Toxic Effects of Polychlorinated Biphenyls in Myogenic Cells

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Polychlorinated biphenyls (PCBs) are molecules structurally related to dioxins and were widely used in the past in industrial applications. Their chemical stability and high lipophilicity make them persistent pollutants and dangerous occupational contaminants. Our previous results showed that "low concentrations" of PCBs (≤ 10 μg/ml, using the commercial mixture *Aroclor* 1254) inhibit *in vitro* hormonal induced myogenic differentiation. Here we extend the notion of PCBs as inhibitors of myogenic differentiation induced by lower serum medium. *Aroclor* 1254 treatment of myogenic cells, induced to differentiate in low serum medium, inhibits (at concentrations ≤ 10 μg/ml) the extent of fusion and the size of the myotubes as well as the accumulation of sarcomeric myosin. We also investigated whether the cell mortality observed at *Aroclor* 1254 concentrations ≥ 10 μg/ml is due to necrosis or to apoptosis. Using different approaches, we observed that *Aroclor* 1254 causes necrosis but not apoptosis of myogenic cells in a dose–dependent manner. In addition, we report that *Aroclor* 1254 induces release of the intracellular enzymes lactate dehydrogenase (LDH) and creatine kinase (CK) in a dose–dependent manner. These results may explain the CK serum elevation observed in patients exposed to high doses of PCBs.

Key words —— polychlorinated biphenyls, myoblast differentiation, apoptosis, necrosis

INTRODUCTION

Polychlorinated biphenyls (PCBs) are a family of halogenated aromatic hydrocarbons used as mixtures of congeners characterised by different numbers and positions of the chlorine substitutions. Due to their thermal stability and high dielectric constant, these synthetic chemicals were used since 1929 as lubricants, insulating materials, and cooling fluids for industrial transformers, capacitors and other electrical equipment. PCB production stopped in the United States in 1977 because of growing evidence of their widespread environmental and human toxicity.[1)] In the following years other countries have adopted the same measures. Nevertheless, extensive use and improper disposal practices make PCBs ubiquitous environmental contaminants. Because of their chemical stability and lipophilic nature, these compounds accumulate in the food chain, inducing injurious effects in living organisms. Besides being considered as putative carcinogenic compounds, PCBs are toxic to the nervous, immune, endocrine, and reproductive systems, both in animals and humans.[2)] Embryos, fetuses, and newborns appear to be particularly susceptible to PCBs. Differet to adult animals in which low doses of contaminants may produce no apparent effects, offspring born to exposed mothers may be seriously compromised, some effects being manifest at birth (*e.g.* anatomical abnormalities), others becoming evident during postnatal or adult life (*e.g.* cognitive disorders and reduced fertility, respectively).[3)] Growth is reduced in animals exposed to PCBs during development[4)] and the incidence of malformations is increased.[5,6)] In humans, prenatal exposure to PCBs has been associated with lower body weight,[7)] shorter height, and decreased total lean mass.[8)] Since skeletal muscle significantly contributes to body mass and PCBs accumulate in skeletal muscle,[9)] a direct toxicity of PCBs in this tissue may be hypothesized. This hypothesis is further supported by our previous data showing that PCBs specifically inhibit the differentiation of both skeletal myogenic cell lines and pri-

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mary cultures of fetal myoblasts.[10] The effects of PCBs on myogenic cell differentiation occur in the absence of overt toxicity although ultrastructural analysis revealed subtle alterations compatible with cellular responses to xenobiotics.[11] In the same study, we also compared the effects of three commercial PCB mixtures (*Aroclor* 1232, *Aroclor* 1254 and *Aroclor* 1262, the last two digits indicating the approximate percentage of chlorine content) on muscle differentiation. We have shown that *Aroclor* 1254 has an intermediate effect compared to the other mixtures. *Aroclor* 1254 is widely used for *in vitro* studies because its content of more than 60 PCB congeners is representative of PCB environmental pollution.[12] For these reasons, we chose *Aroclor* 1254 to further analyse the effects of PCBs on skeletal muscle cells.

In the present study, we investigated the biological effects of low doses of *Aroclor* 1254 on the myogenic differentiation obtained by culturing L6C5 cells in low serum medium. We also studied the effects on cell viability of higher doses of *Aroclor* 1254 (> 10 μg/ml). In particular, we addressed the point of the possible mechanism of myoblast and myotube PCB-dependent toxicity.

