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The use of primary cultures of adult rat hepatocytes to study induction of enzymes and DNA synthesis: Effect of nafenopin and electroporation

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Summary. Primary cultures of adult rat hepatocytes maintained in a well-differentiated state, in a chemically defined medium containing 2% DMSO, have been utilized to study the effect of non-mutagenic hepatocarcinogens such as the peroxisome proliferator nafenopin. The parameters chosen in this in vitro system were those that paralleled the major in vivo effects of nafenopin on the liver, mainly: the proliferation of the endoplasmic reticulum and induction of cytochrome P-452, the proliferation of the peroxisome compartment and the induction of cyanide-insensitive β-oxidation of fatty acids and the stimulation of liver growth as measured by the DNA synthetic activity of the hepatocytes.

In this review, we also describe the morphology of hepatocyte cultures prepared from previously electroporated hepatocytes and the potential for the use of electroporation to introduce growth related genes into hepatocyte cells to study the mechanisms of hepatocyte growth at the molecular level. In addition we describe the formation of endoplasmic reticulum whorls in these cultures as a consequence of nafenopin treatment. 'Whorl formation' by hepatotrophic chemicals has been previously shown to occur in vivo; in this report, it is described for the first time in vitro.

Key words. Hepatocyte cell cultures; cytochrome P-450; peroxisome proliferation; DNA synthesis; nafenopin; electroporated hepatocytes; DMSO.

Introduction

Considerable efforts are currently being exerted to reduce the use of animals for toxicity testing. Consequently, primary cultures of adult rat hepatocytes, as an alternative to the whole animal, are now widely employed for assessment of genotoxic potential and cytotoxicity mechanisms[4, 12]. Other parameters of interest to us and others are those concerning the hepatocarcinogenic or tumor promotive action of chemicals which were negative in standard tests of genotoxicity[16]. Many of these chemicals induce liver growth accompanied by increases in specific enzyme activities. For example, the long-term administration of the hypolipidemic agent nafenopin and other peroxisomal proliferators has been found to cause liver tumors[15–17] accompanied by liver growth, proliferation of the endoplasmic reticulum and the peroxisomal compartment[7, 18, 25], induction of microsomal cytochrome P-452 and the peroxisomal cyanide insensitive β-oxidation system[1, 14]. The relationship between such effects and tumor development is not clearly understood, although a correlation between peroxisome proliferation and hepatocarcinogenesis has been suggested[15, 16]. Since the target organ is the liver, hepatocyte cultures provide an excellent in vitro system to assess the effects of nafenopin and similar chemicals on the above-mentioned parameters and to study the cellular mechanisms of these toxic manifestations with the minimal use of animals.

However, the use of hepatocyte cultures for such studies has been hindered by the short life-span of the cells in culture (3–4 days), the loss of differentiated functions and the rapid and continual decline in their cytochrome P-450 content under a variety of culture conditions[21, 22]. A major advancement in hepatocyte culture techniques was the introduction of serum-free chemically defined media, designed specifically for the maintenance of liver cells in culture. With such systems it is possible to per-

form mechanistic studies without interference from unidentified factors in the serum [19]. Recently, Isom et al.[8] used a serum-free chemically defined medium, termed HCD, and were able to maintain adult hepatocytes in culture in a well-differentiated state for up to 40 days by supplementing the medium with 2% DMSO one day after plating. We have therefore characterized this culture system, in terms of its response to nafenopin. In addition, we describe here our observations on the long-term effects of nafenopin in normal and electroporated hepatocytes maintained in this HCD medium supplemented with EGF and without DMSO.

Induction of cytochrome P-450 in DMSO-treated hepatocytes

Addition of 2% DMSO to hepatocyte cultures in serum-free chemically defined medium on day 1 considerably retarded the cytochrome P-450 decline between days 1 and 13 (fig. 1), although an initial decline in cytochrome P-450, similar to previous reports [21, 22], occurred on one day, before and directly after the addition of DMSO. The maintenance of constant and measurable levels of cytochrome P-450 from day 2 to day 6 is advantageous when compared with previously used culture systems. Thus, a better assessment of the inducing properties of chemicals on cytochrome P-450-dependent enzyme activities is feasible. Figure 2 shows that when DMSO-treated hepatocytes were exposed to phenobarbital, 3-methylcholanthrene and nafenopin during this period of time (from day 3 to day 6), net increases in cytochrome P-450,

