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Genetic Variation in AhR Gene Related to Dioxin Sensitivity in the Japanese Field Mouse, *Apodemus speciosus*

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1. Introduction

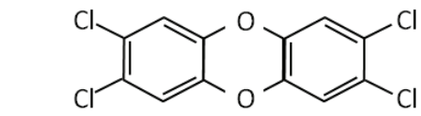
Human beings have developed many tools, technologies, and chemicals for their convenience and comfort. For example, herbicides and/or insecticides that are sprayed on crop lands prevent damage from pests and have resulted in remarkable increases in crop yield. Polychlorinated biphenyls (PCBs), which have insulating properties and are incombustible, were widely used in the past in electronic instruments and by the electric industry. However, some of these chemicals have harmful effects on organisms. In Seveso, Italy, a large amount of dioxins was emitted by explosion of an agrochemical factory. This accidental release of dioxins killed a lot of farm animals and many people living near the factory developed skin inflammation due to exposure to the high concentrations of dioxins. As seen above, many similar chemical spill disasters have occurred and new chemicals are still being produced. Dioxins are one of the most toxic groups of manmade chemicals known. Dioxins are not only highly toxic, but they also insidiously disrupt reproductive function by mimicking the actions of hormones in the body. Their effects on reproduction, such as reducing the number of sperm and affecting the sex ratio in offspring, may impair the fitness of individuals. Decreased reproductive success of individuals in a population may result in the extinction of local populations and eventually species extinction.
In this chapter, we describe the effects of the most toxic chemical pollutant, dioxins, on the Japanese field mouse, *Apodemus speciosus*. We also discuss the diversity of dioxin sensitivity and attempted to identify dioxin sensitivity in mice using a molecular indicator. Our findings suggest that it is important to take into consideration the differences in dioxin response in each mouse for an accurate estimation of the impact of the pollution.

1.1 Dioxins, benzofurans, and PCBs

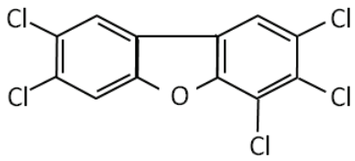
1.1.1 Physical and chemical properties

Dioxin is a generic term for polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (co-PCBs), all of which are halogenated

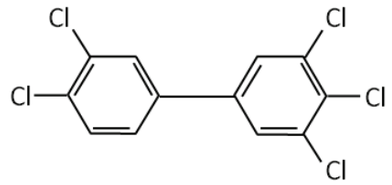
aromatic compounds. Among them, PCDDs are comprised of two benzene rings interconnected by two oxygen bridges. PCDFs also consist of two benzene rings interconnected by a carbon bond and an oxygen bridge. The generic structures of PCDDs and PCDFs are shown in Figure 1a and b. PCDDs and PCDFs have 75 and 135 congeners, respectively. Each compound differs in the number and position of the chlorine atoms. PCBs comprise two benzene rings joined by a carbon bond and have 209 congeners (Fig. 1c). Among these, the congeners with a coplanar conformation that shows chlorine substitution in the non-*ortho* (2, 2′, 6, and 6′) or mono-*ortho* position are called dioxins. Dioxins exhibit extremely low water solubility but are highly soluble in organic solvents. Their lipophilic and hydrophobic properties explain their high concentrations in lipids and organic compounds, and consequently their high degree of biomagnification through food webs.



(a) 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD)



(b) 2,3,4,7,8-pentachlorodibenzofuran (2,3,7,8-PCDF)



(c) 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126)

Fig. 1. Chemical structures of a dioxin, dibenzofuran, and co-PCB

1.1.2 Overview of pollution

Dioxins are unintentionally generated chemical compounds. They were present in minute amounts as a byproduct in previous herbicides. The first time dioxins were recognized worldwide was the Vietnam War. Between 1961 and 1971, nearly 19.5 million gallons (approximately 78 million liters) of herbicides were aerially-sprayed in Vietnam by the United States Armed Forces for tactical defoliation and crop destruction (Stellman et al., 2003). The most commonly used herbicide was Agent Orange, which was constructed of esters of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The 2,4,5-T contained in herbicides included 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and resulted in serious harm to human health, such as birth defects. Thereafter, many accidental seepages and spillages during the production of chlorine and organochlorine compounds, such as bleach, herbicides, and pesticides, were reported (Roland et al., 2008). Furthermore, solid residues emitted by chemical companies have been discharged into landfills and dumps. Some landfills and dumps that were not adequately prepared to prevent pollution leaked PCDD/Fs into the environment. Hazardous waste incinerators and other thermal processes produce high proportions of of PCDD/Fs from these precursors, resulting in a considerable impact on the local environment by high emission of PCDD/Fs. Although new cases of pollution due to dioxin emissions rarely occur these days, previous contaminations have not yet been adequately remediated because it is not easy to completely eliminate a pollutant once it has been emitted.

1.2 Effect of dioxin on organisms is mediated by AhR

Dioxin absorption into the body results in various toxic effects, such as induction of various drug metabolizing enzymes, wasting syndrome, immune suppression, tumor promotion, inflammation, teratogenesis, homeostasis disruption, alterations in cell proliferation, apoptosis, adipose differentiation, and endocrine disruption (Masunaga, 2009; Pohjanvirta & Tuomisto, 1994; Poland & Knutson, 1982; Puga et al., 2000; Stevens et al., 2009; Vos et al., 2000). Although the toxic effects of dioxins are widespread, most are controlled by one protein, aryl hydrocarbon receptor (AhR).

AhR is a ligand-activated transcription factor which mediates most of the dioxin-derived toxic effects (Sogawa & Fujii-Kuriyama, 1997). AhR initially forms a complex with heat shock protein 90 (HSP90), X-associated protein 2 (XAP2), and telomerase binding protein (p23) in cytoplasm (Fig. 2). When an AhR ligand such as TCDD enters the cytoplasm and binds to AhR, the activated AhR translocates into the nucleus, where it dissociates from chaperone proteins and interacts with a number of different proteins.

