK1/2 pathway inhibitor), The HTR-8/SVneo and HPT-8 cells (2×10^6) were exposed to Co-PCBs (80 μ mol/l) for 24 h. A: The relative gC1qR gene expression levels are shown for the HTR-8/SVneo and HPT-8 cells. The gC1qR expression levels were analysed by real-time PCR. ** $p < 0.01$ versus PCBs group. B: The gC1qR protein expression levels were measured in the HTR-8/SVneo and HPT-8 cells using western blot analyses. The graph depicts the relative gC1qR protein levels normalised to β -actin. The results are expressed as the means $\pm$ SD of three separate experiments ($N = 3$). *** $p < 0.001$, ** $p < 0.01$ versus the PCBs group. C: The numbers of apoptotic cells were determined by flow cytometric analysis for the detection of apoptotic death. The cells were treated with plain medium, 20 μ M SB203580 (a p38 MAPK pathway inhibitor) or 30 μ M PD098059 (a ERK1/2 pathway inhibitor) for 25 h, The HTR-8/SVneo and HPT-8 cells double-stained with annexin V-FITC and PI. The early and the late apoptotic cells were distributed in the Q1_LR and Q1_UR regions, respectively. The necrotic cells were located in the Q1_UL region. The results are expressed as the means $\pm$ SD from 3 independent experiments ($N = 3$). D: After 1-h pretreatment with 20 μ M SB203580 (a p38 MAPK pathway inhibitor) or 30 μ M PD098059 (a ERK1/2 pathway inhibitor), The HTR-8/SVneo and HPT-8 cells (2×10^6) were exposed to Co-PCBs (80 μ mol/l) for 24 h. The numbers of apoptotic cells were determined by flow cytometric analysis for the detection of apoptotic death. The cells were double-stained with annexin V-FITC and PI. The results are expressed as the means $\pm$ SD from 3 independent experiments ($N = 3$). * $p < 0.05$ versus the PCBs group.

Roles of p38 MAPK and ERK1/2 in PCB-induced cytotoxicity in EVCT-derived transformed cells lines

To further elucidate the roles of the p38 MAPK and ERK1/2 signaling pathways in the cytotoxicity of Co-PCBs to the EVCT-derived transformed cells lines, after 1-h pretreatment

with 20 μ M SB203580 (a p38 MAPK pathway inhibitor) or 30 μ M PD098059 (a ERK1/2 pathway inhibitor), HTR-8/SVneo and HPT-8 cells were treated with 80 μ mol/L Co-PCBs for 24 h, SB203580 and PD098059 are maintained in the medium during the 24h Co-PCBs treatment. gC1qR gene and protein expression levels were analysed by real-time PCR and western blotting (Fig. 4A-B). The results demonstrated that the gC1qR mRNA and protein levels were notably decreased in the PCBs + SB203580 and PCBs + PD098059 groups compared to the PCBs alone group.

To fully understand the modulation of the apoptosis process by SB203580 or PD098059, the effect of the 2 drugs were tested in parallel in HTR-8/SVneo and HPT-8 cells that not treated with Co-PCBs. We performed annexin V and PI double-staining experiments followed by flow cytometric analyses. These results indicated the numbers of apoptotic cells were no significantly changed by SB203580 or PD098059 treatment relative to the plain medium group (Fig. 4C).

After 1-h pretreatment with 20 μ M SB203580 (a p38 MAPK pathway inhibitor) or 30 μ M PD098059 (a ERK1/2 pathway inhibitor), HTR-8/SVneo and HPT-8 cells were treated with 80 μ mol/L Co-PCBs for 24 h. We next performed annexin V and PI double-staining experiments followed by flow cytometric analyses. The findings indicated that Co-PCB

treatment caused significant apoptotic death of HTR-8/SVneo and HPT-8 cells; interestingly, the numbers of apoptotic cells were decreased by SB203580 or PD098059 treatment relative to the PCB-treated group (Fig. 4D).

Discussion

Co-PCBs exposure-induced toxicity has been linked to the pathophysiologies of diseases that including cancer, liver disease, type 2 diabetes and obesity [16]. Epidemiological research assessing the effects of maternal exposure to PCBs/dioxins on pregnancy outcomes has found that such exposure increases the proportions of adverse outcomes related to spontaneous abortion, stillbirth and preterm delivery, foetal growth restriction, and low birth weight [17-18]. Several earlier studies also reported evidence supporting a relationship between exposure to Co-PCBs and spontaneous abortion [19-20], whereas other earlier studies did not support this association [21-22]. The inconsistencies between these early studies were largely due to sample size, indirect exposure assessments, study design or Co-PCB exposure levels. The findings from our primary analysis support the existence of an association between exposure to Co-PCBs and spontaneous abortion. Moreover, it has been reported that serum concentrations of Co-PCBs are increased in women with foetal loss (miscarriages or stillbirths) and that high levels of Co-PCBs can directly increase apoptosis and cytotoxicity in human extravillous trophoblast cells. These results agree with those of the present study in which we demonstrated that treatment with Co-PCBs concentration-dependently triggered apoptosis in the human EVCT-derived transformed cell lines HTR-8/SVneo and HPT-8. The cytotoxicity of Co-PCBs on HTR-8/SVneo and HPT-8 cells was manifested as the apoptotic features of blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation.

