Suppression of Liver Cell Apoptosis In Vitro by the Non-Genotoxic Hepatocarcinogen and Peroxisome Proliferator Nafenopin

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Abstract. Suppression of apoptosis has been implicated as a mechanism for the hepatocarcinogenicity of the peroxisome proliferator class of non-genotoxic carcinogens. The ability of the peroxisome proliferator nafenopin to suppress or delay the onset of liver apoptosis was investigated using primary cultures of rat hepatocytes and the Reuber hepatoma cell line FaO. 50 μM nafenopin reversibly maintained the viability of primary rat hepatocyte cultures which otherwise degenerated within 8 d of establishment. The maintenance of viability of hepatocyte monolayers was associated with a significant decrease in the number of cells exhibiting chromatin condensation patterns typical of apoptosis. Apoptosis could be induced in hepatocytes by administration of 5 ng/ml TGFβ. Co-addition of 50 μM nafenopin significantly reduced TGFβ1-induced apoptosis by 50–60%.

TGFβ1 (1–5 ng/ml) also induced apoptosis in the FaO rat hepatoma cell line. Cell death was accompanied by detachment of FaO cells from the monolayer and detached cells exhibited chromatin condensation and non-random DNA fragmentation patterns typical of apoptosis. Co-addition of 50 μM nafenopin to TGFβ1-treated FaO cultures significantly reduced the number of apoptotic cells detaching from the monolayer at 24 h. In contrast, nafenopin had no significant effect on FaO apoptosis induced by the DNA damaging agents etoposide and hydroxyurea. We conclude that suppression of liver cell death by apoptosis may play a role in the hepatocarcinogenicity of the peroxisome proliferators, although the extent of this protection is dependent on the nature of the apoptotic stimulus.

The hypolipidemic drug nafenopin is one of a diverse group of non-genotoxic carcinogens termed peroxisome proliferators (19) that cause hepatic peroxisome proliferation and liver enlargement in rodents (20, 14). These changes are accompanied by the elevated transcription of genes encoding the microsomal enzyme cytochrome P4504A1 (17) and the peroxisomal enzymes of β-oxidation, acyl-CoA oxidase, and peroxisomal bifunctional enzyme (11, 15). More importantly, despite their apparent lack of genotoxic activity, the peroxisome proliferators are potent rodent liver carcinogens (7, 26). Since the molecular mechanisms responsible for peroxisome proliferator-induced hepatocarcinogenesis are not yet defined, the risk posed to man by this diverse group of compounds cannot yet be assessed.

A number of mechanisms have been proposed to explain the hepatocarcinogenicity of the peroxisome proliferators (reviewed in 14, 18). The peroxisome proliferator-activated receptor is activated by these chemicals and as a result has been implicated in the carcinogenic process (9, 10, 25). It has also been suggested that excess hydrogen peroxide, produced as a result of increased peroxisomal activity, may cause DNA damage in hepatocytes by oxidative stress (21). The sustained replicative DNA synthesis induced by peroxisome proliferators in hepatocytes could act to promote DNA-damaged hepatocytes and other previously initiated cells (13). Complementary to sustained proliferation, another factor in peroxisome proliferator-induced hepatocarcinogenesis may be the suppression or retardation of hepatocyte apoptosis (programmed cell death), allowing the survival of potentially tumorigenic cells that would otherwise be eliminated (5). Evidence in support of the latter hypothesis has been obtained from in vivo studies (3, 4, 23): other non-genotoxic carcinogens such as cyproterone acetate or phenobarbital induce liver cell hyperplasia in rodents (23). Withdrawal of these compounds causes regression of the hyperplastic liver with a concomitant increase in cell death by apoptosis. This liver regression is reversible and can be inhibited by readministration of the relevant compound (3). Interestingly, the apoptosis that follows withdrawal of cyproterone acetate can also be inhibited by the peroxisome proliferator nafenopin (4). While these results indicate that nafenopin may suppress apoptosis in the liver, there is as yet
no conclusive evidence that this suppression plays a role in the hepatocarcinogenicity of the peroxisome proliferators. In order to address this important question, quantitative analysis of the ability of peroxisome proliferators to modulate liver cell apoptosis is required.