MATERIALS AND METHODS

PCBs —— The commercial PCB mixture routinely used throughout this study (*Aroclor* 1254) was purchased from Ultra Scientific (North Kingston, RI, U.S.A.), originally dissolved in iso-octane. After solvent evaporation, obtained by gentle nitrogen flow, PCBs were redissolved in dimethyl sulfoxide (DMSO) (1000x final concentration). Solvent alone was added to controls (0.1%, v/v final concentration).

Cell Cultures —— L6C5 cells,[13] a sub-clone of the rat myogenic L6 cell line[14] originally obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), were used throughout this study. Myoblasts were plated at the density of 1.0–1.3×10^4 cells/cm^2 in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO, U.S.A.) supplemented with 50 μg/ml gentamycin and 20 mM Hepes/NaOH (pH 7.4) and 10% fetal bovine serum. After 24 hr, sub-confluent cultures were switched to the differentiation medium (DM: DMEM supplemented with 2% horse serum), and PCBs were added as appropriate. Media were renewed every 2 days during the cultures. Fetal bovine serum and horse serum were purchased from Euroclone Ltd. (Wetherby, U.K.).

Measurement of Myoblast Viability —— Twenty-four hours after plating, L6C5 myoblasts were shifted to DM and cultured in the absence or presence of different concentrations of PCBs. After 24 hr the spent media, containing cells spontaneously detached from the substrate, were collected, centrifuged at 800 *g* and the cell pellets were resuspended in 200 μl of phosphate buffered saline (PBS). Aliquots of the cell suspensions were stained with trypan-blue (TB, 0.4%, v/v) and the cells counted using a Thoma chamber, performing 10 counts for each specimen. Conversely, the cells still adherent to the dish surface were repeatedly washed with PBS, fixed in 4% formaldehyde in PBS for 30 min at 4°C, and stained with Hoechst 33342 for 10 min at room temperature. Fluorescent nuclei were counted by evaluating ten randomly selected microscopic fields on each specimen at 340–380 nm using a Zeiss microscope (Göttingen, Germany).

Myoblast Differentiation Studies —— L6C5 cells were cultured in DM in the absence or presence of PCBs. After 6 days, myotubes were washed and fixed in a solution of 0.2% Triton X-100 and 4% formaldehyde in PBS for 20 min at 4°C. Each sample was incubated for 1 hr at room temperature with undiluted anti-sarcomeric myosin monoclonal antibody MF-20[15] and then stained using an immunoperoxidase labelling kit (Histostain-Plus kit, Zymed Laboratories Inc., South San Francisco, CA, U.S.A.) as detailed in De Lellis.[16]

Ultrastructural Analysis —— L6C5 cells, cultured in DM either in the presence or absence of PCBs, were processed and analysed by transmission electron microscopy (TEM) as previously reported.[17] Briefly, the cells were embedded in Epon 812 following a standard procedure; fragments were excised from the Epon layers, re-embedded and sectioned perpendicularly to the cell monolayers at a nominal thickness of 50 nm. After staining with uranyl acetate and lead hydroxide, the specimens were photographed with a Hitachi H7000 electron microscope (Tokyo, Japan).

Morphological Studies of Necrosis and Apoptosis —— Necrosis was induced by exposing L6C5 cells to 20 mM H_2O_2 in DM.[18] After a 36 hr treatment in DM containing 100 ng/ml insulin-like growth factor 1 (IGF-1), apoptosis was triggered by culturing L6C5 cells in serum-free medium, a treatment able to induce apoptosis in approximately 60% of cultured cells.[19] Both treatments were carried out ei-

ther for 1 or 48 hr. At the end of the 1 hr treatments, the cells were washed and incubated for 7 min at 37°C in the presence of 10 μg/ml Hoechst 33342 in PBS. Thereafter, the cells were washed, incubated with 5 μM propidium iodide (PI) in PBS for 10 min at room temperature, repeatedly washed, and fixed in a solution of 4% formaldehyde in PBS for 30 min at 4°C. Stained specimens were observed and photographed under a fluorescence microscope (using the 340–380 nm filter for Hoechst 33342 and the 530 nm filter for PI). By this procedure only nuclei of necrotic cells show a red fluorescence when observed with the PI filter.[20] At the end of the long term incubation (48 hr), spontaneously detached cells were gathered by collecting spent media, and adherent cells were stripped using a 0.25% trypsin-EDTA solution. Ethanol was added to the cell suspension (1 : 3, v/v) for 15 min at 4°C. After centrifugation at 800 *g*, the cell pellets were fixed in an ethanol-acetic acid solution (5 : 1, v/v) and aliquots, stained with Hoechst 33342, were analysed by fluorescence microscopy.

