DIRECT, CONTINUOUS MONITORING OF AIR POLLUTION BY TRANSGENIC SENSOR MICE RESPONSIVE TO HALOGENATED AND POLYCYCLIC AROMATIC HYDROCARBONS

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Abstract

The aryl hydrocarbon receptor (AhR) plays crucial roles in toxicological responses of animals to halogenated and polycyclic aromatic hydrocarbons. To achieve direct, continuous risk assessment of air pollution using biological systems, we generated transgenic sensor mice that produce secreted alkaline phosphatase (SEAP) under the control of AhR. In response to single, oral exposure to AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (5 μg/kg body weight), the established senor mice exhibited dramatic (> 1,000 fold) and sustained (> 84 days) elevation of serum SEAP with a peak at 3 - 5 weeks. The mice also responded sensitively to 3-methylcholanthrene, benzo[*a*]pyrene and β-naphthoflavone. The increase of SEAP was dose-dependent, and male mice responded more sensitively than female mice. RT-PCR analysis revealed that the main responding organ was the liver. Using the sensor mice, we attempted to monitor air pollution caused by cigarette smoke that contains substantial levels of AhR agonists. Mice were placed everyday in an experimental smoking room for 3 h. Every time following exposure, the sensor mice exhibited transient, reversible elevation of serum SEAP. Our result is the first success to demonstrate that direct, comprehensive and real-time monitoring of air pollution is feasible using genetically engineered mammals.

Introduction

Monitoring of environmental pollution by halogenated and polycyclic aromatic hydrocarbons (HAHs and PAHs) is important for promotion and maintenance of human health. It is because that those chemicals cause a broad spectrum of toxicity mediated by aryl hydrocarbon receptor (AhR) in mammals, including carcinogenesis, teratogenesis and immune dysfunction[1]. Pollution of foods, water, soil and air by aromatic hydrocarbons is monitored using physicochemical methods, *e.g.*, gas chromatography - mass spectrometric analysis, to measure the levels of individual pollutants. However, the conventional approach has obvious limitations to estimate the real risk of environmental pollution in humans. For example, environmental samples contain numerous toxic and non-toxic substances, but the concentration approach cannot take consideration of absorption, metabolism, synergism and antagonism of these substances in mammals. Furthermore, the current monitoring method does not allow for direct assessment of accumulative and integrative influences of xenobiotic substances taken through respiratory, ingestive and transdermal routes. To overcome these problems, we attempted to generate transgenic sensor mice that produce secreted alkaline phosphatase (SEAP) under the control of AhR.

Materials and Methods

Generation of transgenic mice.

The *Mul*I-*Sal*I fragment of pDRE-SEAP[2] was microinjected into the pronuclei of fertilized oocytes of C57BL/6J mice, and the injected embryos were transferred into pseudopregnant mice using standard techniques. Transgenic pups were screened by PCR. Two transgenic lines DRESSA24 and DRESSA25 were established, and adult male DRESSA25 (heterozygous) were generally used for studies.

Administration of mice with AhR agonists.

Mice (9 - 10 weeks) were orally administered with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) [0.1 - 5 μg/kg body weight (BW) in corn oil] or other AhR agonists including 3-methylcholanthren, benzo[*a*]pyrene or β-naphthoflavone (10 mg/kg BW) using feeding needles. Blood (~20 μl) was sampled from the tail veins to evaluate levels of serum SEAP, as described before[3].

Exposure of mice to polluted air.

Mice were placed in an experimental smoking room (60 x 40 x 35 cm) for 3 h. During the study, the room was ventilated continuously by fresh air using a mini-pump (1 L /min). Mainstream smoke (250 ml) prepared from a 14 mg-tar cigarette (Seven Star; Japan Tobacco, Inc.) was injected into the room every 30 min (total 6 times/day) during the exposure period. After the exposure to polluted air for 3 h, the mice were maintained for the following 21 h outside the smoking room. This procedure was repeated every 24 h for up to 4 days. Blood sampling was performed before and 0, 3, 6, 9 and 18 h after the exposure to polluted air. Animal experiments were performed following the regulation and guideline at University of Yamanashi.

Chemiluminescent assay.

Activity of SEAP in serum was evaluated by a chemiluminescent method using Great EscAPe SEAP detection kit (Clontech), as described before[4].

RT-PCR.

Total RNA was extracted using TRIzol (Invitrogen), EZ1 RNA kit (Qiagen) and BioRobot EZ2 (Qiagen). One microgram of total RNA was subjected to reverse transcription using Omniscript Reverse Transcriptase (Qiagen). PCR was performed using TaKaRa Ex Taq Hot Start Version (Takara) with the following primers purchased from Sigma-Aldrich Japan. The used primers were SEAP (5'- CAGGACATCGCTACGCAGCTCATCT -3', 5'- GTAAGCCCTGCTTTCATGATGACCA -3') and GAPDH (5'- ACCACAGTCCATGCCATCAC -3', 5'- TCCACCACCCTGTTGCTGTA -3').

