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Peroxisome Proliferation and its role in Carcinogenesis

Views and expert opinions of an IARC Working Group
Lyon, 7–11 December 1994

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1. CONSENSUS REPORT

1.1 Introduction

A large number of chemicals have been shown to induce peroxisome proliferation in the livers of mice and rats. Such chemicals include hypolipidaemic and other drugs, some herbicides, plasticizers, solvents, food flavours and natural products. Since humans are exposed to peroxisome proliferators to a significant extent, assessment of the adverse biological effects of this group of compounds, and particularly their potential carcinogenicity, has become an important issue.

At a meeting in December 1993 (IARC, 1993) to identify priorities for *IARC Monographs* in 1995–2000, several groups of agents were identified that may have specific mechanisms of action. One such group was chemicals that induce proliferation of peroxisomes, particularly in hepatocytes of rats and mice. The present meeting on peroxisome proliferation and its relationship to carcinogenesis was convened as a result of a recommendation of that meeting to discuss generic mechanisms of carcinogenicity before certain groups of chemicals were evaluated.

The *IARC Monographs* programme aims to identify chemicals and other agents and mixtures that are carcinogenic to humans; the evaluations are agreed upon by groups of invited experts in relevant fields. Most of the information considered during this process is derived from studies of human epidemiology and experimental carcinogenicity; information that may be relevant to the mechanism by which the putative carcinogen acts is also considered, as it may be helpful in making an overall evaluation. At a meeting on the mechanisms of carcinogenesis, held in Lyon in 1991 (Vainio *et al.*, 1992), information relevant to an evaluation of carcinogenic risk was considered to include evidence of: genotoxicity (i.e. structural changes at the level of the gene), effects on the expression of relevant genes (i.e. functional changes at the intracellular level), effects on cell or tissue morphology or behaviour and time and dose relationships of carcinogenic effects and interactions between agents. That advisory meeting concluded that the available data on mechanisms should be summarized and the strength of the evidence for the action of certain mechanisms and their relevance to carcinogenicity should be evaluated. Information on mechanism of action may show that similar effects occur in humans and experimental animals, or it could suggest species specificity. The ways in which such information might be used to modify an evaluation of carcinogenicity are described in the Preamble to each volume of *Monographs* since Volume 54 (IARC, 1992).

1.2 Characteristics of peroxisome proliferation

1.2.1 Peroxisomes

Peroxisomes are single, membrane-limited, cytoplasmic organelles that are found in cells of animals, plants, fungi and protozoa. In rat hepatocytes, they are normally spherical or oval, about 0.5 μm in diameter and contain a finely granular matrix with a crystalline nucleoid core (Cohen & Grasso, 1981); it has been suggested that peroxisomes are not discrete organelles but actually exist as a continuous reticulum. They account for about 2% of the cytoplasmic volume and total cellular protein (Cohen & Grasso, 1981; Reddy & Lalwani, 1983; Mannaerts & Van Veldhoven, 1993). They are characterized by their content of catalase and a number of hydrogen peroxide-generating oxidases (Cohen & Grasso, 1981; Reddy & Lalwani, 1983), and, like mitochondria, they contain a fatty acid β-oxidation enzyme system. The half-life of peroxisomal enzymes is generally about 36 h. Peroxisomal disorders can have serious consequences for the organism (Mannaerts & Van Veldhoven, 1993; Reddy & Mannaerts, 1994).

1.2.2 Peroxisome proliferation in rats and mice

Peroxisome proliferation can be defined as a cellular process characterized by increases in the volume density of peroxisomes and of peroxisomal fatty acid β-oxidation activity. Determination of peroxisome proliferation may be based on either but preferably both of these end-points. A **peroxisome proliferator** can be defined as a chemical that increases peroxisome proliferation. In judging whether a chemical is a peroxisome proliferator, due attention should be given to adequacy of experimental design, conduct and analysis.

A characteristic structural feature of many, but not all, peroxisome proliferators is the presence of an acidic function (Lake & Lewis, 1993), which is normally a carboxyl group and either occurs free in the parent structure or is generated by metabolism. Marked differences in the potencies of peroxisome proliferators have been demonstrated in several studies. For example, the hypolipidaemic agent ciprofibrate is orders of magnitude more potent than the plasticizer di(2-ethylhexyl)phthalate (Reddy *et al.*, 1986). Potent compounds can increase peroxisome volume density (peroxisome volume as a proportion of cytoplasmic volume) in liver of rats and mice from around 2% to 20–25%, whereas smaller changes may be produced by weaker compounds and by physiological factors, including certain high-fat diets and vitamin E deficiency.

