Polychlorinated Biphenyls Activate Caspase-3-Like Death Protease *in Vitro* but Not *in Vivo*

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We prove here that serum albumin inhibits apoptosis induced by polychlorinated biphenyls (PCBs), confirming that serum albumin binds to PCB, and that the albumin–PCB complexes inhibit apoptosis in HL-60 cells. We found that PCB (50 μM) increased the activity of caspase-3-like protease when HL-60 cells, as well as splenocytes, were cultured in "serum-free medium." Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) inhibited apoptosis in cells cultured in the serum-free medium containing 50 μM PCB. To elucidate whether or not PCBs induce apoptosis *in vivo*, we examined apoptosis of splenocytes by administering PCB to ICR mice (100, 500, 1000 mg·kg^{-1}·d^{-1}) for 5 d and characterizing splenocytes. Interestingly, splenocytes treated with PCB did not show any changes characteristic of apoptosis. These results demonstrate that PCB activates the caspase-3-like death protease *in vitro* in serum-free medium, but does not induce apoptosis of splenocytes *in vivo*, suggesting that blood serum may mask the apoptosis induced by PCB.

Key words polychlorinated biphenyl; caspase-3-like protease; serum albumin; splenocyte

PCB (polychlorinated biphenyl) was ubiquitous and widely used in a variety of industrial purposes for several decades. Because of its extensive use and chemical stability, PCB has been identified in human and wildlife serum, adipose tissue, and breast milk.[1,2] It had immunotoxic effects on antibody production against T-dependent sheep red blood cell antigen, primary activation of T cells by mixed lymphocyte response, and lymphocyte proliferation induced by various mitogens. The responses of PCB resemble those of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds.[3] PCB, including other environmental toxicants such as TCDD, is now also considered an endocrine disruptor as well as an apoptosis inducer.[4—7]

It is well known that apoptosis is cellular suicide, and is functionally the opposite of mitosis. It plays a key role in development, homeostasis, and removal of damaged and premalignant cells. Apoptosis can be induced by different stimuli, and apoptotic cell death is characterized by common features including chromatin condensation, cell shrinkage, and DNA fragmentation. During apoptosis, many cellular proteins undergo caspase-dependent degradation.[8] In particular, caspase-3-like proteases are responsible for the cleavage of their substrates at the onset of apoptosis. Although the relevance of cleavage of structural proteins, like poly-adenosyl ribose polymerase (PARP), an inhibitor of caspase-activated deoxyribonuclease (ICAD), DNA fragmentation factor (DFF), gelsolin, fodrin, actin, and lamin, is easily conceivable,[9—11] the functional importance of these and other cleavages, such as those of signaling molecules, is not yet understood. It is, however, widely assumed that the caspase-specific cleavage of these proteins is responsible for the various hallmarks of apoptosis: nuclear fragmentation, cytoplasmic membrane blebbing, and DNA fragmentation.[9]

We and others previously reported that PCB increased DNA fragmentation in a dose- and time-dependent manner in splenocytes, suggesting that apoptosis was attributable to the toxicity of PCB in murine splenocytes.[12,13] We also reported the protective effect of serum albumin in PCB-induced apoptosis might be due to non-specific binding of PCB to proteins in serum rather than due to serum components such as growth factors.[12]

In this study, the major finding is that PCB induces apoptosis *via* a caspase-3-like protease-dependent mechanism *in vitro*, but does not act as an apoptosis inducer *in vivo*.

MATERIALS AND METHODS

Cells and Reagents HL-60 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml of penicillin and streptomycin. The cells were seeded at a concentration of 5.0×10^5 cells/ml and maintained by passage every 2 d. Spleen cells were prepared from mouse spleen as described previously.[12]

