Assessment of Dioxin-like Effect of Ash Extracts - Comparison of Chemical Analysis and *In Vitro* Bioassay -

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Introduction

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/DFs) are formed by the thermal processes of waste combustion, and are detected in emissions of municipal waste incinerator [1), 2)]. Regulatory control of emission and risk assessment of PCDD/DFs were carried out by the toxic equivalency factor/TCDD equivalent (TEF/TEQ) approach [3)]. It was reported that good correspondence between TEQ and toxicities *in vivo* were demonstrated in PCDD/DFs mixture [4)].

Whereas it is reviewed [3)] that complex mixtures containing compounds that act through multiple pathways to give both similar and different toxic responses as PCDD/DFs, the TEF/TEQ approach may not be appropriate. According to the investigations done by more complex chemical mixtures, such as extracts of fish [5)] and ash [6)], the estimated TEQ observed in bioassay were higher than calculated values. These differences were partially suggested by the presence of bioassay active components that are not detected by GC-MS analysis [5)]. It has been reported that some toxic components except for PCDD/DFs, PCBs and polycyclic aromatic hydrocarbons (PAHs) could be present in incinerator fly ash [6)]. We also reported that unknown organohalogen compounds are higher by orders of magnitude than PCDD/DFs in ash samples based on analysis of extractable organic halogens and PCDD/DFs [7)]. It is also speculated that the unknown compounds are included as persistent organic pollutants (POPs), and some of them may have dioxin-like toxicity. As far as hazard and risk assessment of arylhydrocarbon receptor (AhR) mediated-effect on humans and wildlife, bioassay is likely to be more practical than calculated TEQ values.

In this study, we demonstrated to clarify the relationship between chemical analysis and two bioassays to evaluate the toxicity of ash extracts that contain complex mixtures of POPs.

Materials and Methods

Subjects and Chemical Analysis - Three kinds of ash samples were collected from medical and municipal waste incinerators located in Japan. Extraction, clean-up and GC-MS analysis were carried out as described elsewhere [7)]. Crude extract and PCDD/DFs fraction were redissolved in dimethyl sulfoxide (DMSO) and used for bioassay.

Chemicals - 2,3,7,8-TCDD (1mg; crystalline form) was obtained from AccuStandard (New Haven, CT) and dissolved in DMSO. All other chemicals used were analytical grade.

Cell lines - Human hepatoma cell line HepG2 were grown in DMEM supplemented with 10% (v/v) fetal bovine serum in an incubator maintained at 5% CO_2 95% air and 100% humidity at 37°C.

Ethoxyresorufin O-Deethylase (EROD) Assay - HepG2 cells were seeded on 24-well culture plate and incubated for 24 hours . Ash extracts or TCDD were added to the culture medium in various concentration until the concentration of DMSO did not exceed 0.5% (v/v) . After 24 hours, cells were washed by PBS and then EROD activity was determined using the method adapted from the procedure described by Kennedy and Jones [8].

Plasmid Construction - prXRE7-Luc, in which seven copies of synthesized rat Glutathione *S*-transferase xenobiotic response element (XRE) [9] were subcloned into upstream of the firefly luciferase gene in pGL2-promotor vector and used as reporter gene.

Transfection - HepG2 cells were cotransfected with prXRE7-Luc and pTARGET vector and selected with Geneticin (GIBCO BRL). Among stable transfectants, the clone showed luciferase activity in the presence of 10^{-9} M TCDD were selected and used for further assay.

Luciferase Assay - The cloned cells were seeded on 24-well culture plate. Incubation and exposure of ash extracts or TCDD were the same as the above. After 24 hours, cells were harvested and luciferase activity was determined with luciferase assay system (Promega) and luminometer (LB9501, Berthold) .

Protein Assay - Protein concentration was measured with BCA protein assay reagent (Pierce) using bovine serum albumin as standard.

Statistical Analysis - Statistics were performed by one-way ANOVA least significant difference test.

Results and Discussion

The data of chemical analysis was summarized in Table 1. All ash samples showed relatively high TEQ values.