Analysis of Cell Apoptosis by Flow Cytometry ——— L6C5 cells were cultured for 24 hr in DM without or with PCBs. After trypsinization the cells were resuspended in 200 μl of PBS, mixed with 10 volumes of ice-cold 70% ethanol, and incubated for 30 min at 4°C. Cells were harvested by centrifugation at 800 *g* and resuspended in 800 μl of PBS containing 0.1 mg/ml of ribonuclease (RNase) and 40 μg/ml of PI for 30 min at 4°C. DNA-content analysis was performed on about 500000 cells in 0.5 ml of PBS per sample using a cytofluorometer (Calibur FACS, Becton Dickinson, NJ, U.S.A.) equipped with an argon-ion laser tuned to 488 nm.

Quantitative Analysis of Extracellular Lactate Dehydrogenase (LDH) and Creatine Kinase (CK) Release ——— Necrotic damage induced by PCBs was assessed in myoblasts by measuring extracellular lactate dehydrogenase (LDH) activity, and in myotubes by measuring the extracellular LDH and creatine kinase (CK) activities. L6C5 myoblasts or myotubes were exposed for 1 or 5 hr in DM without or with PCBs. Spent media were collected, centrifuged at 800 *g* and the supernatants were added to commercially available reagents to test the extracellular activities of LDH (Boehringer Mannheim Systems, Indianapolis, IN, U.S.A.) and CK (Roche Diagnostics, Indianapolis, IN, U.S.A.). Enzymatic reactions were performed for 10 min at 37°C and spectrophotometrically evaluated using a BM/Hitachi 717 chemistry analyser (Boehringer Mannheim GmbH, Germany).

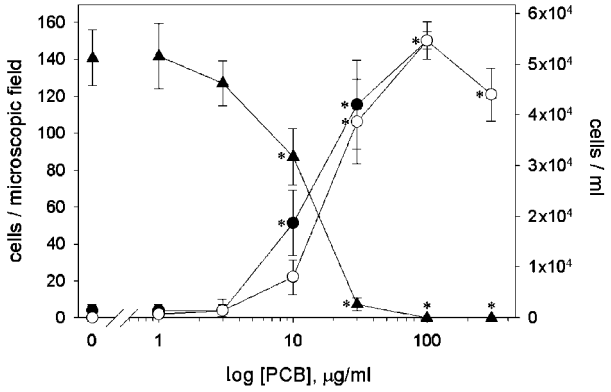

Fig. 1. Measurement of the Number of Viable, Spontaneously Detached, and Dead Cells in L6C5 Cultures Exposed to PCBs

L6C5 myoblasts were cultured for 24 hr in DM in the absence or presence of increasing concentrations of PCBs. Spontaneously detached (●) and dead trypan-blue stained cells (○) were measured by Thoma chamber and expressed as cells per milliliter of spent media ± SEM. Viable myoblasts (▲), still adherent to the dishes, were counted in ten randomly selected fields and expressed as the average of the total number of cells per microscopic field ± SEM. *Significantly different from control values ($p \leq 0.05$).

Statistical Analysis ——— Data are presented as the mean ± SEM. Statistical significance was assessed using Student's *t*-test.

RESULTS

Effects of PCBs on L6C5 Cells Adhesion and Viability

L6C5 cells were cultured for 24 hr in the presence of *Aroclor* 1254 at concentrations ranging from 0 to 300 μg/ml. Exposure of the cells to PCB concentrations up to 3 μg/ml did not affect cell density (Fig. 1). Treatment with PCBs at concentrations higher than 3 μg/ml induced a dose–dependent decrease in cell density, with the half-maximal effective concentration (EC_{50}) being approximately 20 μg/ml (Fig. 1). Concomitantly the number of floating-free cells increased in proportion with the rise in PCB concentration. Since cell detachment could be the consequence of cell death, the viability of the suspended cells was assessed by the TB-test. As shown in Fig. 1, most of the myoblasts floating free in the spent media were found to be dead by TB staining.