1.2.1 Induction of various drug metabolizing enzymes

The ligand-activated AhR taken up into the nucleus forms a heterodimer with AhR nuclear translocator (Arnt) (Fig. 2). AhR-Arnt heterodimers bind to the xenobiotic responsive element (XRE), and activate transcription of various drug metabolizing genes such as the cytochrome P450-1A1 (CYP1A1), -1A2, and -1B1, uridine diphosphate glycosyltransferase 1 family polypeptide A1 (UGT1A1), glutathione S-transferase (GST)-Ya subunit and NADPH-quinon-oxidoreductase (Denison et al., 1988; Elferink et al., 1990; Fujisawa-Sehara et al., 1987, 1988) (Fig. 2). The CYP1-family (-1A1, -1A2, and -1B1) consists of phase I drug metabolizing enzymes and they oxidize extraneous substances like dioxins to metabolites and finally excrete them from the body. However, in the process of metabolism, oxidation by CYP enzymes activates xenobiotics and produces reactive oxygen species (ROS), which cause cellular oxidative stress and ultimately result in DNA damage by DNA-single strand break and sometimes cancer promotion (Dalton et al., 2002; Nebert et al., 2000). Furthermore, Latchoumycandane et al. (2002) reported a TCDD dose-dependent reduction of sperm number in rats by oxidative stress. The sperm plasma membrane is rich in polyunsaturated fatty acids so lipid peroxidation of the polyunsaturated fatty acids adversely affects sperm.

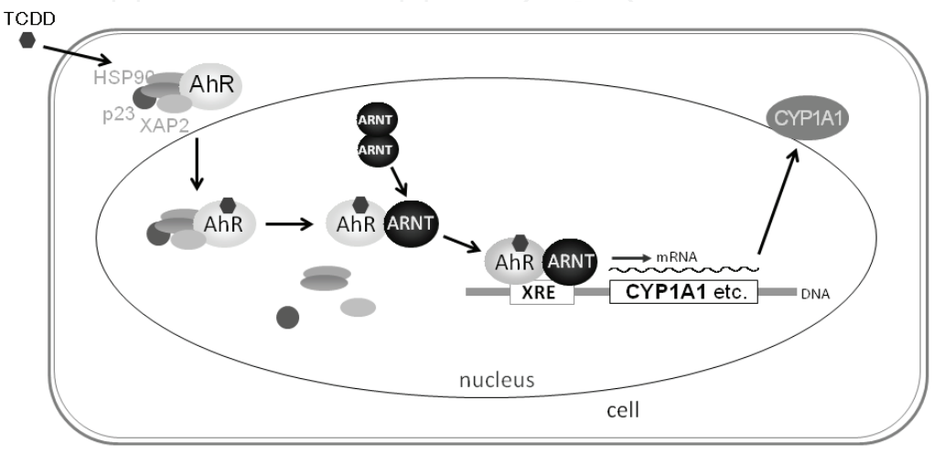


Fig. 2. Simplified scheme of gene regulation by AhR in terms of drug metabolizing enzymes

1.2.2 Disruption of reproductive function

Originally it was believed that AhR regulated the expression of ovarian cytochrome P450 aromatase (CYP19), which is a key enzyme in estrogen synthesis (Baba et al., 2005). AhR regulates the ovarian biological clock and ultimately governs the estrus cycle with AhR repressor (AhRR), which is a negative feedback modulator of the AhR. The AhR also regulates the expression of testicular cytochrome P450 side chain cleavage (P450scc), which is a key enzyme in testosterone synthesis (Fukuzawa et al., 2004). If TCDD invades the cell and binds to AhR, it would disrupt the functions of AhR, such as hormonal synthesis. Furthermore, AhR acts on steroid receptors, such as the oestrogen receptor (ER) and androgen receptor (AR), by two opposite pathways (Ohtake et al., 2003, 2007). In one pathway, ligand-activated AhR activates the expression of ER and AR mediated target genes without hormonal stimulation, while in the other pathway, ligand-activated AhR degrades ER and AR through an ubiquitin-proteasomal system. These actions of AhR induced by TCDD disrupt usual hormonal action and ultimately reproductive function as well.

1.2.3 Cell cycle regulation, tumor promotion, and apoptosis

AhR dimerizes with the RelA subunit of nuclear factor-kappa B (NF-κB). NF-κB/Rel transcription factors regulate many genes involved in control of cellular proliferation, neoplastic transformation, and apoptosis (Kim et al., 2000). Also, AhR and RelA cooperate to activate c-myc oncogene, which is associated with cellular proliferation and tumor promotion. Furthermore, it is known that AhR promotes progression of the cell through the cell cycle (Puga et al., 2002). The gap1 (G1) phase of the cell cycle is inhibited by TCDD-induced AhR. In the DNA synthesis (S) phase, AhR forms protein-protein complexes with the retinoblastoma protein (RB), which is critical for transfer into S-phase. Thus, AhR directly affect cell cycle regulation. Therefore, exposure to TCDD is likely to disrupt the cell cycle and stimulate tumorigenesis.

1.2.4 Cellular inflammatory response

AhR is involved in cellular inflammatory signaling through a non-genomic pathway (Matsumura, 2009). This non-genomic pathway does not require dimer formation with Arnt. The ligand activated AhR regulates rapid increases in intracellular Ca2+ concentration, as well as increases in enzymatic activation of cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-2 (Cox-2). These factors are associated with inflammatory action and cause wasting syndrome and hydronephrosis.

1.2.5 Immunotoxicity

The immune system is one of the most sensitive targets of dioxin (Birnbaum and Tuomisto, 2000). However, conflicting findings have been reported regarding the immunological effects of dioxin. One adverse effect is suppression of the immune system. TCDD inhibits immunoglobulin secretion and decreases resistance to bacterial, viral, and parasitic infections in TCDD-exposed animals (Birnbaum and Tuomist, 2000; Holsapple et al., 1991; Nohara et al., 2002). Also, AhR has been recognized as a key factor in the immunotoxicity of dioxin because AhR plays an important role in the development of liver and the immune system (Fernandez-Salguero et al., 1995). Meanwhile, another advantageous effect is its promotion of immunity. AhR regulates regulatory T (T_{reg}) cells and interleukin (IL)-17-producing T (T_H17) cell differentiation in a ligand-specific manner (Quintana et al., 2008).