It is well known that low doses of Co-PCBs have stimulatory effects on the immune system, whereas high doses exhibit suppressive effects [23]. We reported that PCB-associated spontaneous miscarriage is related to the ability of Co-PCBs to induce gC1qR expression in EVCT-derived transformed cells [6]. Increasing evidence indicates that gC1qR can modulate various physiological cell responses; e.g., growth perturbations, morphological abnormalities and the initiation of apoptosis [24]. The findings of our previous study revealed that gC1qR is overexpressed in HTR-8/SVneo and HPT-8 cells and that this overexpression mediates cell apoptosis. Moreover, gC1qR expression increased in the EVCT-derived transformed cells in a dose-dependent manner in response to treatment with Co-PCB concentrations ranging from 0 to 120 $\mu\text{mol/L}$. gC1qR expression reached its highest level in response to the 120 $\mu\text{mol/L}$ treatment with Co-PCBs. But we chose the concentration of 80 $\mu\text{mol/L}$ Co-PCBs for subsequent experiments.

Previous reports about information available on the Co-PCBs toxicity in human cells are very few, due to the nature of absorption of different human tissues. To obtain such information, Ghosh S [25] chose 70 μM of Co-PCBs to expose human liver cells and 80 μM of Co-PCBs to expose human kidney cells to detect the cellular viability and apoptosis, and concluded that liver cells initiate the absorption of Co-PCBs, however, the concentration reaches its maximum level much earlier in kidney cells in human tissues. The reasons are as follows: the liver is mainly responsible for the uptake, storage, disposal of drug and toxins physiologically, while the kidney mainly regulates the mineral composition, body's fluid volume, re-absorption of water and inorganic electrolytes [26-27]. In present experiment, the maternal blood and placenta/decidual tissue were chosen as target to study the toxicity of Co-PCBs exposures, and according to the above analysis and our previous experimental data, we chose the 80 μM concentration of Co-PCBs as the proper concentration to mimic environmental Co-PCBs exposure to matrix in subsequent experiments.

MAPK signaling pathways mediate the intracellular signaling associated with a variety of cellular physiological process, including the morphological and functional differentiation of villous trophoblasts, and have been proven to be important for the induction of cytotoxicity

responses [28-31]. Thus, to identify the potential mechanisms that lead to cytotoxic responses of HTR-8/SVneo and HPT-8 cells to Co-PCBs, we demonstrated the roles members of the MAPK signal cascades, including ERK1/2 and p38 kinase. It has been verified that the gC1qR expression, the p38 MAPK and ERK1/2 signaling pathway were significantly increased in spontaneous abortion villous tissues in our experiments. Accumulating evidence indicates that the silencing of the gC1qR gene decreases Co-PCB-induced activations of phospho-p38 and phosphor-ERK1/2. Meanwhile, our results revealed that SB203580 (a p38 MAPK pathway inhibitor) or PD098059 (a ERK1/2 pathway inhibitor) decreased PCBs-induced expression of gC1qR levels, which blocked the cytotoxic effect of Co-PCBs on apoptosis in HTR-8/SVneo and HPT-8 cells. Thus, the p38 MAPK/ERK1/2 pathway seems to be responsible for the gC1qR-regulated apoptosis of EVCT-derived transformed cells that is induced by Co-PCBs.

In conclusion, we demonstrated a mechanism by which Co-PCBs regulate cell apoptosis that is dependent on the gC1qR gene. The present study also proved that the p38 MAPK/ERK1/2 signaling pathway is involved in Co-PCB-induced apoptosis of HTR-8/SVneo and HPT-8 cells. The cytotoxicities of Co-PCBs suggest that increased gC1qR levels are important in the apoptosis of EVCT-derived transformed cells and that gC1qR induces apoptosis through the p38 MAPK/ERK1/2 signaling pathway in EVCT-derived transformed cells.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (Contract grant number: No. 81202039), the Nanjing Medical Science and Technique Development Foundation (Contract grant number: No. QRX11112), the Medical Science and Technology Development Foundation, Nanjing Department of Health (Contract grant number: No. 201308041), the Nanjing Medical University Natural Science Foundation (Contract grant number: No. 2013NJMU136) and Science and Technology Commission Foundation of Huangdao District of Qingdao (Contract grant number: No. 2014-1-97).

