Review Article

PPARα- and DEHP-Induced Cancers

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Di(2-ethylhexyl)phthalate (DEHP) is a widely used plasticizer and a potentially nongenotoxic carcinogen. Its mechanism had been earlier proposed based on peroxisome proliferator-activated receptor α (PPARα) because metabolites of DEHP are agonists. However, recent evidence also suggests the involvement of non-PPARα multiple pathway in DEHP-induced carcinogenesis. Since there are differences in the function and constitutive expression of PPARα among rodents and humans, species differences are also thought to exist in the carcinogenesis. However, species differences were also seen in the lipase activity involved in the first step of the DEHP metabolism, which should be considered in DEHP-induced carcinogenesis. Taken together, it is very difficult to extrapolate the results from rodents to humans in the case of DEHP carcinogenicity. However, PPARα-null mice or mice with human PPARα gene have been developed, which may lend support to make such a difficult extrapolation. Overall, further mechanical study on DEHP-induced carcinogenicity is warranted using these mice.

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1. INTRODUCTION

Di(2-ethylhexyl)phthalate (DEHP) a plasticizer around the world, suggesting that many people come across this chemical every day. Animal studies showed that this chemical is a nongenotoxic carcinogen. Metabolites of DEHP, mono- and dicarboxylic acids, transactivate peroxizome proliferator-activated receptor α (PPARα), which has been thought to result in nongenotoxic carcinogenesis [1, 2]. However, the latest studies also showed the involvement of non-PPARα pathways; multiple pathways might be involved in the pathway of DEHP-induced carcinogenicity [3]. There are species differences in the functional activation or constitutive expression of rodent and human PPARα, and that in humans is thought to be less active and expressive than those of rodents. Recently, inflammation-related carcinogenesis has drawn attention [4, 5]. PPARα is involved not only in the induction of target genes such as β-oxidation enzymes of fatty acids but also in anti-inflammation signaling [6, 7], suggesting that PPARα also may protect against carcinogenesis. Species differences in lipase activity (DEHP-metabolizing enzyme) among mice, rats, and marmosets have been also reported recently [8], suggesting that this kinetic difference should be considered in the species differences in DEHP-induced carcinogenesis. In this review, we focused on DEHP-induced hepatic carcinogenesis in relation to PPARα-dependent and PPARα-independent pathways, and discussed the science policy.

2. PPARs

PPARs are involved in a member of the nuclear hormone receptor superfamily, and consist of three subunits: PPARα, PPARβ/δ, and PPARγ [9]. These three isoforms have been identified at the organ-specific level. In the respective organ, PPARs function as transcription factors through the classic ligand-dependent nuclear hormone receptor mechanism. Upon binding to their ligands, PPARs undergo conformational changes that allow corepressor release [10]. The PPAR-ligand complex binds to direct repeat 1 elements or peroxisome proliferator response elements (PPREs), usually located upstream of the target genes, which results in the induction of fatty acid transport and metabolism, glucose metabolism, and also elicitation of anti-inflammatory effects [6, 11].

As one of the three isoforms, PPARα is mainly expressed in organs that are critical in fatty acid catabolism, such as liver, heart, and kidney [7]. Thus, this nuclear receptor is
primarily involved in the regulation of fatty acid metabolism. In addition to this function, PPARα also has various functions including the promotion of gluconeogenesis, lipogenesis, ketogenesis, and anti-inflammatory effects [6].

3. PPARα LIGANDS

The ligands of PPARα represent a diverse group of chemicals including not only endogenous ligands but also exogenous synthetic ligands with a high likelihood of clinical, occupational, and environmental exposure of humans to chemicals [1, 12]. The primary endogenous ligands are fatty acids, mainly the 18–20 carbon polyunsaturated fatty acids and eicosanoids [7, 13–17]. As exogenous ligands, fatty acids and thiazoldinediones are involved. Additionally, the general population is exposed to environmental chemicals such as plasticizers (e.g., phthalates), solvents (e.g., tetrachloroethylene and trichloroethylene), perfluorooctanoic acid and herbicides (e.g., 2, 4-dichlorophenoxyacetic acid, diclofop-methyl, haloxyp, lactofen, and oxidiazon).

Of these ligands, the toxicity of DEHP is well established in relation to PPARα. This chemical is used as a plasticizer to improve the plasticity and elasticity of polyvinyl chloride products that have become ubiquitous in our daily living. These products are widely used in building materials, wallpaper and flooring, wire covering, vinyl sheeting for agriculture, food packages, and medical devices such as intravenous and hemodialysis tubing and blood bags. The recent production of DEHP in Japan has approached 14 000 tons per year, which accounts for about 54% of all plasticizers used [11]. It is noted that mono- and dicarboxylic acid metabolites of DEHP, not DEHP itself, act as ligands for PPARα [18] and have potentially adverse effects on liver, kidney, heart, and reproductive organs though monocarboxylic acid, mono(2-ethylhexyl) phthalate (MEHP), also binds to PPARy [18].