The former cells suppress autoimmune conditions such as encephalomyelitis, while the latter cells increase the conditions. Since AhR activation by TCDD has been shown to induce functional T_{reg} cells and improve immunity, AhR is being focused on as a unique property for therapeutic immune-modulation.

1.3 AhR diversity

As above mentioned, AhR is a vital factor because its functions are wide-ranging, from cell cycle regulation to hormonal synthesis. In fact, AhR have been present for a long time in various organisms such as invertebrates, fish, birds, and mammals. Meanwhile, in any single species, the genetic diversity of AhR, which may alter protein function, is always maintained.

1.3.1 History of AhR

Many mammal, bird, fish, insect, and nematode species possess AhR (Hahn, 2002). In invertebrates like nematodes and flies that have ancestral AhR protein, AhR homologs lack specific binding ability to dioxin-like compounds (Butler et al., 2001). Originally in evolutionary history, AhR only had a physiological function without ligand-induced activity. This is why invertebrates are not affected by dioxin related chemicals. AhR subsequently developed into a ligand-inducible biotransformation system in some lineages. Vertebrates like birds and fish have at least two AhR genes, designated AhR1 and AhR2 (Hahn, 2002). In fish AhR genes, AhR1 shows a lower mRNA expression level than AhR2. Also, AhR1 has inactive or reduced TCDD sensitivity as compared with AhR2 (Hansson & Hahn, 2008). Therefore, AhR 2 has been considered as the predominant form in fish. In birds, meanwhile, AhR2 is a recessive form and AhR1 is the major form (Yasui et al., 2007). Although both forms exhibited specific binding to TCDD and induced genes, AhR2 showed a lower binding efficiency than AhR1. In mammals, just a single AhR gene has been confirmed. The mammalian AhR exhibited high binding affinity to TCDD in laboratory rodents to marine wild mammals such as beluga whales and harbor seals (Hahn, 2002).
These studies on AhR indicated that ancestral AhR was duplicated in at least the fish lineage with dioxin-binding ability and disappearance of one AhR gene in the mammalian lineage. The AhR gene was widely conserved among animals, but has evolved by acquiring a new function or functions in each lineage.

1.3.2 AhR structure and function

AhR is a member of the structurally similar gene family with structural motifs designated as bHLH (basic helix–loop–helix) and PAS (Per, Arnt/AhR, Sim homology) (Gu et al., 2000; Taylor & Zhulin, 1999). In the NH2-terminal region, AhR proteins contain a bHLH motif, which is involved in DNA binding and hetero- or homodimerization (Fig. 3). bHLH includes both a nuclear localization signal (NLS) and a nuclear export signal (NES) (Davarinos & Pollenz, 1999; Lees & Whitelaw, 1999). The sequence of nearly 250 amino acids adjacent to the COOH-terminus of the bHLH region is called the PAS domain, which was initially identified as a sequence conserved among *Drosophila* PER, human ARNT and *Drosophila* SIM (Gu et al., 2000; Taylor & Zhulin, 1999). The PAS domain consists of the two imperfect repeats of 50 amino acids, PAS-A and PAS-B, and has been considered to function as an interactive surface for hetero or homodimer formation. The ligand binding domain of AhR is located in the sequence overlapping in part with the PAS-B region, and also with the binding site for Hsp90 which keeps AhR structurally competent to bind a ligand

(Coumailleau et al., 1995). The Hsp90 interacts with the bHLH region to mask the NLS of AhR, resulting in the cytoplasmic maintenance of AhR. Ligand binding to AhR protein changes the conformation of the Hsp90/AhR complex to expose the NLS of AhR, leading to nuclear translocation of the complex (Lees & Whitelaw, 1999). The COOH-terminal half of AhR possesses transactivation activity that is mediated through CBP/p300 and RIP140 coactivators (Sogawa et al., 1995).

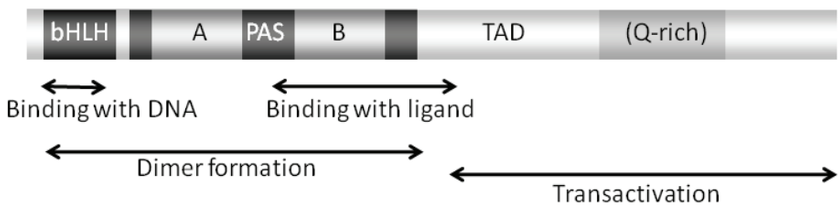


Fig. 3. Schematic representation of functional domain of AhR
bHLH (basic helix–loop– helix); PAS (Per– Arnt– Sim) domain; A and B (PAS A and B repeats); TAD (Transactivation domain); Q-rich (glutamine rich) region. The locations of functional domains are indicated by bars.

1.3.3 Dioxin sensitivity and AhR polymorphism

Remarkable differences in sensitivity to TCDD have been reported among species and strains (Bello et al., 2001; Enan et al., 1996; Kleeman et al., 1988; Pohjanvirta et al., 1993; Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982). For example, the lethal dose 50 % (LD50) values vary from 1 μg/kg for guinea pig, the most sensitive animal, to >5000 μg/kg for hamster, the most resistant (Poland and Knutson, 1982). Also, aquatic birds including the common tern (*Sterna hirundo*) are up to 250-fold less sensitive to dioxins than the typical avian model, the domestic chicken (*Gallus gallus*) (Hoffman et al., 1998; Lorenzen et al., 1997). In the case of same species, LD50 value varies from 182 μg/kg for dioxin-sensitive C57/BL6 strain mice to 2570 μg/kg for mouse DBA strain that is resistant (Poland and Knutson, 1982).

Some of these differences have been explained by genetic variations in AhR which are related to significant protein function. In congeneric mouse strains, C57/BL6 and DBA, the difference in sensitivity is due to a difference in ligand-binding affinity from the difference in the primary structures by only one amino acid substitution (Ema et al., 1994). Similar findings have been reported in 2 bird species, chicken and common tern (Karchner et al., 2006). Furthermore, deletion of 38 or 43 amino acids in the transactivation domain due to one base substitution in an intronic region resulted in different susceptibility to TCDD in congeneric rat strains (Pohjanvirta et al., 1998).