These data indicate that the exposure of L6C5 cells to PCBs reduces cell density and induces cell death.

Effects of Low Doses of PCBs

Our previous results showed that low concentrations of *Aroclor* 1254 (≤ 10 μg/ml) inhibit myoblast differentiation in a synthetic medium containing hormones like arginine-vasopressin (AVP) and IGF-1.[21] In order to verify that this effect on myogenic differentiation was not due to impairment of the hormonal signal transduction, L6C5 cells were induced to differentiate in low serum medium in the absence or presence of *Aroclor* 1254. Myoblast fusion into multinucleated myotubes and sarcomeric myosin accumulation, both parameters of cell terminal differentiation, were simultaneously evaluated by immunoperoxidase morphological analysis using an anti-myosin antibody. As shown in Fig. 2, the addition of 0 (Fig. 2a), 4 (Fig. 2b), and 8 μg/ml (Fig. 2c) of *Aroclor* 1254 induced a dose–dependent reduction in myotube number and size. Moreover, PCBs dose–dependently inhibited intracellular myosin accumulation. In the sample exposed to a moderately toxic concentration (10 μg/ml) of PCBs (Fig. 2d), no myotubes were detectable and the initial decrease in cell number was appreciable.

These results were confirmed and extended by transmission electromicroscopy. The ultra-structural effects of a non-toxic low concentration (4 μg/ml) of PCBs on L6C5 myoblasts differentiating in low serum for 24 hr showed inhibition of initial sarcomerogenesis and signs of toxicity, such as the accumulation of vacuolar structures in the cytoplasm (Fig. 3b).

Taken together these data confirm that doses of PCBs lower than 10 μg/ml inhibit the differentiation of L6C5 cells cultured in low-serum medium.

Effects of High Doses of PCBs

To assess the type of PCB-induced cell death, L6C5 cells were incubated for 1 hr in DM alone (control), in serum-free complete medium (apoptosis condition), in DM containing high concentrations of H_2O_2 (necrosis condition), and in DM plus 30 μg/ml PCBs. As shown in Fig. 4, cells cultured in DM did not show any uptake of PI into the nuclei. Conversely, PI-stained nuclei were clearly detectable in the cells exposed to H_2O_2 (Fig. 4d) and in the PCB-treated cells (Fig. 4h) but not in the cells undergoing apoptosis (Fig. 4f).

Since no sign of apoptosis was evident after 1 hr, all treatments were prolonged. After 48 hr, most of the H_2O_2-treated cells, serum-free cultured cells, and PCB-treated cells spontaneously detached from the dish surface. As shown in Fig. 5a, nuclei of DM

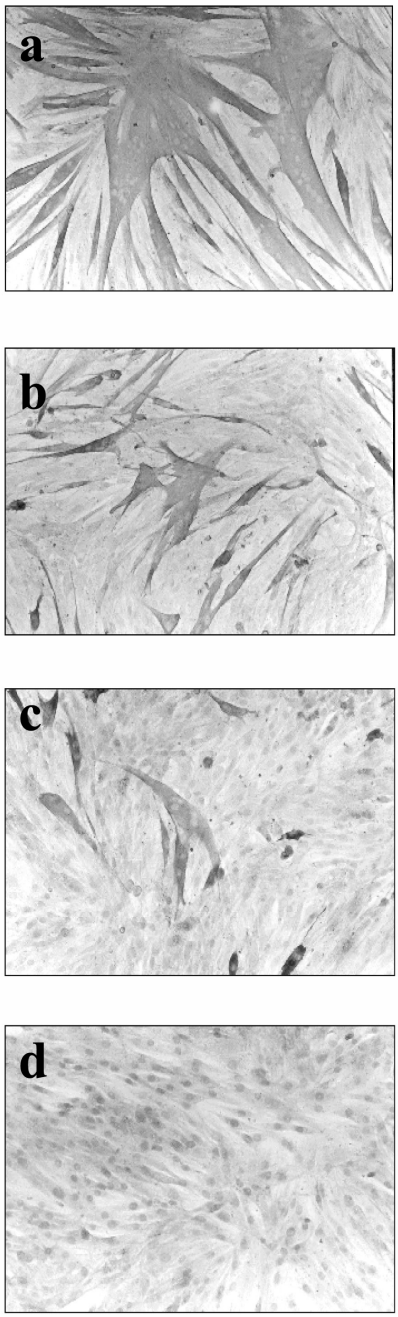

Fig. 2. Morphological Analysis of the Effects of Different Concentrations of PCBs on Myoblast Differentiation