Liver enlargement induced by peroxisome proliferators is due to both hepatocyte hyperplasia (increased replicative DNA synthesis and cell division) and hypertrophy. Morphological examination reveals increased peroxisome volume density, which results from an increase primarily in the number of peroxisomes, although size may also be increased. The major biochemical alteration is induction of the activities of peroxisomal enzymes of the fatty acid β-oxidation system and of CYP4A subfamily isoenzymes. The activity of the peroxisomal fatty acid β-oxidation cycle is normally determined by measuring overall activity (e.g. as cyanide-insensitive palmitoyl-coenzyme A oxidation) or by assaying the first rate-limiting enzyme of the cycle, namely acyl-coenzyme A oxidase (Mannaerts & Van Veldhoven, 1993). There is differential induction of peroxisomal enzyme activities, in that while that of the β-oxidation cycle enzymes can be markedly induced, smaller increases are observed in the activities of other peroxisomal enzymes, such as catalase. The stimulation of microsomal fatty acid-oxidizing enzymes (normally measured as lauric acid 12-hydroxylase) is due to induction of cytochrome P450 isoenzymes in the CYP4A subfamily (Gibson, 1989). The activity of carnitine acetyltransferase can also be markedly induced by peroxisome proliferators (Cohen & Grasso, 1981; Reddy & Lalwani, 1983); however, as this enzyme is found in peroxisomal, mitochondrial and microsomal fractions, enzyme induction may reflect stimulation of activity in more than one subcellular compartment (Cohen & Grasso, 1981; Moody *et al.*, 1991). The morphological and biochemical changes described above persist at the steady-state level for as long as the peroxisome proliferator is administered; reversal depends on the half-lives of both the induced enzymes and the rate of elimination of the chemical.

Other reported effects of peroxisome proliferators in hepatocytes of rats and mice include mitochondrial proliferation (with changes in enzyme activities), increase in the number of lysosomal bodies (with changes in enzyme activities and lipofuscin deposition) and effects on UDPglucuronosyltransferase activities. Peroxisome proliferators have also been reported to induce microsomal and cytosolic epoxide hydrolase activities, to modulate intracellular calcium concentrations, to stimulate protein kinase C and to reduce the activities of glutathione peroxidase, glutathione *S*-transferase and superoxide dismutase (Reddy & Lalwani, 1983; Bentley *et al.*, 1993; Grasso, 1993; Lake, 1993, 1995).

While marked effects may be observed in hepatocytes, only small increases in mRNA levels of peroxisomal fatty acid β-oxidation enzymes have been observed in certain other tissues, such as the kidney, intestine and heart.

1.2.3 Peroxisome proliferation in vitro

Peroxisome proliferation has been demonstrated *in vitro* in primary rat and mouse hepatocyte cultures in a number of different laboratories, with standard hepatocyte media and culture conditions (Lock *et al.*, 1989; Moody *et al.*, 1991; Lake & Lewis, 1993; Foxworthy & Eacho, 1994). The factors responsible for the induction of peroxisome proliferation have been shown in these studies to be intrahepatic, and they are retained in cell culture. The characteristics of peroxisome proliferation *in vivo*, including stimulation of DNA synthesis, increased peroxisome numbers, changes in morphology and differential induction of enzyme activities, have also generally been observed in cultured hepatocytes.

In most studies, the effects of chemicals on the activities of peroxisomal and microsomal fatty acid-oxidizing enzymes have been examined in hepatocytes cultured for 3–4 days; however, peroxisome proliferation has also been reported in long-term (> 7 days) hepatocyte cultures and in other systems, including certain liver-cell lines, hepatocyte spheroids and liver slices (Lake, 1995). Peroxisome proliferation has also been demonstrated in hepatocytes transplanted into subcutaneous fat or the anterior chamber of the eye of rats and in hepatocytes induced in rat pancreas (Reddy & Mannaerts, 1994).

1.2.4 Mechanisms of induction of peroxisome proliferation

Hypotheses to explain the initiation of peroxisome proliferation in hepatocytes include: the involvement of a receptor, substrate overload and peroxisome proliferators serving as substrates for peroxisomal enzymes (Reddy & Lalwani, 1983; Bentley *et al.*, 1993). Attention has focused mainly on identifying receptors and on the effect of peroxisome proliferators on lipid metabolism. These two hypotheses are not mutually exclusive (Bentley *et al.*, 1993). In the hypothesis of substrate overload, peroxisome proliferation is considered to be an adaptive response to perturbation of lipid metabolism, and induction of CYP4A isoenzymes is involved. Certain peroxisome proliferators inhibit fatty acid oxidation, form coenzyme A esters, increase levels of fatty acids and displace fatty acids from the cytosolic fatty acid-binding protein (Lock *et al.*, 1989; Bentley *et al.*, 1993).

Green and coworkers (Issemann & Green, 1990) have cloned a peroxisome proliferator-activated receptor (PPAR) from mouse liver. This protein is a member of the steroid hormone receptor superfamily and, after activation, acts as a transcription factor. When a chimaeric receptor expression vector containing regions that encode the putative ligand-binding domain of this mouse PPAR and the DNA-binding domain of the human oestrogen receptor was transfected into COS 1 cells, it could be activated by peroxisome proliferators, leading to transcriptional activation of a gene containing an oestrogen response element. Several PPARs have now been described in the mouse, rat, frog and human beings. They may also be activated by certain fatty acids, and their target genes encompass those for peroxisomal, microsomal, mitochondrial and cytosolic enzymes, all of which are involved in fatty acid metabolism (Desvergne & Wahli, 1994; Reddy & Mannaerts, 1994; Lake, 1995), underlining the important physiological role of these PPARs in lipid metabolism. At the molecular level, PPARs form a heterodimer with RXR that binds to a specific response element in the target gene. The transcriptional effect is further modulated through interaction of PPAR or PPAR-RXR with other transcription factors, which act either positively, like Sp1 (Krey *et al.*, 1995), or are repressive, such as COUP-TF (Reddy & Mannaerts, 1994; Lake, 1995). Some PPARs may be dominant repressors of other forms (Kliewer *et al.*, 1994).

