Effects of the rodent peroxisome proliferator and hepatocarcinogen, perfluorooctanoic acid, on apoptosis in human hepatoma HepG2 cells

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The effects of perfluorooctanoic acid (PFOA), a potent hepatocarcinogen and peroxisome proliferator in rodents, on human cells have not yet been examined. In the present study we demonstrate that treatment of human hepatoblastoma HepG2 cells with PFOA induces apoptosis, as well as perturbs the cell cycle. This apoptosis was characterized by electron microscopy, which revealed typical nucleosomal fragmentation (also observed as a ‘DNA ladder’ upon electrophoresis on agarose) and was quantitated using propidium iodide staining of cellular DNA and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. This process was dose- and time-dependent: apoptosis manifest with 200 µM and maximal (45% of the cells) upon exposure to 450 µM PFOA for 24 h. Electrophoresis of the DNA from HepG2 cells exposed to 500 µM PFOA for 24 h or to 400 µM PFOA for 48 h revealed a smear typical of non-specific degradation. These findings indicate that in the presence of high concentrations of PFOA, primary and secondary necrosis. Quantitation of trypan blue exclusion supported this conclusion. Flow cytometric analysis revealed that the cell cycle of HepG2 cells was perturbed by exposure to 50–150 µM PFOA. A 50 µM concentration resulted in a significant increase in the proportion of G2/M cells and, simultaneously, a decrease in the number of cells in the S phase, whereas treatment with 100 or 150 µM PFOA increased the proportion of cells in the G0/G1 phase and decreased the number of cells in the G2/M and S phases. Simultaneous flow cytometric analysis of apoptosis-associated DNA strand breaks using the TUNEL procedure and of propidium iodide staining of cellular DNA revealed DNA breaks in HepG2 cells exposed to 150 µM PFOA, prior to nuclear fragmentation.

Introduction

Perfluorinated fatty acids and their derivatives have many commercial uses based on their anti-wetting and surfactant properties, as well as on their chemical and thermal stabilities. These compounds are used as water repellents, corrosion inhibitors, heat exchangers and hydraulic fluids (1). In light of their relative metabolic inertness, perfluorinated fatty acids that leak out into the general environment are likely to be persistent there.

Extensive studies on perfluorinated compounds, including perfluorooctanoic (PFOA) and perfluorodecanoic acids, in our laboratory have demonstrated that these compounds are extremely potent peroxisome proliferators (2–4). They induce, among other things, hepatomegaly, proliferation of peroxisomes and up-regulation of peroxisomal β-oxidation of fatty acids (1–7). With respect to the dose required to obtain maximal effects, we have found that PFOA is the most potent known peroxisome proliferator in mice.

It is well documented that long term-treatment with PFOA and other peroxisome proliferators—including various hypolipidemic drugs (e.g. clofibrate, ciprofibrate and nafenopin), industrial chemicals (e.g. phthalates) and herbicides (e.g. 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid)—results in the development of liver tumors in rodents (8–11). Although the mechanism(s) underlying this carcinogenesis have not been clarified, a number of hypotheses have been proposed (12–14). Peroxisome proliferation may result in elevated levels of hydrogen peroxide, which may in turn initiate tumorigenesis via oxidative DNA damage (15, 16). At the same time, peroxisome proliferators may act as tumor promoters by stimulating DNA replication (17–19). In addition, peroxisome proliferators may act to inhibit the normal process of apoptosis in the liver (20).

It is not yet clear whether peroxisome proliferators are also carcinogenic in humans. Epidemiological studies (although not always statistically significant) have reported the development of cancer in some patients chronically treated with fibrates (21, 22). Such studies may become quite important in light of the fact that certain groups in the population are exposed to relatively high levels of peroxisome proliferators, including farmers using certain herbicides and pesticides, workers in the chemical industry who are exposed to PFOA and phthalates, and patients with heart problems who receive hypolipidemic drugs.

The possibility that the hepatocarcinogenicity of peroxisome proliferators in rodents reflects inhibition of apoptosis is of special interest to us because of the following reports: treatment of primary rat hepatocyte cultures with nafenopin (a hypolipidemic drug and peroxisome proliferator) resulted in increased DNA synthesis and decreased apoptosis (23). In addition, Perrone et al. (24) have found that apoptosis induced in rat hepatocytes by transforming growth factor β can be inhibited by clofibrac acid and ciprofibrate.