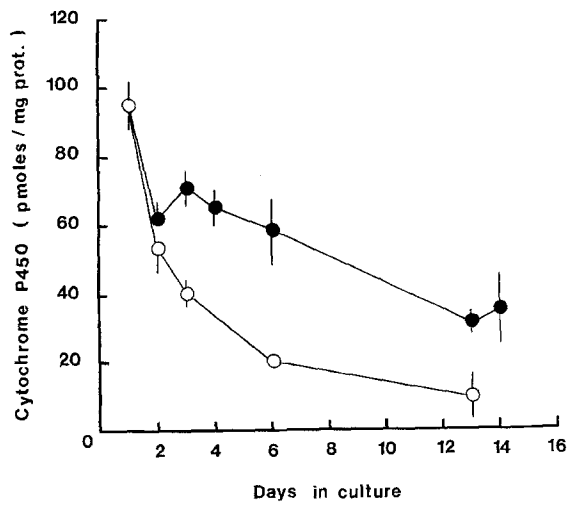

Figure 1. Partial maintenance of cytochrome P-450 in hepatocytes cultured in serum-free medium containing 2% DMSO. Cultured hepatocytes were maintained either in the absence (–O–) or in the presence of 2% DMSO (–●–) added at day 1 after seeding. Cytochrome P-450 was determined in the 10,000 × g supernatant [13]. Values are expressed as means ±SEM of 3–6 independent experiments. Where not shown, the SEM is smaller than the diameter of the symbol. The cytochrome P-450 content of fresh hepatocytes was 169 ± 18 pmoles/mg protein in 8 separate experiments. (Reprinted by permission from Exp. Cell Res. *171* (1987) 37–51, Academic Press)

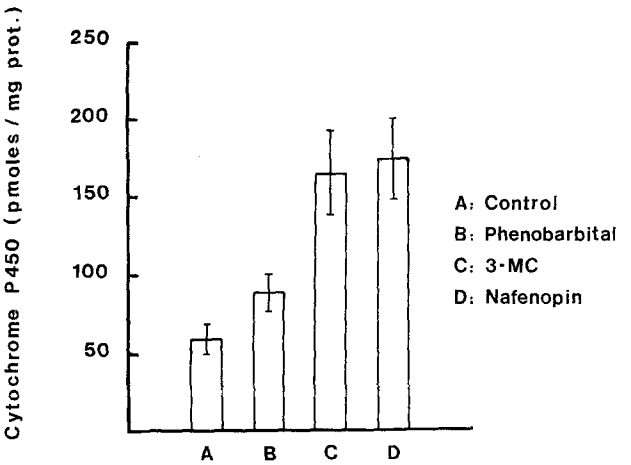

Figure 2. Induction of cytochrome P-450 in hepatocytes cultured in serum-free medium supplemented with 2% DMSO. Phenobarbital (0.5 mM), 3-methylcholanthrene (2 μM) and nafenopin (32 μM) were added at day 3 after plating. At day 6 the cells were harvested and the cytochrome P-450 content was determined. Values are expressed as means ±SEM of 3–6 independent experiments. Control cytochrome P-450 at day 3 prior to the addition of the inducers was 69 ± 5 pmoles/mg protein. (Reprinted by permission from Exp. Cell Res. *171* (1987) 37–51, Academic Press)

P-448 and P-452 were observed [13]. However, it should be borne in mind that the cytochrome-P-450 content maintained under these culture conditions may not be homogeneous since firstly, changes in the concentrations of the cytochrome P-450 isozymes can occur depending on the composition of the medium [23] and secondly, although DMSO may maintain cytochrome P-450 content simply by increasing the synthesis of its heme moiety, the possibility that it is selectively inducing certain isozymes cannot be ignored [13].

Another advantage of maintaining cytochrome P-450 content for longer periods of time in culture is that it may enable more effective studies with chemicals which require metabolic activation by cytochrome P-450 before they are able to exert their toxic effect.