Figure 1 shows the results of TCDD induced EROD and luciferase activity. Both activities were dose-dependent manner and maximal induction was observed in the concentration of 1610 pg/ml culture medium (5nM).

Table 1 Results of Chemical Analysis

Incinerator	Medical Waste		Municipal
Sample	fly ash	bottom ash	fly ash
Name	MedSW1-FA	MedSW2-BA3	MSW7-FA
Recovery (%)	42.9	70.9	69.2
TEQ (ng/g ash)	14.6	13.9	10.2

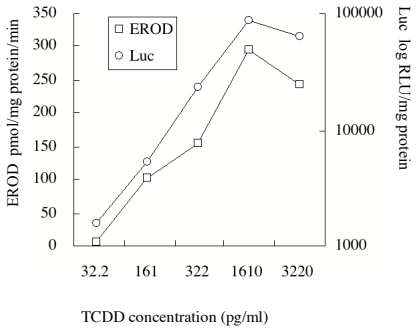

Fig.1 TCDD standard curve for EROD and XRE-mediated Luciferase Assay

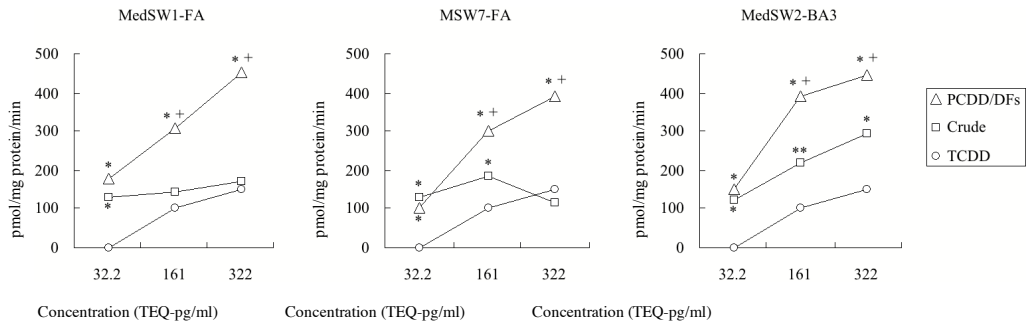

Fig. 2 EROD activity induced by TCDD, Crude Extract and PCDD/DFs Fraction
* Significant difference from TCDD of the same TEQ concentration ($p<0.05$)
** Significant difference from TCDD of the same TEQ concentration ($p<0.10$)
\+ Significant difference from Crude Extractn of the same TEQ concentration ($p<0.05$)

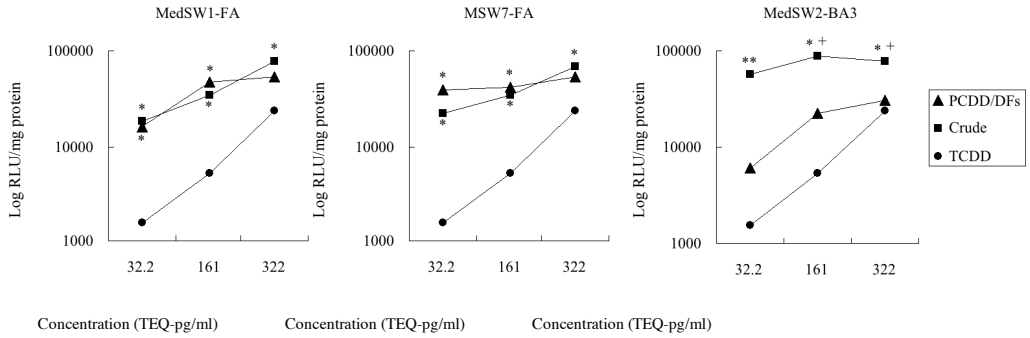

Fig. 3 XRE-mediated luciferase activity induced by TCDD , Crude Extract and PCDD/DFs Fraction
* Significant difference from TCDD of the same TEQ concentration ($p<0.05$)
** Significant difference from TCDD of the same TEQ concentration ($p<0.10$)
\+ Significant difference from PCDD/DFs Fraction of the same TEQ concentration ($p<0.05$)

