*Korean J. Toxicol.*
Vol. 13, No. 3, pp. 187-191, 1997

# Apoptotic Cell Death of Mouse Splenocytes by Polychlorinated Biphenyls and Its Prevention by Serum

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*(Received Jine 4, 1997)*
*(Accepted August 11, 1997*

***ABSTRACT*** *: Cell death induced by polychlorinated biphenyls (PCBs), environmental toxicant, was investigated in mouse splenocytes. The fragmentation of intact DNA, a parameter of apoptotic cell death, was evaluated qualitatively by agarose gel electrophoresis analysis and quantitatively by diphenylamine reaction method. PCBs induced apoptotic cell death of splenocytes in a dose- and time- dependent manner. The effect of serum on the apoptotic cell death induced by PCBs was also investigated. The DNA fragmentation induced by PCB treatment in serum-free medium was clearly inhibited by an addition of serum to the culture medium. The decrease of DNA fragmentation due to serum addition was accompanied with the increase of cell viability.*

***Key Words*** *: PCBs, Apoptosis, Splenocyte, Serum*

## I. INTRODUCTION

There are two distinct types of cell death, apoptosis and necrosis, which differ morphologically and biochemically (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). Apoptosis is an active process of cellular self-destruction, whereas necrosis is essentially an accidental process (Cohen, 1993). Apoptotic cells exhibit characteristic changes, including chromatin condensation and membrane blebbing. The most common feature of apoptosis is the fragmentation of DNA into nucleosomal size, which yields a ladder pattern in agarose gel and is a hallmark of apoptotic cell death (Wyllie *et al.*, 1984).

Apoptotic cell death is induced not only by physiological stimuli, but also by nonphysiological stimuli, such as irradiation (Barry *et al.*, 1990; Stephens *et al.*, 1991), stimulation of hormones (Wyllie, 1980; Nicto *et al.*, 1992), toxins (Barry *et al.*, 1990; Morimoto and Bonavida, 1992), and anticancer drugs (Li and Kaminskas, 1987). In recent times, it has been reported that environmental toxicants, such as chromium (Rajaram *et al.*, 1995), mercury (Aten *et al.*, 1995), alkylating agents (O'Connor *et al.*, 1991), or TCDD (McConkey *et al.*, 1988) also induced apoptosis of various cells.

The apoptotic cell death of lymphocytes is induced by external signals, such as glucocorticoids and γ-irradiation (Wyllie *et al.*, 1980), or by the depletion of serum factor(s) (Jiang *et al.*, 1993). Serum-deprivation has been reported to induce apoptotic cell death in lymphocytes and fibroblasts (Lucas *et al.*, 1991; Tamm and Kikuchi, 1991). The mechanism and significance of apoptosis induction by serum deprivation in cell culture are yet to be understood. However, it has been proposed that dependence on external factors for cell survival could be an effective control mechanism for cell number homeostasis *in vivo* (Raff, 1992).

Polychlorinated biphenyls (PCBs), ubiquitous environmental contaminants, elicit a variety of biological effects in laboratory animals, including hepatotoxicity (Parkinson and Safe, 1987), immunotoxicity (Silkworth *et al.*, 1986), carcinogenesis (Hayes, 1987), neurotoxicity (Rogan and Gladen, 1992), and birth defects (Safe, 1986). Although the mechanism involved in PCB-induced immunotoxicity remain unknown, immunotoxicity of PCBs have been widely investigated (Davis and Safe, 1989; Ganey *et al.*, 1993).

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In this study, we investigated the effects of PCBs on murine splenocytes. We have also examined the effect of serum on the PCB-induced cell death of splenocytes. Our study revealed that PCB treatment induced the cell death of splenocytes via apoptotic pathway, and that the apoptotic cell death of splenocytes induced by PCB treatment was prevented by serum addition.

## II. MATERIALS AND METHDOS

### 1. Chemicals

Commercial mixture of polychlorinated biphenyls, Aroclor 1254, was purchased from Chem-Service (West Chester, Pennsylvania), and a stock solution was prepared in DMSO. The final culture concentration of DMSO in all experiments was 0.1% (v/v) or less. DNA molecular marker and RNase were obtained from Behringer Mannheim. FCS was purchased from HyClone.

### 2. Cell culture and treatment

ICR-Kist mice were obtained from Genetic Engineering Research Institute, KIST, Korea. Splenic lymphocytes were obtained by gently disrupting the spleen with syringe plunger, and cultured in RPMI 1640 medium (GIBCO Lab., Grand Island, NY) supplemented with or withoout 10% fetal calf serum and 50 μM 2-mercaptoethanol. Splenocytes were cultured in 24 well plates (Costar, Cambridge, MA) at a density of $1\times10^7$ cells/well in 1 m*l* of culture medium. PCBs were directly added to the culture medium at various concentrations.