4. SPECIES DIFFERENCES IN PPARα

Since there are species differences in the toxicity of PPARα agonists, the expression levels or functions of the receptor are thought to be different among species. Several explanations for the species differences in response to the ligands have been suggested [19, 20]. One of the major factors was considered to be due to di differences in the level of PPARα expression [21, 22] although other possibilities include differences in ligand affinity between rodent and human PPARα, differences in cellular context of PPARα expression, and those in PREP sequences found upstream of critical target genes [23, 24]. Indeed, PPARα expression in humans is about 1/10 times less than that in rodents [25]. In addition, micro-RNA expression regulated by PPARα has been recently reported to be unchanged in wild-type mice, but not in mice with human PPARα gene [26]; Wy-14,643 inhibited a micro-RNA let-7C which is involved in suppression of tumorigenesis in wild-type mice, but neither in PPARα-null mice nor in mice with human PPARα gene. Mice with human PPARα gene are resistant to hepatocellular proliferation though they respond to Wy-14,643 in β-oxidation and serum triglycerides [27]. These results suggest that the function of the PPARα signaling in liver proliferation and tumorigenesis by the chemical exposure is not always similar in mice and humans.

In regard to the species differences in the PPREs, the lack of acyl CoA oxidase (ACO) induction in studies on liver biopsies from humans treated with hypolipidemic drugs or primary human hepatocytes treated with Wy-14,643 may be attributable to an inactive functional PPRE since the sequence of a PPRE for the ACO gene from a small number of human liver biopsy samples was found to be different from that of the rats [28]. However, Reddy remarked at a panel discussion that, although the sequence of ACO gene promoter in the mouse was also different from that in the rat, both rodents are responsive to some peroxisome proliferators in ACO induction [20]. In addition, differences in the ability of rodents and human PPARα to recognize and bind PPRE are unlikely since the DNA binding domains of the human and rodent PPARα are 100% homologous [29, 30]. Though characterized from only a limited number of individuals, the prevalence in the population of defective PPAR alleles cannot be determined at this point [31]. The species difference in the sequence of PPRE may not be involved in the difference in response to ligands between rodents and humans.

In addition to the lower expression levels of PPARα in human, there was a truncated, inactive form of PPARα in human liver, suggesting that the expression of full-length functional PPARα was very low. These inactive forms of PPARα may be insufficient to bind PPRE because PPREs may be occupied in vivo by other nuclear receptors that bind to similar sequences, thus affecting responsiveness to ligands [25].

5. SPECIES DIFFERENCES IN DEHP METABOLISM

In addition to the species differences in PPARα functions or expression levels, we should also be mindful of the importance of those in the metabolism of DEHP between rodents and humans. DEHP absorbed in the body is first metabolized by the catalytic action of lipase to produce MEHP and 2-ethylhexanol (2-EH) [32]. Some MEHP is then conjugated with UDP-glucuronide by UDP-glucuronosyltransferase (UGT) and excreted in the urine. The remaining MEHP is excreted directly in the urine or is oxidized by cytochrome P450 4A, then further oxidized by alcohol dehydrogenase (ADH) or aldehyde dehydrogenase (ALDH) to dicarboxylic acid or ketones. 2-EH is metabolized mainly to carboxylic acid (mainly 2-ethylhexanoic acid (2HEA)) via 2-ethylhexanal by catalytic action of ADH and ALDH. Thus, lipase may be an essential enzyme to regulate the DEHP metabolism; knowing the species difference in the lipase activity may be an important tool to clarify the species difference in metabolism.

Recently, the activities of lipase, UGT, ADH, and ALDH for DEHP metabolism in several organs were measured and compared among mice, rats, and marmosets [8]. Marmosets were used as a reference to human. Clear-cut species differences were seen in the activities of the four enzymes involved in the DEHP metabolism among mice, rats, and
marmosets. The most prominent difference was observed in the lipase activity with an almost 148- to 357-fold difference between the highest activity in mice and the lowest in marmosets (Figure 1). These differences were comparable to those in the kinetic parameter, Vmax. These results suggest that the constitutive levels of lipase were greater in the mice and rats than in marmosets. Indeed, lipase-mRNA levels in livers from mice or rats were much higher than those in the kinetic parameter, Vmax.

Thus, concentrations of MEHPs in marmoset (Figure 2). Thus, concentrations of MEHPs in livers from mice or rats were much higher than those in primates; cumulative 14C excretion in urine of African green monkey following bolus injection of 14C-DEHP leached into autologous plasma occurred earlier than in human [34].