Binding of peroxisome proliferators to PPAR has not been demonstrated but cannot be excluded. Peroxisome proliferators have been shown to increase the level of certain PPARs in mouse and rat liver (Reddy & Mannaerts, 1994; Lake, 1995). Activation of PPARs by peroxisome proliferators may require metabolism (e.g. to a coenzyme A ester or other derivative) or may occur by displacement of fatty acids from their cytosolic binding protein (Desvergne & Wahli, 1994). Several mechanisms could account for tissue and species differences in response to peroxisome proliferators. These include differences in the metabolism of peroxisome proliferators, interaction of PPARs with different sets of transcription factors, and species differences in the regulatory element of a given target gene (see Reddy & Rao, this volume). Moreover, tissue differences in the distribution of PPARs have been documented (Reddy & Mannaerts, 1994; Lake, 1995; Zhu *et al.*, 1995).

1.2.5 Hepatocellular proliferation induced by peroxisome proliferation

Not only peroxisome proliferation but also hepatocyte proliferation is an important response in the livers of rats and mice receiving peroxisome proliferators. Acute hepatocyte proliferation, which involves about 50% of hepatocytes, is seen to begin about 48 h after the beginning of administration of a peroxisome proliferator. This early proliferative response subsides after several days. Chronic hepatocyte proliferation has been seen in the livers of rats and mice after administration of some, but not all, peroxisome proliferators. This replicative response may continue for the duration of exposure to the chemical. While the replication rate is less than that seen in the acute phase, the total proliferative response is much greater. Preferential hepatocyte proliferation occurs in the tumours that develop in rats and mice after administration of peroxisome proliferators and is seen in lesions that are the direct progenitors of tumours.

As enhanced hepatocyte proliferation is associated with administration of peroxisome proliferators to rats and mice, and in view of the importance of cell proliferation in carcinogenesis, hepatocyte proliferation is included in any evaluation of peroxisome proliferators, such as an assessment of species differences.

1.2.6 Species differences

Differences between species with regard to hepatic peroxisome proliferation have been investigated *in vitro* and *in vivo* (Cohen & Grasso, 1981; Stott, 1988; Lock *et al.*, 1989; Moody *et al.*, 1991;

Bentley *et al.*, 1993). Factors that must be taken into account in such studies include the metabolism, disposition and dose of the chemical, sex differences and intrahepatic differences in response; *in vitro*, the functional viability of the hepatocyte preparations must also be considered.

Rats and mice are clearly responsive to peroxisome proliferators; although differences among strains have been observed, these are minor in comparison with the magnitude of species differences in response. On the basis of the activities of marker enzymes (e.g. cyanide-insensitive palmitoyl-coenzyme A oxidation, lauric acid 12-hydroxylase, carnitine acetyltransferase) and ultrastructural examination, Syrian hamsters appear to exhibit an intermediate response, whereas in most studies guinea pigs are either unresponsive or refractory. There is no evidence of significant peroxisome proliferation in either New (e.g. marmoset) or Old (e.g. rhesus) World monkeys (Lock *et al.*, 1989; Bentley *et al.*, 1993; Lake, 1995) *in vivo*, although high doses of ciprofibrate and DL-040 were reported to induce hepatic peroxisome proliferation in cynomolgus and rhesus monkeys (Reddy *et al.*, 1984; Lalwani *et al.*, 1985).

The results of studies with cultured primary hepatocytes from rats, mice, Syrian hamsters, guinea pigs and primates *in vitro* mirror those obtained *in vivo* in the same species (Bentley *et al.*, 1993; Lake, 1995): less effect is observed in Syrian hamster than in rat or mouse hepatocytes, and little or no effect is seen in guinea pig and primate hepatocytes, even though such preparations responded to other chemical challenges.

Comparatively few investigations have been conducted to evaluate species differences in cell replication. Although both nafenopin and Wy-14,643 are potent mitogens in rat liver, they do not appear to produce any significant stimulation of replicative DNA synthesis in the hepatocytes of Syrian hamsters treated *in vivo* either acutely or chronically (Lake, 1995). Similarly, methylclofenapate increased replicative DNA synthesis in rat hepatocytes *in vitro*, but no effect was observed in guinea pig or marmoset hepatocytes (Elcombe & Styles, 1989).

For data on human hepatocytes and human volunteers, see section 1.5, Peroxisome proliferators, human response and hazard.