Apoptotic cells in vivo are rapidly recognized and phagocytosed by neighboring cells (8). Moreover, in contrast to necrosis, apoptosis occurs in single cells scattered throughout the organ and only a relatively small number of cells are detectable as apoptotic at any one time (8). Taken together, these factors make it difficult to quantitatively analyze the amount of apoptosis occurring in the liver in vivo.

Primary rat hepatocytes degenerate rapidly following their isolation and only survive in culture for a limited period of time (22). We and others have shown that exposure of these hepatocyte cultures to nafenopin can reversibly maintain their viability. In this paper we present the results of experiments designed to determine whether this maintenance of hepatocyte viability is a result of suppression of hepatocyte apoptosis. In light of recent reports that the liver growth regulator TGFβ induces apoptosis in primary hepatocytes and a number of cell lines in vitro (12, 16, 27), we have also examined the ability of nafenopin to protect hepatocytes from TGFβ-induced cell death.

We have performed complementary studies of the effect of nafenopin on liver apoptosis using the FaO hepatoma cell line, since these cells are easier to manipulate in culture than primary hepatocytes. We previously validated FaO as a suitable model system for this application since it expresses markers of differentiated liver function, responds to nafenopin via induction of cytchrome P450A1 and readily undergoes apoptosis in response to a number of stimuli (1). Here we have investigated whether nafenopin also protects FaO cells from apoptosis induced by TGFβ. To address the question of whether nafenopin prolongs cell survival regardless of the cell death stimulus, we have also investigated the effects of co-addition of nafenopin with the DNA damaging cytotoxic agents etoposide and hydroxyurea.

**Materials and Methods**

**Materials**

Nafenopin (2-methyl-2-[1,2,3,4-(tetrahydro-1-naphthyl)]-phenoxyl]proionic acid) was a gift from Ciba-Geigy (Basel, Switzerland). TGFβ, from human platelets was from Sigma (Poole, UK), Acridine orange, Hoechst 33258, and ethidium bromide were from Molecular Probes Inc. (Junction City, Oregon). Ham's Nutrient Mixture F12 was from Imperial Laboratories (Andover, UK) and all other tissue culture reagents were purchased from Gibco Ltd. (Uxbridge, UK). All other chemicals were from Sigma (Poole, UK). FaO cells were a gift from Dr. Mary Weiss (Pasteur Institute, Paris).

**Preparation of Primary Hepatocyte Cultures**

Rat hepatocytes were isolated from 180-200 g male Alderley Park rats (Winter derived) by a two stage collagenase perfusion method as described previously (24). Briefly, livers of rats under terminal anaesthesia were perfused with 0.05% collagenase and a hepatocyte suspension was obtained by passing digested livers through a 0.125 mm gauze. Cells were washed in buffered saline (pH 7.2) and centrifuged at 200 g. Cells were then resuspended in 20 ml medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Flasks were pre-coated with rat tail collagen to aid attachment of hepatocytes. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Medium was changed 4, 24, and every 48 h after seeding.

**Culture of FaO Hepatoma Cells**

FaO cells were cultured in Ham's Nutrient Mixture F12 supplemented with 10% fetal bovine serum, 2 mM t-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were routinely seeded at 1 x 10⁶ cells/75 cm² flask in 10 ml medium on day 0 and passaged every 5 d by trypsinization. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and medium was changed every 48 h.

**Assessment of Cell Viability by Trypan Blue Exclusion**

Equal volumes of cell suspension and trypan blue (0.3% in phosphate buffered saline) were mixed and cell counts were performed on a hemocytometer. At least 100 cells were counted per data point. Viable cells were identified as those which excluded trypan blue and were expressed as a percentage of total cell number.