### 3. Cell viability

Cell viability was measured by hemocytometry using trypan blue exclusion method. One volume of Trypan blue (0.4%, Sigma) was added to 5 volumes of cell suspension harvested from cultures. The cells were examined by inverted light microscopy. Cell viability was expressed as percent of control.

### 4. Agarose gel electrophoresis

Splenocytes ($1\times10^7$ cells) were harvested from 24 well microplates and centrifuged at 200×g for 10 min. Cell pellets were lysed with 400 μ*l* of lysing buffer (0.2% Triton X-100, 10 mM Tris, and 1 mM EDTA, pH 8.0). Cell lysates were centrifuged for 10 min (13,000×g), and the supernatant containing small DNA fragments was separated from the pellet containing intact DNA; half was used for agarose gel electrophoresis, and the other half, as well as the pellets were used for quantitation of fragmented DNA by diphenylamine reaction.

Agarose gel electrophoresis was performed as described (Illera *et al.*, 1993). The supernatants (200 μ*l*) were extracted with an equal volume of absolute isopropyl alcohol at -20°C overnight. The pellets were completely dried and then resuspended in 100 μ*l* of TE solution (10 mM Tris HCl, 1 mM EDTA, pH 7.4) and 50 μ*l* of loading buffer (15 mM EDTA, 2% SDS, 50% glycerol, 0.5% bromophenol blue, 10 μg/m*l* RNase). The samples were then heated at 65°C for 10 min and analyzed by electrophoresis at 50 V for 40 min on a 1.5% agarose gel containing 0.71 μg/m*l* of ethidium bromide for 40 min with TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0). DNA ladders were visualized by UV light and photographed with a Polaroid MP-4 land camera.

### 5. Quantitation of fragmented DNA

The quantitation of DNA fragmentation was determined by the diphenylamine (DPA) method. Briefly, the pellet containing intact DNA was resuspended in 200 μ*l* of lysing buffer. After addition of perchloric acid to the pellet suspension and the supernatant (remaining 200 μ*l*) at the final concentration of 0.5N, sample tubes were heated at 95°C for 15 min. The content of DNA in both the pellet and the supernatant was determined by diphenylamine reaction. Two volumes of DPA reagent (0.088 M DPA, 98% v/v glacial acetic acid, 1.5% v/v sulfuric acid, and 0.5% v/v of 1.6% acetaldehyde solution) were added to the sample. After overnight incubation at room temperature, aliquots (200 μ*l*) were transferred to 96-well polystyrene microtiter plates and OD at 595nm was measured on ELISA readr (Molecular Devices). The percentage of DNA

fragmentation was expressed as follows :

$$\text{DNA fragmentation (\%)} = \frac{2(\text{OD of supernatant})}{\text{OD of pellet} + 2(\text{OD of supernatant})} \times 100$$

## III. RESULTS AND DISCUSSION

Apoptosis is characterized by DNA fragmentation due to the activation of endonuclease, which cleaves the nuclear DNA into nucleosome-sized units (Bursch *et al.*, 1990; Arends *et al.*, 1990; Arends and Wyllie, 1991). To assess whether PCBs induce the charateristic apoptotic internucleosomal DNA cleavage in murine splenocytes, the DNA samples from the both control and PCB-treated cells were analyzed quantitatively by the diphenylamine method, and qualitatively by the agarose gel electrophoresis.

The splenocytes were maintained in serum-free medium and treated with PCBs at the various concentrations for 4h. Fig. 1 shows a dose-response curve of PCB effect on DNA fragmentation of splenocytes over a 4h culture. Whereas little DNA fragmentations were showed in control cells and splenocytes treated with doses of PCBs as low as 10 μM, the extents of DNA fragmentation in splenocytes were largely increased by the treatment with PCBs above 20 μM. However, at much higher dose (50 μM), the degree of DNA fragmentation was reduced, which might be due to the increase of cell death.

It was confirmed by electrophoretic analysis that the DNA fragmentation produced by PCB treatment was due to the apoptotic cleavage. The electrophoretic analysis of DNA isolated from PCB-treated splenocytes revealed a ladder of DNA fragments with lengths of nucleosomal size, which is typical of apoptotic cell death (Fig. 2). The slight fragmentation of DNA appears in control cells (lane 2), which is considered by the spontaneous apoptosis of splenocytes.