1.3 Hepatocarcinogenicity in experimental animals

1.3.1 Concordance with peroxisome proliferation

Prolonged administration of members of the structurally diverse class of peroxisome proliferators has been shown in many studies to produce liver tumours in rats and mice (Cohen & Grasso, 1981; Reddy & Lalwani, 1983; Bentley *et al.*, 1993), but the long-term effects of these compounds in other species have been examined in only a few studies. Clobuzarit, which induces peroxisome proliferation in rat and mouse liver (Orton *et al.*, 1984), did not induce tumours in Syrian hamsters in a two-year study (Tucker & Orton, 1995). Di(2-ethylhexyl)-phthalate, administered either by inhalation or by intraperitoneal injection, also did not induce tumours in this species (Schmezer *et al.*, 1988), although the doses and treatment regimens may not have been appropriate for an assessment of hepatocarcinogenicity. Both nafenopin and Wy-14,643 induce liver enlargement and sustained peroxisome proliferation in Syrian hamsters, but neither induced liver tumours after 80 weeks (Lake, 1995). Although peroxisome proliferation is therefore not sufficient for tumour induction, it may be necessary in the sequence of events that leads to the carcinogenicity of these compounds.

Some long-term studies have been performed in primates with ciprofibrate, clobuzarit and clofibrate. Although none was of lifetime duration, a 6.5-year study of clofibrate in marmosets covered about half of the expected lifespan of that species (Tucker & Orton, 1993). Increased relative liver weight was observed in some studies, but there was no evidence for significant peroxisome proliferation or peroxisome proliferator-induced liver lesions (Tucker & Orton, 1993; Graham *et al.*, 1994). These results suggest that the peroxisome proliferator-induced altered hepatic foci typically observed in rats and mice are not induced in Syrian hamsters or primates.

A comparison of 39 paired sets of data on carcinogenicity and peroxisome proliferation for 18 agents that have been shown to possess an intrinsic ability to induce peroxisome proliferation in the livers of rats and mice indicates a strong concordance (80%) between peroxisome proliferation and hepatocarcinogenicity after long-term exposure to these chemicals (Table 1), providing further support for the validity of peroxisome proliferation as an early biomarker for carcino-

genesis in the liver (Reddy *et al.*, 1980; Reddy & Rao, 1992).

Peroxisome proliferators are generally inactive in a wide range of short-term tests for genotoxicity *in vitro* and *in vivo*, including mutagenicity in *Salmonella*, unscheduled DNA synthesis, DNA damage as measured by ^{32}P-postlabelling, intrachromosomal deletion and recombination in yeast (Warren *et al*, 1980; Reddy & Rao, 1992; Ashby *et al*, 1994). A recent, critical evaluation of the results of various tests for the mutagenicity of peroxisome proliferators thus eliminated intrinsic genotoxicity as the unifying mechanism of action for this class of carcinogenic chemicals (Ashby *et al*, 1994). It should be noted, however, that the properties of peroxisome proliferation and genotoxicity are not necessarily mutually exclusive, and some of these chemicals have genotoxic activity *in vitro*. A few hepatocarcinogenic peroxisome proliferators induced weak cytogenetic and/or cell transforming effects in cultured cells (Table 2), although this is not a consistent property of peroxisome proliferators. Several peroxisome proliferators also inhibited intercellular communication in rat and mouse hepatocytes and in hamster cells in culture (Table 2).

As genotoxicity is not a primary biological effect of peroxisome proliferators, any genetic alteration that may be necessary for the carcinogenic action could occur indirectly during long-term exposure to these agents as a result of biological alterations; consequently, the process of initiation would be rather slow and not as drastic as observed with genotoxic carcinogens. Structurally diverse peroxisome proliferators induce characteristic responses in hepatocytes by interacting with members of the PPAR subfamily. Thus, the hepatocarcinogenicity of these chemicals is strongly associated with the induction of these predictable and highly characteristic responses. Caution should be exercised, however, in drawing a mechanistic inference from this receptor-mediated response, despite the concordance between peroxisome proliferation and hepatocarcinogenicity. Knowledge of the carcinogenic mechanisms of peroxisome proliferators is incomplete; however, the characteristic biochemical composition of peroxisomes and the fact that peroxisome proliferation is associated with disproportionate changes in the levels of peroxisomal enzymes, leading to production of excess hydrogen peroxide concentrations in liver, may provide clues to the involvement of this organelle in carcinogenesis.

1.3.2 Plausible mechanisms

Several mechanisms have been proposed for the induction of hepatocellular tumours in rats and mice.

1.3.2.1 Receptor-mediated responses and oxidative stress

This hypothesis relies on experimental evidence that the biological effects of peroxisome proliferators are confined predominantly to hepatic cells, that peroxisome proliferation is associated with disproportionate increases in the activities of enzymes that generate and degrade hydrogen peroxide, perturbing the hydrogen peroxide balance in the liver, and that tumours develop in this organ, which is also the main organ that responds to peroxisome proliferation. Peroxisomes in liver contain at least five distinct oxidases, which use a variety of substrates to generate hydrogen peroxide. Of these, urate oxidase, a liver-specific peroxisomal oxidase, and peroxisomal fatty acyl-coenzyme A oxidase, the first enzyme in the peroxisomal oxidation system, are of interest. Urate oxidase is expressed in the livers of most mammals, including rats and mice, but not in humans or higher primates. It is a pivotal enzyme in the metabolism of uric acid, a naturally occurring, potent biological antioxidant. The metabolic degradation of uric acid to allantoin by urate oxidase leads to the generation of hydrogen peroxide and accounts for the low serum levels of uric acid in these animals (Ames *et al.*, 1981). The activity of urate oxidase is two to three times higher in livers with peroxisome proliferation than in normal livers, with a consequent reduction in the concentration of uric acid in serum.