1.3.4 Significance of genetic diversity

As mentioned above, AhR has high diversity among and within species. These genetic variations are linked to differences in protein function that had an important effect on ecological processes such as population recovery from a disturbance (Pearman & Garner, 2005; Reusch et al., 2005), interspecific competition (Booth & Grime, 2003; Yoshida et al., 2003), and local adaptation (Kron & Husband, 2006; de Roode et al., 2005). If an environment is changed by an accident like chemical pollution and warming temperatures, native

organisms would find it difficult to survive in such as altered environment. However, if organisms can maintain genetic diversity that is reflected in differences in response to environmental change, local adaptation would occur and the species would be maintained.
Dioxin pollution is one example of environmental change. Although many toxic effects of dioxin have been clarified, the disruption of reproductive function may have a serious impact on the offspring of adult animals and ultimately may cause local extinction. AhR is a useful gene with which to conduct field studies of dioxin pollution because the functional cascade of AhR is well-known and actual variations including functional differences have been reported. Furthermore, because AhR plays a very important role in a variety of biological processes, AhR variation that alters the action of a protein would have a strong effect on an organism. If we can find a mutation related to AhR ability, the mutation may be a useful molecular indicator for differentiating between dioxin sensitivity and insensitivity.

1.4 Present situation and past examples of dioxin pollution in Japan

1.4.1 Dioxin Pollution in Japan

Large amounts of dioxins have been released into the Japanese environment since the 1950s (Masunaga et al., 2001, 2003; Yoshida & Nakanishi, 2003). The major sources of pollution from the 1960s to 1970s were derived from two herbicides used in rice paddy fields, pentachlorophenol (PCP) and chlornitrofen (CNP). Since the 1980s, however, municipal and industrial waste incinerators have become the major sources of dioxin emissions. Illegal incinerators and dumpsites built in lowland areas in particular have released large amounts of dioxins into the environment and thereby may have seriously affected wildlife living in the vicinity of these polluted sites.

1.4.2 Japanese field mouse

The Japanese field mouse, *Apodemus speciosus*, is broadly distributed in secondary forest in Japanese lowlands, including polluted areas where a lot of dioxins have been illegally released by herbicide spraying and illegally constructed waste incinerators. Furthermore, this species possesses the species-specific characteristic of accumulating higher levels of dioxins in the liver than higher order predators in the same food web (Ministry of Environment, Government of Japan, 2008; Yasuda et al., 2003). According to a previous study, the dioxin concentration in the Japanese field mouse was 4,900 pg-TEQ/g-lipid, which was higher than the Japanese weasel (2,900 pg-TEQ/g-lipid) and the red fox (2,300 pg-TEQ/g-lipid) (Yasuda et al., 2003). Additionally, it is easier to develop a genetic study because the Japanese field mouse is phylogenetically closer to other mice that have been used as a model animal in the field of life science. Therefore, this species is useful when studying the physiological and ecological effects of dioxin on wildlife.
The aim of this study is to clarify the effects of dioxin pollution on Japanese wildlife in terms of genetic background. Therefore, our focus was mutation of the Aryl hydrocarbon receptor gene as a molecular marker that reflects the degree of response to dioxin. We also selected the Japanese field mouse as a bioindicator because of their high accumulation of dioxin, broad distribution in the Japanese environment, and ease of genetic analysis.
First, we identified polymorphisms of *Apodemus speciosus* AhR (*As*-AhR) and a critical mutation related to functional differences. Then we estimated the toxic effect of dioxin on Japanese field mice in terms of the degree of dioxin sensitivity using AhR mutation as a molecular indicator.

2. Searching for critical mutation in *As*-AhR

2.1 Analysis of *As*-AhR sequence and polymorphisms

We examined the full-length of the *As*-AhR sequence and found a lot of variation in the nucleotide sequences. *As*-AhR consists of 857 to 875 amino acids with calculated molecular masses of 96.0 to 97.9 kDa, and exhibited the highest degree of similarity to the mouse AhR by a database search (DDBJ). The variations in sequence length were due to the insertion of 8 to 23 repeats of glutamines (Gln) at codon 596 in the transactivation domain (TAD). In comparison to mouse C57BL/6J strain AhR (Ema et al., 1992), *As*-AhR showed a high similarity (approximately 88.2%) of the amino acid sequence. Also, it shared 100% sequence identity in the basic helix-loop-helix (bHLH) motif (aa 36 - 82 for *As*-AhR and mouse AhR), and high sequence identity of 98 % in the Per-AhR/Arnt-Sim homology (PAS)-A and 95.2 % in the PAS-B domains (PAS-A, aa 130 - 186 for *As*-AhR, aa 130 - 182 for mouse AhR; PAS-B, aa 292 - 343 for *As*-AhR, aa 288 - 339 for mouse AhR). As for TAD (aa 384 – stop codon for *As*-AhR, aa 381 – stop codon for mouse AhR), the sequence homology between *As*-AhR and mouse AhR was 82.9 %.

As-AhR exhibited a variety of polymorphisms in the coding region. Seventy-one SNPs were found within 63 individuals that underwent sequencing. Forty-four of 71 SNPs were synonymous, while 27 non-synonymous changes produced 25 amino acid substitutions. The N-terminal half of *As*-AhR, aa 1 - 383 including bHLH and PAS domains, contained 27 SNPs and 8 amino acid substitutions. On the other hand, the C-terminal half of *As*-AhR, aa 384 - stop codon including TAD, had 44 SNPs and 17 amino acid substitutions. Variations of Gln repeats were found in TAD. Like the Japanese field mouse, such a large number of variations in a species living in the wild have never been reported. For example, the human AhR variation which was studied in various ethnic groups around the world had only four amino acid substitutions (Harper et al., 2002).