6. MECHANISM OF DEHP-INDUCED CANCER

DEHP causes tumors, especially in liver when chronically administered to rats and mice [35–39], similar to the other peroxisome proliferators such as Wy-14643. Table 1 shows that DEHP induces hepatic tumors in mice and rats. From the viewpoint of percentage in feed, the lowest-observed effect-level (LOEL) of DEHP carcinogeticity in the rat was 0.6%, and the no-observed effect-level (NOEL) was 0.1% [2]. In the mouse, the corresponding values may be 0.05% for LOEL and 0.01% for NOEL because the study in which male mice were exposed to 0.05% DEHP for 78 weeks exhibited a significant increase in the hepatic tumor incidence rate compared with controls, but not when exposed to 0.01% DEHP [40].

DEHP also has potential for carcinogenesis in other organs; pancreatic acinar cell adenoma and mononuclear cell leukemia incidences were significantly increased in male F344 rat but not in F344 female rat and B6C3F1 mouse of both sexes after DEHP exposure [35, 36, 44]. The reason why these cancers are not observed in female rat has not been identified.

Chronic treatment with PPARα agonist results in an increased incidence of liver tumors which were thought to have occurred through a PPARα-mediated mechanism as revealed by the resistance of PPARα-null mice to liver cancer induced by Wy-14,643 exposure for 11 months [46]. All the wild-type mice fed with 0.1% Wy-14643 diet for 11 months had multiple hepatocellular neoplasms, including adenomas and carcinomas, while the PPARα-null mice fed with the 0.1% Wy-14643 diet for the same duration were unaffected. Ward et al. [47] reported that exposure for only six months to 12,000 ppm DEHP caused induction of peroxisomal enzymes, liver enlargement, and histopathological increases in eosinophil counts and peroxisomes in the cytoplasm of wild-type mice, while there were no such toxic findings in the liver of PPARα-null mice. Thus, DEHP-derived
| Author | Species, strain | Sex | Route | Duration | Dosage | Type of tumor | Tumor frequency (%) |
|--------|----------------|-----|-------|----------|--------|---------------|---------------------|
| [39]   | Rat F344       | M   | Feed  | 103 w    | 0.00%  | Hepatic tumors | 6                   |
|        |                |     |       |          | 0.60%  |               | 12                  |
|        |                |     |       |          | 1.20%  |               | 24                  |
|        |                | F   | Feed  | 103 w    | 0.00%  | Hepatic tumors | 0                   |
|        |                |     |       |          | 0.60%  |               | 12                  |
|        |                |     |       |          | 1.20%  |               | 26                  |
| [41]   | Rat F344       | F   | Feed  | 2 y      | 0.00%  | Hepatic tumors | 0                   |
|        |                |     |       |          | 0.03%  |               | 6                   |
|        |                |     |       |          | 0.10%  |               | 5                   |
|        |                |     |       |          | 1.20%  |               | 30                  |
| [42]   | Rat F344       | M   | Oral  | 24 m     | 0 (water) | Liver carcinoma | 4                   |
|        |                |     |       |          | 0 (vehicle) |               | 12                  |
|        |                |     |       |          | 2EH 50 mg/kg |               | 6                   |
|        |                |     |       |          | 2EH 150 |               | 6                   |
|        |                |     |       |          | 2EH 500 |               | 2                   |
|        |                | M   | Oral  | 24 m     | 0 (water) | Liver adenoma | 0                   |
|        |                |     |       |          | 0 (vehicle) |               | 0                   |
|        |                |     |       |          | 2EH 50 mg/kg |               | 0                   |
|        |                |     |       |          | 2EH 150 |               | 0                   |
|        |                |     |       |          | 2EH 500 |               | 0                   |
|        |                | F   | Oral  | 24 m     | 0 (water) | Liver carcinoma | 0                   |
|        |                |     |       |          | 0 (vehicle) |               | 2                   |
|        |                |     |       |          | 2EH 50 mg/kg |               | 2                   |
|        |                |     |       |          | 2EH 150 |               | 4                   |
|        |                |     |       |          | 2EH 500 |               | 0                   |
| [42]   | Mouse B6C3F1   | M   | Oral  | 18 m     | 0 (water) | Liver carcinoma | 8                   |
|        |                |     |       |          | 0 (vehicle) |               | 12                  |
|        |                |     |       |          | 2EH 50 mg/kg |               | 12                  |
|        |                |     |       |          | 2EH 200 |               | 14                  |
|        |                |     |       |          | 2EH 750 |               | 18                  |
|        |                | M   | Oral  | 18 m     | 0 (water) | Liver adenoma | 0                   |
|        |                |     |       |          | 0 (vehicle) |               | 0                   |
|        |                |     |       |          | 2EH 50 mg/kg |               | 0                   |
|        |                |     |       |          | 2EH 200 |               | 0                   |
|        |                |     |       |          | 2EH 750 |               | 2                   |
|        |                | F   | Oral  | 18 m     | 0 (water) | Liver carcinoma | 2                   |
