

Review Article

# Oxidative Stress Induced by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: An Application of Oxidative Stress Markers to Cancer Risk Assessment of Dioxins

Rie YOSHIDA\* and Yasutaka OGAWA

National Institute of Industrial Health, 21-1, Nagao 6-chome, Tama-ku, Kawasaki 214-8585, Japan

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**Abstract:** Dioxins are known to be a class of highly toxic and persistent environmental contaminants. Among them the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been the most intensively studied, and it has been classified as a human carcinogenic substance by the International Agency for Research on Cancer (IARC). Although the mechanism of carcinogenesis by TCDD is unclear, it is now considered to have act a cancer promoter. In this review, we discuss the ability of TCDD to induce oxidative stress *in vivo*, the mechanism of the oxidative stress induction, and how oxidative stress relates to the development of cancer. We then discuss the advantages of measuring the level of oxidative stress in people exposed to dioxins in epidemiological studies for cancer risk assessment. We also discuss several methods of measuring the level of oxidative stress in humans.

**Key words:** Dioxins, TCDD, Molecular epidemiology, Cancer risk assessment, Oxidative stress markers

## Introduction

Dioxins are known to be a class of highly toxic, broadly dispersed, and persistent environmental contaminants. Dioxins include 75 polychlorinated dibenzo-*p*-dioxins (PCDDs), 135 polychlorinated dibenzofurans (PCDFs), and 12 non-ortho and mono-ortho polychlorinated biphenyls (coplanar PCBs), which are structurally similar to PCDDs and PCDFs<sup>1, 2)</sup>. The dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been intensively studied and is known to be the most toxic compound among these compounds<sup>1)</sup>. It also has the greatest affinity for the aryl hydrocarbon receptor (AhR)<sup>1)</sup>. Since the term “dioxins” is used to refer to the compounds that act as AhR ligands and elicit dioxin-like effects, their toxicity relative to that of TCDD can be determined as a toxic equivalency factor (TEF)<sup>2-4)</sup>. The total

toxic equivalent (TEQ) is the sum of the magnitude of exposure to dioxins multiplied by its respective TEF and is used for risk assessment<sup>2, 4)</sup>.

It is well known that TCDD induces cancer in rats, mice, and hamsters<sup>1, 5, 6)</sup>. TCDD also has several other toxic effects, including dermal, immune, reproductive, developmental, and endocrine effects<sup>1, 7)</sup>. Although there have been no epidemiological cancer studies on humans exposed to pure TCDD, there have been numerous studies suggesting that cancer is associated with exposure to chemicals contaminated with TCDD<sup>8)</sup>. Several cohort studies have revealed an increased incidence of death from many kind of cancer, such as lymphoma and soft tissue sarcoma, related to accidental exposure to TCDD<sup>9)</sup> or to chemicals contaminated with TCDD during manufacture<sup>10, 11)</sup>. Since TCDD is carcinogenic to experimental animals, and there are some lines of evidence from epidemiological studies that it is also carcinogenic to humans, as well as because the mechanism of human

\*To whom correspondence should be addressed.

carcinogenesis is similar to the mechanism in experimental animals, the IARC considers TCDD to be a human carcinogen<sup>1)</sup>.

However, the relation between carcinogenesis by TCDD and the biochemical effects or changes in humans exposed to TCDD is not fully understood, and further investigations are needed. In this review, we discuss the participation of oxidative DNA damage in carcinogenesis by TCDD, and we also discuss some biomarkers that can be used for cancer risk assessment in people exposed to dioxins.

## Mechanism of Carcinogenesis by TCDD

Although the mechanism of carcinogenesis by TCDD is obscure, it may include a several-steps process starting with the binding to AhR<sup>1)</sup>. Generally, three stages have been proposed for chemical carcinogenesis: initiation, promotion, and progression<sup>12, 13)</sup>. Initiation involves the induction of an irreversibly altered cell and is frequently equated with a mutational event. Promotion is the experimentally defined process by which the incited cell clonally expands into a visible tumor. Progression is the process that leads to malignant cancer, and it occurs in multiple stages, because most colorectal cancers have three or more altered genes, and it has been estimated that 10 or more mutational changes occur in cancer<sup>13)</sup>. As shown by the colorectal-tumor development model described by Vogelstein *et al.*, multiple genetic changes must occur after promotion of the initiated cell<sup>14)</sup>.