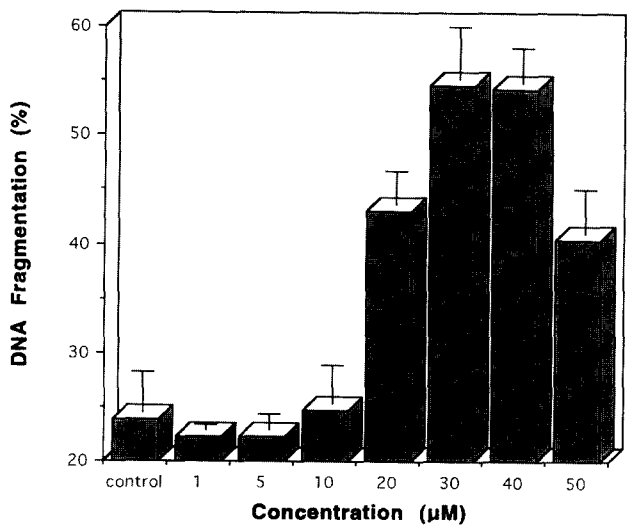


**Fig. 1.** Effect of PCB treatment on the DNA fragmentation of splenocytes. Splenocytes were incubated in serum-free medium with various concentrations of PCBs for 4h. After incubation, splenocytes were harvested, and the amount of DNA fragmentation was determined by diphenylamine reaction as described in Materials and Methods.

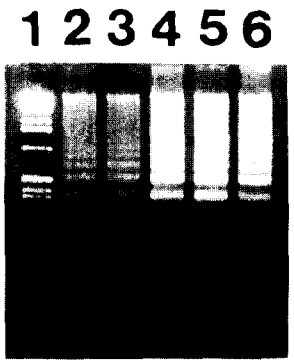


**Fig. 2.** Agarose gel electrophoresis of DNA extracts from PCB-treated splenocytes. Splenocytes were incubated in serum-free medium with various concentrations of PCBs for 4h. After incubation, splenocytes were harvested, and the DNA fragmentation was analyzed in 1.5% agarose gel electrophoresis. Lane 1, 1kb DNA ladder as marker; lane 2, control; lane 3, 10 μM PCBs; lane 4, 20 μM PCBs; lane 5, 30 μM PCBs; lane 6, 40 μM PCBs.

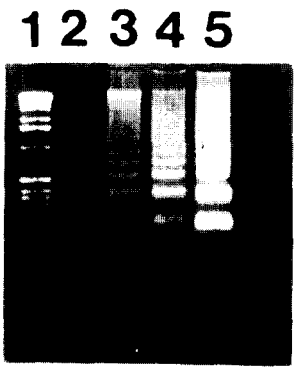


**Fig. 3.** Time course of DNA fragmentation in PCB-treated splenocytes. Splenocytes were incubated in serum-free medium containing 30 μM PCBs for 0, 2, 4, and 24 h (lane 2-5, respectively). At indicated times, splenocytes were harvested and the DNA fragmentation was analyzed as described in Fig. 2. Lane 1 represents 1kb DNA ladder as marker.

Splenocytes treated with 10 μM PCBs also reveal a slight fragmentation like control cells (lane 3). The ladder is clearly detectable in splenocytes treated with 20, 30, and 40 μM (lane 4, 5, and 6, respectively). Fig. 3 shows the time course of DNA fragmentation in splenocytes treated with 30 μM PCBs under serum-free condition. The intensity of ladder bands increased in a time-dependent manner. The present results have demonstrated that splenocytes treated with PCBs in the absence of serum displayed the increased DNA fragmentation in a dose- and time-dependent fashion.

It has been reported that serum-deprivation induced apoptotic cell death in lymphocytes and fibroblasts (Lucas *et al.*, 1991; Tamm and Kikuchi, 1991). Although the mechanisms of apoptosis induction by serum-deprivation are no clearly elucidated, it is considered that the induction of apoptosis in serum-deprived cells could be due either to nutrient deprivation or to absence of specific cytokines that are necessary for survival of cells. It is also known that chronic mitogenic stimulation of growth cultures and adding of promoters may act as negative regulators of cell death induced by different factors (Tomei *et al.*, 1985).