|        |                |     |       |          | 0 (vehicle) |               | 0                   |
|        |                |     |       |          | 2EH 50 mg/kg |               | 2                   |
|        |                |     |       |          | 2EH 200 |               | 2                   |
|        |                |     |       |          | 2EH 750 |               | 6                   |
|        |                |     |       |          | 2EH 750 |               | 10                  |
| [43]   | Rat F344       | M   | Feed  | 2 y      | 0 ppm   | Hepatocellular carcinoma | 2                   |
|        |                |     |       |          | 6000 ppm |               | 2                   |
|        |                |     |       |          | 12000 ppm |              | 10                  |
|        |                | M   | Feed  | 2 y      | 0 ppm   | Hepatocellular neoplastic nodule | 4                   |
|        |                |     |       |          | 6000 ppm |               | 10                  |
|        |                |     |       |          | 12000 ppm |              | 14                  |
| Author Species, strain | Sex | Route | Duration | Dosage | Type of tumor          | Tumor frequency (%) |
|------------------------|-----|-------|----------|--------|------------------------|---------------------|
| F Feed 2 y             |     |       |          | 0 ppm  | Hepatocellular carcinoma | 0                   |
|                        |     |       |          | 6000 ppm| 4                       |
|                        |     |       |          | 12000 ppm| 16                     |
|                        |     |       |          | 0 ppm  | Hepatocellular neoplastic nodule | 0                   |
|                        |     |       |          | 6000 ppm| 8                       |
|                        |     |       |          | 12000 ppm| 10                     |
| [43] Mouse B6C3F1      | M   | Feed 2 y |          | 0 ppm  | Hepatocellular carcinoma | 18                  |
|                        |     |         |          | 3000 ppm| 29                      |
|                        |     |         |          | 6000 ppm| 38                      |
|                        |     |         |          | 0 ppm  | Hepatocellular adenoma | 10                   |
|                        |     |         |          | 3000 ppm| 23                      |
|                        |     |         |          | 6000 ppm| 20                      |
| F Feed 2 y             |     |       |          | 0 ppm  | Hepatocellular carcinoma | 0                   |
|                        |     |       |          | 3000 ppm| 14                      |
|                        |     |       |          | 6000 ppm| 34                      |
|                        |     |       |          | 0 ppm  | Hepatocellular adenoma | 2                    |
|                        |     |       |          | 3000 ppm| 2                      |
|                        |     |       |          | 6000 ppm| 2                      |
| [40] Rat F344          | M   | Diet 79 w |          | 0 ppm  | Hepatocellular carcinoma | 10                  |
|                        |     |         |          | 2500 ppm| 0                       |
|                        |     |         |          | 12500 ppm| 40                     |
|                        |     |         |          | 0 ppm  | Hepatocellular adenoma | 10                   |
|                        |     |         |          | 2500 ppm| 10                     |
|                        |     |         |          | 12500 ppm| 10                     |
| F Diet 79 w            |     |       |          | 0 ppm  | Hepatocellular carcinoma | 0                   |
|                        |     |       |          | 2500 ppm| 0                       |
|                        |     |       |          | 12500 ppm| 20                     |
|                        |     |       |          | 0 ppm  | Hepatocellular adenoma | 0                    |
|                        |     |       |          | 2500 ppm| 0                      |
|                        |     |       |          | 12500 ppm| 10                     |
| M Diet 105 w           |     |       |          | 0 ppm  | Hepatocellular carcinoma | 1                   |
|                        |     |       |          | 100 ppm | 0                       |
|                        |     |       |          | 500 ppm | 2                       |
|                        |     |       |          | 2500 ppm| 5                       |
|                        |     |       |          | 12500 ppm| 30                     |
|                        |     |       |          | Recovery| 13                      |
|                        |     |       |          | 0 ppm  | Hepatocellular adenoma | 5                    |
|                        |     |       |          | 100 ppm | 10                      |
|                        |     |       |          | 500 ppm | 5                       |
|                        |     |       |          | 2500 ppm| 12                      |
|                        |     |       |          | 12500 ppm| 26                     |
|                        |     |       |          | Recovery| 22                      |
|                        |     |       |          | 0 ppm  | Hepatocellular carcinoma | 0                   |
|                        |     |       |          | 100 ppm | 2                       |
|                        |     |       |          | 500 ppm | 0                       |
|                        |     |       |          | 2500 ppm| 2                       |
|                        |     |       |          | 12500 ppm| 18                     |
|                        |     |       |          | Recovery| 7                      |
Table 1: Continued.