Interestingly, in human primary cultures and cell lines, no such effect has been found. If anything, peroxisome proliferators seem to have the opposite effect on human cells. Thus, treatment of human hepatocytes with clofibrac acid induced apoptosis. Furthermore, Canuto et al. (25) have demonstrated that human hepatoblastoma cell lines undergo massive apoptosis upon exposure to high concentrations of clofibrac.
Since little is known about whether the hepatocarcinogenic potency of PFOA involves inhibition of apoptosis, the aim of the present investigation was to determine the effect of this metabolically rather inert compound on apoptosis in the human hepatoblastoma cell line, HepG2. In addition, the possibility that PFOA enhances mitosis in these cells was also examined, since such an enhancement might also promote the carcinogenic effects of other xenobiotics. We have used a wide range of PFOA concentrations, which either had relatively little effect on the viability of the cells or resulted in cytotoxicity.

Materials and methods

Materials

Propidium iodide, trypan blue, RNase A, DNase I, proteinase K, phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris, ethylene-diamine tetracetic acid (EDTA), bromophenol blue and ethidium bromide were purchased from Sigma (St Louis, MO). Molecular Biology Certified Agarose and DNA-standard AmpliSize Molecular Ruler, 50–2000 bp ladder were procured from Bio-Rad (Hercules, CA). PFOA was bought from Aldrich (SteinHeim, Germany). All other chemicals were of analytical grade and purchased from Fluka (Switzerland). All tissue culture reagents, i.e. Dulbecco’s minimal essential medium containing Earl’s salts and 1-glutamine, fetal bovine serum, sodium pyruvate, non-essential amino acids, penicillin, streptomycin and trypsin/EDTA solution were procured from Gibco BRL–Life Technologies (Grand Island, NY). Cell culture flasks and dishes were obtained from Falcon (Becton Dickinson, San José, CA).

Cell culture

The HepG2 cell line [which is derived from a human hepatoblastoma (26)] was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). The HepG2 cells were maintained in Dulbecco’s minimal essential medium with Earl’s salts and 1-glutamine in a humidified incubator under 5% CO₂/95% air at 37°C. This medium was supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, 1% (v/v) non-essential amino acids and 100 IU of penicillin and 100 μg of streptomycin per ml.

Treatment of HepG2 cells with PFOA

HepG2 cells were seeded at a density of 1.0x10⁶ onto 25 cm² tissue flask and thereafter grown to subconfluence (as judged from light microscopy), which required 3 days. The medium was changed every 24 h during growth. Subsequently, HepG2 cells were exposed to different concentrations of PFOA (0–550 μM) by adding an appropriate volume of the stock solution of PFOA in dimethyl sulfoxide (DMSO) to the culture medium. The period of incubation with PFOA was routinely 24 h. Control cells were cultured in medium containing 0.2% DMSO, i.e. the concentration of DMSO obtained when the largest volume of the PFOA stock solution was added. A separate investigation of the effects of DMSO on the parameters studied here revealed no differences between DMSO-treated cells and untreated cells.

In cell-cycle experiments, untreated cells were used as control and DMSO-treated cells were designated as cells treated with PFOA at a concentration of 0 μM. In time-course experiments, HepG2 cells were exposed to 400 μM PFOA for 12, 24, 36 or 48 h.

At the end of the treatment, cultures were rinsed twice with phosphate-buffered saline (PBS) at 37°C and thereafter harvested employing 0.05% trypsin and 0.02% EDTA. The medium and rinsing solutions containing floating cells were kept for later analysis. Trypsinized and spontaneously floating cells were collected, counted and mounted in aceto-orcein and centrifugation at 150 g. The cell pellet was washed twice with PBS and subsequently was used for analyses.

Determination of cell viability by trypan blue exclusion

A common test for cell viability is based on the ability of a cell with an intact plasma membrane to exclude the dye trypan blue. Cells unstained and stained with trypan blue (0.4% in PBS) were counted under the light microscope using a Menzel-Glaser haemocytometer (Germany). In each sample, 400 cells were counted and the results expressed as the percentage of all the cells which stained with trypan blue.