**Analysis of Hepatocyte Nuclear Morphology**

Primary hepatocyte monolayers were fixed in ice cold methanol/acetic acid (3:1) for 5 min. Cells were then stained with Hoechst 33258 (5 µg/ml in H₂O) for 10 min and washed in distilled water. Cells were mounted in a solution of 20 mM citric acid, 50 mM d-sodium orthophosphate, and 50% glycerol (pH 5.5) to achieve optimum fluorescence and were examined at a wavelength of 350-460 nm using an Olympus microscope with fluorescence attachment. 10 randomly chosen fields, representing at least 800 cells, were analyzed for each data point. Apoptotic cells were identified as those with brightly staining condensed chromatin and were expressed as a percentage of total cell number.

**Analysis of FaO Nuclear Morphology**

The morphology of detached and attached FaO cells was examined by acridine orange staining and fluorescence microscopy as described previously (1). Briefly, a 6 µl sample of cell suspension was mixed on a slide with an equal volume of acridine orange solution (10 µg/ml in phosphate buffered saline). Green fluorescence was detected between wavelengths 500-525 nm using an Olympus microscope with fluorescence attachment. Apoptotic cells were identified as those with brightly staining condensed nuclei and were expressed as a percentage of total cell number. Images were recorded using a Biorad MRC 600 confocal microscope.

**Analysis of DNA Integrity by Agarose Gel Electrophoresis**

The DNA from attached and detached FaO cells was analyzed by agarose gel electrophoresis as described previously (1). Briefly, 10⁶ cells were pelleted at 174 g and resuspended in 20 µl lysis buffer (10 mM EDTA, 50 mM Tris/HCl containing 0.5% sodium lauryl sarcosinate, and 0.5 mg/ml protease K). 10 µl low gelling temperature agarose, melted at 70°C, was added before loading the samples onto a 2% agarose gel containing 10 µg/ml ethidium bromide. The gel was flooded with tris-phosphate-EDTA running buffer and DNA was subjected to electrophoresis at 40 V for 3 h. The gel was then incubated for 3 h with TPE running buffer containing 0.1 mg/ml RNase and DNA was visualized under UV epi-illumination.

**Treatment of Cells with Nafenopin**

Nafenopin was added to the medium of primary hepatocytes and FaO cells from a 20 mM stock dissolved in dimethylformamide (DMF). Control flasks were treated with DMF to the same final concentration of 0.25% vol/vol.

**Treatment of Cells with TGFβ**

Freshly isolated hepatocytes were seeded as described above. FaO cells were seeded at 3 x 10⁶ cells/25 cm² flask in 4 ml Ham's F12 medium containing 10% charcoal-stripped serum. After 24 h, cells were washed and...
medium replaced with serum free Williams E or Ham's F12 medium containing 0.2% BSA. After a further 24 h 1 ng/ml or 5 ng/ml TGFβ1 was added from a 2 μg/ml stock in 4 mM HCl. Control flasks were treated with HCl to the same final concentration of 0.1% vol/vol. The viability of primary hepatocytes was analyzed 24 and 48 h after TGFβ1 addition by Hoechst 33258 staining. FaO cell viability was assessed daily over 3 d after TGFβ1 addition by trypan blue exclusion, acridine orange staining of nuclear morphology, and gel electrophoresis of DNA from attached and detached cells.

**Treatment of FaO Cells with VP-16 and Hydroxyurea**

FaO cells were seeded at 1 × 10⁵ cells/25 cm² flask in 4 ml complete Ham's F12 medium. 24 h after seeding, 50 μM nafenopin or DMF was added After a further 24 h, 10 μM VP-16 (from a 4 mM stock in DMF) or 1 mM hydroxyurea (from a 100 mM stock in Ham's F12 medium) were added. Cell viability was assessed daily for 3 d after drug addition by trypan blue exclusion, acridine orange staining of nuclear morphology, and gel electrophoresis of DNA from attached and detached cells.