| Species, strain | Sex | Route | Duration (w) | Dosage (ppm) | Type of tumor | Tumor frequency (%) |
|-----------------|-----|-------|--------------|--------------|---------------|---------------------|
| [40] Mouse B6C3F1 | F   | Diet  | 105          | 0            | Hepatocellular adenoma | 0                   |
|                 |     |       |              | 100          |               | 6                   |
|                 |     |       |              | 500          |               | 2                   |
|                 |     |       |              | 2500         |               | 3                   |
|                 |     |       |              | 12500        |               | 10                  |
|                 | M   | Diet  | 79           | 0            | Hepatocellular carcinoma | 0                   |
|                 |     |       |              | 100          |               | 0                   |
|                 |     |       |              | 500          |               | 0                   |
|                 |     |       |              | 1500         |               | 0                   |
|                 |     |       |              | 6000         |               | 7                   |
|                 | F   | Diet  | 79           | 0            | Hepatocellular adenoma | 7                   |
|                 |     |       |              | 100          |               | 10                  |
|                 |     |       |              | 500          |               | 20                  |
|                 |     |       |              | 1500         |               | 10                  |
|                 |     |       |              | 6000         |               | 7                   |
|                 | M   | Diet  | 105          | 0            | Hepatocellular carcinoma | 6                   |
|                 |     |       |              | 100          |               | 8                   |
|                 |     |       |              | 500          |               | 14                  |
|                 |     |       |              | 1500         |               | 31                  |
|                 |     |       |              | 6000         |               | 22                  |
|                 |     |       |       | Recovery     | Hepatocellular adenoma | 6                   |
|                 |     |       |              | 100          |               | 17                  |
|                 |     |       |              | 500          |               | 20                  |
|                 |     |       |              | 1500         |               | 22                  |
|                 |     |       |              | 6000         |               | 27                  |
|                 |     |       |       | Recovery     | Hepatocellular adenoma | 5                   |
|                 | F   | Diet  | 105          | 0            | Hepatocellular carcinoma | 4                   |
|                 |     |       |              | 100          |               | 3                   |
|                 |     |       |              | 500          |               | 5                   |
|                 |     |       |              | 1500         |               | 15                  |
|                 |     |       |              | 6000         |               | 23                  |
|                 |     |       |       | Recovery     | Hepatocellular adenoma | 42                  |
|                 |     |       |              | 100          |               | 3                   |
|                 |     |       |              | 500          |               | 6                   |
|                 |     |       |              | 1500         |               | 14                  |
|                 |     |       |              | 6000         |               | 49                  |
|                 |     |       |       | Recovery     | Hepatocellular adenoma | 24                  |
| Author Species, strain | Sex | Route | Duration | Dosage | Type of tumor | Tumor frequency (%) |
|------------------------|-----|-------|----------|--------|---------------|---------------------|
| [43] Rat F344          | M   | Feed  | 2 y      | 0, 6000, 12000 ppm | Pituitary adenoma or carcinoma | Decrease in highest dose |
|                        | F   | Feed  | 2 y      | 0, 6000, 12000 ppm | Pituitary adenoma or carcinoma | Decrease in lower dose |
|                        | M   | Feed  | 2 y      | 0, 6000, 12000 ppm | Thyroid C-cell adenoma or carcinoma | Decrease in highest dose (unclear) |
|                        | M   | Feed  | 2 y      | 0, 6000, 12000 ppm | Testis interstitial cells tumor | Decrease in highest dose |
|                        | F   | Feed  | 2 y      | 0, 6000, 12000 ppm | Mammary gland | Decrease in highest dose |
| [36] Rat F344          | M   | Diet  | 78 w     | 0 ppm | Interstitial cells tumor or testes | 90 |
|                        |     |       |          | 2500 ppm |                     | 100 |
|                        |     |       |          | 12500 ppm |                    | 30 |
|                        | M   | Diet  | 104 w    | 0 ppm | Interstitial cells tumor or testes | 92 |
|                        |     |       |          | 100 ppm |                     | 90 |
|                        |     |       |          | 500 ppm |                     | 91 |
|                        |     |       |          | 2500 ppm |                    | 92 |
|                        |     |       |          | 12500 ppm |                   | 31 |
|                        | F   | Diet  | 104 w    | 0 ppm | Interstitial cells tumor or testes | 23 |
|                        |     |       |          | 100 ppm |                     | 26 |
|                        |     |       |          | 500 ppm |                     | 29 |
|                        |     |       |          | 2500 ppm |                    | 49 |
|                        |     |       |          | 12500 ppm |                   | 42 |
|                        |     |       |          | 0 ppm | Mononuclear cell leukemia | 0 |
|                        |     |       |          | 100 ppm |                     | 0 |
|                        |     |       |          | 500 ppm |                     | 0 |
|                        |     |       |          | 2500 ppm |                    | 0 |
|                        |     |       |          | 12500 ppm |                   | 8 |
| [44] Rat F344          | M   | Diet  | 79 w     | 0 ppm | Interstitial cells tumor or testes | 90 |
|                        |     |       |          | 12500 ppm |                     | 30 |
|                        |     |       |          | 0 ppm | Mononuclear cell leukemia | 0 |
|                        |     |       |          | 12500 ppm |                    | 10 |
Table 1: Continued.