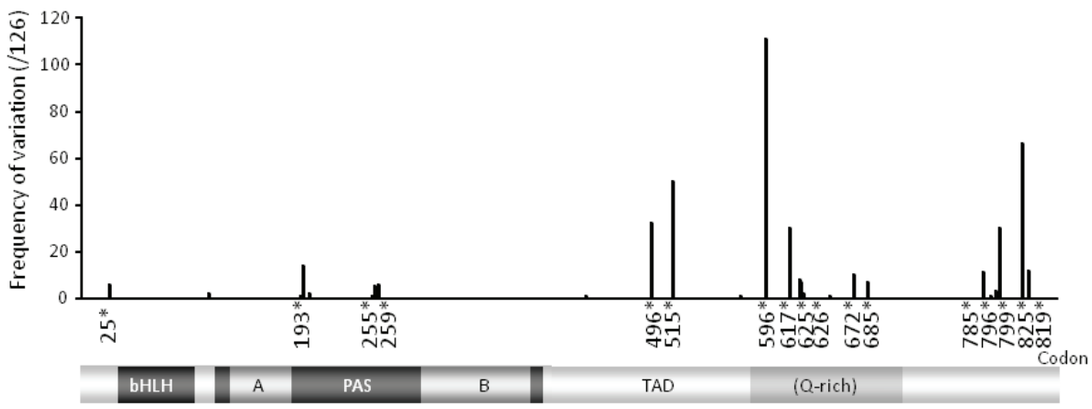


Fig. 4. Frequency of mutation
These bars indicate the number of mutations on the basis of major sequences among 63 individuals analyzed (126 *As*-AhR genes). Horizontal axis shows codon number of *As*-AhR.
* indicates significant mutation site calculated by binominal test with probability 1/850 and Bonferroni correction

Next, to identify the key region for mutation in *As*-AhR, the frequencies of mutations at each codon were counted (Fig. 4). In the N-terminal half of AhR, genes which had a mutation at

each codon were very few, although four sites showed a significant difference by binomial test. In TAD, many codons had a significantly higher mutation rate. In codon 596 having Gln repeats especially, the frequency of variation was the highest (88.1 %). The bHLH, PAS-A, and PAS-B regions that shared high sequence identity with mouse AhR revealed no amino acid variations (Fig. 4). These regions were highly conserved within and among species. In contrast, almost all amino acid substitutions were found in TAD and the region between PAS-A and -B. These results agree with previous studies on AhR variations among laboratory strains of rodent species (Hahn et al., 2004; Harper et al., 2002; Thomas et al., 2002). bHLH and PAS domains in the AhR may be under a physicochemical constraint that does not permit amino acid substitutions. Therefore, a mutation in these regions may potentially cause a critical change in protein function. On the other hand, a mutation in bHLH and PAS domains always implies extreme risk since these regions of AhR are essential for survival. In TAD, many mutations were observed and provided diversity of AhR. In *As*-AhR, nearly 70 % amino acid substitution was observed in TAD. The highest frequency of variation was Gln repeats at codon 596 in which 8 to 23 repeats of poly-Gln were found. Poly-Gln repeats encoded on DNA have been recognized as a medically important trigger because some neurological disorders were found to be associated with unstable expanded trinucleotide repeats, which are called trinucleotide repeat diseases, examples of which are fragile X syndrome and spinobulbar muscular atrophy. These diseases develop when the number of uninterrupted repeats exceeds a constant number and thereby lead to a worsening phenotype into subsequent generation by repeat expansion (Cummings & Zoghbi, 2000). On the other hand, the extended CAG repeats in androgen receptors (AR) have been known to prevent a decrease in sperm number and loss of DNA integrity that were caused by persistent organohalogen pollutant (POP) exposure as a beneficial effect of poly-Gln repeats in human male reproductive function (Giwercman et al., 2007). The expanding Gln repeats found in *As*-AhR might cause a functional change in activity of *As*-AhR protein, as suggested by trinucleotide repeat diseases and CAG repeats in AR gene.

2.2 Functional analysis of *As*-AhR polymorphism *in vitro*

To identify mutations that play a critical role in functional differences in *As*-AhR protein, we initially clarified functional domains altering protein activity by mutation. Comparison of the protein activity of *As*-AhR between the N-terminal and C-terminal regions by reporter assays revealed that mutations detected in the N-terminal region had no functional differences while mutations in the C-terminal region caused functional differences in protein activity (Ishiniwa et al., 2010). Therefore, we focused on polymorphisms in the C-terminal region including TAD which had a higher variation rate than the PAS region. We constructed expression plasmids fused to the C-terminal region (aa 423 to C-terminus) of 9 *As*-AhR alleles into the 3' end of GAL4 DNA binding sequence (pGAL4DBD-*As*-AhR-TAD), which covered all 17 amino acid mutations detected in TAD. The transcriptional activity of the transactivation domain of AhR (*As*-AhR-TAD) was then measured (Fig. 5).

A significant difference in transactivation was observed among the *As*-AhR-TAD alleles (one-way ANOVA, $F=3.806$, $p=0.002$). Insertion of different numbers of Gln repeats into codon 596 had no apparent effect on the transactivation activity (Fig. 5). Also, comparison of alleles that showed higher and lower activity in reporter assay revealed a residue was common in three alleles which exhibited lower activity, allele 7, 8, and 9 (Fig. 5). The shared

residue was arginine (Arg) at codon 799. On the other hand, other alleles, allele 1 to 6, which showed higher activity, shared glutamine (Gln) at codon 799. Therefore, we focused on the substitution at codon 799.