### Lack of direct genotoxicity

Although a few reports based on the investigations of genotoxicity of TCDD by conventional genotoxicity tests, such as mutagenicity tests using *salmonella typhimurium* or mouse lymphoma L5178Y cells, the unscheduled DNA synthesis test, sister chromatid exchange, micronucleus test, and chromosomal aberrations, concluded that the results were positive, many reports concluded that they were negative<sup>1, 5, 15)</sup>. In addition, there have been several investigations showing that TCDD plays specific role in cancer promotion<sup>16–18)</sup>, for example, a study showing an increase in the incidence of hepatocellular carcinomas in rats subcutaneously injected with TCDD or given *N*-nitrosodiethylamine (initiator) intragastrically<sup>16)</sup>. Therefore, the carcinogenicity of TCDD has been considered not to include genotoxicity, which contributes to initiation, but to include a promotional effect.

In general, however, there are many possible mechanisms for inducing mutations indirectly, for example<sup>13)</sup>:

- 1) chemical—DNA (adduct)—mutation,
- 2) chemical—microtubule—DNA—mutation,
- 3) chemical—O<sub>2</sub> (activated)—DNA—mutation,
- 4) chemical—receptor—enzyme—O<sub>2</sub> (activated)—DNA—mutation,
- 5) chemical—receptor—protein (recombinase)—DNA—mutation,
- 6) chemical—receptor—protein—DNA synthesis/cell division (normal)—mutation,
- 7) chemical—receptor—protein—DNA synthesis/cell division (abnormal)—mutation,
- 8) chemical—receptor—protein—DNA synthesis/cell division—tumor—mutation

Therefore, it can not be ruled out that TCDD exhibits indirect genotoxicity undetected by conventional mutagenicity tests.

### Oxidative DNA damage

In spite of many studies showing a lack of direct genotoxicity, oxidative DNA damage was detected *in vivo* and *in vitro* after exposure to TCDD as follows. DNA breaks were detected in the liver of SD female rats<sup>19–21)</sup>, in the liver of C57BL/6 mice<sup>22)</sup>, in rat peritoneal lavage cells<sup>23)</sup>, in mouse fetuses and placenta<sup>24, 25)</sup>, and in mouse brain tissue<sup>26)</sup>. Moreover, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) has been detected in the liver of SD female rats liver<sup>27)</sup> and in the urine of C57BL/6J inbred mice<sup>28)</sup>. Park *et al.*<sup>29)</sup> reported increased rate of excretion of 8-oxoguanine (8-oxoGua), the major repair product of 8-oxodG<sup>29)</sup>, in the culture medium of hepatoma Hepa1c1c7 cells.

Lipid peroxidation or an increase in the oxidized (GSSG) glutathione/reduced (GSH) glutathione ratio, which is a marker of oxidative stress<sup>30)</sup>, was also observed when DNA damage occurred<sup>21, 27, 29)</sup>, and increased O<sub>2</sub><sup>-</sup> production has been detected in peritoneal lavage cells of SD rats<sup>23)</sup> and peritoneal exudate cells of C57BL/6J and DBA/2 mice<sup>31)</sup>. It is well known that reactive oxygen species (ROS), such as the OH radical ( $\cdot$ OH) and O<sub>2</sub><sup>-</sup>, induce DNA breaks, 8-oxodG, lipid peroxidation<sup>32)</sup>, and increase of the GSSG / GSH ratio<sup>30)</sup>. A dose-response relation was observed in some of these studies<sup>20, 23, 26, 31)</sup>, and the dose regimens are shown in Table 1. Considering all of these results as a whole, while TCDD did not induce DNA damage in most genotoxicity tests, it did induce oxidative DNA damage or increase oxidative stress in several situations, which shows that TCDD is able to induce ROS, even if indirectly.