Detection of DNA fragmentation by agarose gel electrophoresis

Low molecular weight DNA was extracted using a modification of the classical procedure described by Wyllie (27). Cells (5x10⁶) were lysed in 10 mM Tris–HCl pH 8.0, 10 mM EDTA and 0.5% Triton X-100 for 30 min on ice. This lystate was then centrifuged at 15 000 g for 15 min at 4°C to separate the fragmented DNA (supernatant) from the intact chromatin (pellet). The supernatant was incubated with RNase A (5 μg/ml) for 1 h at 37°C, followed by a 2 h incubation at 50°C with proteinase K (200 μg/ml) and 0.5% sodium dodecyl sulfate. After extraction of the supernatant with phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris pH 8.0 and 1 mM EDTA) fragmented DNA was precipitated with 100% cold ethanol and 5 M NaCl and subsequently air-dried.

This DNA sample was dissolved in 10 mM Tris–HCl pH 8.0, containing 1 mM EDTA and then mixed with 6 vol DNA loading buffer (40% sucrose in 50 mM EDTA–0.25% bromophenol blue) and loaded onto a 1.8% agarose gel containing 0.2 μg/ml ethidium bromide. Electrophoresis was conducted in the running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0) at 5 V/cm. DNA-standard AmpliSize Molecular Ruler, 50–2000 bp ladder was run in parallel. Finally, the DNA bands were visualized by UV illumination.

Quantification of apoptosis by flow cytometry

Detection of DNA strand breaks by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

HepG2 cells were fixed with 1% paraformaldehyde in PBS (freshly prepared) for 20 min on ice and then, after washing in PBS, were resuspended in 70% cold (~20°C) ethanol and transferred to the freezer, where they were stored for up to 3 days.

After rehydration of cells in PBS, they were subjected to the DNA strand breaks labeling procedure using a kit called ‘Apoptosis Detection System, Fluorescetin’ available from Promega (Madison, WI). Using this kit, fluorescein-12-dUTP is incorporated into DNA strand breaks by the enzyme terminal deoxynucleotidyl transferase. This procedure was performed as described in the protocol provided by the supplier. Negative control cells were processed through all the same steps of analysis using all reagents except exogenous terminal deoxynucleotidyl transferase. Positive control cells were prepared by treating the cells with DNase I.

These samples were analysed using a FACSCalibur Flow Cytometer (Becton Dickinson) equipped with an air-cooled argon-ion laser emitting at 488 nm. The green fluorescence emitted by fluorescein-12-dUTP in the DNA of individual cells was collected through a 530/30 nm bandpass. The data from 10 000 cells were stored and analyzed on a logarithmic scale using CellQuest Software.

Propidium iodide staining of cellular DNA

The method employed in this case was essentially that described by Nicoletti et al. (28). Adherent and floating cells were pooled, washed twice with cold PBS, then fixed in 5 ml 70% ice-cold ethanol with vortexing and, finally, maintained at ~20°C for at least 4 h. Following two more washes with PBS, the cell pellet was stained with the fluorescent probe solution containing PBS, 1% Triton X-100, 50 μg/ml propidium iodide and 0.5 mg/ml RNAse A for 1 h at room temperature in the dark. Cells were then analyzed using a FACSCalibur cytometer (Becton Dickinson) with excitation at 488 nm, with gating out of doublets and clumps using pulse processing and collection of fluorescence emission >620 nm. The percentage of cells undergoing apoptosis was obtained from the percentage of cells in the distinct subdiploid region of the DNA distribution histograms. Simultaneous flow cytometric analysis of the TUNEL assay of apoptosis-associated DNA strand breaks and of staining of cellular DNA with propidium iodide, which was done between fluorescence emitted by fluorescein-12-dUTP was collected through a 530/30 nm bandpass and the orange fluorescence of propidium iodide was collected simultaneously through a 585/42 nm bandpass. Electronic compensation was employed to eliminate spectral overlap. CaliBRITe beads (Becton Dickinson) were used to determine the appropriate compensation setting. All other conditions were as described above.