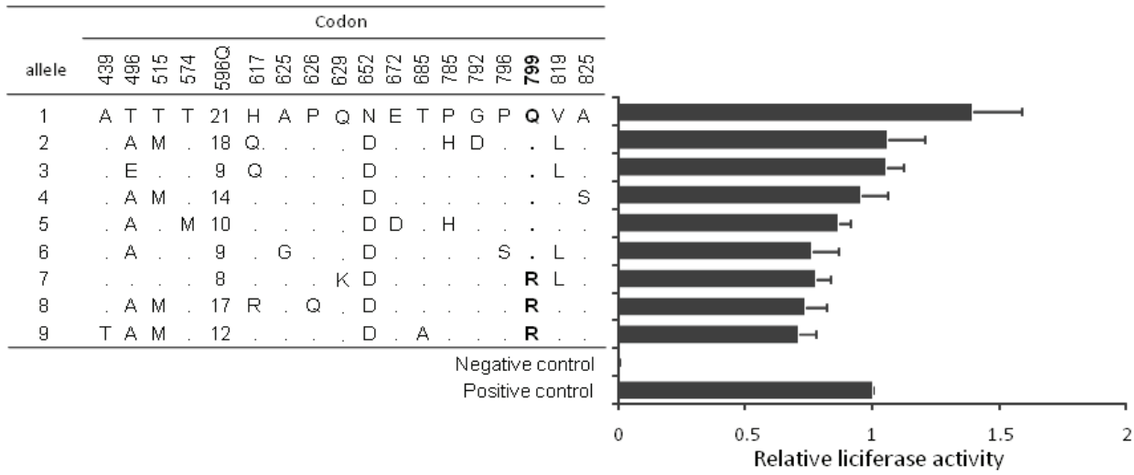


Fig. 5. Functional analysis of *As*-AhR-TAD *in vitro*
The left diagram shows the *As*-AhR-TAD alleles used in the functional analysis. A dot indicates the same residue as the top sequence. The numbers in the 596Q line show the Gln repeat number at codon 596. The right bars indicated the degree of transactivation mediated by *As*-AhR-TAD alleles. HeLa cells were transfected with pG5E-luc, pBOS-LacZ along with expression constructs for mouse AhR-TAD (Positive control), *As*-AhR-TAD, or no TAD (Negative control.; empty GAL4DBD vector). Luciferase activity was measured after 44 h. Relative luciferase activity was calculated by normalizing firefly luciferase activity to the control of mouse AhR-TAD. The values are expressed as the mean and standard error calculated from seven replicates (one-way ANOVA, p=0.002).

To compare the functional difference between Gln-799 and Arg-799 mutants in the same background, pBOS-HA-*As*-AhR-Gln-799 and Arg-799 were constructed and ligand-inducible luciferase expression was quantified. The reporter activity of Gln-799 showed significantly higher activity than Arg-799 (Fig. 6; t-test, p=0.015).
According to Ko et al. (1997), the mouse AhR-TAD can be divided into three subdomains: acidic-rich (aa 515 - 583), proline-rich (aa 643 - 740), and serine-rich (aa 726 - 805) regions. Similarly, the human AhR-TAD also contains three subdomains: an acidic subdomain (aa 500 - 600), a Q-rich subdomain (aa 600 - 713), and a P/S/T subdomain (aa 713 - 848) (Rowlands et al., 1996). In *As*-AhR-TAD, codon 799 is localized in a region orthologous to the serine-rich subdomain of mouse AhR-TAD and P/S/T subdomain of human AhR-TAD. Both of these subdomains act as a repression domain of AhR transactivation (Ko et al., 1997; Kumar et al., 2001). Although the protein structure of the AhR-TAD region is not yet fully understood, differences in the chemical properties between Gln and Arg might change the protein structure and interaction with coactivators, and result in repressive function in transcription. In summary, we succeeded in finding a critical point mutation in *As*-AhR that causes a functional difference in protein activity *in vitro*, which may be related to dioxin sensitivity.

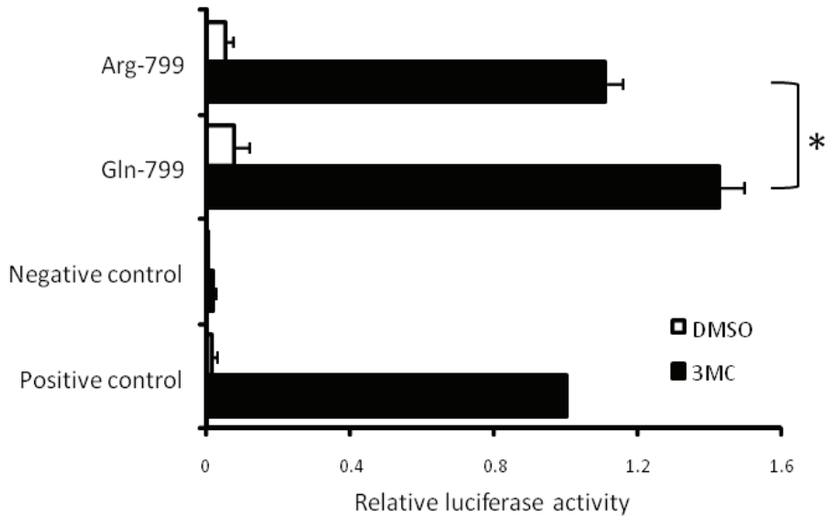


Fig. 6. Functional analysis of mutant AhR, Arg-799 and Gln-799
HeLa cells were transfected with pXREtk-Luc, mouse Arnt, and pBOS-LacZ along with expression constructs for mouse AhR; positive control, As-AhR (Arg-799 and Gln-799), or no AhR (negative control). Transfected cells were treated with DMSO or 3MC (3 mM final concentration), and luciferase activity was measured after 44 h. Relative luciferase activity was calculated by normalizing firefly luciferase activity to the control of mouse AhR. The values are expressed as the mean and standard error calculated from three replicates. A significant difference between mutant As-AhRs was detected by the t-test (*$p < 0.05$).

2.3 Functional analysis of *As*-AhR polymorphism *in vivo*

Does the mutation found *in vitro*, Gln and Arg at codon 799, cause differences in dioxin sensitivity *in vivo*? We examined this question by TCDD administration to Japanese field mice, whose genotype has been identified. Male mice (n=14) were divided into the two genotypes, Q/Q and R/R, by restriction fragment length polymorphism (RFLP) -PCR. TCDD was then administered orally by gastric sonde with an initial loading dose of 200 ng TCDD/kg body weight or an equivalent volume of sesame oil (vehicle) as control, followed by a weekly maintenance dose of 40 ng TCDD/kg body weight or an equivalent volume of sesame oil for three weeks. One week after the last exposure, male mice were deeply anesthetized with diethyl ether, and then the major organs including the testis, epididymis, and liver were removed.
The CYP1A1 mRNA expression level was measured in liver. Here, CYP1A1 was used as a marker enzyme to evaluate the toxic effects of dioxins because it has been reported that CYP1A1 was a sensitive dioxin induced gene (Hirakawa et al., 2007; Watanabe et al., 2005). In both genotypes, an increase in hepatic CYP1A1 mRNA expression was observed at a dose of 200/40 (Fig. 7). However, a comparison between genotypes showed that Q/Q was higher in CYP1A1 expression than R/R at doses of 200/40 ng/kg body weight and a significant difference in the expression between Q/Q and R/R was observed (Mann-Whitney U-test, $p<0.05$). Furthermore, we evaluated the reproductive effects caused by oxidative stress through the AhR-CYP1A1 pathway. In histological analysis, morphological abnormality and the number of single strand DNA breaks in the testis were not observed, while the number of spermatozoa in the epididymis showed a clear difference between genotypes.

