Development of risk assessment for dioxin analogues and PAHs in the environmental samples by the two kind of *in vitro* bioassay

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Introduction

Polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDF) and non-ortho coplanar PCBs (Co-PCBs) are three classes of structurally and toxicologically similar persistent environmental contaminants. These compounds are formed as by-products in various chemicals and combustion processes and are now global environmental contaminants. Recently, much importance has been attached to the problem of environmental pollution by PCDDs, PCDFs and Co-PCBs released from municipal solid waste (MSW) incinerators in Japan, because the numbers of MSW incinerators and industrial waste incinerators were 1841[1)] and over 20000 in our country, respectively. Therefore, the residents around the incinerator are incessantly taken by a feeling of unrest, and further it has been concerned for adverse effect of their health condition. Actually, to perform the environmental risk assessment for the residents, it was required to determine the concentration of dioxin analogues in many samples (bloods, mother milk, soils and flue gasses from the incinerator etc.) by GC-MS analyses. However, from the standpoints of high cost and tedious method demand for the above dioxin analysis, it is very difficult to evaluate the environmental risk around all incinerators in Japan. On the other hand, from the observation result that a remarkable correlation was found between the binding affinity of PCDDs to the Ah receptors[2)], the authors tried to develop the rapid biological evaluation method for environmental risk assessment; the induction of 7-ethoxyresorufin O-deethylase (EROD) activity in human cancer cells, reflecting induction of P450IA1, was chosen as a sensitive biological activity of dioxin analogues. In addition, polynuclear aromatic hydrocarbons (PAHs) which detect in the same environmental samples around the incinerators, can also induce EROD activity. In contrast, it is well known that dioxin analogues indicate negative for the mutagenesity, but PAHs indicate

positive. Therefore, it is very important to develop risk assessment method synthetically judged from EROD activity and mutagenic activity, separating the toxic contribution by dioxin analogues from that by PAHs.

In the present work, we tried to compare with EROD activity by human liver cancer cells Hep G2, and mutagenic activity by *Salmonella typhimurium* TA1535/pSK 1002, which induced by dioxin analogues and PAHs in environmental samples. Thus, by using the actual environmental soil samples after GC-MS analyses, we investigated whether their concentrations was well correlated with the data obtained from the above two bioassay.

Materials and Methods

1) Culture of human liver cancer cell Hep G2 and rat liver cancer cell H4IIE

Hep G2 cells (ATCC, HB-8065) and H4IIE cells (ATCC, CRL-1548) were grown in 24-well plates in Eagle's MEM, containing 10% fetal bovine serum (FBS). The cells were incubated at 37 °C with 5% CO_2 in a CO_2 incubator. After 24hr in culture, medium was replaced by fresh medium, and standards of various dioxin analogues or extracts of soil samples were added to wells. Then, the both cells were used for test of EROD activity.

2) Primary culture of rat hepatocytes

Male SD rats (5weeks) were fed standard diet for one week. Animals were anesthetized by administration of sodium pentobarbital (50 mg/kg, i.p.). Hepatocytes were prepared by recirculing collgenase perfusion of the liver in situ according to the methods of Seglen[3]. Culture conditions and treatments described for Hep G2 and H4IIE cells.

3) EROD activity expressed by liver cells

Cells were washed with cold PBS (phosphate buffered saline, pH7.4), and placed for 1 hr, after the addition of 0.1% Triton X-100 in PBS. Then, the cell preparation solution was centrifuged at 4 °C. The supernatant was finally designified as enzyme solution for the test of EROD activity. Activity of 7-ethoxyresorufin O-deethylase (EROD) was assayed according to Bruke and Mayer method[4].

4) The *umu* test: mutagenic activity (SOS-inducing activity; ß-galactosidase activity) by *Salmonella typhimurium* TA1535/pSK 1002

The *umu* test was performed according to the methods of Oda et al.[5], using a tester strain of *S. typhimurium* TA1535/pSK 1002; it was determined that *umu* gene expression induced by the dioxin analogues and/or PAHs in the crude extracts of soil samples.

5)Levels of dioxin analogues and PAHs in soil samples by GC-MS analysis

Determination of dioxin analogues and PAHs were performed according to the methods of Ohta et al.[6] and the modification methods of US-EPA (1988), respectively. Finally, to compare the toxic level by PCDDs, PCDFs and Co-PCBs in analyzed soil samples, the values of 2,3,7,8-TCDD toxic equivalent quantity (TEQ) were calculated for PCDDs and PCDFs using international 2,3,7,8-TCDD Toxicity Equivalence Factors (I-TEFs)[7] and for Co-PCBs using TEFs[8]. Similarly, to compare the toxic level by PAHs, the values of B(a)P toxic equivalent quantity (B-TEQ) were calculated for 14 kinds of PAHs using B(a)P Toxicity Equivalence

Factors (B-TEFs) postulated by Nisbet and Lagoy[9].