Cell-cycle analysis

The proportions of the cells in the G₀/G₁, S and G₂/M phases were calculated from DNA content histograms obtained by flow cytometric analysis of cells stained with propidium iodide. The conditions used for this analysis were the same as described in the protocol provided by the supplier. Negative control cells were stained with propidium iodide and 0.5 mg/ml RNAse A for 1 h at room temperature in the dark. Cells were then analyzed using a FACSCalibur cytometer (Becton Dickinson) with excitation at 488 nm, with gating out of doublets and clumps using pulse processing and collection of fluorescence emission >620 nm. The percentage of cells undergoing apoptosis was obtained from the percentage of cells in the distinct subdiploid region of the DNA distribution histograms. Simultaneous flow cytometric analysis of the TUNEL assay of apoptosis-associated DNA strand breaks and of staining of cellular DNA with propidium iodide, which was done between fluorescence emitted by fluorescein-12-dUTP was collected through a 530/30 nm bandpass and the orange fluorescence of propidium iodide was collected simultaneously through a 585/42 nm bandpass. Electronic compensation was employed to eliminate spectral overlap. CaliBRITe beads (Becton Dickinson) were used to determine the appropriate compensation setting. All other conditions were as described above.

Cell-cycle analysis

The proportions of the cells in the G₀/G₁, S and G₂/M phases were calculated from DNA content histograms obtained by flow cytometric analysis of cells stained with propidium iodide. The conditions used for this analysis were the following: the sample tube contained ~2x10⁶ cells/ml; the flow rate was low, then the number of events was ~300k; the amplifier mode was linear and the coefficient of variation for the G₁ peak was <4%. Cell doublets were eliminated from the data. This was achieved by setting a gate around the singlet population, which was identified on a dot-plot of the integrated area of the fluorescence versus the width of the fluorescent signal. Data thus collected were analysed using the CellQuest Software.

Morphological examination by electron microscopy

While still on the plate, HepG2 cells were rinsed twice at 37°C in 0.15 M sodium cacodylate buffer (pH 7.3) and then scraped off into 1.5% glutaraldehyde (in sodium cacodylate buffer). The cells were fixed in this solution for 24 h at 4°C and subsequently, after pelleting by centrifugation at 1700 g for 5 min in 0.15 M sodium cacodylate buffer and then resuspended in this same buffer. For post-fixation of the cells, 1% osmium tetroxide (in sodium phosphate buffer) was used. After these treatments the cells were rinsed twice as described above and then pelleted by centrifugation at 5000 r.p.m. for 5 min. The pellets were then dehydrated in ethanol and
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Fig. 1. Demonstration of DNA fragmentation in HepG2 cells exposed to PFOA by agarose gel electrophoresis. HepG2 cells were treated with different concentrations of PFOA for 24 h. (a) Lane 2, 0.2% DMSO; lane 3, 250 µM PFOA; lane 4, 300 µM PFOA; lane 5, 400 µM PFOA; lane 6, 450 µM PFOA. (b) Lane 2, 0.2% DMSO; lane 3, 200 µM PFOA; lane 4, 500 µM PFOA; lane 5, 350 µM PFOA. The left lane contains molecular weight markers. The ladder pattern of fragmentation was observed in DNA samples from cells treated with PFOA, but not in cells treated with DMSO alone. A smear was observed in DNA samples from cells treated with 500 µM PFOA. The results shown are representative of three separate experiments. For further methodological details, see Materials and methods.

Statistical analysis
Data are expressed as means ± SD. The mean values were compared using Student’s t-test. Differences for which $P < 0.05$ are considered to be statistically significant.

Results

Demonstration of PFOA-induced apoptosis by electrophoresis to obtain a ‘DNA ladder’
DNA fragments extracted from HepG2 cells treated with 0.2% DMSO or different concentrations of PFOA for 24 h were subjected to agarose gel electrophoresis (Figure 1a and b). A typical ‘DNA ladder’ was observed, with bands separated by multiples of ~200 bp. This indicates the presence of DNA fragments the size of single nucleosomes and oligonucleosomes (29), which are considered to be characteristic biochemical markers for apoptotic cells (27).

As can be seen, the extent of DNA fragmentation (i.e. intensity and number of bands) is dose dependent. The lowest concentration of PFOA at which a ‘DNA ladder’ was observed was 200 µM. The highest level of DNA fragments was observed in HepG2 cells exposed to 400–450 µM PFOA. The DNA isolated from HepG2 cells treated with 500 µM PFOA exhibited a smear typical of non-specific degradation, i.e. necrosis. No fragmentation or other forms of degradation was observed in the DNA extracted from DMSO-treated cells.