Caspase-3-Like Protease Assay HL-60 cells were cultured with PCB (50 μM) in a 24-well plate at a concentration of 2.0×10^6 cells/ml in serum-free medium or with 10% serum. For the *in vivo* toxicity assays, splenocytes from ICR mice were isolated and used throughout the experiment. Polychlorinated biphenyls (PCBs, Aroclor 1254, Aldrich Chemical Co., Milwaukee, WI, U.S.A.), and PCB 126 as a congenor (Aldrich Chemical Co., Milwaukee) were dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium at a final concentration of 50 μM or administered to mouse as an *in vivo* study. Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) as a specific caspase inhibitor was purchased from Bachem Chemical Co., Ltd. (Bubendorf, Switzerland) and dissolved in DMSO (final 0.1%). The fluorogenic substrate, DEVD-AFC, used as a specific caspase-3 substrate for protease activity was obtained from Bachem. The activities of caspases were measured using a fluorogenic assay kit (R & D Systems, Inc., Minneapolis, MN, U.S.A.).

DNA Fragmentation Assay DNA laddering typical of apoptosis was visualized on agarose gel electrophoresis as previously reported with a slight modification.[12] In brief, the

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supernatant containing small DNA fragments was separated from the pellet containing intact DNA; half of it was used for agarose gel electrophoresis and the other half, as well as the pellets, was used for quantitative analysis of fragmented DNA by diphenylamine reaction.[14)]

TLC Analysis RPMI medium was added to the PCB solution (50 μM) and applied to a TLC plate (Silica gel-60, F254, 20×20 cm, 0.25 mm thickness, Merck Co.) with 100% normal hexane in a TLC chamber. Ten percent sulfuric acid was sprayed on the plate.

Aryl Hydrocarbon Receptor (AhR) Experiment The spleen cells were collected from DBA2 [AhR(+/+)] and C57BL [AhR(−/−)] mice and washed twice with PBS. They were resuspended in RPMI medium without serum at a concentration of 5×10^5 cells/ml, and transferred to 24-well plates. PCB (10, 50, 100 μM of PCB 1254 as a mixture; 1, 5, 25 μM of PCB 126 as a congener) was added to the individual wells, and the cells were incubated for 6 h. PCB-treated cells were centrifuged twice with saline, and DNA fragmentation determined by the DPA method.

***In Vivo* PCB Study** PCB was diluted in corn oil to a concentration of 200 mg/ml and a single i.p. injection of 100, 500, or 1000 $mg\cdot kg^{-1}\cdot d^{-1}$ was administered to each ICR mouse for 5 d until 6 h before sacrifice. Splenocytes were collected and examined for PCB-induced apoptosis, indicated by caspase-3 activity, and DNA fragmentation as evidence of *in vivo* toxicity. Positive control used was doxorubicin at a concentration of 10 μg/ml.

RESULTS AND DISCUSSION

Because caspase-3-like protease is known as a pivotal downstream molecule that is involved in the final execution of apoptosis,[8,9,15,16)] it is very important to examine the expression/activity of the apoptotic marker(s) induced by PCB for the biological monitoring and evaluation of hazardous environmental pollutants/toxicants.

To assess the effect of PCBs on HL-60 cell death prior to *in vivo* study, the cells were treated with (Fig. 1A, slashed columns) or without (Fig. 1A, white columns) PCB (50 μM) in serum-depleted RPMI medium, and incubated for 3 h (Fig. 1A, columns 3—4) or 6 h (Fig. 1A, columns 7—8). As a result, 45% of the DNA was fragmented by 3 h exposure, whereas 80% of the DNA was fragmented when the cells were exposed on PCB for 6 h (Fig. 1A, column 5 and data partially not shown). Percentages of DNA fragmentation did not change when cells were incubated in medium with serum for up to 6 h. We therefore concluded that optimal exposure time of PCB was 6 h. These results indicate that PCB in serum-depleted medium induces DNA fragmentation of HL-60 cells in a time-dependent manner.