Results and discussion

It is well known that metabolism of liver cancer cells are fairy different from that of normal liver cells. However, we considered to fully use liver cancer cells as toxic indicator for environmental samples if kinetic behavior expressed by liver cancer cells against toxic compounds is similar to that of normal liver cells. Fig. 1 shows the comparisons of EROD activity induced by primary cultures of rat hepatocytes, rat cancer cell line H4IIE and human cancer cell line HepG2 in the presence of 2,3,7,8-TCDD as high toxic compound (Fig. 1A) and 1,3,6,8-TCDD as low toxic compound (Fig. 1B). With increasing the concentrations, the EROD activities of three kind of liver cells was also increased. Further, when similar experiment was performed by additions of 2,3,7,8-TCDF and OCDF, the difference of their kinetic behavior among three liver cells was not observed. Based on the above results, we further studied the environmental risk assessment by using human cancer cell line HepG2 because of its high sensitivity for dioxin analogues. Actually, when it estimate the environmental risk or their TEQ concentration from the levels of EROD activity, inducing by the extract of various environmental samples, the big problem still remained; there are a lot of other environmental pollutants such as PAHs and agricultural chemicals in the environment, which can also highly induce EROD activity. Therefore, it needed to adopt another biological evaluation method, which indicate negative for dioxin analogues, but positive for PAHs or other chemicals. Thus, SOS-inducing activity by *Salmonella typhimurium* TA1535/pSK 1002 was used as the indicator of other pollutants except dioxin analogues.

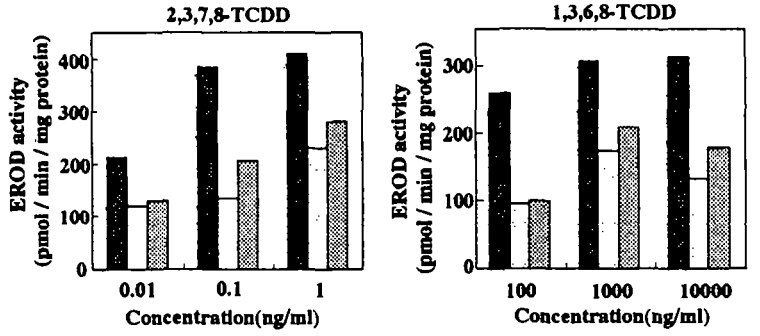

Fig.1 Comparisons of EROD activity by 2,3,7,8-TCDD and 1,3,6,8-TCDD in primary cultures of rat hepatocytes (□), rat cancer cell line H4E(▨) and human cancer cell line HepG2 (■)

Fig. 2 shows the comparisons of SOS-inducing activity (ß-galactosidase activity) in *S. typhimurium* induced by 36 kinds of mixtures of 2,3,7,8-TCDD and Benzo(a)pyrene (B(a)P). We tested for the mixture samples of 2,3,7,8-TCDD and Benzo(a)pyrene, represented as dioxin analogues and PAHs, respectively. As a results, ß-galactosidase activity induced by all samples was completely dependent on B(a)P concentration, in the presence or absence of TCDD. Fig. 3

shows the comparisons of EROD activity in HepG2 cells induced by 36 kinds of mixtures of 2,3,7,8-TCDD and B(a)P. In the presence of over 100 ng/ml of B(a)p or 100 pg/ml of TCDD, EROD activity was increased. Interestingly, it was observed not only additive effect but synergistic effect on EROD activity by the additions of the mixture of TCDD (1000～10000 pg/ml) and B(a)P(1000～10000 ng/ml). Finally, we tried to demonstrate the availability of environmental risk assessment based on two kind of bioassay; by using various soil samples; it was compared the estimated TCDD and B(a)P concentration judged from two bioassay (as shown in Fig 2 and Fig 3) with the true TCDD and B(a)P concentration calculated from GC-MS analysis. As a result, it was recognized that their concentrations in tested all samples was fairly correlated, and that these bioassay was not only qualitative but semi-quantitative.

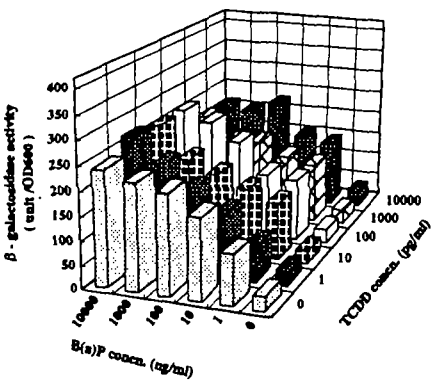

Fig. 2 Effects of β - galactosidase activity in *Salmonella typhimurium* TA1535/pSK1002 induced by 36 kind of mixtures of 2,3,7,8-TCDD and Benzo(a) pyrene

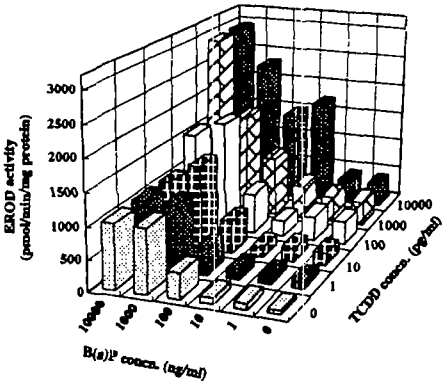

Fig. 3 Effects of EROD activity in HepG2 cells induced by 36 kind of mixtures of 2,3,7,8-TCDD and Benzo(a)pyrene

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