Specifically, genotype Q/Q showed a 20 % reduction in the number of spermatozoa after TCDD exposure, while R/R showed no response (data not shown). In genotype Q/Q, the reduction of the number of spermatozoa was most likely due to the high activity of AhR protein observed in CYP1A1 expression. Meanwhile, reproduction in genotype R/R was not affected by TCDD exposure because the activity of AhR protein would be low, as shown in terms of CYP1A1 expression.

As a result, a single mutation at the gene level caused a difference in reproductive function between the two genotypes through an AhR mediated physiological response. Thus, genotype R/R was dioxin-resistant and Q/Q had high sensitivity, which indicates that the Japanese field mouse has a diverse TCDD sensitivity that is mediated by AhR. The mutation in *As*-AhR would be a useful indicator for making a decision about whether a mouse is susceptible or resistant to dioxin.

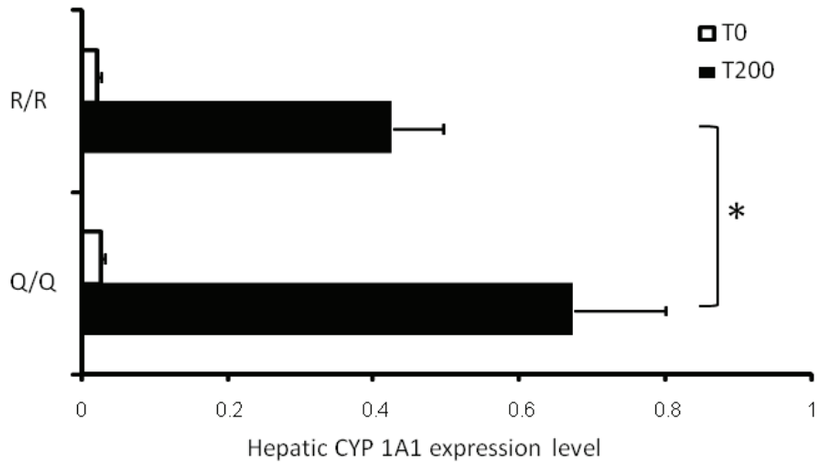


Fig. 7. Induction of CYP1A1 mRNA expression level in genotype Q/Q and R/R mouse
The animals were treated with 0 and 200 ng TCDD/kg as an initial dose followed by weekly maintenance doses of 0 and 40 ng TCDD/kg. The mRNA levels were corrected by β-actin expression. The values are expressed as the mean ± SE for 7 mice per treatment group. A significant difference between Q/Q and R/R at each exposure dose was detected by the U-test (*P<0.05)

3. Application of the critical mutation in *As*-AhR to field study

3.1 Overview of study sites

The sampling was conducted from 2003 to 2004 at six different sites in Japan. Four of six sites were chosen as dioxin-polluted sites. Sanwa (San) in Niigata Prefecture and Kunugi-yama (Kun) in Saitama Prefecture were near garbage incineration plants. Nagaoka (Nag) in Niigata Prefecture was located downstream of industrial waste disposal plants. Sakata (Sak) in Niigata Prefecture was a lagoon contaminated by an influx of agrichemicals. The remaining two sites, Kakuda (Kak) which is secondary forest located in Niigata Prefecture and Nukumidaira (Nuk) which is a primary beech forest in Yamagata Prefecture, were chosen as non-polluted background sites. At each site, the mice were captured using Sherman-type live traps baited with sunflower seeds and soil samples were obtained. A total of 92 mice were caught; 55 at polluted sites and 37 at non-polluted sites.

The dioxin concentrations in soil were 78.5 ± 2.3 pg-TEQ/g dw-ignition-loss (average ± standard error) in non-polluted sites (n=2) and 1140.6 ± 749.5 pg-TEQ/g dw-ignition-loss in polluted sites (n=4). The concentrations in liver were 411.6 ± 6.4 pg-TEQ/g-lipid in non-polluted sites (n=2) and 1847.3 ± 547.6 pg-TEQ/g-lipid in polluted sites (n=4). The CYP1A1 expression as physical reaction to dioxin pollution showed 0.05 ± 0.01 in non-polluted sites (n=37) and 0.11 ± 0.01 in polluted sites (n=55). These values are presented as levels of CYP1A1 mRNA relative to control. A significant difference in the CYP1A1 level was observed between polluted and non-polluted sites (Mann-Whitney U-test, p=0.007).
Polluted sites had higher dioxin levels in both soil and mice. Furthermore, higher CYP1A1 expression as a toxic reaction was observed in polluted sites. However, these chemical and physiological analytical results were not considered to represent the diversity of mouse sensitivity to dioxin. We decided to determine what would happen if these results were re-analyzed using AhR polymorphism as a molecular indicator.