Induction of peroxisomal β-oxidation in DMSO-treated hepatocytes

The advantage of the use of the DMSO-treated hepatocyte cultures to study the peroxisomal β-oxidation of fatty acids and its induction by various peroxisome proliferators, including nafenopin, is again due to the better survival of the hepatocytes over longer periods of time and the relative stability of the basal peroxisomal β-oxidation activity compared with other culture systems [2, 6, 11]. Thus, addition of nafenopin to the culture medium on day 1 caused a net increase in this activity which was time-dependent and maintained for at least 14 days in culture (fig. 3). When nafenopin was added from day 3 to 6, the hepatocytes retained the ability to induce the peroxisomal β-oxidation activity by 4-fold when measured on day 6 [13]. Under the same experimental condi-

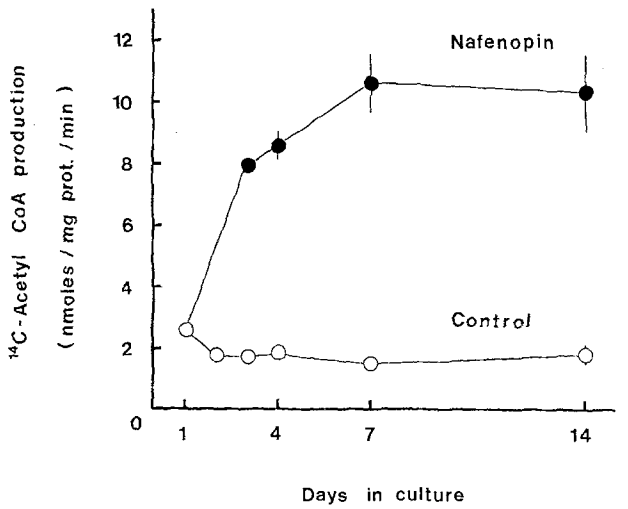

Figure 3. Induction of cyanide-insensitive palmitoyl-CoA oxidation by nafenopin in rat hepatocytes cultured in serum-free medium containing 2% DMSO. After 24 h cultures were maintained without (–O–) or with addition of nafenopin (32 μM) (–●–). After various times the cells were harvested and palmitoyl-CoA oxidation was determined. Values are expressed as means ±SEM of 4 independent experiments. The control value at day 1 was determined just prior to commencement of treatment. (Reprinted by permission from Exp. Cell Res. *171* (1987) 37–51, Academic Press)

tions, we have recently shown that the levels of mRNA for peroxisomal fatty acyl-CoA oxidase and the peroxisomal bifunctional enzyme also increased in a time-dependent manner when measured 1, 8 and 20 h after nafenopin addition with a 5-fold increase over control values at 20 h [3]. Thus this culture system will facilitate mechanistic studies at both the cellular and the molecular level.

Induction of DNA synthesis in DMSO-treated hepatocytes

When adult hepatocytes are maintained in culture, in a chemically defined medium, they can be stimulated to undergo a wave of DNA synthesis as well as a limited amount of cell proliferation in response to certain hormones and growth factors, such as insulin, glucagon and epidermal growth factor (EGF) [9, 10]. Thus, they provide a good in vitro model for studying the many factors that regulate liver growth. Consequently, the effect of chemicals on liver growth can also be investigated.

In the presence of 2% DMSO in the medium, the basal DNA synthetic activity of the cultured hepatocytes is decreased by 40% (table), but the hepatocytes retain their ability to respond to hormones such as angiotensin II, to the growth factor EGF and to chemicals such as nafenopin (table and fig. 4). We have noted that the peak of the wave of DNA synthesis induced by addition of EGF to the culture medium at day 1 occurred at a later time (at day 4, instead of day 3) when 2% DMSO was present in the medium.

This demonstrates the usefullness of adult hepatocyte cultures in studies relevant to liver regeneration and carcinogenicity. We have recently extended the application of these cultures to investigate mechanisms of liver growth at the molecular level. Using the method of electroporation of isolated hepatocytes [26], we introduced the c-myc gene, an oncogene expressed during liver regeneration and carcinogenesis [5], into isolated hepatocytes to investigate the effect of this gene on the stimulation of DNA synthesis by EGF in culture. The results showed that the c-myc gene product was expressed in the electroporated cells and that this expression coincided with a significant transient enhancement of the EGF-induced DNA synthesis activity [27].