**Statistical Analysis of Results**

The statistical significance of the difference between the mean results of at least three independent experiments was calculated using the Students t test.

**Results**

**Maintenance of Hepatocyte Viability by Nafenopin**

Primary hepatocyte cultures degenerated by approximately 8 d after their establishment. Analysis of degenerating hepatocyte monolayers by light microscopy revealed that hepatocytes lost their regular polygonal shape, increased their number of nuclei and detached from the monolayer (Fig. 1 a). The viability of hepatocyte cultures was maintained by the addition of 50 μM nafenopin to the culture medium (Fig. 1 b). Hepatocyte viability was maintained for at least 6 wk but withdrawal of nafenopin from the cultures lead once again to the onset of degeneration and cell death about 8 d later (Fig. 1 c).

**Effect of Nafenopin on Hepatocyte Apoptosis**

Fluorescence microscopy of fixed hepatocyte monolayers stained with Hoechst 33258 was used to enumerate cells with chromatin condensation patterns typical of apoptosis. In the absence of nafenopin, apoptotic cells were detected in hepatocyte monolayers as early as 24 h after isolation and were detected at levels of 0.5–1% of total cell number on each of the following 8 d (Fig. 2 a). In six independent experiments, the number of apoptotic cells counted in nafenopin-treated hepatocyte cultures during the 8 d after establishment was significantly lower (P < 0.1) than the number seen in untreated controls (Fig. 2 b).

**Effect of Nafenopin on TGFβ₁-induced Hepatocyte Apoptosis**

Exposure of serum-free primary hepatocyte cultures to 1–5 ng/ml TGFβ₁, for 48 h resulted in apoptotic cell death. Treatment with 5 ng/ml TGFβ₁ caused a significant five- to six-fold increase in the number of apoptotic cells detected above levels seen in untreated controls (P < 0.001). The TGFβ₁ solvent, HCl, had no significant effect on cell viability. Co-addition of 50 μM nafenopin to cultures treated with TGFβ₁ (5 ng/ml) significantly reduced by 40 and 75% the number of apoptotic cells counted after 24 and 48 h, respectively (P < 0.1, Fig. 3). The addition of the nafenopin solvent DMF had no significant effect on apoptotic cell number.

**Induction of Apoptosis in FaO Hepatoma Cells by TGFβ₁**

Treatment of serum-free FaO cultures with 1–5 ng/ml TGFβ₁ led to a concentration-dependent induction of cell death which was accompanied by detachment of cells from the monolayer. For example, in three independent experi-
ments, a 48-h exposure of FaO cells to TGFβ1, resulted in a mean cell loss from the monolayer of 89%. This cell loss was shown to be the result of cell death by apoptosis since 70–80% of detached cells, collected at 2-h intervals over the course of the experiment, exhibited chromatin condensation patterns characteristic of apoptosis (Fig. 4 a and b). Moreover, gel electrophoresis of DNA from these detached cells revealed a DNA “ladder” indicative of the internucleosomal DNA cleavage reported for apoptosis in many cell types (Fig. 4 c). The TGFβ1, solvent, HCl, had no effect on FaO viability.

Effect of Nafenopin on TGFβ1-induced FaO Apoptosis
Co-addition of 50 μM nafenopin to FaO cultures treated with 1–5 ng/ml TGFβ1, significantly increased the number of viable cells that remained attached to the monolayer above the number counted in cultures treated with TGFβ1 alone (P < 0.05). For example, Fig. 5 a shows the percent increase in viable cell number induced by the co-addition of nafenopin in cultures treated with TGFβ1 (1 ng/ml). This increase represented a rescue of 10–20% of total cells from death. No significant increase in viable cell number was seen in DMF solvent-treated control cultures. The increase in viable cell number detected in the presence of nafenopin was associated with a significant decrease in the rate of detachment of cells (Fig. 5 b).