| Author, Species, strain | Sex | Route | Duration | Dosage | Type of tumor | Tumor frequency (%) |
|-------------------------|-----|-------|----------|--------|---------------|---------------------|
|                         | M   | Diet  | 105 w    | 0 ppm  | Interstitial cells tumor or testes | 92 |
|                         |     |       |          | 12500 ppm | 31 |
|                         |     |       |          | Recovery | 32 |
|                         | M   | Diet  | 105 w    | 0 ppm  | Mononuclear cell leukemia | 23 |
|                         |     |       |          | 12500 ppm | 42 |
|                         |     |       |          | Recovery | 53 |
| [35] Mouse B6C3F1      | M,  | Diet  | 78 w,    | 0 ppm  | No data about tumors |  |
|                         |     |       |          | 100 ppm, 1500 ppm |  |
|                         |     |       |          | 6000 ppm |  |
| [44] Rat F344          | F   | Diet  | 79 w     | 0 ppm  | No data about tumors |  |
|                         |     |       |          | 12500 ppm |  |
| [44] Mouse B6C3F1      | M,  | Diet  | 79 w     | 0 ppm  | No data about tumors |  |
|                         |     |       |          | 6000 ppm |  |
| [45] Mouse 129/Sv, PPARα-null | M  | Diet  | 21 m    | 0%     | Liver tumors (hepatocellular adenoma, hepatoceleular carcinoma, cholangiocellular carcinoma) |  |
|                         |     |       |          | 0.01%  | Wild-type PPARα-null | 0 4 |
|                         |     |       |          | 0.05%  |                          | 9 4 |
|                         |     |       |          |        |                          | 10 25.8 |

carcinogenicity was thought to be mediated by PPARα, similar to Wy-14,643, and DEHP was considered to cause primarily PPARα-dependent carcinogenicity in rodents, but it is considered to be relatively safe in humans, similar to other ligands [2]. However, Ward et al. [47] could not directly observe DEHP-derived tumors in the wild-type mice, because exposure to DEHP for 6 months may not be sufficient to induce hepatic tumors, as suggested by Marsman et al. [48]; they reported that DEHP tumorigenesis required longer exposure periods than Wy-14,643. It is doubtful whether DEHP definitively induces hepatic tumors via PPARα.

As mentioned above, the following simple mechanism has been proposed for the DEHP-induced hepatocarcinogenesis; when DEHP was administered to rats and mice, the chemical caused an increase in cell proliferation and peroxisome proliferation [49]. The latter is accompanied by an increase in both peroxisomal and mitochondrial fatty acid metabolizing enzymes such as ACO. As a byproduct of fatty acid oxidation, enzymes involved with β-oxidation generate H₂O₂, resulting in elevated oxidative stress. DEHP also causes an increase in proinflammatory cytokines and inhibition of apoptosis [2, 24].

DEHP-induced liver carcinogenesis in rodents, however, appears to involve more complex pathways as described in the following events whereby various combinations of the molecular signals and multiple pathways may be involved [3]. DEHP is metabolized to bioactive metabolites which are absorbed and distributed throughout the body; they might induce PPARα-independent activation of macrophages and production of oxidants, and also activate PPARα and sustained induction of target genes. The inductions lead to enlargement of hepatocellular organelles, an increase in cell proliferation, a decrease in apoptosis, sustained hepatomegaly, chronic low-level oxidative stress and accumulation of DNA damage, and selective clonal expansion of the initiated cells. Finally, preneoplastic nodules might be induced and might result in adenomas and carcinoma.

Peraza et al. [10] also suggest that PPARα is the only receptor in PPARs that is known to mediate carcinogenesis, while the prevailing evidence suggests that PPARβ, PPARγ, and their ligands appear to be tumor modifiers that inhibit carcinogenesis, albeit there is still controversy in the field. Melnick [50] also addressed non-PPARα mechanisms for DEHP-induced carcinogenicity as follows. (1) Peroxisome proliferator-induced tumorigenesis is related to the genes involved in cellular proliferations of, for example, p38 mitogen-activated protein kinase, which is not involved in peroxisome proliferations [51]. (2) DEHP and other peroxisome proliferators stimulated growth regulatory pathways such as immediate early genes for carcinogenesis (c-jun, c-fos, junB, egr-1), mitogen-activated protein kinase, extracellular signal-regulated kinase, and phosphorylation of p38, which were dissociated from PPARα activation in rat primary cultures [52–54]. These findings also support the view that peroxisome proliferators, including DEHP, may have the potential for tumorigenesis via non-PPARα signal pathways.

In recent years, an inflammation-associated model of cancers has been given attention [4, 5]. PPARα exerts anti-inflammation effects by repressing nuclear factor kappa B (NFκB) [55], which inhibits inflammation signaling and subsequent cancer [4].