Disclosure Statement

The authors declare that they have no conflict of interest.

References

- 1 Arisawa K, Uemura H, Hiyoshi M, Satoh H, Sumiyoshi Y, Morinaga K, Kodama K, Suzuki T, Nagai M, Suzuki T: Dietary intake of PCDDs/PCDFs and coplanar PCBs among the Japanese population estimated by duplicate portion analysis: a low proportion of adults exceed the tolerable daily intake. *Environ Res* 2008;108:252-259.
- 2 Kiguchi O, Saitoh K, Ogawa N: Simultaneous extraction of polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and coplanar polychlorinated biphenyls from contaminated soil using pressurized liquid extraction. *J Chromatogr A* 2007;1144:262-268.
- 3 Christian MS, Brent RL, Calda P: Embryo-fetal toxicity signals for 17alpha-hydroxyprogesterone caproate in high-risk pregnancies: a review of the non-clinical literature for embryo-fetal toxicity with progestins. *J Matern Fetal Neonatal Med* 2007;20:89-112.
- 4 Tsukimori K, Tokunaga S, Shibata S, Uchi H, Nakayama D, Ishimaru T, Nakano H, Wake N, Yoshimura T, Furue M: Long-term effects of polychlorinated biphenyls and dioxins on pregnancy outcomes in women affected by the Yusho incident. *Environ Health Perspect* 2008;116:626-630.
- 5 Chiusolo M, Ivaldi C, Procopio E, Greco GM, Lodato M, Cadum E: Human biomonitoring and variation of haematic parameters in a population exposed to PCB and dioxin near a steel plant in the lower Susa Valley. *Epidemiol Prev* 2012;36:24-33.

- 6 Gu PQ, Gao LJ, Li L, Liu Z, Luan FQ, Peng YZ, Guo XR: Endocrine disruptors, polychlorinated biphenyls-induced gC1qR-dependent apoptosis in human trophoblast cell line HTR-8/SVneo. *Reprod Sci* 2012;19:181-189.
- 7 Tsuji M, Aiko Y, Kawamoto T, T Hachisuga, Kooriyama C, Myoga M, Tomonaga C, Matsumura F, Anan A, Tanaka M: Polychlorinated biphenyls (PCBs) decrease the placental syncytiotrophoblast volume and increase Placental Growth Factor (PLGF) in the placenta of normal pregnancy. *Placenta* 2013;34:619-623.
- 8 Prakash M, Kale S, Ghosh I, Kundu GC, Datta K: Hyaluronan-binding protein 1 (HABP1/p32/gC1qR) induces melanoma cell migration and tumor growth by NF-kappa B dependent MMP-2 activation through integrin $\alpha(v)\beta(3)$ interaction. *Cell Signal* 2011;23:1563-1577.
- 9 Peerschke EI, Petrovan RJ, Ghebrehiwet B, Ruf W: Tissue factor pathway inhibitor-2 (TFPI-2) recognizes the complement and kininogen binding protein gC1qR/p33 (gC1qR): implications for vascular inflammation. *Thromb Haemost* 2004;92:811-889.
- 10 Meenakshi J, Anupama SK, Datta K: Constitutive expression of hyaluronan binding protein 1 (HABP1/p32/gC1qR) in normal fibroblast cells perturbs its growth characteristics and induces apoptosis. *Biochem Biophys Res Commun* 2003;300:686-693.
- 11 Gao LJ, Gu PQ, Zhao W, Ding WY, Zhao XQ, Guo SY, Zhong TY: The role of globular heads of the C1q receptor in HPV 16 E2-induced human cervical squamous carcinoma cell apoptosis is associated with p38 MAPK/JNK activation. *J Transl Med* 2013;11:118.
- 12 Wada T, Penninger JM: Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* 2004;23:2838-2849.
- 13 Yang F, Chen H, Liu Y, Yin K, Wang Y, Li X, Wang G, Wang S, Tan X, Xu C, Lu Y, Cai B: Doxorubicin caused apoptosis of mesenchymal stem cells via p38, JNK and p53 pathway. *Cell Physiol Biochem* 2013;32:1072-1082.
- 14 Guo R, Su Y, Liu B, Li S, Zhou S, Xu Y: Resveratrol suppresses oxidized low-density lipoprotein-induced macrophage apoptosis through inhibition of intracellular reactive oxygen species generation, LOX-1, and the p38 MAPK pathway. *Cell Physiol Biochem* 2014;34:603-616.
- 15 Choy MY, Manyonda IT: The phagocytic activity of human first trimester extravillous trophoblast. *Hum Reprod* 1998;13:2941-2949.
- 16 Arrebola JP, Fernández MF, Olea N, Ramos R, Martin-Olmedo P: Human exposure to p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE) in urban and semi-rural areas in southeast Spain: a gender perspective. *Sci Total Environ* 2013;458-460:209-216.
- 17 Revich B, Aksel E, Ushakova T, Ivanova I, Zhuchenko N, Klyuev N, Brodsky B, Sotskov Y: Dioxin exposure and public health in Chapaevsk, Russia. *Chemosphere* 2001;43:951-66.
- 18 Yorifuji T, Kashima S, Tokinobu A, Kato T, Tsuda T: Regional impact of exposure to a polychlorinated biphenyl and polychlorinated dibenzofuran mixture from contaminated rice oil on stillbirth rate and secondary sex ratio. *Environ Int* 2013;59:12-15.
- 19 Khanjani N, Sim MR: Maternal contamination with PCBs and reproductive outcomes in an Australian population. *J Expo Sci Environ Epidemiol* 2007;17:191-195.
- 20 Nieuwenhuijsen MJ, Dadvand P, Grellier J, Martinez D, Vrijheid M: Environmental risk factors of pregnancy outcomes: a summary of recent meta-analyses of epidemiological studies. *Environ Health* 2013;12:6.
- 21 Axmon A, Rylander L, Strömberg U, Hagmar L: Miscarriages and stillbirths in women with a high intake of fish contaminated with persistent organochlorine compounds. *Int Arch Occup Environ Health* 2000;73:204-208.
- 22 Sugiura-Ogasawara M, Ozaki Y, Sonta S, Makino T, Suzumori K: PCBs, hexachlorobenzene and DDE are not associated with recurrent miscarriage. *Am J Reprod Immunol* 2003;50:485-489.
- 23 Spector JT, De Roos AJ, Ulrich CM, Sheppard L, Sjödin A, Wener MH, Wood B, McTiernan A: Plasma polychlorinated biphenyl concentrations and immune function in postmenopausal women. *Environ Res* 2014;131:174-180.
- 24 Liu Z, Su YJ, Gu PQ, Ji ZY, Wang XG, Gao LJ: The Role of the Globular Heads of C1q Receptor (gC1qR) Gene in Regulating Apoptosis of Human Cervical Squamous Cell Carcinoma. *Cell Physiol Biochem* 2012;30:1181-1190.
- 25 Ghosh S, De S, Chen Y, Sutton DC, Ayorinde FO, Dutta SK: Polychlorinated biphenyls (PCB-153) and (PCB-77) absorption in human liver (HepG2) and kidney (HK2) cells in vitro: PCB level and cell death. *Environ Int* 2010;36:893-900.