Effect of Nafenopin on FaO Apoptosis Induced by DNA-damaging Agents
Treatment of FaO cells with 10 μM etoposide led to rapid cell loss from the monolayer, with 63% cell death after 48 h. Similarly, treatment with 1 mM hydroxyurea resulted in 84% detachment and cell death at 48 h. In both cases, detached cells collected at 2-h intervals exhibited chromatin condensation and non-random DNA fragmentation patterns typical of apoptosis. 24 h after addition of 10 μM etoposide 48% of detached cells exhibited chromatin condensation patterns typical of apoptosis, as revealed by AO staining, whereas only 10% of detached cells were permeable to trypan blue. The corresponding values for cells detaching after 1 mM hydroxyurea treatment were 37 and 4%, respectively. In contrast to the results of corresponding experiments using TGFβ1, the presence of 50 μM nafenopin in hydroxyurea- and VP-16–treated FaO cultures had no significant effect on the viable cell number. (Fig. 6 a and b).

Discussion
The promotion of tumorigenic lesions occurs not only by sustained hyperplasia but also by suppression of apoptosis.
Figure 4. Morphology of viable (a) and apoptotic (b) FaO cells following treatment with 5 ng/ml TGFβ1. (Apoptotic cells which detached from the monolayer were collected 2 h after addition of fresh medium to the flask); (c) agarose gel electrophoresis of DNA from attached (lane 2) and detached (lane 3) FaO cells 24 h after addition of TGFβ1. Lane 1 contains HaeIII DNA markers.

Figure 5. (a) Effect of 50 μM nafenopin on FaO cell viability in the presence of 1 ng/ml TGFβ1; (ii) TGFβ1 + 50 μM nafenopin; (iii) nafenopin solvent DMF control (0.25% vol/vol). Data points represent the mean and standard error of five independent experiments in which the number of viable cells remaining attached after TGFβ1 addition were counted at one timepoint per day. Results are presented as a percentage of the cell number in cultures treated with TGFβ1 alone. (b) Effect of 50 μM nafenopin on the detachment of FaO cells from the monolayer following treatment with 1 ng/ml TGFβ1; (ii) Untreated control; (iii) 1 ng/ml TGFβ1 (ii) TGFβ1 + DMF (nafenopin solvent 0.25% vol/vol); (iv) TGFβ1 + 50 μM nafenopin. Data points represent the mean and standard error of three independent experiments in which the number of detached cells was counted at 2-h intervals for 30 h after drug addition. Results are presented as a cumulative percentage of the total cell number on addition of TGFβ1.

Considerable emphasis has been placed on investigating the importance of increased mitogenesis in peroxisome proliferator-induced hepatocarcinogenesis, but few studies have addressed the role of suppression of apoptosis, for which little direct evidence exists. The peroxisome proliferator nafenopin has been reported to inhibit apoptosis during the liver involution that follows withdrawal of the tumor promoter cyproterone acetate (4). However, in view of the short half-life of apoptotic hepatocytes in vivo, it is difficult to determine accurately the level of apoptosis occurring at any one particular time. Further elucidation of the effect of peroxisome proliferators on liver apoptosis would be facilitated by the use of in vitro model systems that can be more readily manipulated. We have previously validated FaO as a suitable cell type for this purpose (1). In this study we examined the effect of nafenopin on apoptosis using both FaO cells and monolayer cultures of primary rat hepatocytes.

We have shown here that nafenopin reversibly maintains the viability of primary rat hepatocytes for at least 6 wk. Interestingly, hepatocyte cultures can be maintained by nafenopin for as long as 18 mo (C. Elcombe, personal communication). In the absence of nafenopin, hepatocyte cultures degenerate within 8 d of their establishment. We de-
Figure 6. Effect of 50 μM nafenopin on FaO cell viability in the presence of (a) 10 μM etoposide and (b) 1 mM hydroxyurea. Data points represent the mean and standard error of three independent experiments in which the number of viable cells remaining attached after drug addition was counted at one timepoint per day; (c) control; (d) etoposide (a) or hydroxyurea (b); (e) etoposide (a) or hydroxyurea (b) + 50 μM nafenopin; (f) etoposide (a) or hydroxyurea (b) + DMF (nafenopin solvent) (0.25% vol/vol).