### Oxidative stress and carcinogenesis

Oxidative stress may lead to carcinogenesis via different

**Table 1. Experimental designs to detect oxidative stress induced by TCDD**

Markers	Organs (animal species)	Dose of TCDD	References
DNA breaks	Liver (SD rat)	40 µg/kg/day (1 day)	19)
		0, 25, 50, 100 µg/kg/day (1 day)	20)
		100 µg/kg/day (1 day)	21)
	Liver (C57BL/6J mouse)	125 µg/kg/day (1 day)	22)
	Peritoneal lavage cell (SD rat)	0, 25, 50, 75, 100, 125 µg/kg/day (1 day)	23)
	Fetus, pracentia (CF1 mouse)	30 µg/kg/day (1 day)	24)
	Fetus, pracentia (C57BL/6J, DBA mouse)	30, 60 µg/kg/day (1 day)	25)
Brain (B6C3 F1 mouse)	0, 0.45, 1.5, 15, 150 ng/kg/day (13 weeks)	26)	
8-oxodG	Liver (SD rat)	125 ng/kg/day (30 weeks)	27)
	Urine (C57BL/6J mouse)	5 µg/kg/day (1 day)	28)
8-oxoGua	Culture medium of Hepa 1c1c cell	500 pM (2 days)	29)
Lipid peroxidation	Brain (B6C3 F1 mouse)	0, 0.45, 1.5, 15, 150 ng/kg/day (13 week)	26)
	Liver (SD rat)	0, 25, 50, 100 µg/kg/day (1 day)	20)
GSSG/GSH ratio	Liver (C57BL/6J mouse)	5 µg/kg/day (1 day)	28)
O <sub>2</sub> <sup>-</sup>	Peritoneal lavage cell (SD rat)	0, 5, 25, 50, 125 µg/kg/day (1 day)	23)
	Peritoneal exudate cell (C57BL/6J, DBA/2 mouse)	0, 5, 25, 50, 125 µg/kg/day (1 day)	31)

mechanisms in the three stages of chemical carcinogenesis: initiation, promotion, and progression. Oxidative DNA modification causes DNA mispairings<sup>33,34</sup>, which can lead to inheritable mutations, and these mutations may alter the function of genes, such as oncogenes<sup>32</sup> or tumor suppressor genes<sup>35</sup>, which are involved in the evolution of most kinds of cancer<sup>13</sup>. A mechanism of promotion is defined as an effect on gene expression primarily through perturbation of the signal transduction pathways<sup>12</sup>. Accordingly, the promotional effect of TCDD may depend on oxidative stress, because oxidative stress can modulate many signaling pathways. Modulated signaling pathways, which are considered to have a promotional effect, include the following examples: 1) hypomethylation resulting from 8-oxodG formation, 2) accumulation of intracellular calcium by alteration of membrane structure and function as a result of oxidative damage to lipids and proteins, 3) protein kinase C (PKC) activation triggered by calcium and protein oxidation, and 4) inhibition of gap junction intercellular communication regulated by PKC and membrane oxidation<sup>35</sup>. Another mechanism of promotion is enhancement of specific gene expression in initiated cells by either increasing DNA synthesis or decreasing apoptosis<sup>35</sup>. Oxidative stress can activate genes regulating DNA synthesis or apoptosis, i.e., *c-mic*, *c-fos*, *c-jun*, or *p53*<sup>35</sup>. This also suggests that oxidative stress has promotional effects. While tumor progression represents the biological changes from

the neoplastic stage to the neoplasm, oxidative stress may play a role in tumor progression by producing persistent alterations in signaling pathways, leading to uncontrolled growth, genomic instability, chemotherapy resistance, invasion, and metastasis<sup>35</sup>. This suggests that oxidative DNA damage or oxidative stress is implicated in the mechanisms of carcinogenesis by TCDD.

Oxidative stress probably contributes to many other toxic responses produced by TCDD<sup>36</sup>. For example, Alsharif *et al.* showed induction of oxidative stress at the near LD 50 dose<sup>22</sup>, and thus oxidative stress by TCDD may contribute to acute and lethal tissue damage other than carcinogenesis. The IARC considers effects at doses below the lethal dose to be related to the mechanisms of carcinogenesis<sup>1</sup>, and thus the experiment showing only oxidative stress and no lethal effect at the low dose of 5 µg/kg by Shertzer<sup>28</sup> and 125 ng/kg by Tritscher<sup>27</sup> are proper subjects for investigation of the contribution of ROS to carcinogenesis related to TCDD.