Statistical analysis.

Data were expressed as means ± SE (n≥4). Statistical analysis was performed using the non-parametric Mann-Whitney *U* test to compare data in different groups. *P* value < 0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Generation of dioxin response element (DRE)-based sensing via SEAP (DRESSA) mice. We previously reported a fast, sensitive bioreporter assay DRESSA that can detect and quantify the levels of HAHs and PAHs[2,5]. Using the same gene construct that contains a SEAP gene under the control of DRE, we generated transgenic sensor mice (DRESSA mice). Two transgenic lines DRESSA24 and DRESSA25 were established. To examine responses of the sensor mice to AhR agonists, wild-type mice and DRESSA mice were orally administered with 2,3,7,8-TCDD (5 μg/kg BW), and the levels of serum SEAP were evaluated at day 3. As shown in **Fig. 2**, the serum SEAP level was markedly elevated (approximately 50 fold) in DRESSA25, while the level in wild-type mice was not increased. We also confirmed that DRESSA mice could respond to other AhR ligands including 3-methylcholanthrene, benzo[*a*]pyrene and β-naphthoflavone (10 mg/kg BW) (data not shown). In contrast, DRESSA24 showed high levels of basal SEAP activity, which may be caused by integration of the transgene downstream of some housekeeping gene promoter(s). However, responsiveness to 2,3,7,8-TCDD was still preserved in DRESSA24.

Characterization of responses to dioxin in DRESSA mice. As an *in vivo* reporter molecule, SEAP has several advantages. Although reporter mice using β-galactosidase, green fluorescent protein (GFP) or luciferase need to be sacrificed and/or internal organs must be exposed or excised for analyses (*e.g.*, for photo imaging, protein/RNA extraction or tissue sectioning)[6-10], the mice using SEAP require only 5μl of serum to evaluate the activity of SEAP. The serum SEAP can be measured easily, quickly and sensitively by using conventional chemiluminescent methods. These properties enable continuous assessment of serum SEAP activity in individual animals[4]. In contrast to secreted luciferase, activity of SEAP is not affected by serum components[3]. While the half-lives of CAT, β-galactosidase and GFP are 20 - 50 h, that of serum SEAP is 2 h in mice[11]. It allows real-time assessment of certain promoter activity *in vivo*[4]. Based on these previous data, we examined kinetics of serum SEAP in male DRESSA25 after oral administration with 2,3,7,8-TCDD (5 μg/kg BW). As shown in **Fig. 3**, significant elevation of serum SEAP activity was observed within 12 h. Unexpectedly, during the course of the study, the level of SEAP continuously increased until day 28 without additional exposure to dioxin. The induction rate at day 28 was approximately 1,100 fold over the basal level. The level of SEAP was then gradually declined, but significant elevation of SEAP was still detectable until day 84. Administration with corn oil alone did not cause elevation of serum SEAP.

We examined dose responses of DRESSA mice to dioxin. DRESSA25 were orally exposed to 0.1 - 5 μg/kg

BW 2,3,7,8-TCDD, and activity of serum SEAP was evaluated until day 21. As shown in **Fig. 4**, the level of serum SEAP increased in response to dioxin dose-dependently, and the detection limit was approximately 0.1 μg/kg BW.

Previous reports indicated that gender-dependent differences in responses to xenobiotics may be present in mice[6]. We therefore compared responses of male and female DRESSA25 to 2,3,7,8-TCDD. Interestingly, the increase in serum SEAP was not different between males and females at day 1. However, although significant elevations of serum SEAP continued in females for at least 3 weeks, the degree of elevation was much lower than that in males (**Fig. 5**). These results elucidated that male mice respond to aromatic hydrocarbons more sensitively and are suitable as sensing animals to detect environment pollution.

To identify major organs responsible for the production of SEAP, we examined expression of *SEAP* mRNA by RT-PCR in indicated tissues after oral administration with 2,3,7,8-TCDD. As shown in **Fig. 6,** under the untreated condition, a low level of *SEAP* mRNA was detectable in the brain, but not in other organs tested. At day 3 and day 21, expression of *SEAP* was induced markedly in the liver, and modestly in the brain.

Monitoring of air pollution caused by cigarette smoke using DRESSA mice. Using *in vitro* DRESSA bioassay[2,5], we recently reported that cigarette smoke strongly activates the AhR - DRE pathway[12]. We therefore examined whether air pollution caused by cigarette smoke can be detected and monitored directly and successively using the established DRESSA mice. For this purpose, DRESSA mice were exposed to cigarette smoke in experimental smoking room, as described in *Materials and Methods*. As shown in **Fig. 7**, after the exposure to the polluted air, serum levels of SEAP were significantly elevated. This result was reproducible, and every time following exposure to the polluted air, DRESSA mice exhibited similar kinetics of serum SEAP. In contrast, DRESSA mice placed in the room for 3 h without exposure to polluted air did not show any increases in the level of serum SEAP. These results suggested that direct, comprehensive and real-time monitoring of air pollution is feasible using genetically engineered, SEAP-based sensor mice only via simple blood sampling.