Flow cytometric quantification of PFOA-induced apoptosis employing the TUNEL assay and propidium iodide staining of cellular DNA
Histograms of flow cytometric quantitation of apoptosis-associated DNA strand breakage using the TUNEL assay in HepG2 cells exposed to 350 or 450 µM PFOA for 24 h are shown in Figure 2b and c. The number of cells containing detectable DNA strand breaks was increased to 17 and 43%, respectively, after incubation with these concentrations of PFOA. The corresponding value for cells treated with 0.2% DMSO was ~1.7%.

Fig. 2. Histograms of flow cytometric quantitation of apoptosis-associated DNA strand breakage using the TUNEL assay. HepG2 cells were exposed to 350 µM (b) or 450 µM PFOA (c) for 24 h. Negative control cells in (a) were processed through all steps of the analysis, but without addition of the exogenous terminal deoxynucleotidyl transferase. Positive control cells in (a) were prepared by treating the cells with DNase I. The results shown are representative of three separate experiments. For further methodological details see Materials and methods.
Flow cytometric analysis of propidium iodide-stained HepG2 cells treated with different concentrations of PFOA for 24 h revealed typical DNA content histograms (Figure 3). The distinct subdiploid region in these histograms represents the cells undergoing apoptotic DNA degradation (30). PFOA-treated cells demonstrated a much larger hypodiploid DNA peak than did DMSO-treated HepG2 cells did (Figure 3). The size of this peak is dose dependent, the maximum being observed with 450 μM PFOA.

**Dependence of the pathway of PFOA-induced death in HepG2 cells upon concentration**

The percentages of apoptotic and necrotic cells in HepG2 cultures exposed to different concentrations of PFOA are presented in Figure 4. The percentages of sub-G0/G1 (apoptotic) cells were calculated from the DNA histograms obtained by flow cytometry, while the percentages of necrotic cells were determined on the basis of permeability to trypan blue, as seen under the light microscopy. In control, untreated HepG2 cells the percentage of trypan blue-excluding cells was always >97.4% and the percentage of apoptotic cells was <1.69%. Treatment of cells with 0.2% DMSO did not significantly alter these values: 2.96% of the cells were permeable to trypan blue and 1.9% appeared apoptotic.

The relative numbers of apoptotic and trypan blue-positive HepG2 cells were not affected by exposure to 50–150 μM PFOA. The percentage of apoptotic cells significantly exceeded the control level with 200 μM PFOA, reached maximum with 450 μM and was decreased at higher concentrations. Upon treatment with 350 μM PFOA, the number of trypan blue-positive cells was not significantly higher than among control cells, but this number increased dramatically to 80% at higher concentrations of PFOA.

**Morphological characterization of HepG2 cells after treatment with PFOA**

Since HepG2 cells treated with 400 μM PFOA for 24 h exhibited maximal apoptosis, whereas treatment with 500 μM PFOA resulted in cells with a disrupted plasma membrane and randomly fragmented DNA, these two concentrations were chosen for morphological characterization by electron microscopy. HepG2 cells treated with DMSO appeared well-differentiated, much like normal liver cells (Figure 5a). After treatment with the lower concentration, these cells exhibited morphological changes characteristic of apoptosis, i.e. condensed chromatin within the nucleus, pronounced nuclear lobulation and nuclear fragmentation to form apoptotic bodies typical of advanced stages of apoptosis (Figure 5b). After treatment with 500 μM PFOA, three types of cells were observed, i.e. apoptotic cells with nuclear fragmentation; cells exhibiting several features typical of necrosis, such as extensive degeneration of cytoplasmic structure, vacuolization and disruption of the plasma membrane; and cells at a late stage...
Fig. 5. Morphological changes induced in HepG2 cells by PFOA. The cells were treated with 0.2% DMSO alone (a) or 400 µM (b) or 500 µM PFOA (c) (added in DMSO) for 24 h. After treatment with 400 µM PFOA, the cells revealed condensed chromatin within the nucleus, pronounced nuclear lobulation and nuclear fragmentation to form apoptotic bodies. After treatment with 500 µM PFOA, three different types of cells are seen: apoptotic cells with nuclear fragmentation; cells with several features of necrosis, i.e. extensive degeneration of cytoplasmic structure, vacuolization and disruption of the plasma membrane; and cells at a late stage of cytolysis, with disintegration of nuclei and organelles. Magnification ×3300.
propidium iodide-stained cells (Figure 7). The size of the cells in the S phase, concomitant with an elevation in the first peak.