### Oxidative Stress and Female Specific Carcinogenesis by TCDD

Female-specific carcinogenesis in the liver has been reported when TCDD was administered to SD rats<sup>5,15,37</sup>. Furthermore, in a two-stage model that is a short-term bioassay capable of distinguishing promotion from initiation<sup>17</sup>, TCDD induced preneoplastic foci in the liver

of female rats but not in the liver of male rats or in the liver of ovariectomized rats<sup>38</sup>). Accordingly, the presence of estrogens which are ovarian hormones, contributes to hepatocarcinogenesis by TCDD<sup>38</sup>). This female-specific estrogen-related hepatocarcinogenesis may result from oxidative DNA damage, because considerably more 8-oxodG was detected in the liver of female rats compared to the liver of ovariectomized rats after exposure to TCDD<sup>27</sup>).

Hamster models may explain the mechanism of estrogen-related carcinogenesis. Estrogen-induced kidney tumorigenesis in Syrian hamsters is useful for mechanistic studies of estrogen-induced carcinogenicity, because a 100% tumor incidence is routinely achieved with the natural hormone 17 $\beta$ -estradiol (E2) or estrone, without coadministration of any other carcinogen<sup>39</sup>). E2 was also observed to induce oxidative DNA damage, such as DNA breaks<sup>40</sup>) and 8-oxodG<sup>41</sup>) in hamsters. In addition, several synthetic steroidal estrogens have been found only weak carcinogens in animal models, in spite of retaining hormonal potency. Accordingly, estrogen's carcinogenicity probably does not require the hormonal potency of estrogen but the metabolic activation of estrogens to reactive intermediates, which may be a key event in the development of estrogen-induced tumors<sup>39</sup>). The major estrogenic hormone, E2, originates in the ovary and is metabolized to 2-hydroxyestradiol(2-OHE2) by cytochrome P450 CYP1A1(CYP1A1)<sup>42-44</sup>) or to 4-hydroxyestradiol(4-OHE2) by cytochrome P450 CYP1B1(CYP1B1)<sup>44</sup>). Although these metabolites, 2-OHE2 and 4-OHE2, do not induce DNA damage per se<sup>45,46</sup>), Li *et al.*<sup>45</sup>) reported that they induce strand breaks in isolated DNA in the presence of Cu(II) and that this damage is inhibited by catalase. In addition, a significant increase in 8-oxodG in isolated DNA was induced by 4-OHE2 in the presence of liver microsomes<sup>46</sup>), and free radicals were detected by the electron spin resonance method (ESR) when 2-OHE2 or 4-OHE2 was incubated with Cu(II)<sup>47</sup>). These results suggest the following mechanism to explain the induction of DNA damage by 2-OHE2 or 4-OHE2<sup>45-47</sup>). 2-OHE2 or 4-OHE2 is oxidized to semiquinone by internal metal ions, such as Cu(II), or by enzymes in the microsomes. Redoxcycling then occurs, and the semiquinone is oxidized to quinone, and the the quinone in turn is reduced to semiquinone. Superoxide ( $O_2^-$ ) is produced during this process, and it is converted to  $H_2O_2$  by disproportionation<sup>48</sup>). In the presence of transition metal ions, such as Fe(II) or Cu(I),  $H_2O_2$  is converted to a potent oxidizing radical,  $\cdot OH$ , via the Fenton reaction<sup>49</sup>). This  $\cdot OH$  probably induces the DNA damage.

It has also been reported that the levels of CYP1A1 and CYP1A2 increased dose-dependently<sup>38, 50</sup>) and that the transcription of mRNA of CYP1B1 is enhanced<sup>27</sup>) in the liver when female SD rats are exposed to TCDD. Moreover, the average levels of CYP1B1 mRNA are significantly higher in the kidneys and liver of TCDD-treated females than in those of similarly treated males<sup>51</sup>). These finding imply that metabolism of the ovarian hormone estrogen to the catechol estrogens 2-OHE2 or 4-OHE2 is enhanced by exposure to TCDD, and that the level of catechol estrogens in the liver of female SD rats increased as a result. Furthermore, Wahba *et al.* reported that the level of the metals Cu and Mg increased in the liver of TCDD-treated SD rats<sup>52</sup>). In view of this finding, after metabolism of E2 to 2-OHE2 or 4-OHE2, the 2-OHE2 or 4-OHE2 may react with metals, such as Cu, in the liver and allow free radicals to induce oxidative DNA damage in the liver of female SD rats. Accordingly, the possible mechanisms of hepatocarcinogenesis by TCDD in the liver of female rats include oxidative DNA damage through induction of CYP1A1 or CYP1B1, enhancement of E2 metabolism, a rise in transition metal concentration and, as a result, induction of ROS.