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Figure 1. Schematic representation of the reporter construct introduced into mice.

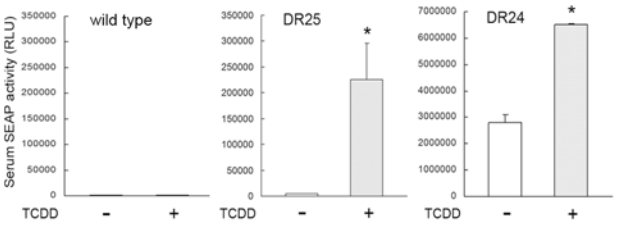

Figure 2. Responses of serum SEAP activity after exposure to dioxin in wild-type and DRESSA mice.

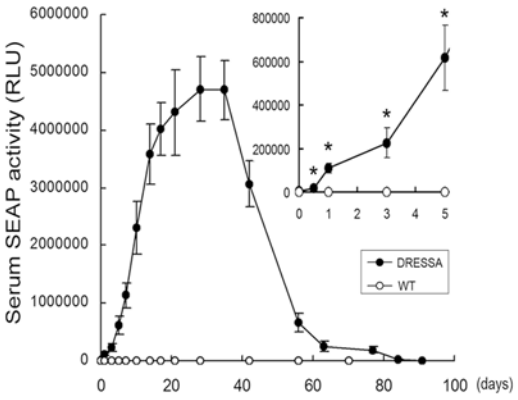

Figure 3. Kinetics of serum SEAP in DRESSA mice exposed to dioxin.

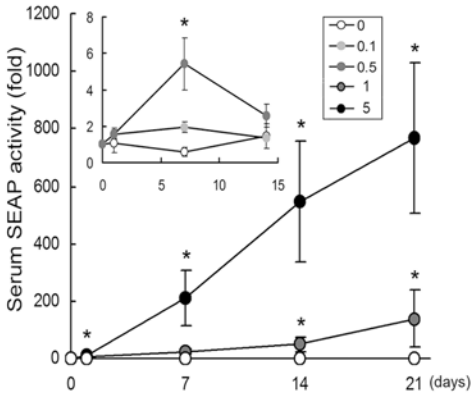

Figure 4. Dose-dependent responses of serum SEAP in DRESSA mice exposed to dioxin.

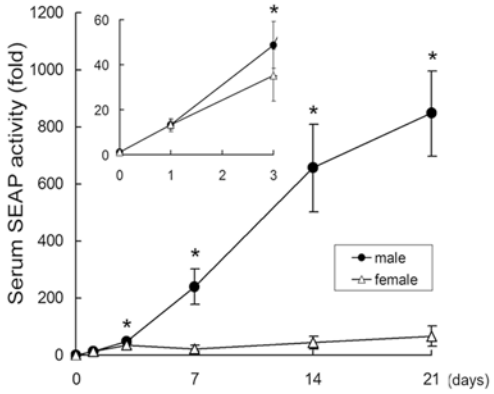

Figure 5. Gender-dependent difference in the response to dioxin.

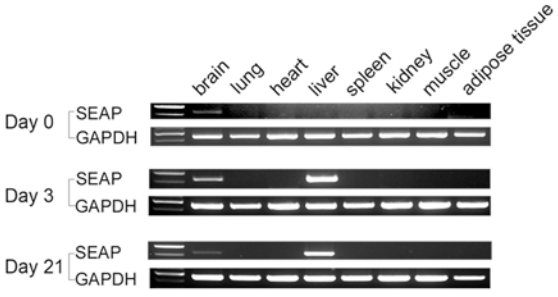

Figure 6. RT-PCR analysis of SEAP expression in various organs after oral administration with dioxin.

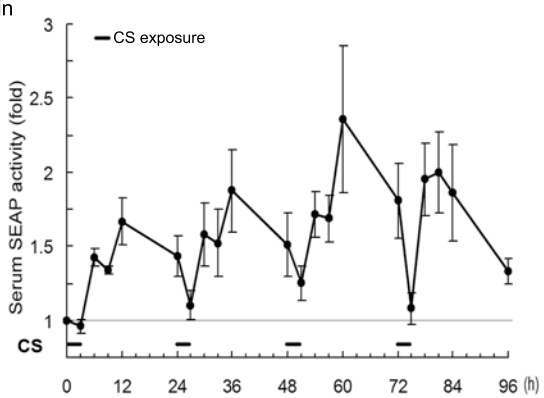

Figure 7. Monitoring of air pollution caused by cigarette smoke using DRESSA mice.