In these DNA strand breaks using the TUNEL procedure and propidium for 48 h, PFOA for 12 h, PFOA for 24 h, PFOA for 36 h, and PFOA for 48 h.

Fig. 7. Time dependence of PFOA-induced DNA fragmentation in HepG2 cells. HepG2 cells were incubated with 400 µM PFOA for 12–48 h (lane 3, 12 h; lane 4, 24 h; lane 5, 36 h; lane 6, 48 h) or 0.2% DMSO for 48 h (lane 2). The left lane contains molecular weight markers. The ladder pattern of DNA fragmentation was observed in cells treated with PFOA for 12–36 h, but not in cells treated with DMSO. A smear of DNA was observed in cells treated with PFOA for 48 h. The results shown are representative of two separate experiments.

of cytolysis, with disintegration of nuclei and organelles (Figure 5c). These morphological observations are in agreement with our other results concerning apoptosis and necrosis.

Time-dependence of PFOA-induced death of HepG2 cells

DNA fragments isolated from HepG2 cells treated with PFOA for 12–48 h were subjected to electrophoresis (Figure 6). The first evidence of a ladder pattern was observed after 12 h of incubation with 400 µM PFOA. The highest level of DNA fragments was observed in cells incubated with PFOA for 24–36 h. DNA isolated from cell samples after incubation for 48 h exhibited a smear typical of non-specific degradation. Again, no DNA fragments were obtained from cells treated with DMSO.

The time-dependence of PFOA-apoptosis in HepG2 cells was also examined using flow cytometric analysis of propidium iodide-stained cells (Figure 7). The size of the hypodiploid peak varied with time of exposure to PFOA in the same manner as the DNA ladder pattern obtained by electrophoresis, with one exception: the apoptotic peak observed by flow cytometry was still present after a 48 h exposure of HepG2 to 400 µM PFOA.

Effect of different concentrations of PFOA on the proportion of HepG2 cells in different phases of the cell cycle

As can be seen from Figure 3, treatment of HepG2 cells with PFOA changed the DNA frequency distribution histogram. Treatment with 200–500 µM PFOA led to a reduction in the proportion of cells in the S and G2/M phases of the cell cycle. The proportion of G0/G1 cells decreased upon exposure to 450–500 µM PFOA.

The effects of different concentrations of PFOA on the distribution of HepG2 cells in the different phases of the cell cycle are illustrated in Figure 8. The frequencies of cells in the G0/G1, S and G2/M phases were expressed as percentages of the corresponding values for control cells. Untreated HepG2 cells demonstrated a relatively normal distribution pattern, with most cells in the G0/G1 phase of the cycle (63.8%) and fewer in the S phase (18.0%) and G2/M (18.2%) phases.

A significant increase in the number of cells in the G2/M phase (up to 116%) and a simultaneous significant decrease in the number of S phase cells (down to 84%) were observed in HepG2 cells treated with 50 µM PFOA. However, there was no increase in the total number of cells per plate after treatment with this concentration of PFOA for 24 h (data not shown). With 100 and 150 µM PFOA, the proportion of G0/G1 cells was increased up to 112–119%; while the number of S phase cells remained at ~85%.

A significant decrease in the proportion of HepG2 cells in the S phase was observed with all PFOA concentrations used, with a maximal decrease to 45% with 400–450 µM PFOA. Incubation of HepG2 cells with 200 µM PFOA significantly decreased the proportion of G2/M cells, coincident with the appearance of a discrete sub-G0/G1 peak, as can be seen from Figure 3. The proportion of cells in the G0/G1 phase was only affected by the highest concentrations of PFOA (Figure 8), which induced the maximal level of apoptosis in HepG2 cells.