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such as nafenopin may stem, in part, from this ability to suppress apoptosis, more detailed investigations are required in order to validate this hypothesis.

TGFβ1 is a negative regulator of liver growth, implicated in the control of liver homeostasis (2). Recent studies have indicated that, in addition to inducing apoptosis in vivo and in vitro, cytoplasmic TGFβ1 latent protein is also increased in apoptotic hepatocytes (6). Although it is not yet clear whether this upregulation is controlled at the pre- or posttranslational level, it has been proposed that TGFβ1 may be involved in the induction of liver apoptosis (6). According to this proposal, cells which sustain some form of damage would exhibit elevated cytoplasmic levels of TGFβ1 protein, either by enhanced synthesis or increased uptake of TGFβ1 from other cells, and would consequently become programmed to die by apoptosis. The ability of nafenopin to block TGFβ1-induced cell death would thus provide a mechanism by which pre-neoplastic cells could survive long enough to accumulate additional transforming mutations. This hypothesis is supported by results from our studies of the effect of nafenopin on TGFβ1-induced hepatocyte and FaO cell death. In the absence of nafenopin, hepatocyte degeneration occurred within 48 h of TGFβ1 treatment. The rapid onset of cell death was reflected in the increased percentage of apoptotic cells (3-8%) identified in the monolayer at any one timepoint. TGFβ1 also rapidly induced FaO cell death which was accompanied by detachment of cells from the monolayer. In contrast to detached primary hepatocytes, which were always highly degraded, detached FaO cells initially excluded trypan blue and exhibited chromatin condensation and non-random DNA fragmentation patterns typical of apoptosis. In both cell systems, the level of TGFβ1-induced apoptosis was reduced significantly by co-addition of nafenopin. Interestingly, nafenopin did not prevent totally TGFβ1-induced hepatocyte or FaO cell death. Nevertheless, suppression of cell death in only a small percentage of hepatocytes in vivo could be relevant to hepatocarcinogenesis, particularly if any surviving cells have previously sustained genetic mutations. The prolonged viability of these potentially tumorigenic cells could allow the accumulation of further transforming lesions, and ultimately the development of liver tumors.

Our studies demonstrate that the ability of nafenopin to protect liver cells from chemically induced apoptosis is dependent on the nature of the apoptotic stimulus. Both the topoisomerase II inhibitor, etoposide, and the antimetabolite, hydroxyurea, rapidly induced cell death in FaO cells. This cell death was shown to occur predominantly via apoptosis; there was no evidence of necrotic cell death as defined by morphology and the low percentage of cells with plasma membranes permeable to trypan blue. Etoposide induces protein-associated DNA double strand breaks and hydroxyurea, by preventing the synthesis of DNA bases, indirectly damages DNA resulting in single strand breaks. The onset of FaO apoptosis induced by these DNA damaging agents was not inhibited or retarded by co-addition of nafenopin. A possible explanation for this is that the extent of cellular damage following exposure to these genotoxic agents is too extreme to be prevented by nafenopin. Alternatively, it may be that peroxisome proliferators such as nafenopin interfere with specific cell death signals with the result that only certain forms of cellular damage can be prevented.
It is improbable that one mechanism alone is responsible for peroxisome proliferator-induced hepatocarcinogenesis. It is more likely that a number of different processes work in tandem to promote pre-neoplastic liver cells. While the exact relationship between these different mechanisms remains speculative, the results of this paper certainly give further support to the theory that suppression of apoptosis may be, at least partially, responsible for the hepatocarcinogenic nature of this important class of non-genotoxic carcinogens.

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