L6C5 cells were cultured for 6 days in DM in the absence (a) or presence of 4 μg/ml (b), 8 μg/ml (c), and 10 μg/ml (d) of PCBs. Immunocytochemistry was performed to detect myosin expression.

treated cells, enzymatically detached from the substrate, appeared normal in size and showed homogenous chromatin. PCB- and H_2O_2-treated cells (Figs. 5b and 5d) showed uniformly stained nuclei with no picnotic chromatin, whereas only the nuclei of myoblasts cultured in serum-free medium presented signs of apoptosis such as nuclear fragmen-

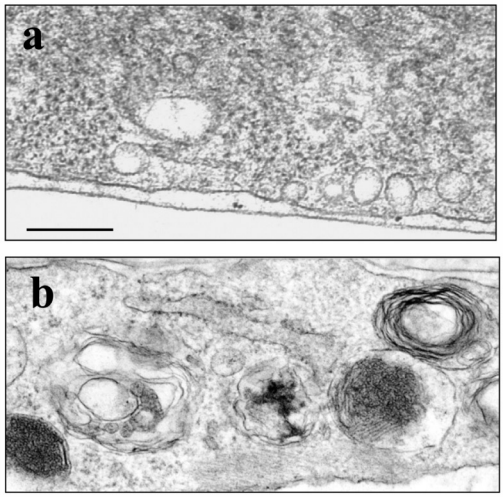

Fig. 3. Ultrastructural Analysis of the Effects of a Low Concentration of PCBs on L6C5 Myoblasts

Cells were cultured for 24 hr in DM without (a) or with 4 μg/ml PCBs (b). Each specimen was sectioned perpendicularly to the dish surface and observed by transmission electromicroscopy. Bar represents 0.2 μm.

tation and DNA condensation (Fig. 5c).

Apoptotic cells have a reduced amount of DNA and this feature can be evaluated measuring the percentage of the cells in the M1 population (a subpopulation with a DNA content lower than diploid) by cytofluorimetry. For this reason L6C5 myoblasts, cultured for 24 hr in DM in the presence of 0, 8, and 30 μg/ml of *Aroclor* 1254, were analysed by cytofluorimetry and M1 populations were compared. As shown in Fig. 6, there was no increase in the M1 population in the PCB-treated samples compared to the control.

The loss of cell membrane integrity is a central event in necrosis phenomena and the measurement of the activity of intracellular enzymes (such as LDH or CK) released in the culture medium is a quantitative method to measure cellular necrosis. Fig. 7a shows that L6C5 myoblasts released LDH in the extracellular medium in the presence of increasing concentrations of PCBs (0–300 μg/ml). The increase in LDH activity was clearly evident after 1 hr and reached its maximum after 5 hr.

In order to assess whether PCBs can induce cell damage in differentiated L6C5 cells, CK and LDH activities were measured in the extracellular medium of myotubes cultured for 5 hr in DM plus *Aroclor* 1254 (0–300 μg/ml). PCB treatment markedly increased the release of both LDH and CK from myotubes, with a maximal effect at 100 μg/ml (Fig. 7b). These results suggest that PCBs have direct toxic effects on both L6C5 myoblasts and myotubes, inducing necrosis without triggering programmed cell death.