Ito et al. [45] proposed possibility of DEHP tumorigenesis via a non-PPARα pathway using PPARα-null mice. They compared DEHP-induced tumorigenesis in wild-type and
PPARα-null mice treated for 22 months with diets containing 0, 0.01, or 0.05% DEHP. Surprisingly, the incidence of liver tumors was higher in PPARα-null mice exposed to 0.05% DEHP (25.8%) than in similarly exposed wild-type mice (10%), while the incidence was 0% in wild-type mice and 4% in PPARα-null mice without DEHP exposure. The levels of 8-hydroxydeoxyguanosine increased dose-dependently in mice of both genotypes, but the degree of increase was higher in PPARα-null mice than in wild-type mice. NFkB levels also significantly increased in a dose-dependent manner in PPARα-null mice. The proto-oncogene c-jun-mRNA was induced, while c-fos-mRNA tended to be induced only in PPARα-null mice fed with 0.05% DEHP-containing diet. These results suggest that chronic low-level oxidative stress induced by DEHP exposure may lead to the induction of inflammation and/or the expression of proto-oncogenes, resulting in a high incidence of tumorigenesis in PPARα-null mice. Moderate activated PPARα might protect from p65/p50 NFkB inflammatory pathway caused by chronic DEHP exposure in wild-type mice. Although cross-talk of PPARγ, but not PPARα, with cyclooxygenase 2 (Cox-2), which also was related with inflammation-induced hepatocellular carcinoma, has been suggested [56], there was neither induction of Cox-2 nor PPARγ in both genotyped mice of that study (data not shown).

Additionally, we compared the mechanisms of tumorigenesis between wild-type mice and PPARα-null mice using hepatocellular adenoma tissues of both genotyped mice [57]. The microarray profiles showed that the up- or downregulated genes were quite different between hepatocellular adenoma tissues of wild-type mice and PPARα-null mice exposed to DEHP, suggesting that their tumorigenesis mechanisms might be different. Interestingly, the gene expressions of apoptotic peptidase activating factor 1 and DNA-damage-inducible 45α (Gadd45α) were increased in the hepatocellular adenoma tissues of wild-type mice exposed to DEHP, whereas they were unchanged in corresponding tissues of PPARα-null mice. On the other hand, the expressions of cyclin B2 and myeloid cell leukemia sequence 1 were increased only in the hepatocellular adenoma tissues of PPARα-null mice. Taken together, DEHP may induce hepatocellular adenomas, partly via suppression of G2/M arrest regulated by Gadd45α and caspase 3-dependent apoptosis in PPARα-null mice. However, these genes may not be involved in tumorigenesis in wild-type mice. In contrast, the expression level of Met was notably increased in the liver adenoma tissue of wild-type mice, which may suggest the involvement of Met in DEHP-induced tumorigenesis in wild-type mice. However, we could not determine whether DEHP promoted the spontaneous liver tumor in PPARα-null mice because spontaneous hepatocellular tumors are known to occur in these mice at 24 months of age [58], while we observed DEHP-induced tumorigenesis at 22 months of age. To clarify this, gene expression profiles of liver tumors in the control group must be analyzed.

Taken together, the mechanisms of DEHP-induced carcinogenesis do not consist of only a simple pathway such as PPARα-mediated peroxisome proliferation as mentioned by Rusyn et al. [3]. PPARα-independent pathways may also exist and, by contrast, activated PPARα may protect against DEHP-induced carcinogenesis. The valance of the production of oxidative stress via the transactivation of PPARα and subsequent DNA damages versus the effective exertion of anti-inflammation by activating the receptor may determine the incidence of DEHP-induced tumors.

7. FUTURE INVESTIGATIONS

To determine the mechanism of species difference in response to peroxisome proliferators, a mouse line with human PPARα was produced and designated hPPARαTetOff [27]. This mouse line expresses the human receptor in liver in a PPARα-null background by placing the hPPARα cDNA under control of the Tet-Off system of doxycycline control with the liver-specific LAP1 (C/EBPβ) promoter. Interestingly, the hPPARαTetOff mice express the human PPARα protein at levels comparable to those expressed in wild-type mice; so we should not need to consider the species differences in the expression of PPARα between mice and humans. Treatment of this mouse line with Wy-14,643 revealed induction of genes’ encoding peroxisomal lipid-metabolizing enzymes, including ACO, bifunctional enzyme and peroxisomal thiolase, and the fatty acid transporter CD36 at a level comparable to that in wild-type mice, expressing native mouse PPARα. This suggested that human PPARα is functionally active. Upon treatment with Wy-14,643, hPPARαTetOff mice also had lower levels of fasting serum total triglycerides similar to wild-type mice. However, hPPARαTetOff mice did not show any significant hepatocellular proliferation, nor did they have an induction of cell cycle control genes, in contrast to Wy-14,643-treated wild-type mice where a significant increase in mRNAs encoding PCNA, cMYC, cJUN, CDK1, CDK4, and several cyclins was found after treatment with Wy-14,643. hPPARαTetOff mice were also found to be resistant to Wy-14,643-induced hepatocarcinogenesis after 11 months of Wy-14,643 feeding in contrast to a 100% incidence in the wild-type mouse group [59].