Livers of rats and mice with peroxisome proliferation show a 20- to 40-fold increase in the activity of peroxisomal fatty acyl-coenzyme A oxidase, owing to transcriptional activation of the responsible gene, whereas catalase activity is increased by less than twofold. These disproportionate increases in hydrogen peroxide generating and hydrogen peroxide degrading peroxisomal enzymes, together with reductions in the overall cellular capacity to detoxify hydrogen peroxide, provide a plausible biological basis for the role of peroxisome proliferation-associated oxidative stress in hepatocarcinogenesis. Livers of animals with massive peroxisome proliferation have a biochemical milieu that is consistent with the presence

Table 1. Database for examining the concordance between hepatocellular peroxisome proliferation (PP) and hepatocarcinogenicity (HC) in rats and mice (M, male; F, female)

Compound	CAS No.	Rats					Mice				
		Strain	PP		HC		Strain	PP		HC	
			M	F	M	F		M	F	M	F
Benzylbutyl phthalate	85-68-7	F344		–		–					
Cinnamyl anthranilate	87-29-6	F344	–	+	–	–	B6C3F1	+	+	+	+
Ciprofibrate	52214-84-3	F344	+		+		C57Bl	+		+	
Clobuzarit	22494-47-9	Wistar	+		–		C57Bl	+		+	
Clofibrate	637-07-0	SD	+	+	+	+	C57Bl	+		–	
		F344	+		+		Swiss	+		–	
		Wistar	+		–						
Di(2-ethylhexyl)adipate	103-23-1	F344	+	+	–	–	B6C3F1	+	+	–	+
Di(2-ethylhexyl)phthalate	117-81-7	F344	+	+	+	+					
Di-isononyl phthalate	28553-12-0	F344	–	–	–	–					
Gemfibrozil	25812-30-0	SD	+		+						
Lactofen	3513-60-4						CD–1	+	+	+	+
LY 171883	88107-10-2						B6C3F1		+		+
Methylclofenapate	21340-68-1	F344	+		+						
Nafenopin	3771-19-5	F344	+		+						
Tetrachloroethylene	127-18-4	F344	–	–	–	–	B6C3F1	+	+	+	+
Tibric acid	37087-94-8	F344	+		+						
Trichloroacetic acid	76-03-9						B6C3F1	+		+	
Trichloroethylene	79–01–6	F344	–		–		B6C3F1	+		+	
		Osborne-Mendel	–		–						
Wy-14,643	50892-23-4	F344	+		+						

Adapted from Ashby *et al.* (1994)

Table 2. Overall activity of hepatocarcinogenic peroxisome proliferators in assays for morphological cell transformation and gap-junctional intercellular communication

Compound	Cell transformation	Intercellular communication
Clofibrate	+	+
Di(2-ethylhexyl)adipate	–	
Di(2-ethylhexyl)phthalate	+	+
Methylclofenapate		–
Nafenopin		+
Trichloroethylene	+	+
Trichloroacetic acid		+
Wy-14,643	+	+

+, Most assays with the compound gave a positive response (i.e. induced cell transformation or decreased intercellular communication).
–, Most assays with the compound gave a negative response (i.e. did not induce cell transformation or did not decrease intercellular communication).

of a sustained increase in the levels of hydrogen peroxide (Reddy & Rao, 1992). Increased amounts of lipofuscin and high levels of conjugated dienes have been reported in livers of rats treated for long periods with peroxisome proliferators. Furthermore, fatty acyl-coenzyme A oxidase-rich peroxisomes isolated from the livers of rats treated with a peroxisome proliferator induced DNA strand breaks *in vitro* in one study; this finding was not reproduced in other studies. Taken together with the observation that livers with chronic peroxisome proliferation show a two- to fourfold increase in the level of 8-hydroxydeoxyguanosine in DNA, these reports can be construed as evidence for the role of receptor-mediated transcriptional activation of hydrogen peroxide-generating peroxisomal fatty acyl-coenzyme A oxidase in the carcinogenesis associated with peroxisome proliferation. A recent study (Cattley & Glover, 1993), however, demonstrated that the increase in 8-hydroxydeoxyguanosine is dependent on the method of DNA isolation, suggesting that it occurs in the homogenate and thus reflects changes in mitochondrial rather than nuclear DNA.

In rats given long-term treatment with both ciprofibrate, a peroxisome proliferator, and ethoxyquin, an antioxidant, a marked reduction in hepatocellular tumour development is seen, despite the increases in liver enlargement, hepatocellular proliferation and peroxisome proliferation (Rao *et al.*, 1984). Conflicting results were obtained, however, in studies of the modulating effects of vitamin E deficiency and peroxisome proliferation-induced carcinogenesis (Glauert *et al.*, 1990; Lake *et al.*, 1991).

It is less likely that the level of oxidative stress caused by sustained induction of peroxisome proliferation would yield measurable DNA breakage similar to that resulting from exposure to genotoxic chemicals. Induction of DNA damage by free radicals occurs relatively commonly in mammalian DNA, and, although it is rapidly repaired, it is implicated in spontaneous initiation (Saul & Ames, 1986; Loeb, 1989). As mentioned above, several lines of evidence indicate that induction of peroxisome proliferation is not the sole factor involved in tumour development. The lack of a consistent quantitative association between induction of peroxisome proliferation, DNA oxidation and tumour response also indicates that additional factors must operate.