It is well known that camptothecin induces apoptosis *via* a caspase-3-dependent mechanism in various cancer cells, which is suppressed by a caspase inhibitor.[16—19)] We therefore investigated whether or not the caspase-3-like protease is involved in PCB-induced apoptosis in HL-60 cells. As a positive control, we confirmed that camptothecin (0.1 μg/ml) induced apoptosis in HL-60 cells, but addition of Z-VAD-fmk, a general inhibitor of caspases, inhibited DNA fragmentation. The inhibitor strongly blocked apoptosis in the PCB-treated cells (Fig. 1B, columns 3—8 for PCB treated with

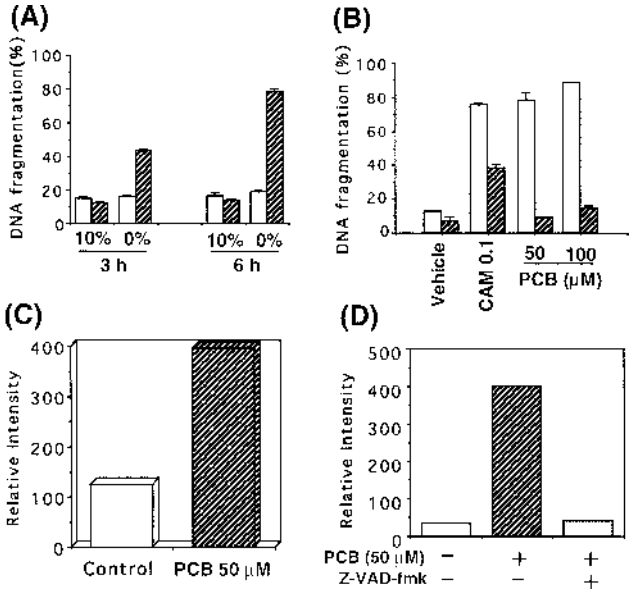

Fig. 1. Polychlorinated Biphenyls Trigger Caspase-3-Like Death Protease Activity

(A) PCB (50 μM) induced apoptosis in serum-free RPMI medium. HL-60 cells were cultured and then incubated for 24 h in 24-well plates. After washing with serum-free RPMI medium, PCBs (50 μM) were added to each plate for a 3 or 6 h-incubation in 0 or 10% serum-supplemented medium. Cells with (slashed columns) or without (white columns) were harvested to determine DNA fragmentation by the diphenylamine method.[16)] Bars indicate the S.D. of three independent determinations. (B) The specific inhibitor Z-VAD-fmk inhibited PCB-induced apoptosis. As a positive control, camptothecin (0.1 μg/ml) was used. When cells were treated with 50 or 100 μM PCBs alone (white columns), 80—90% of the DNA was fragmented, but Z-VAD-fmk (slashed columns) strongly suppressed apoptosis to less than 10%. Bars indicate the S.D. of three independent determinations. (C) Caspase-3-like protease is associated with PCB-induced apoptosis in HL-60 cells. To examine the involvement of caspase-3-like protease during PCB-induced apoptosis, a fluorogenic assay was used as described in Materials and Methods. (D) PCB induced caspase-3-like protease activity in spleen cells. Spleen cells were isolated and purified as described previously.[12)] In spleen cells, Z-VAD-fmk (dotted column) strongly suppressed PCB-induced caspase-3-like death protease activity, whereas PCBs alone (slashed column) showed high relative intensity in the assay. In (C) and (D), the data represent the mean value of two determinations in one of three independent experiments.

(slashed) or without (white) the inhibitor). When we used the specific substrate (DEVD-AFC) for caspase-3 activity, the activity of PCB-treated cell lysates was 3.2 fold higher than that of control cell lysates (Fig. 1C). The inhibition of PCB-induced apoptosis by Z-VAD-fmk is very specific and potent compared to that of camptothecin-induced cell death in the cells. These results may indicate that the PCB signal related to caspase-3-like protease is more potent than that by camptothecin. In Fig. 1D we confirmed that caspase activation in splenocytes is triggered by PCB, but inhibited by Z-VAD. These data show that PCB triggered the activity of the caspase-3-like protease in HL-60 cells.