Simultaneous flow cytometric analysis of apoptosis-associated DNA strand breaks using the TUNEL procedure and propidium iodide staining of cellular DNA

The major advantage of the in situ TUNEL assay appears to be its ability to reveal DNA breaks at a relatively early stage of apoptosis, prior to any significant loss of DNA or to nuclear fragmentation. Indeed, this procedure is well suited for the identification of cells at very early stages of apoptosis (31,32). By combining the TUNEL procedure with staining of cellular DNA by propidium iodide, these investigators were able to determine the phase of the cell cycle in which the DNA breaks occurred (31).

We applied this same approach to examine apoptosis in PFOA-treated HepG2 cells (Figure 9). Our data clearly confirm the utility of this approach in identifying the phase of the cell cycle in which DNA breaks occur.

The proportion of cells containing detectable DNA strand breaks was increased to 5.24% after incubation with 150 µM PFOA, even though no population of apoptotic cells with loss of DNA content (which would give rise to a discrete sub-G0/G1 population) was yet apparent. Exposure of HepG2 cells to 200–300 µM PFOA resulted in a reduction in the proportion of cells in the S phase, concomitant with an elevation in the
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Fig. 8. Effects of different concentrations of PFOA on the proportions of HepG2 cells in different phases of the cell cycle. The proportions of treated cells in the G0/G1 (filled circles), S (filled squares) and G2/M (filled triangles) phases are expressed as percentages of the corresponding values for control cells. Control HepG2 cells demonstrate a distribution pattern in which most cells are in the G0/G1 phase of the cycle (63.8%) and fewer are in the S (18%) and G2/M (18.2%) phases. The values presented are means ± SD (bars) of data obtained from four or five independent experiments. *P < 0.05, i.e. significantly different from the corresponding value for control cells.

Discussion

PFOA, a hepatocarcinogen and potent peroxisome proliferator in rodents, has been shown here to induce apoptosis in the human hepatoblastoma cell line HepG2. This induction of apoptosis was dose dependent; low concentrations had no obvious effect, concentrations >200 µM became increasingly apoptotic, and maximal apoptosis was obtained with exposure to 450 µM PFOA. This process was also time dependent, reaching a maximal value after ~36 h of exposure.

The induction of apoptosis in primary human hepatocytes by clofibrate acid and ciprofibrate (24), as well as in HepG2 cells by clofibrate (25) has been reported previously. Similar responses have been observed in studies involving intermediately differentiated (HTC) and poorly differentiated (RH1) human liver cell lines, in which nafenopin suppressed cell growth by markedly inhibiting DNA synthesis, with associated increased levels of apoptosis (33). Opposite effects have been observed in well-differentiated FAO (rat) cell lines, as well as in primary cultures of rodent hepatocytes and in vivo, i.e. nafenopin suppresses apoptosis and induces replicative DNA synthesis in these systems (33–35). Together with these other data, our findings suggest that PFOA and other peroxisome proliferators may not elicit hepatocarcinogenic processes in humans, at least not by inhibiting apoptosis.

HepG2 cells are capable of undergoing apoptosis through the basic/common-signaling pathway. p53 and c-Myc play an important role in the apoptosis-signaling pathway in HepG2 cells treated with a number of apoptosis-inducing compounds (36). Expression of c-fos and c-jun and the DNA binding activity of activating protein-1 were up-regulated by hypoxia in HepG2 cells, and 6-thioguanine-sensitive protein kinase(s) may be involved in the signaling pathway leading to apoptosis in these hypoxic HepG2 cells (37).

The mechanism by which PFOA causes apoptosis in HepG2 cells remains to be elucidated. Perhaps the ability of this compound to uncouple oxidative phosphorylation (38) or PFOA, a hepatocarcinogen and potent peroxisome proliferator increase production of reactive oxygen species (9,15,39, 40–43) plays a role in this context.

Another interesting conclusion that can be drawn from our studies is that a high concentration of, and prolonged incubation with, PFOA cause HepG2 cells to undergo rapid primary and secondary necrosis. This process occurs in response to a relatively small increase in the concentration of PFOA. Other apoptosis-inducing agents studied in our laboratory have not shown such a sharp transition (44). Together with these other data, our findings suggest that PFOA and other peroxisome proliferators may not elicit hepatocarcinogenic processes in humans, at least not by inhibiting apoptosis.