### **Oxidative Stress by Metabolism of Xenobiotics and Its Enhancement by TCDD**

CYP1A1/2 or CYP1B1 induced by TCDD may produce ROS by a mechanism different from that described in the previous section. Park *et al.*<sup>29</sup>) reported that Hepa1c1c7 cell excretion of 8-oxoGua into the growth medium increased with CYP1A1 induction after exposure to TCDD. However, there is no significant increase of 8-oxoGua in the medium of c4 cells, which are AhR defective, or in the presence of 7'8-BF, which is an inhibitor of CYP 1A1<sup>29</sup>). This suggests that induction of CYP 1A1 is required to generate 8-oxoGua in the growth medium of Hepa1c1c7 cells. The P450 catalytic cycle, after binding to a certain substrate, involves hydroxylations, epoxidations, and heteroatom oxidations of the substrate<sup>53</sup>), and certain substrates also participate in the redox chemistry during these oxidations by P450. Activation of molecular oxygens occur during these reaction. The formation of hydrogen peroxide as a product of CYP catalysis is described in the previous section<sup>54</sup>). Another possible mechanism of production of ROS is reported to be the oxidation of substrate by cytochrome P450 and reduction of  $O_2$  to  $O_2^-$ <sup>54, 55</sup>). As stated in the previous section, the  $O_2^-$  can spontaneously dismutate and generate hydrogen peroxide<sup>48</sup>), and if transition metals are present, hydroxyl

radicals will be generated<sup>49</sup>). Accordingly, hydroxyl radicals will be generated during the biotransformation of xenobiotics. For example, paraquat (1,1'-dimethyl-4,4'-bipyridinium), which has been used as a herbicide, enters redox cycling and generates ROS via metabolism by P450<sup>56</sup>. Paraquat is metabolized by NADPH cytochrome P-450 reductase to a reduced form, which is a paraquat radical. Reaction of the paraquat radical with O<sub>2</sub> generates O<sub>2</sub><sup>-</sup> and regenerates paraquat, which can be reduced again, and the O<sub>2</sub><sup>-</sup> can generate H<sub>2</sub>O<sub>2</sub> and ·OH, as mentioned above. In addition, a major metabolic pathway of benzo [a] pyrene(B[a]P) to 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (B[a]P diol epoxide) also leads to the formation of free radicals that cause oxidative DNA damage<sup>57</sup>. CYP1A1 and 1A2 show their highest activity on conversion of polycyclic aromatic hydrocarbons, such as B[a]P, to diol metabolites<sup>53</sup>. It is well known that environmental pollutants, such as tobacco smoke and smog, contain B[a]P<sup>58</sup>). Accordingly, exposure to TCDD may increase ROS production *in vivo* via biotransformation of xenobiotics through induction of P450 enzymes.

### **Oxidative Stress by Other Mechanisms and Its Enhancement by TCDD**

**(Induction of TNF-alpha, activation of the enzymes involved in the production of ROS, and inhibition of enzymes involved in the removal of reactive oxygen species)**

Exposure of Ah-responsive mice (Ahbb) to TCDD results in a dose-dependent increase in the concentration of TNF-alpha in the serum<sup>59</sup>), and TNF-alpha has been found to promote free radical formation under certain condition<sup>35</sup>). Alsharif *et al.*<sup>22</sup>) reported that anti TNF-alpha inhibits oxidative DNA damage in peritoneal lavage cell in mice. This suggests that TNF-alpha activates phagocytic cells and that these cells release ROS. Accordingly, release of TNF-alpha following exposure to TCDD may play a specific role in activating phagocytic cells and contribute to oxidative stress *in vivo*. TNF-alpha, however, might be induced as only an acute toxic response to toxicants, because the exposure dose in this study was high, near the LD 50<sup>22</sup>).