In a previous report we investigated the effect of PCB on cell viability, suggesting that PCB (100 μM) decreased cell viability by up to 20% of the HL-60 cells and murine splenocytes.[12)] The major finding of the present study is that PCB induces caspase-3-like protease *in vitro*, but does not act as an apoptosis inducer *in vivo*. In support of this, we first showed by TLC analysis that PCB might bind to serum albumin. As shown in Fig. 2A, PCB alone showed a clear spot (lane 1). By increasing the amount of albumin in the medium (0—10%), the spot disappeared (lanes 2—6), suggesting that PCB bound to albumin and did not show the spot. To confirm the involvement of serum albumin on apoptosis induction in

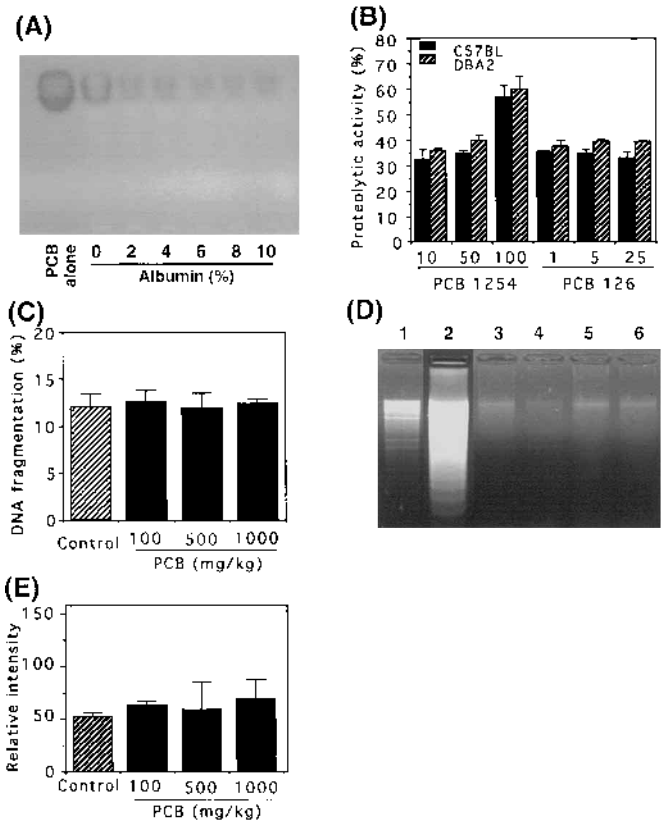

Fig. 2. Polychlorinated Biphenyls Do Not Induce Apoptosis *in Vivo*

(A) TLC analysis shows that polychlorinated biphenyls bind to serum albumin. The RPMI medium supplemented with various concentrations of serum albumin was added to PCB (50 μM), and the complex was applied to a TLC plate (Silicagel-60 F_{254}, 20× 20 cm, 0.25 mm thickness, Merck Co.) with 100% normal hexane in a TLC chamber. Ten percent sulfuric acid was sprayed on the plate. (B) AhR is not associated with PCB-induced apoptosis *in vitro*. The spleen cells were collected from the mice, DBA2 [AhR(+/+)] and C57BL[AhR(−/−)], and washed twice with PBS. The cells were resuspended in RPMI medium without serum at a concentration of 5×10 cells/ml, and transferred to 24-well plates. PCB (10, 50, 100 μM of PCB 1254 as a naturally occurring form); 1, 5, 25 μM of PCB 126 as a congener) was added to the wells, and incubated for 6 h. PCB-treated cells were centrifuged twice with saline, and DNA fragmentation determined. (C) PCB is not an apoptosis inducer *in vivo*. PCB was diluted in corn oil to a concentration of 200 mg/ml. Single i.p. injections of 100, 500, or 1000 $mg \cdot kg^{-1} \cdot d^{-1}$ for 5 d and double i.p. injections of 500 $mg \cdot kg^{-1} \cdot d^{-1}$ at day 0 and day 3 were administered to each ICR mouse, and the mice (n=5) were sacrificed after 6 h. The spleen was minced, and splenocytes were collected for further study. (D) DNA fragmentation assay shows that PCB did not affect apoptosis *in vivo*. There was no detection of caspase-3-like protease activation in splenocytes induced by PCB *in vivo*. Lane 1; size marker of *Hin*dIII digest of λDNA, lane 2; 0.1 μg/ml camptothecin treated, lane 3; control, lane 4; 100 $mg \cdot kg^{-1} \cdot d^{-1}$, lane 5; 500 $mg \cdot kg^{-1} \cdot d^{-1}$, lane 6; 1000 $mg \cdot kg^{-1} \cdot d^{-1}$, lane 7; PCB treated. (E) No change in caspase-3-like protease activity induced by PCB *in vivo*. The relative intensity was measured as described in Materials and Methods.