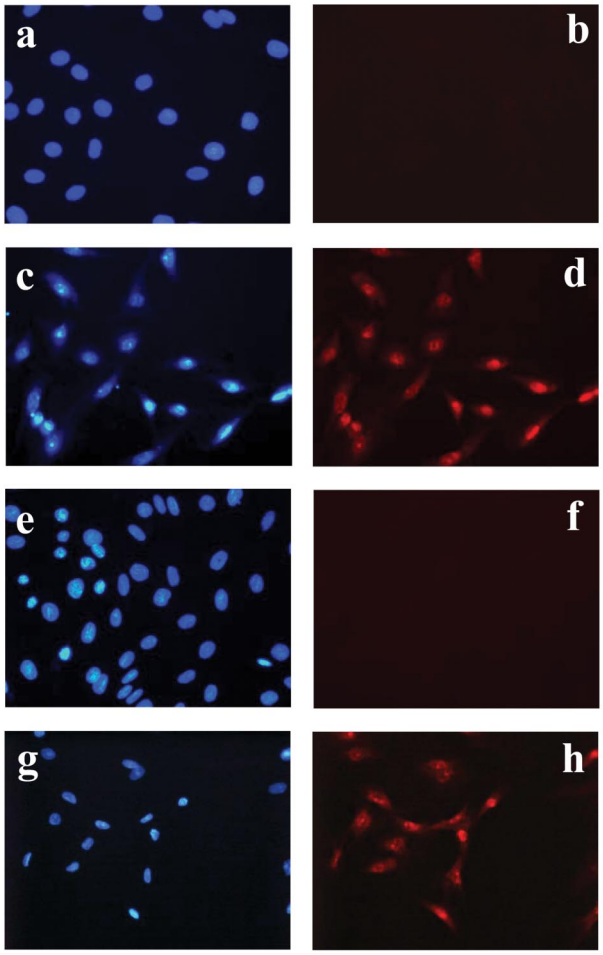

Fig. 4. Double Staining Analysis of Short Time Effects of High Concentrations of PCBs

L6C5 cells at 80% confluence were incubated in DM alone as control (a, b); in DM + 20 mM H_2O_2 as necrosis positive control (c, d); in serum free medium (after 36 hr treatment in DM with 10^{-9} M IGF-1) as apoptosis positive control (e, f); and in DM supplemented with 30 μg/ml PCBs (g, h). After 1 hr of these treatments, the cultures were fixed and stained. For each sample, the same microscopic field was observed using the Hoechst filter (left pictures) and the 530 nm filter for PI (right ones).

DISCUSSION

The results obtained in this study extend our previous findings showing that low doses of PCBs inhibit L6C5 myoblast differentiation, achieved by culturing cells in serum-free medium added to supraphysiological concentrations of AVP and IGF-1. Since we observed a decrease in differentiation in L6C5 cells cultured in low serum medium in

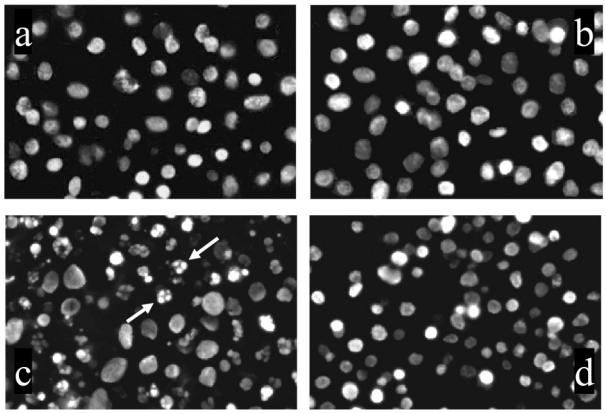

Fig. 5. L6C5 Nuclear Features after Long Term Treatment with High Concentrations of PCBs

Sub-confluent cells were treated in DM alone (a); in DM added to 20 mM H_2O_2 (b); in serum free medium after 36 hr treatment in DM with 10^{-9} M IGF-1 (c); and in DM supplemented with 30 μg/ml PCBs (d). After 48 hr, spontaneously and enzymatically detached cells were collected, fixed, stained with Hoechst, and observed using the appropriate filter. Arrows indicate apoptotic nuclei.

the presence of *Aroclor* 1254 (Fig. 2), we hypothesize that PCBs can interfere with the molecular machinery of L6C5 differentiation rather than inhibiting AVP and IGF-1 hormonal signalling. Since several of the biological effects of PCB congeners are mediated by the aryl hydrocarbon receptor,[22] a transcription factor belonging to the basic helix-loop-helix family,[23] the involvement of such a receptor in the skeletal muscle response to PCBs is currently under investigation in our laboratory.

Beyond inhibiting L6C5 differentiation, PCB treatment induced cell damage (Fig. 3), which became progressively more evident at *Aroclor* 1254 doses ≥ 10 μg/ml, leading to cell death. Two main types of cell death, apoptosis and necrosis, characterized by different mechanisms and physiological roles,[24] have been described so far. Apoptosis is a slow process, actively controlled by the cell through a sequence of programmed events such as condensation and fragmentation of chromatin and formation of apoptotic bodies. Phagocytosis of apoptotic fragments by macrophages allows recycling of useful substrates without involving surrounding cells, thereby contributing to organism homeostasis. Apoptosis can be elicited both by physiological (*e.g.* cell aging) and pathological (*e.g.* gp120 HIV glycoprotein, DNA damage induced by ionizing radiations, *etc.*) stimuli. *In vitro* it can be induced by the lack of those factors which are physiologically required for cell survival (growth factors, hormones, nutrients).