Stimulation of [methyl-^{3}H]-thymidine incorporation into DNA in hepatocyte cultures maintained in HCD medium supplemented with 2% DMSO by nafenopin and angiotensin II

Treatment[a]	Without DMSO	With DMSO
	cpm $\times 10^{-3}$/μg DNA[b]	
None	54.7 ± 10	34.2 ± 2
Nafenopin (32 μM)	90.7 ± 13	66.3 ± 6
Angiotensin II (10^{-8}M)	123.7 ± 11	56.6 ± 2

[a] Twenty-four hours after plating, hepatocytes were fed HCD medium +/– DMSO and treated as described in the table. Radioactivity in DNA was determined 72 h after plating and 24 h after addition of [methyl-^{3}H]-thymidine as described in Muakkassah-Kelly et al.[13]. [b] Values are means ±SE of three petri dishes.

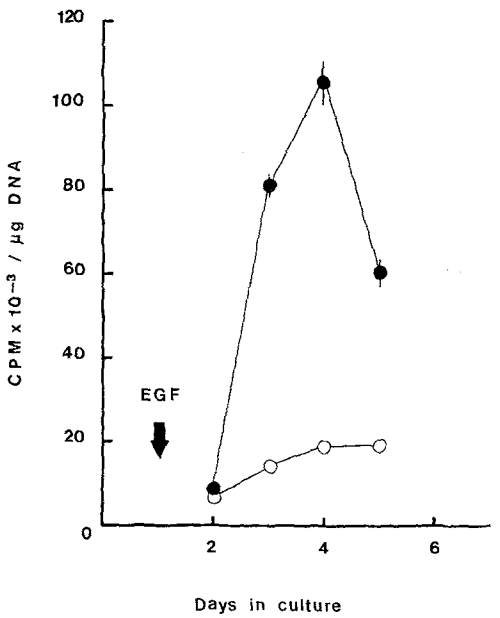

Figure 4. Stimulation by EGF of [methyl-^{3}H]-thymidine incorporation into DNA in hepatocytes cultured in HCD medium supplemented with DMSO (2%). Hepatocyte cultures were treated with DMSO at day 1 without (–O–) or with addition of EGF (50 ng/ml) (–●–). Cells were harvested at the times indicated in the figure, 24 h after thymidine addition. Values are means ±SEM of three triplicate culture dishes. (Reprinted by permission from Exp. Cell Res. *171* (1987) 37–51, Academic Press)

Morphological observations

In this report we briefly discuss the morphological changes we have observed when the electroporated hepa-

tocytes were maintained in culture in the chemically defined medium in the presence of EGF without addition of DMSO.

We have previously shown [13] that hepatocytes cultured in the presence of DMSO responded to nafenopin exposure by 1) an increase in the number of peroxisomes, and 2) and enhanced survival of cells. In addition, the occurrence of megamitochondria was observed after prolonged cultivation periods (i.e. 37 days), irrespective of the presence of nafenopin. In the absence of DMSO, nafenopin treatment of hepatocytes also caused proliferation of peroxisomes (figs 5 and 7) and formation of megamitochondria (fig. 5). Furthermore, some hepatocytes contained large aggregates of concentrically arranged smooth membranes, i.e. membranous whorls or 'fingerprints' (fig. 6). Similar structures were described to occur in hepatocytes of nafenopin-treated rats [24]. Many structurally unrelated hepatotropic compounds elicit the formation of such concentric membrane arrays; their physiological significance is not understood, although they might be engaged in cholesterol biosynthesis [20]. In cultures that were prepared from electroporated hepatocytes, the presence of a non-hepatocytic cell type was a common feature. These elongated cells contained abundant polysomes and rough endoplasmic reticulum (fig. 7) and thus showed some characteristics of fibroblasts. They often occurred at the periphery of patches or territories that were constituted by hepatocytes (fig. 7). At the moment, we are unable to offer a meaningful explanation for this phenomenon.

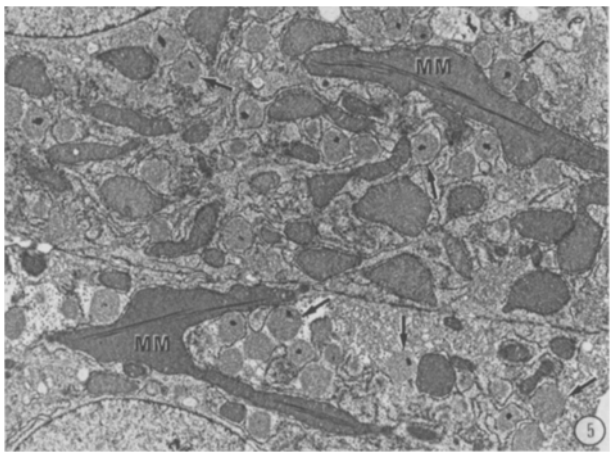

Figure 5. Rat hepatocytes cultured for 14 days in the presence of EGF (25 ng/ml) and nafenopin (10 µg/ml). Note the striking proliferation of peroxisomes (arrows). As previously described for hepatocytes cultured in presence of 2% DMSO [13], megamitochondria (MM) appeared. × 7920.