The availability of transgenic cells containing the rat peroxisomal fatty acyl-coenzyme A oxidase gene has provided an opportunity to examine the role of increased expression of this hydrogen peroxide-generating peroxisomal protein in cell transformation (Chu *et al.*, 1995). These and other transgenic cells and transgenic animals that overexpress fatty acyl-coenzyme A oxidase should serve as useful systems for further elucidating the relative roles of peroxisome proliferation and oxidative stress in hepatocarcinogenesis.

1.3.2.2 Increased cell proliferation

Cell proliferation has been linked experimentally and conceptually to carcinogenesis induced by both genotoxic and nongenotoxic carcinogens (Rajewsky, 1972; Ames & Gold, 1990; Cohen & Ellwein, 1990). In tumorigenesis induced by genotoxic carcinogens, cell proliferation is a crucial event in converting DNA damage to heritable mutations and causing clonal expansion of mutated cell populations (Grisham *et al.*, 1983). Cell proliferation induced by genotoxic carcinogens is compensatory and may be associated with cytotoxic cell injury; this type of cell proliferation tends to augment the process of carcinogenesis (Columbano *et al.*, 1987). Peroxisome proliferator-induced hepatocellular proliferation is not preceded by hepatocellular injury (Reddy *et al.*, 1979); and Columbano *et al.* (1987) reported that this primary mitogenic proliferation is not as effective as compensatory hyperplasia in the carcinogenic process, although the conditions of the experiments were different from those in long-term bioassays. The suggestion that peroxisome proliferator-induced carcinogenesis is mediated by hepatocellular proliferation is based in part on the observed mitogenic properties of peroxisome proliferators, such as nafenopin, Wy-14,643, ciprofibrate and clofibric acid (Moody *et al.*, 1977; Reddy *et al.*, 1979; Marsman *et al.*, 1988; Yeldandi *et al.*, 1989; Marsman *et al.*, 1992), and on the hypothesis that the chronic mitogenic response has an indirect carcinogenic effect in that it increases the probability that endogenous DNA damage will be converted into mutations (Ames & Gold, 1990; Cohen & Ellwein, 1991).

The initial, acute cell proliferation is unlikely to have an important effect on carcinogenesis, for several reasons. First, very little cell proliferation occurs during this phase in comparison with that which takes place during the two years of a typical bioassay in rats or mice. Second, the acute phase of cell proliferation is finished before induction of peroxisome proliferation has been completed, so that acute hepatocyte proliferation cannot play a

role in enhancing the DNA damage that may occur secondary to induction of peroxisome proliferation. The increased hepatocellular proliferation observed during continued treatment with peroxisome proliferators may, however, contribute to the carcinogenic process. Chronic enhancement of hepatocyte proliferation is correlated with the greatest tumour responses (Marsman *et* al., 1988, 1992), suggesting a role in carcinogenesis. Proliferation of non-preneoplastic hepatocytes may not be sufficient to elicit carcinogenesis (Reddy & Rao, 1992; Ashby *et al.*, 1994; Reddy & Rao, this volume).

1.3.2.3 Preferential growth of preneoplastic lesions

It has been proposed that peroxisome proliferators induce preferential growth of altered hepatocytes in developing liver tumours (Schulte-Hermann *et al.*, 1981, 1983; Marsman & Popp, 1994), and to a much greater degree than other nongenotoxic carcinogens, such as phenobarbital. As this growth depends on continued administration of a peroxisomal proliferating agent, it is not an inherent property of the preneoplastic cells. Support for the conclusion that these agents act by inducing preferential growth is provided by the observation that more tumours develop in older than younger rats fed nafenopin or Wy-14,643, although tumours were found in both two-month- and 15-month-old rats fed these compounds (Cattley *et al.*, 1991; Kraupp-Grasl *et al.*, 1991). In studies with ciprofibrate, a potent peroxisome proliferator, however, no appreciable difference in tumour incidence was seen between six- and 12-month-old rats (Rao *et al.*, 1990). Peroxisome proliferators can induce liver tumours in both young and old rats, and, like any other carcinogen, genotoxic or nongenotoxic, they can act to some extent at any stage of the process of carcinogenesis. The observation of *ras* gene mutations in B6C3F1 mouse liver tumours (Hegi *et al.*, 1993) is not consistent with the notion that peroxisome proliferators act solely as promoters of spontaneous carcinogenesis. The spectrum and frequency of the activating mutations detected in the H-*ras* and K-*ras* genes differ significantly between ciprofibrate-induced and spontaneously occurring liver tumours. These observations further support the suggestion that ciprofibrate and other peroxisome proliferators are genotoxic by indirect means, such as oxidative stress.

Several other possible carcinogenic mechanisms of action of peroxisome proliferators have been proposed, including stimulation of protein kinase C, uncoupling of oxidative phosphorylation and interference with intercellular communication (Keller *et al.*, 1993; Bayly *et al.*, 1994; Bojes & Thurman, 1994; Krutovskikh *et al.*, 1995).