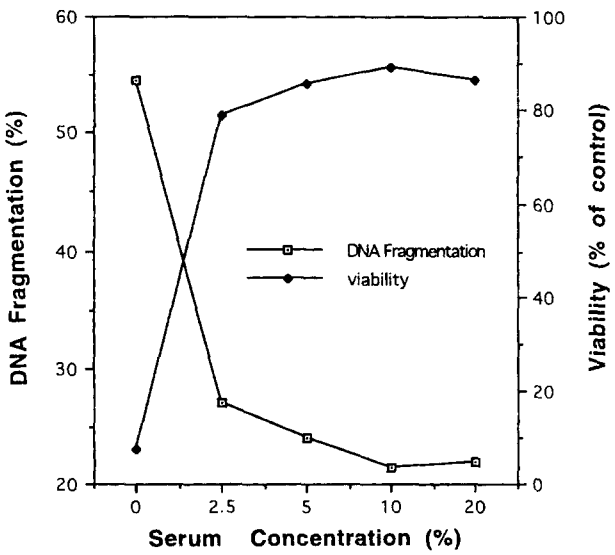


**Fig. 4.** Effect of serum concentrations on the DNA fragmentation and cell viability by PCB treatment. After incubation of splenocytes in serum-free medium containing 30 μM PCBs for 4 h, splenocytes were harvested. DNA fragmentation and cell viability were determined by diphenylamine reaction and trypan blue exclusion method, respectively.

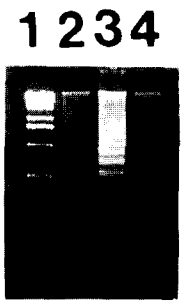


**Fig. 5.** Effect of serum addition on PCB-indued apoptosis. Splenocytes were incubated with 30 μM PCBs in culture medium without serum (lane 3) or with 10% serum (lane 4) for 4 h. After incubation, splenocytes were harvested, and the DNA fragmentation was analyzed in 1.5% agarose gel electrophoresis. Lane 1 represents 1kb DNA ladder as marker and lane 2, control.

In this background, we investigated whether the presence of serum might affect the PCB-induced apoptotic cell death of splenocytes. As shown in Fig. 4, the increase of serum concentration in culture medium containing 30 μM of PCBs led to the decrease of DNA fragmentation and also the increase of viability of splenocytes. Agarose gel electrophoresis also confirmed that DNA fragmentation of splenocytes treated with 30 μM PCBs in the absence of serum was strongly inhibited by serum addition (Fig. 5). These results suggested that growth factors in serum might play an important role in the viability of splenocytes treated with PCBs. However, the prevention of PCB-induced apoptosis of splenocytes by the addition of serum might be due to the non-specific binding of PCBs to serum component. This possibility will be examined in our further studies.

## ACKNOWLEDGEMENTS

We thank Y.H. Kim and D.H. Hong for technical assistance. This work was supported in part by a research grant from Kyonggi University and HAN project of Ministry of Science and Technology, Republic of Korea.

## REFERENCES

Arends, M.J., Morris, R.G. and Wyllie, A.H. (1990): Apoptosis. The role of the endonuclease, *Am. J. Pathol.*, **136**, 593-608.

Arends, M.J. and Wyllie, A.H. (1991) : Apoptosis : mechaniss and roles in pathology, *Int. Rev. Exp. Pathol.*, **32**, 223-254.

Aten, J., Prigent, P., Poncet, P., Blanpied, C., Claessen, N., Druet, P. and Hirsch, F. (1995) : Mercuric chloride-induced programmed cell death of a murine T cell hybridoma. I. Effect of the proto-oncogene Bcl-2, *Cell. Immunol.*, **161**, 98-106.

Barry, M.A., Behnke, C.A. and Eastman, A. (1990) : Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins, and hyperthermia, *Biochem. Pharmacol.*, **40**, 2353-2362.

Bursch, W., Kleine, L. and Tenniswood, M.(1990) : The biochemistry of cell death by apoptosis, *Biochem. Cell Biol.*, **68**, 1071-1074.

Cohen, J.J.(1993) : Overview : Mechanisms of apoptosis, *Immunol. Today*, **14**, 126-130.

Davis, D. and Safe, S. (1989) : Dose-response immunotoxicities of commercial polychlorinated biphenyls (PCBs) and their interaction with 2,3,7,8-tetrachlorodibenzo-p-dioxin, *Toxicol. Lett.*, **48**, 35-43.

Ganey, P.E., Sirois, J.E., Denison, M., Robinsone, J.P. and Roth, R.A. (1993) : Neutrophil function after exposure to polychlorinated biphenyls *in vitro*, *Environ. Health Perspect.*, **101**, 430-434.

Hayes, M.A. (1987) : Carcinogenic and Mutagenic Effects of PCBs. Springer-Verlag, New York.

Illera, V.A., Perandones, C.E., Stunz, L.L., Mower, D.A. Jr. and Ashman, R.F. (1993) : Apoptosis in splenic B lymphocytes. Regulation by protein kinase C and IL-4. *J. Immunol.*, **151**, 2965-2973.