3.2 Effects of dioxin pollution on the Japanese field mouse –Reanalysis using molecular indicator related to dioxin sensitivity-

To clarify differences in the response to dioxin exposure between dioxin-sensitive mice and dioxin-resistant mice, we divided the mice into three genotypes, Q/Q, Q/R, and R/R at codon 799 of *As*-AhR by RFLP-PCR. The CYP1A1 level in each genotype between non-polluted and dioxin-polluted sites was analyzed again. For genotype Q/Q, the Japanese field mice collected from polluted sites showed significantly higher CYP1A1 mRNA expression levels than those from non-polluted sites (Fig. 8, U-test, p=0.009). For genotype Q/R, mice from polluted sites showed higher CYP1A1 expression levels than those from non-polluted sites, although a significant difference was not observed. For genotype R/R, there was no difference between the two sites. Also, the difference in CYP1A1 mRNA expression level between polluted and non-polluted sites became smaller from genotype Q/Q to R/R (Fig. 8). The result which pooled data from all genotypes in section 3.1 was similar to the result for genotype Q/Q mice because both showed remarkable differences in CYP1A1 expression between non-polluted and polluted sites. Then, after calculating the frequency of each genotype at non-polluted and polluted sites, it was revealed that both sites were occupied by the genotypes Q/Q and Q/R (Fig. 9). At dioxin-polluted sites, genotype Q/Q constituted more than half of all individuals, while the frequency of genotype R/R was very low at both sites.
These results suggest that the difference in CYP1A1 expression level between non-polluted and polluted sites in pooled data of all genotypes was due to the proportion of genotype Q/Q and Q/R to the total population. In this study, the mice from dioxin-polluted sites were predominantly genotype Q/Q, which had a high sensitivity to dioxin, thereby revealing a critical difference between non-polluted and polluted sites. If genotype R/R is a major constituent member in mice from polluted sites, CYP1A1 expression levels will not appear to be so high, leading us to conclude that the mice were not exposed to dioxin exposure. Therefore, when comparing the toxic effect of dioxin exposure among various populations, information concerning population structure with respect to dioxin sensitivity is important for discussing the result because the implication of the response to dioxin exposure is different between mice that are susceptible and resistant to dioxin.

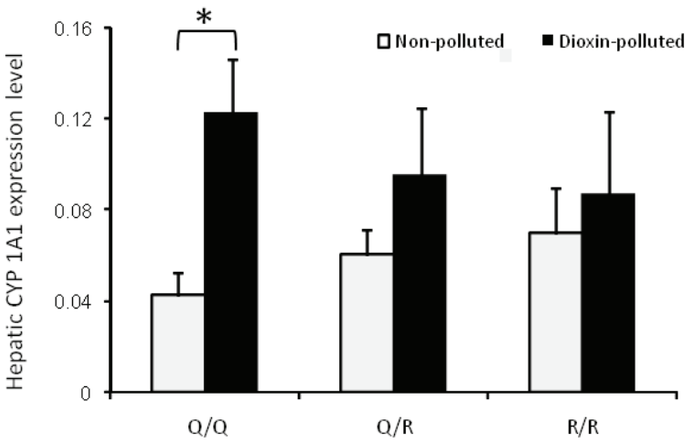


Fig. 8. Hepatic CYP1A1 mRNA expression level of Japanese field mice captured in non-polluted and polluted forests
Hepatic CYP1A1 mRNA expression levels were estimated by quantitative real-time RT-PCR analysis. The histogram represents relative levels of CYP1A1 to β-actin mRNA. The values are expressed as the mean ± standard error. Q/Q, Q/R, and R/R indicate the genotypes at codon 799 of As-AhR. A significant difference between non-polluted and polluted forests was detected by the U-test (*$p < 0.05$)

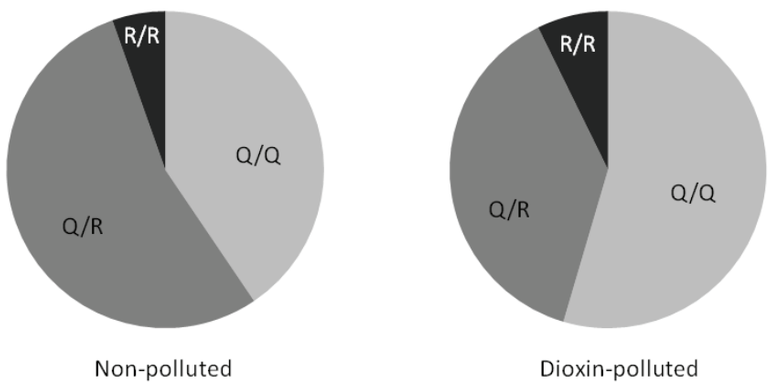


Fig. 9. Frequency of genotype Q/Q, Q/R, and R/R in dioxin polluted and non-polluted sites

4. Conclusion

In this chapter, we have described the toxic reaction to dioxin pollution in Japanese field mice based on their genetic background related to dioxin sensitivity. As a molecular indicator, we focused on the AhR gene, which plays an important role in dioxin-induced toxicity and consequently detected mutations, Q and R, at codon 799 in *As*-AhR that resulted in functional differences between alleles *in vitro* and *in vivo*. Mice with the Q allele showed high dioxin sensitivity, while those with the R allele showed resistance. Furthermore, we applied the mutation to wild mice and found that mice collected from dioxin-polluted sites exhibited a significantly higher toxic reaction than mice from non-polluted sites because mice from polluted sites were predominantly genotype Q/Q. AhR

polymorphism was useful as an indicator for evaluating the effects of dioxin pollution on wildlife.

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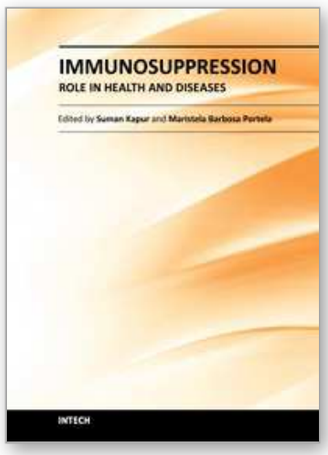

Immunosuppression - Role in Health and Diseases
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A need for a book on immunology which primarily focuses on the needs of medical and clinical research students was recognized. This book, "Immunosuppression - Role in Health and Diseases" is relatively short and contains topics relevant to the understanding of human immune system and its role in health and diseases. Immunosuppression involves an act that reduces the activation or efficacy of the immune system. Therapeutic immunosuppression has applications in clinical medicine, ranging from prevention and treatment of organ/bone marrow transplant rejection, management of autoimmune and inflammatory disorders. It brings important developments both in the field of molecular mechanisms involved and active therapeutic approaches employed for immunosuppression in various human disease conditions. There was a need to bring this information together in a single volume, as much of the recent developments are dispersed throughout biomedical literature, largely in specialized journals. This book will serve well the practicing physicians, surgeons and biomedical scientists as it provides an insight into various approaches to immunosuppression and reviews current developments in each area.

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