- 26 Vezina CM, Walker NJ, Olson JR: Subchronic exposure to TCDD, PeCDF, PCB126, and PCB153: effect on hepatic gene expression. *Environ Health Perspect* 2004;112:1636-1644.
- 27 Tsukimori K, Uchi H, Mitoma C, Yasukawa F, Fukushima K, Todaka T, Kajiwarra J, Yoshimura T, Hirata T, Wake N, Furue M: Comparison of the concentration of polychlorinated biphenyls and dioxins in mothers affected by the Yusho incident and their children. *Chemosphere* 2011;84:928-935.
- 28 Morales-Prieto DM, Ospina-Prieto S, Chaiwangyen W, Weber M, Hölter S, Schleussner E, Fitzgerald JS, Markert UR: Intracellular crosstalk between extracellular regulated kinase1/2 and signal transducer and activator of transcription 3 regulates JEG-3 choriocarcinoma cell invasion and proliferation. *Sci World J* 2013;2013:259845.
- 29 Tang C, Liang J, Qian J, Jin L, Du M, Li M, Li D: Opposing role of JNK-p38 kinase and ERK1/2 in hydrogen peroxide-induced oxidative damage of human trophoblast-like JEG-3 cells. *Int J Clin Exp Pathol* 2014;7:959-968.
- 30 Wang M, Li Y, Wu M, Wang W, Gong B, Wang Y: WWOX suppresses cell growth and induces cell apoptosis via inhibition of P38 nuclear translocation in cholangiocarcinoma. *Cell Physiol Biochem* 2014;34:1711-1722.
- 31 Xie M, Yi X, Wang R, Wang L, He G, Zhu M, Qi C, Liu Y, Ye Y, Tan S, Tang A: 14-Thienyl methylene matrine (YY18), the derivative from matrine, induces apoptosis of human nasopharyngeal carcinoma cells by targeting MAPK and PI3K/Akt pathways in vitro. *Cell Physiol Biochem* 2014; 33: 1475-1483.