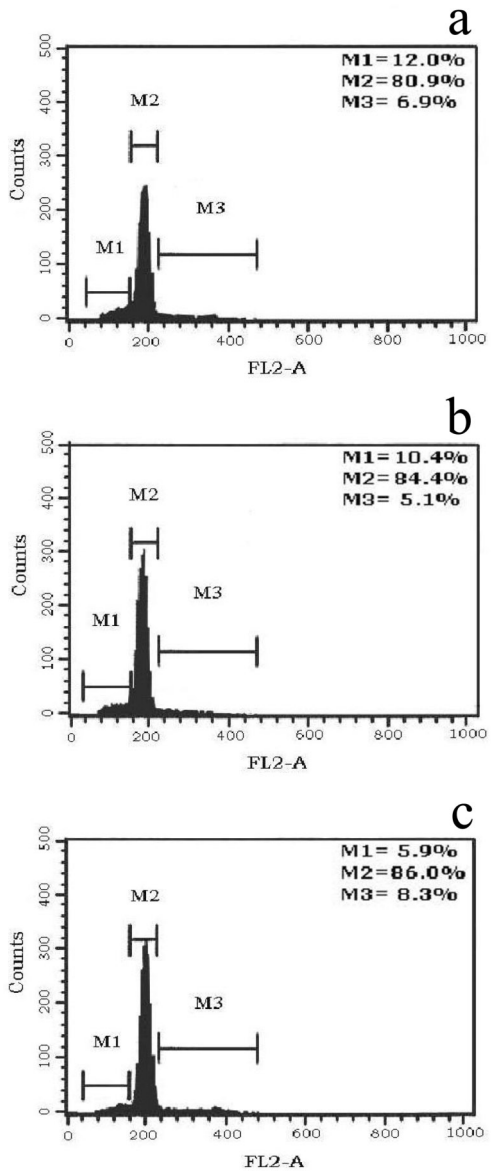

Fig. 6. Analysis of PCB-Induced Apoptosis Performed by Cytofluorimetric Assay

L6C5 cells were cultured for 24 hr in DM with 0 (a), 8 (b), and 30 μg/ml (c) of PCBs. Each cell population was sorted into three subpopulations (M1, M2 and M3) on the bases of different DNA content. M1 counts represent the amounts of apoptotic cells.

On the other hand, necrosis is an acute, pathological phenomenon occurring in cells exposed to various kinds of harmful stimuli, leading to functional impairment of the ionic selectivity of the cell membrane. Hence, various ions (*e.g.* calcium) accumulate in the cytoplasm and cause osmotic swelling of the cell, ultimately resulting in cell disruption and release of its content. In particular the release of lysosomal enzymes directly damages surrounding cells and causes an inflammatory response. It is therefore important to determine whether a patho-

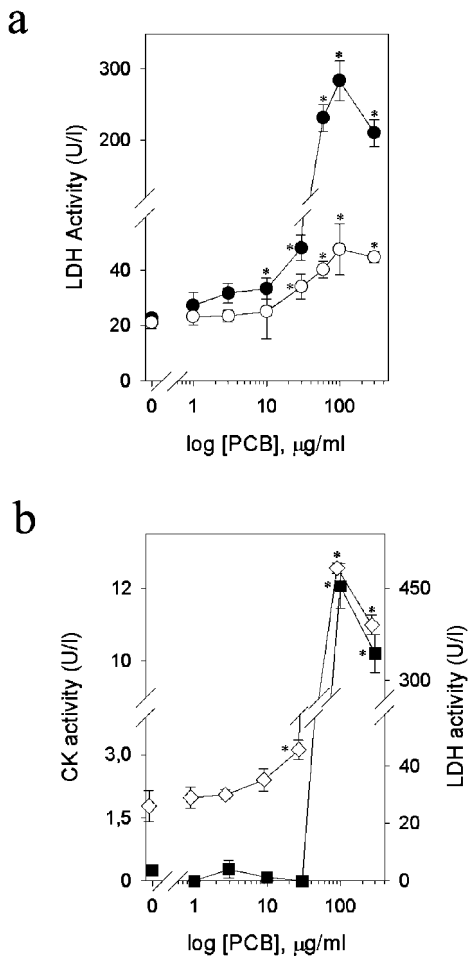

Fig. 7. Quantitative Evaluation of PCB-Induced Necrosis Evaluated by LDH and CK Activities in the Extracellular Medium