TCDD may activate several enzymes, such as xanthine oxidase, that act as oxidants. Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and also catalyzes the oxidation of xanthine to uric acid, resulting in ROS<sup>53</sup>). There have been no reports showing that TCDD activates xanthine oxidase, however, the decreased glutathione levels

following exposure to TCDD are linked to xanthine oxidase activation<sup>36</sup>). Tumor promoters, such as phorbol ester TPA, also increase xanthine oxidase activity<sup>60</sup>). Since TCDD has tumor-promoter activity, the same as TPA, it may promote xanthine oxygenase activity and induce ROS.

Finally, TCDD may influence several enzymes involved in the removal of ROS, i.e., glutathione peroxidase, catalase, and superoxide dismutase. There have been no reports showing depression of catalase activity following exposure to TCDD, but there have been studies showing that the activity of superoxide dismutase and glutathione peroxidase decreases significantly following exposure to TCDD<sup>61, 62</sup>). These phenomena were observed at a high dose of TCDD, however, and inhibition of these enzymes may only have been responsible for the acute toxic responses.

### **Evaluating the Cancer Risk of Humans Exposed to Dioxins**

As discussed in the previous chapters, on the one hand, many lines of evidence obtained in animal experiments show that oxidative stress may be associated with carcinogenesis by TCDD, and on the other hand, TCDD is considered to be carcinogenic to humans. In view all of the above, it seems logical to conclude that the risk of cancer formation in humans attributable to TCDD may be related to the amount of oxidative damage that TCDD induce, and thus, whether TCDD induces oxidative stress in humans has become of great interest to researchers. People are exposed to dioxins in many ways, from environmental exposure to occupational exposure, but they are hardly ever exposed to pure TCDD in any of these situations. Since the toxic effects of other dioxins start with binding to AhR, which is the gate to the toxic pathway of TCDD, it seems reasonable to assume that the toxic mechanisms of TCDD, such as the induction of oxidative stress, are true of the toxicity of other dioxins. Accordingly, if we could control several factors that induce oxidative stress, such as smoking, it would be of value to measure oxidative DNA damage in epidemiological studies designed to evaluate the risk of cancer induced by dioxins. As shown Table 2, in this section, we will review several methods of measuring oxidative stress markers or other markers related to oxidative stress in humans.

#### *Detection of oxidative DNA damage as an oxidative stress marker*

Oxidative DNA damage is a typical oxidative stress marker and is widely used in research fields<sup>63</sup>). Among the many

**Table 2. Biomarkers of oxidative stress related to dioxin-exposure**

Markers	Methods	Specimens sampled by non-invasive methods
Oxidative DNA damage (8-oxodG, Thymine glycol, DNA fragmentation)	HPLC, ELISA, Electrophoresis	Urine Lymphocyte Tissue (Mucosal cell)
Degradative products (Lipid peroxidation, Malonyldialdehyde, Thiol oxidation)	1)	Serum
Non-Enzymatic antioxidants (Glutathione, Bilirubin, Vitamin C, Vitamin E, Uric acid, Ubiquinone)	2)	Serum Urine Red blood cell
Enzymatic antioxidants (Superoxide dismutase, Catalase, Glutathione peroxidase, Glutathione reductase)	3)	Red blood cell Serum
P450 CYP1A1, CYP1B1	PCR, Western blotting	Lymphocyte
TNF-alpha	ELISA	Serum

1) 2) 3): There are many methods to detect these markers. Refer to other textbooks, such as reference 63.

kinds of oxidative damage to DNA bases, 8-oxodG is the form that can be most sensitively measured<sup>64</sup>). In molecular epidemiological studies of the human health effects of several chemicals, 8-oxodG is already being measured in urine<sup>65, 66</sup> and leukocytes<sup>67</sup>) by HPLC and in urine<sup>68</sup>) and oral mucosal cells<sup>69</sup>) by antibody-based enzyme-linked immunosorbent assay (ELISA). In addition, electrophoresis of DNA in lymphocytes, known as comet assay, is also being used to detect DNA damage at alkaline sensitive sites in epidemiological studies<sup>70, 71</sup>). Thus, it is possible to investigate oxidative DNA damage induced by TCDD in humans by using blood or urine specimens.