1.3.3 Neoplasms in organs other than the liver

Some hepatocarcinogenic peroxisome proliferators have been found also to induce tumours in organs other than the liver. Of the 18 peroxisome proliferators listed in Table 1, benzylbutyl phthalate and tetrachloroethylene have been reported to induce mononuclear-cell leukaemia in Fischer 344 rats; cinnamyl anthranilate, clofibrate and gemfibrozil to induce tumours in rat pancreas; and cinnamyl anthranilate, tetrachloroethylene and trichloroethylene to induce renal tumours in male rats. A number of studies have also been carried out to investigate possible promoting activity in two-stage carcinogenesis models. Di(2-ethylhexyl)phthalate was reported to promote renal tumours (Kurokawa *et al.*, 1988) and to act as a second-stage promoter in skin carcinogenesis in SENCAR mice (Diwan *et al.*, 1985). Clofibrate has a promoting effect in urinary bladder carcinogenesis in Fischer 344 rats (Hagiwara *et al.*, 1990). These effects appear not to be related to the peroxisome proliferating properties of the chemicals, but may represent independent properties. (See also the paper by Dybing *et al.*, this volume.)

1.4 Peroxisome proliferation as a biological marker for hepatocarcinogenesis

It has been suggested that the morphological and biochemical phenomenon of peroxisome proliferation in liver cells could serve as a useful biological marker for identifying the potential carcinogenicity of this class of generally nonmutagenic chemicals (Reddy *et al.*, 1980; Reddy & Lalwani, 1983). Short-term biological effects *in vivo* can be evaluated easily by ascertaining the alterations in peroxisome number and volume density in the livers of rats and mice exposed to several doses of the compound and by determining changes in peroxisomal β-oxidation enzyme activity and in specific mRNA levels (Reddy & Rao, 1992). The ability of chemicals to induce peroxisome proliferation in primary cultures of liver cells could also be used to identify peroxisome proliferators *in vitro* and to evaluate possible species differences in response.

The purpose of a short-term biological marker is to identify the potential carcinogenic nature of an agent and not necessarily to serve as a quantitative indicator of carcinogenicity. Nonetheless, if a

chemical can induce maximal peroxisome proliferation (i.e. increase the peroxisome volume to 15–25% of the cytoplasmic volume), it is reasonable to anticipate a high incidence of hepatocellular tumours in rats and mice exposed for long periods.

1.5 Peroxisome proliferators, human response and hazard

The potential human response to agents that induce peroxisome proliferation in rats or mice has been examined both *in vitro*, in cultured human hepatocytes, and *in vivo,* in subjects receiving hypolipidaemic agents.

As in cultured nonhuman primate hepatocytes, peroxisome proliferators do not have significant effects on marker enzyme activities or the number of peroxisomes in cultured human hepatocytes (for reviews, see Bentley *et al.*, 1993; Ashby *et al*, 1994; Foxworthy & Eacho, 1994). The compounds examined in cultured human hepatocytes include beclobric acid, benzbromarone, ciprofibrate, clofibric acid, fomesafen, monoethylhexylphthalate, methylclofenapate and trichloroacetic acid. In many of these studies, the functional viability of the human hepatocyte preparations was confirmed in parallel experiments in which other end-points were determined. Additionally, although replicative DNA synthesis could be induced in these hepatocytes by epidermal growth factor, no significant changes were induced by methylclofenapate or nafenopin (reviewed by Lake, 1995).

The effects of several hypolipidaemic agents, including ciprofibrate, clofibrate, fenofibrate and gemfibrozil (all well documented peroxisome proliferators in mouse and rat liver), have been studied in human volunteers (for reviews, see Bentley *et al.*, 1993; Ashby *et al.*, 1994). While no significant change was seen in most investigations, one study of clofibrate resulted in a 50% statistically significant increase in the mean number of peroxisomes but a nonsignificant, 23% increase in peroxisome volume density (Hanefeld *et al.*, 1983). Since the measurement of volume density rather than the number of peroxisomes is indicative of peroxisome proliferation, the results of this study indicate a negative human response.

The results of studies of human hepatocytes *in vivo* and *in vitro*, together with the data on effects in experimental animals, suggest that there are marked species differences in response to peroxisome proliferators. Although further studies are desirable, the current literature suggests that compounds that are peroxisome proliferators in rats and mice have little, if any, effect on human liver. In a study by Sher *et al.* (1993), however, a human liver PPARα was as effective as mouse PPARα in a trans-activation assay system *in vitro*. The apparent disparity between the effect of peroxisome proliferators in rodent and human hepatocyte cultures may therefore be due to a number of modulating or confounding factors (Ashby *et al.*, 1994; Reddy & Mannaerts, 1994; Lake, 1995).