(a) L6C5 myoblasts were treated for 1 hr (○) and 5 hr (●) in DM alone or supplemented with increasing concentrations of PCBs. LDH extracellular activity was estimated as reported in the Materials and Methods. (b) L6C5 myotubes were treated for 5 hr in DM alone or supplemented with increasing concentrations of PCBs. CK (■) and LDH (◇) extracellular activities were evaluated as described in the Materials and Methods. *Significantly different from control values ($p \leq 0.05$).

genic stimulus activates an apoptotic program or necrosis.

Many studies have been carried out in order to establish which kind of cell death is caused by PCBs. Necrosis induced by PCBs has been reported in hepatocytes[25,26] and bronchiolar Clara cells,[27] both cell types characterized by cytochrome P-450 dependent oxidative metabolism. In these cells, PCBs induce the formation of epoxide compounds, activating downstream events affecting cell survival. Other studies demonstrated that PCBs were able to induce apoptosis in cell cultures of human neuroblastoma,[28] in murine spleen cells[22] and in primary cultured spleen cells of animals fed with PCB contaminated food.[29] In rat hepatocytes, PCBs can also act as anti-apoptotic and mitogenic compounds leading to cell proliferation and hepatic focal lesions.[30]

Our findings demonstrate that the effects of PCBs on L6C5 cells are related to their concentration: at low concentrations (< 10 µg/ml) PCBs inhibit L6C5 myoblast differentiation determining ultrastructural toxic alterations,[10] whereas at high concentrations (≥ 10 µg/ml) *Aroclor* 1254 induces toxic effects leading to extensive or total myoblast death. The dose–dependency of these effects indirectly suggests that necrosis is involved, since apoptosis, being a process activated by a specific molecular mechanism,[31] does not need a threshold to be triggered. Moreover, since flogosis is a central event in necrosis, it is worthwhile considering that PCBs can act as strong pro-inflammatory agents[32] and non-steroidal anti-inflammatory agents (simultaneously administered to PCBs) decrease PCB toxicity in chick embryos.[33] The total lack of apoptotic features in PCB-treated L6C5 cells confirm that these chemicals induce necrosis in cultured myoblasts. The evidence that myotubes exposed to high doses of PCBs release LDH and CK suggests that myotubes are sensitive to PCB toxicity as well as undifferentiated L6C5 cells.

Our results also provide a biological explanation to clinical alterations observed in humans exposed to PCBs. For example, muscle pain has been reported as a common symptom in workers occupationally exposed to PCBs.[34] The victims of two mass poisoning incidents that occurred in Japan in 1968 (Kanemi-Yusho syndrome) and in Taiwan in 1979 (Yu-Cheng syndrome) in which cooking oil was accidentally contamined with high quantities of PCBs and dioxins, developed chloracne, hyperpigmentation of the skin and nails, peripheral neuropathy, and other signs and symptoms of intoxication.[35] Interestingly, recent results of routine medical checkups have shown significant elevation of serum CK in about 20% of the Kanemi-Yusho patients.[36,37] Since the serum increase in CK is often related to hypothyroidism,[38] and PCBs are known as thyroid hormone disruptors,[4] some of their toxic effects on growth and development were suspected to be related to the impairment of thyroid activity.[39] However, no relationships among PCBs, CK, and thyroid hormones were observed in Kanemi-Yusho patients.[36] Conversely, *in vivo* studies showed atrophy of skeletal muscle fibers in PCB-exposed rats but a statistically significant elevation of serum CK was not observed in the same animals.[40,41]

Our data, showing that PCBs induce necrosis of L6C5 myoblasts and myotubes with extracellular release of LDH and CK, could explain the clinical finding of increased serum CK as a marker of skeletal muscle primary damage. More studies are necessary to clarify the long term effects of PCBs on skeletal muscle tissue.

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The first two authors equally contributed to this work.

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