#### *Other end points as oxidative stress markers*

In order to obtain valid results for the level of oxidative stress in humans, it is important for investigators to apply the various types of assay appropriately. Measurements of lipid peroxidation are one of the most general indicators of oxidative stress, and several new assays for lipid peroxidation have been developed. One of the simplest methods of measuring lipid hydroperoxides is the FOX (ferrous oxidation with xylenol orange) assay based on the reaction between ferrous iron ( $\text{Fe}^{2+}$ ) and lipid hydroperoxides, which yields ferric iron ( $\text{Fe}^{3+}$ ). This assay has been reported to be sensitive to the picomole level of lipid peroxidation, and it can be used in a variety of specimens<sup>63</sup>). Depression of antioxidants,

such as reduced glutathione (GSH), ascorbic acid, ubiquinone, and vitamin E, is often used as evidence of oxidative stress<sup>63</sup>), and although measuring them is not very difficult, reduced levels of these substances is not a perfect predictor of oxidative stress because they also have a pro-oxidant action<sup>63</sup>). Changes in the activity of several antioxidant enzymes have also been used as indirect measures of oxidative stress<sup>63</sup>), for example, induction of specific enzymes, such as glutathione peroxidase, superoxide dismutase, and catalase is often measured in humans as the result of oxidative stress<sup>63</sup>).

Bilirubin has recently been found to scavenge reactive oxygen species produced by oxidative stress *in vivo*, and the oxidative metabolites of bilirubin, called "biopyrrins", are excreted in the urine<sup>72</sup>). Shimoharada *et al.*<sup>73</sup>) proposed a method of measuring biopyrrin levels in the urine by ELISA using an anti-bilirubin monoclonal antibody, and this method may become a useful practical clinical test for the estimation of oxidative stress *in vivo*.

#### *Measurement of CYP activity or TNF-alpha levels as stress markers*

Measurement of CYP1A1 or CYP1B1 activity levels in tissues also seems useful for risk assessment of dioxins in epidemiological studies, because increases in CYP1A1 or CYP1B1 activity may affect the amount of oxidative DNA

damage or other damage related to the metabolic action of CYP1A1 or CYP1B1, as mentioned above. Thus far, only one paper has reported the elevation of CYP1B1 levels in humans exposed to TCDD<sup>74</sup>), and thus further study of CYP1B1 levels in human populations is needed to assess the risk of TCDD. In addition, since Alsharif *et al.*<sup>22</sup>) inferred that ROS is induced via TNF-alpha as a result of the acute toxicity of TCDD, measurement of TNF-alpha levels in the serum of humans exposed to dioxins might be useful in assessing human risk.

## Conclusions

Induction of oxidative stress by TCDD has been observed in animals, but the mechanism is still obscure. Since endogenous oxidative DNA damage may play an important role in cancer development, it is important to investigate oxidative stress *in vivo* after exposure to dioxins. TCDD may induce oxidative stress via estrogen metabolism by CYP1A1 or CYP1B1, which are induced by TCDD. In addition, redox cycling after the metabolism of xenobiotics by CYP 1A1 or CYP1B1 may cause or enhance oxidative stress *in vivo*. Other effects of TCDD may also cause oxidative stress, such as TNF-alpha induction in lymphocytes that in turn cause them to release ROS, activate the enzymes involved in production of ROS, or inhibit the enzymes involved in reduction of ROS. Accordingly, oxidative stress induced by TCDD may be associated with the human carcinogenicity of TCDD, and it is important for researchers to investigate oxidative stress in humans exposed to dioxins. Some molecular biomarkers are typically used as indicators of exposure or the effects of certain chemicals, or susceptibility to them<sup>64</sup>). The molecular biomarkers that we mentioned above would be early-effect indicators in the risk assessment of dioxins, especially in the workplace, because the exposure dose level is usually high. If people who experience certain biological alterations in the early stage, such as oxidative DNA damage, enhancement of CYP inductions, or mutations depending on exposure to dioxins could be identified, it would not be difficult to reduce the risk of occupational diseases, including cancer.

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