Several clinical trials have addressed the potential carcinogenicity of therapeutic hypolipidaemic agents in the human population. A meta-analysis of all randomized clinical trials of cholesterol lowering did not reveal excess mortality from cancer at all sites combined among the actively treated subjects (Law *et al.*, 1994). Two randomized studies of the effect of fibrates in preventing coronary heart disease, however, raised concern, as an excess of deaths from cancer was seen in the fibrate-treated subjects. In the five-year WHO study of clofibrate (WHO European Collaborative Group, 1986; Law *et al.*, 1994), 72 deaths from cancer were observed in the group treated with clofibrate and 54 in the group receiving a placebo ($p = 0.12$). The difference disappeared during the follow-up after the intervention. Data on cancer incidence were not available. No difference was seen in the incidence of or mortality from cancer in the Helsinki Heart Study of gemfibrozil (Huttunen *et al.*, 1994) during the five-year intervention period, but an excess of deaths from cancer (20 *versus* 7) was observed in the original gemfibrozil group during the 3.5-year post-trial follow-up, when about 60% of the participants in both trial groups were taking gemfibrozil. The differences disappeared again when the follow-up was extended to 10 years. Cancer incidence did not differ between the two groups during the observation period. It should be noted that the studies of users of cholesterol-lowering drugs have insufficient statistical power to evaluate the risk for hepatocellular cancer.

Increased mortality from cancer has been associated with a low serum cholesterol level in about half of the longitudinal epidemiological studies published so far (Epstein, 1990, 1992; Jacobs *et al.*, 1992; Law *et al.*, 1994). The excess mortality from cancer seen in several studies was confined to deaths occurring within a few years of cholesterol measurements and was attributed to preclinical cancer. In an analysis of all published cohort studies, the association was present on a long-term basis only for cancers of the lung and of

the lymphatic and haematopoietic system (Law & Thompson, 1991). The finding that the association between low cholesterol and lung cancer was restricted to community cohorts and to certain groups within the cohorts was interpreted as suggesting that the relationship is a result of confounding by a factor linked to both low serum cholesterol level and a high risk for lung cancer.

1.6 Conclusions

The responses to the following questions are based on the interpretation of hepatocellular tumour induction in rats and mice, since the mechanisms of carcinogenesis have been evaluated in detail only in liver. The available information on the mechanisms of tumour response elicited by some peroxisome proliferators in rats and mice at sites other than the liver suggests that peroxisome proliferation does not play a role in the formation of tumours at those sites.

1. What mechanisms are critical to peroxisome proliferation?

The evidence suggests that peroxisome proliferation in mouse and rat liver is mediated by activation of peroxisome proliferator-activated receptors, which are members of the steroid hormone receptor superfamily. Receptor activation may be a direct effect of the peroxisome proliferator or may be mediated through perturbation of lipid metabolism. Such receptors have also been identified in humans.

2. Is peroxisome proliferation an indicator of cancer risk in rats and mice?

There is a strong concordance between peroxisome proliferation and hepatocellular carcinogenesis in rats and mice. On the basis of a more limited database, a similar concordance is seen between hepatocellular proliferation induced by peroxisome proliferators and hepatocellular tumour induction.

3. What are the mechanisms of carcinogenesis mediated by chemically induced peroxisome proliferation?

Two major biological responses to peroxisome proliferators are associated with increased cancer induction in rats and mice. One is peroxisome proliferation, and the other is increased hepatocellular proliferation. The proposed mechanisms of peroxisome proliferator-induced hepatocellular carcinogenesis include oxidative stress, increased hepatocellular proliferation and preferential growth of preneoplastic lesions. These mechanisms may not be mutually exclusive.

Hepatocellular carcinogenic peroxisome proliferators are generally inactive in assays for genotoxicity. Some such agents can cause morphological cell transformation and inhibit gap-junctional intercellular communication. These cellular effects appear to be independent of the process of peroxisome proliferation. Chemicals that induce peroxisome proliferation may have additional carcinogenic effects unrelated to that phenomenon.

4. Does peroxisome proliferation also occur in humans, and do the mechanisms of carcinogenesis mediated by peroxisome proliferation in rats and mice also operate in humans?

Data on the effects in humans of peroxisome proliferators are derived from studies of subjects receiving hypolipidaemic drugs and from studies of cultured human hepatocytes. The limited data *in vivo* suggest that therapeutic doses of hypolipidaemic agents produce little if any peroxisome proliferation in human liver. Hypolipidaemic fibrates and other chemicals that induce peroxisome proliferation in rat and mouse hepatocytes when given at high concentrations do not do so in cultured human hepatocytes.

Marginal, statistically nonsignificant increases in hepatocellular peroxisome proliferation in human liver have been reported after exposure to clofibrate, but a comparable increase in peroxisome proliferation was not associated with hepatocellular carcinogenesis in rats or mice.

5. How can data on peroxisome proliferation be used in making overall evaluations of carcinogenicity to humans?

Chemicals that show evidence of inducing peroxisome proliferation should be evaluated on a case-by-case basis. The evaluation of agents by independent expert groups is a matter of scientific judgement.

When the database supports the conclusion that a tumour response in mice or rats is secondary only to peroxisome proliferation, consideration could be given to modifying the overall evaluation, as described in the Preamble to the *IARC Monographs*, taking into account the following evidence:

(a) Information is available to exclude mechanisms of carcinogenesis other than those related to peroxisome proliferation.

(b) Peroxisome proliferation (increases in peroxisome volume density or fatty acid β-oxidation activity) and hepatocellular

proliferation have been demonstrated under the conditions of the bioassay.

(c) Such effects have not been found in adequately designed and conducted investigations of human groups and systems.

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