HL-60 cells, we removed serum albumin in the culture medium, and carried out an apoptosis induction assay. Interestingly, we confirmed that in serum-free medium PCB accelerated apoptosis in a dose- and time-dependent manner through a caspase-3-like protease-dependent mechanism (Fig. 1). These results appear to suggest that the PCB-serum albumin complex may not enter the cells, which results in caspase activation in cells *via* a PCB-induced apoptotic mechanism, if it exists.

To investigate PCB-induced apoptotic signaling, we assume that a membrane-bound receptor(s) or cytoplasmic receptor(s) is involved in PCB-induced apoptotic signaling and triggers the caspase-3-like protease. To investigate the involvement of AhR during PCB-induced apoptosis, we first selected two mouse lineages, DBA2 [AhR(+/+)] and C57BL [AhR(−/−)]. The mice were sacrificed, splenocytes were isolated and were treated with various concentrations of PCB (PCB 1254 as mixture and PCB 126 as a congener). The cell death for PCB was examined by apoptotic DNA fragmentation (Fig. 2B). The results show that DNA fragmentation between the two spleen cells did not show any change with PCB treatment (10—100, 1—25 μg/ml, repectively). The data show that AhR signaling is not associated with PCB-induced apoptosis, which was assayed by DNA fragmentation. It is known that PCB-induced apoptosis is induced by the caspase-3 death protease.[21] One possibility is that the aryl hydrocarbon receptor (AhR) plays an important role in the signal transduction of endocrine disrupters including PCBs and dioxins. Because PCB is very hydrophobic, it may be trapped in the hydrophobic regions in the cell membranes instead of entering the cytoplasm. It is reported that the Ah receptor is not involved in 2,3,7,8-tetra chlorodibenzo-*p*-dioxin (TCDD)-mediated apoptosis in human leukemia T cells.[21] Therefore, we cannot rule out the possibility that another membrane-bound receptor(s), if it exists, is associated with PCB-mediated apoptosis in HL-60 cells.

We next investigated whether PCB directly affects splenocytes *in vivo* in order to examine the acute *in vivo* toxicity. PCB (100, 500, 1000 $mg \cdot kg^{-1} \cdot d^{-1}$) was administered in a single i.p. injection to each ICR mouse for 5 d until 6 h before sacrifice. The splenocytes were tested for cell death by DNA fragmentation and caspase assays. During PCB exposure, DNA fragmentation in PCB treated cells could not be detected as it was in control cells (Fig. 2C by DPA reaction or 2D by gel assay, respectively). In Fig. 2E, relative intensity of caspase activation induced by PCB *in vivo* did not change in a concentration-dependent manner. These results indicate that at least PCB did not induce apoptosis in spleen cells within their 5-d exposure to PCB.

Our present results suggest that PCB will not cause extremely severe problems in human immune cells, because serum albumin will bind to PCB and thus inhibit apoptosis in the cells. Nevertheless, we still need to assess the possibility of apoptosis inhibition in blood cells caused by PCB and blood serum. Because we cannot exclude the possibility of PCB damage in other tissues/organs, further studies on the deposition of PCB in adipose cells/tissues may identify its molecular mechanism(s) if PCB influences acute toxicity and shows many side symptoms in various human tissues.

In conclusion, we have demonstrated that, in serum-free medium, PCB induced apoptosis in HL-60 cells as well as in splenocytes *via* a caspase-3-like protease-dependent mechanism. The precise mechanism by which PCB transduces the signal to caspase-3 and finally executes the death machinery remains to be elucidated. The mechanistic elucidation of the apoptotic signal transduction triggered by PCB will shed light on regulation of the immunotoxicity in cells.

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