EROD activity induced by ash extracts and fractions are shown in Figure 2. As compared with TCDD, significantly higher EROD activity was observed in both crude extract and PCDD/DFs fraction from three ash samples in the concentration of 32.2 pg-TEQ/ml culture medium. PCDD/DFs fraction showed higher activity than TCDD at the same TEQ value in all tested condition. In 3220 pg-TEQ/ml (10nM) or higher concentration of TCDD, EROD activity was not induced dose-dependently (data not shown) . Therefore we speculated that the existence of some kinds of bioactive chemicals which could activate the cytochrome P450IA isoforms (CYPIA1, CYPIA2 : CYPIAs) in different mechanism from TCDD.

XRE-mediated luciferase activity are shown in Figure 3. The effects of crude extracts and PCDD/DFs fractions on luciferase activity were different from those on EROD assay. It was obvious that crude extracts from fly ash samples (MedSW1-FA and MSW7-FA) induced the similar level of luciferase activity to that of PCDD/DFs at the same TEQ. While the EROD activity in crude extract is lower than PCDD/DFs fraction at relatively high concentration (322 pg-TEQ/ml). In bottom ash sample (MedSW2-BA3), the relationship between crude extract and PCDD/DFs fraction was contrary in both assays. It may be the reason that two-bioassays reflect the different signal-pathways. EROD assay showed the catalytic activity of CYPIAs indirectly [10].

CYPIAs are regulated by endogenous promoter that contains XRE and other kinds of response elements, and many transcription intermediate cofactors modulate the gene expression. It was possible that EROD activity was diminished by heterogeneous impurities in crude extract at multiple steps of gene expression. If this is the case, the results of EROD assay will reflect the real effects on humans and wildlife. But in the case of more complex mixtures with relatively high concentration of chemicals, the toxicity will be underestimated.

In luciferase assay, we have been used mammalian cell-line that stably contained prXRE7-Luc. This strategy ensures that the induction of the reporter gene occurs only through XREs. Response element-regulated reporter gene assays are widely used in molecular biology and toxicology for specific signal-pathway investigation [11]. Concerning to assessing XRE-mediated effect, recombinant luciferase assay is likely to be more reliable than EROD assay.

In both bioassays, ash extracts or fractions showed higher enzyme inducibility than TCDD at the same TEQ. This may be contributed in part by unknown organochlorine, bromine and iodine compounds we described before [7]. Incinerated ashes that contain POPs are usually dumped in the landfill. For risk assessment and regulatory control of emission, further study is required to identify the mechanisms of PCDD/DFs toxicity, the compounds responsible for AhR/XRE signal-pathway and its behavior in the environment.

Acknowledgments

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References

1) Olie K, Vermeulen PL and Hutzinger O (1977) *Chemosphere* 6, 445-459

2) Safe S (1990) *Crit Rev Toxicol* 21, 51-88

3) Safe SH (1998) *Environ Health Perspect* 106 (Suppl 4), 1051-1058

4) Eadon G, Kaminsky L, Silkworth J, Aldous K, Hilker D, O'Keefe P, Smith R, Gierthy JF, Hawley J and Kim N (1986) *Environ Health Perspect* 70, 221-227

5) Zacharewski T, Safe L, Safe S, Chittim B, DeVault D, Wiberg K, Bergqvist PA and Rappe C (1989) *Environ Sci Technol* 23, 730-735

6) Till M, Behnisch P, Hagenmaier H, Bock KW and Schrenk D (1997) *Environ Health Perspect* 105 1326-1332

7) Kawano M, Ueda M, Matsui M, Kashima Y, Matsuda M and Wakimoto T (1998) *Organohalogen Compounds* 36, 221-224

8) Kennedy SW and Jones SP (1994) *Anal Biochem* 222, 217-223

9) Rushmore TH, King RG, Paulson KE and Pickett CB (1990) *Proc Natl Acad Sci USA* 87, 3826-30

10) Burke MD, Mayer RT (1974) *Drug Metab Dispos* 2, 583-588

11) Welsh S and Kay SA (1997) *Curr Opin Biotechnol* 8, 617-622