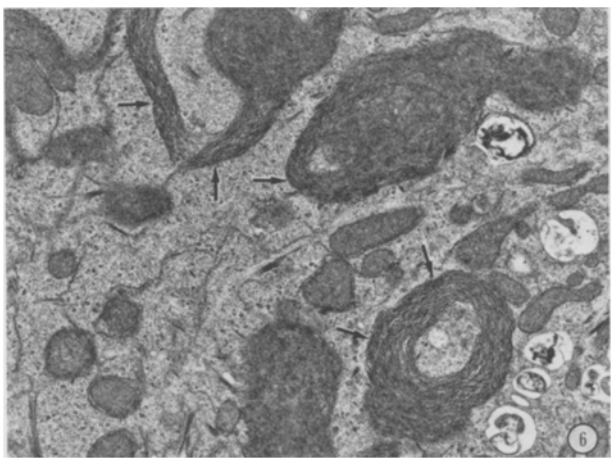

Figure 6. Rat hepatocytes were treated and cultured as outlined in the legend to fig. 5. As can be observed in the in vivo situation [24], nafenopin treatment induced the formation of concentric membrane arrays (i.e. membrane whorls or 'fingerprints') (arrows). × 16,320.

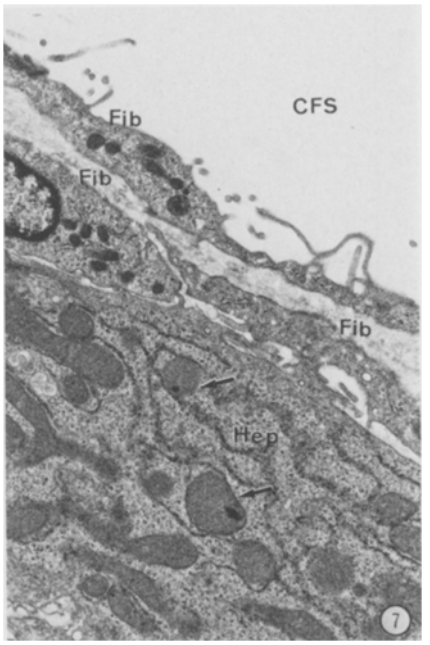

Figure 7. Rat hepatocytes were electroporated in the presence of a control plasmid (pSV_2Neo) and then cultivated for 17 days in the presence of EGF (25 ng/ml) and nafenopin (10 µg/ml). The hepatocytes (Hep) formed patches of territories which were separated by cell-free spaces (CFS). On the periphery of these hepatocyte territories a new cell type (Fib) occurred. These fibroblast-like cells were often elongated and contained numerous polysomes and rough endoplasmic reticulum cisternae. As a response to nafenopin exposure, many peroxisomes (arrows) can be seen in hepatocytes. × 14,620.

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Teratogenicity screening in standardized chick embryo culture: Effects of dexamethasone and diphenylhydantoin *

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Summary. Teratogenic and toxic effects of DXM and DPH were tested using a standardized chick embryo culture. Survival, growth and malformations were scored with respect to the drug concentrations used. DXM ($>10^{-8}$ mol/l) inhibited the differentiation of the extraembryonic blood circulation and induced craniofacial anomalies. DPH ($>1.5\ 10^{-5}$ mol/l) induced cardiomegaly, craniofacial and somitic anomalies. Both drugs were lethal at 10^{-3} mol/l. Comparison of results obtained with 8 drugs shows that the method has a good discriminative power and specificity and that it can be used as a simple, reliable and economical primary screening test, making it possible to reduce the use of animals in toxicological studies.
Key words. Chick embryo; teratogenicity; screening in vitro; dexamethasone; diphenylhydantoin.