Jiang, C., Lu, J., Garcia, G. and Thompson, H.J. (1993) : Spontaneous nucleosomal DNA fragmentation in murine leukemic L1210 cells, *Biochem. Biophys. Res. Commun.*, **194**, 836-841.

Kerr, J.F.R., Wyllie, A.H. and Currie, A. R.(1972) : Apoptosis : A basic biological phenomenon with wide-ranging implications in tissue kinetics, *Br. J. Cancer*, **26**, 239-257.

Li, J.C. and Kaminskas, E. (1987) : Progressive formation of DNA lesions in cultured Ehrlich ascites tumor cells treated with hydroxyurea, *Cancer Res.*, **47**, 2755-2758.

Lucas, M., Solano, F. and Sanz, A. (1991) : Induction of programmed cell death (apoptosis) in mature lymphocytes, *FEBS Lett.*, **279**, 19-20.

McConkey, D.J., Hartzell, P., Duddy, S.K., Hakansson, H. and Orrenius, S. (1988) : 2,3,7,8-tetrachloro- dibenzo-p-dioxin kills immature thymocytes by $Ca^{+2}$-mediated endonuclease activation, *Science*, **242**, 256-259.

Morimoto, H. and Bonavida, B.(1992) : Diphtheria toxin and Pseudomonas A toxin-mediated apoptosis. ADP ribosylation of elongation factor-2 is required for DNA fragmentation and cell lysis and synergy with tumor necrosis factor-alpha, *J. Immunol.*, **149**, 2089-2094.

Nicto, M. A., Gonzalez, A., Gambon, F., Diaz, E.F. and Lopez, R.A.(1992) : Apoptosis in human thymocytes after treatment with glucocorticoids, *Clin. Exp. Immunol.*, **88**, 341-344.

O'Connor, P.M., Wassermann, K., Sarang, M., Magrath, I., Bohr, V. A. and Kohn, K. W.(1991) : Relationship between DNA cross-links, cell cycle, and apoptosis in Burkitt's lymphoma cell lines differing in sensitivity to nitrogen mustard, *Cancer Res.*, **51**, 6550-6557.

Parkinson, A. and Safe, S. (1987) : Mammalian Biologic and Toxic Effects of PCBs. Srpinger-Verlag, New York.

Raff, M.C. (1992) : Social control on cell survival and cell death, *Nature*, **356**, 397-398.

Rajaram, R., Nair, B.U. and Ramasami, T.(1995) : Chromium(III) induced abnormalities in human lymphocyte cell proliferation : Evidence for apoptosis, *Biochem. Biophys. Res. Commun.*, **210**, 434-440.

Rogan, W.J. and Gladen, B.C.(1992) : Neurotoxicology of PCBs and related compounds, *Neuro Toxicology*, **13**, 27-36.

Safe, S.H.(1986) : Comparative toxicology and mechanism of action of polychlorinated dibenzodioxins and dibenzofurans, *Annu. Rev. Pharmacol. Toxicol.*, **26**, 371-399.

Silkworth, J.B., Antrim, L. and Sack, G.(1986) : AH receptor mediated suppression of the antibody response in mice is primarily dependent on the AH phenotype of lymphoid tissue, *Toxicol. Appl. Pharmacol.*, **86**, 380-390.

Stephens, L.C., Ang, K.K., Schultheiss, T.E., Milas, L. and Meyn, R.E.(1991) : Apoptosis in irradiated murine tumors, *Radiat. Res.*, **127**, 308-316.

Tamm, I. and Kikuci, T. (1991) : Activation of signal transduction pathways protects quiescent Balb/c-3T3 fibroblasts against death due to serum deprivation, *J. Cell Physiol.*, **148**, 85-95.

Wyllie, A.H.(1980) : Glucocorticoid-induced thymocyte apoptosis is associated with endogeneous endonuclease activation, *Nature*, **284**, 555-556.

Wyllie, A.H., Kerr, J.F.R. and Currie, A. R. (1980) : Cell death : the significance of apoptosis, *Int. Rev. Cytol.*, **68**, 251-306.

Wyllie, A.H., Morris,R.G., Smith, A.L. and Dunlop, D. (1984) : Chromatin cleavage in apoptosis : association with condensed chromatin morphology and dependence on macromolecular synthesis, *J. Pathol.*, **142**, 67-77.

Tomei, L.D., Shapiro, J.P. and Cope, F.O. (1993) : Apoptosis in C3H/10T1/2 mouse embryonic cells, *Proc. Natl. Acad. Sci.*, **90**, 853-857.