Disruption of the plasma membrane in the presence of high concentrations of PFOA might result from the detergent properties of this compound. It is well known that long-chain fatty acids can damage membranes via their detergent properties. Indeed, when Levitt and Liss (45,46) treated a human B-lymphoblastoid cell line with high concentrations of PFOA or other perfluorinated fatty acids, they observed a detergent effect. It is thus possible that high concentrations of PFOA are directly toxic to the cells so that they undergo both primary and secondary necrosis at the same time.

The second part of our study revealed the effects of different concentrations of PFOA on the HepG2 cell cycle. Treatment of these cells with low concentrations of PFOA (50 µM) results in a significant increase in the number of cells in the G2/M phases and a simultaneous decrease in the number of S-phase cells, whereas treatment with 100 and 150 µM PFOA...
increases the proportion of cells in the G₀/G₁ phase and decreases the numbers of cells in the G₂/M and S phases. It should be remembered in this context that upon exposure of HepG2 cells to 50–150 µM PFOA, there was no detectable increase either in the total cell number or in the proportion of cells with a sub-G₀/G₁ content of DNA (apoptotic cells). These data suggest that treatment with PFOA led to HepG2 cell-cycle arrest in the G₀/G₁ phase. In contrast, Pasilly et al. (47) have reported that the cell cycle of HepG2 cells is not perturbed upon exposure to ciprofibrate, another peroxisome proliferator.

Simultaneous flow cytometric analysis of apoptosis-associated DNA strand breaks using the TUNEL assay and of propidium iodide staining of cellular DNA revealed DNA breaks in HepG2 cells exposed to 150 µM PFOA, prior to nuclear fragmentation. Furthermore, this procedure is well suited for determining the phase of the cell cycle in which the DNA breaks occur (31,32). Exposure of HepG2 cells to 200–300 µM PFOA resulted in a reduction in the proportion of cells in the S phase, concomitant with an increase in the size of the cell population containing DNA breaks. Exposure to 400 µM PFOA led to an almost total loss of S phase cells and a significantly decreased number of cells in the G₂/M and G₀/G₁ phases. These observations suggest that HepG2 cells exposed to PFOA may enter the apoptotic process from any phase of the cell cycle, although cells in S phase may be especially prone to undergo such apoptosis.

Interestingly, TUNEL assay of cells exposed to 400 µM PFOA demonstrated an increase in apoptotic cells clustering with non-apoptotic cells in the G₀/G₁ phase. We suggest that after exposure to high concentrations of PFOA, cells in the G₀/G₁ phase, which would next ordinarily enter the S phase, exit G₁ and become immediately apoptotic. These results are in agreement with those of Breder et al. (48), who reported that certain cytokines cause cell-cycle arrest predominantly in G₁ and that cells thus arrested in G₁ do not enter the S phase, but instead become directly apoptotic. Furthermore, when Leszczynski (49), treated chloroma cells with various doses of hydroxyurea (an agent which causes cell arrest in the G₁ phase), induction of apoptosis was observed within 24–48 h.

Thus, there seem to be indications that an agent which arrests cells in the G₁ phase is also capable of inducing apoptosis. If so, this is an important observation in connection with the development of new anti-cancer drugs. The anti-cancer effect of berberine (an alkaloid found in many plants) on HepG2 cells was shown by Chi et al. (50) to involve a significant decrease in the proportion of cells in the S phase and concomitant arrest in the G₀/G₁ phase. Similarly, the green tea component epigallocatechin-3 has been found to cause cell-

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**Fig. 9.** Simultaneous flow cytometric analysis of apoptosis-associated DNA strand breaks using the TUNEL assay and of propidium iodide staining of cellular DNA. HepG2 cells were exposed to 0.2% DMSO (a), or 150 (b), 250 (c), 350 (d) or 450 µM PFOA (e) for 24 h. The green fluorescence of fluorescein-12-dUTP was collected through a 530/30 nm bandpass and the orange/red fluorescence of propidium iodide through a 585/42 nm bandpass. The results shown are representative of three separate experiments. For further methodological details, see Materials and methods.
cycle arrest in the G0/G1 phase in several human carcinoma cell lines, as well as dose-dependent apoptosis (51).

In conclusion, our findings contrast with the hypothesis that PFOA may have a carcinogenic effect in human cells through suppression of apoptosis. Further investigation is required to elucidate the mechanism by which PFOA induces apoptosis in HepG2 cells. It would also be of great interest to study the effects of PFOA on the cycle.

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References

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