Another transgenic mouse line with human PPARα was generated that has the complete human PPARα gene on a P1 phageartificial chromosome (PAC) genomic clone, introduced onto the mouse PPARα-null background [60]. This new line, designated hPPARαPAC, expresses human PPARα not only in liver but also in kidney and heart. hPPARαPAC mice exhibited responses similar to wild-type mice when treated with fenofibrate lowering of serum triglycerides and induction of PPARα target genes’ encoding enzymes involved in fatty acid metabolism. Treatment of hPPARαPAC mice with fenofibrate did not cause significant hepatomegaly and hepatocyte proliferation similar to hPPARαTetOff mice, suggesting that the resistance to the hepatocellular proliferation found in the hPPARαTetOff mice is not due to lack of expression of the receptor in tissues other than liver.

Until now, there are no reports concerning the interaction between DEHP and hPPARαTetOff or hPPARαPAC. Recently, we have compared the transactivation of mouse and human PPARα by DEHP treatments using wild-type and hPPARαTetOff mice (unpublished observation). A relatively
high dose of DEHP (5 mmol/kg for 2 weeks) clearly activated PPARα in liver of both genotyped mice, but the activation was very little in hPPARα<sup>Teto</sup> mice from the standpoint of the target gene expression as well as triglyceride levels in plasma and liver. Human PPARα response to DEHP may be weak when sufficient human PPARα is expressed in the human liver. Thus, the use of the hPPARα<sup>Teto</sup> mouse model is a very valuable means to solve the species differences in the toxicity of peroxisome proliferators. The results from the typical peroxisome proliferator (Wy-14643) may not always be similar to those of DEHP; a study of each case is needed using hPPARα<sup>Teto</sup> mouse model.

### 8. PROPOSED SCIENCE POLICY STATEMENTS

The International Agency for Research on Cancer downgraded the level of potential health risks of DEHP from 2b (possibly carcinogenic to humans) to 3 (not classifiable as to carcinogenicity to humans) in 2000 [61]. In this report, DEHP carcinogenesis via PPARα was considered not to be relevant to humans because peroxisome proliferator had not been documented either in human hepatocyte cultures exposed to DEHP or in the liver of nonhuman primates. This decision has been variously argued by several scientists in the literature [50, 62, 63]. In contrast, the Japan Society for Occupational Health has maintained the 2B class of DEHP carcinogenicity because of the obvious rodent carcinogenicity [64].

Although the US Environmental Protection Agency (EPA) had classified the risk for DEHP carcinogenicity as B2 (probable human carcinogen) in 1993, recently, the expert panel of EPA report has provided the current scientific understanding of the mode(s) of action of PPARα agonist-induced tumors observed in rodent bioassays that are associated with PPARα agonisms: liver tumors in rats and mice as well as Leydig cell and pancreatic acinar cell tumors in rats—all of which represent limited evidence [65]. Since the key events for the mode of action, which have been causally related to liver tumor formation, include the activation of PPARα, perturbation of cell proliferation and apoptosis, selective clonal expansion, and the PPARα-related key events included in the expression of peroxisomal genes (e.g., palmitoyl CoA oxidase and acyl CoA oxidase) and peroxisome proliferation (i.e., an increase in the number and size of peroxisomes) are reliable markers. Additionally, the evidence obtained from the findings that PPARα agonists did not activate the receptor in human cell culture or biopsy samples, and from epidemiological studies, shows that humans are apparently refractory to the effects of PPARα agonist. However, the EPA maintained the DEHP carcinogenicity criterion.

In 2004, with regard to preclinical and clinical safety assessments for PPAR agonists, the Food and Drug Administration recommended that, due to the prevalence of positive tumor findings of PPAR agonists, two-year carcinogenicity studies on mice and rats are required [66].

Although IARC changed the criterion for DEHP carcinogenicity, other agencies did not because DEHP is a potential rodent carcinogen of liver and the precise mechanism has not been yet understood, though DEHP is a potentially hepatic carcinogen in rodents.

### 9. CONCLUSIONS

As mentioned above, some studies suggest the possibility of DEHP tumorigenesis via a non-PPARα pathway although DEHP also exerts adverse effects via PPARα-dependent pathway. Since there are species differences regarding expression levels, cellular context, and function of PPARα as well as metabolism enzyme activity of DEHP, it is difficult to extrapolate the results from rodents to humans in terms of risk. Recently, hPPARα mice have been developed, which may help to solve these differences. Re-evaluation of the risk of DEHP carcinogenicity may well be warranted if the previous decisions were based on only PPARα-dependent mechanisms.

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