RISK ASSESSMENT OF CHLORINATED ORGANIC COMPOUNDS USING IMMORTALIZED RAT HEPATOCYTES AND TOXICOLOGICAL INFORMATION

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Abstract

Many types of chlorinated organic compounds are detected at low concentration levels in the environment. In this study, we used immortalized rat hepatocytes, which are simpler to handle than primary cultured cells, to investigate the cytotoxic and DNA synthesis effects of nine chlorinated organic compounds detected in industry wastewater and rivers:p-dichlorobenzene(pDCB), o-dichlorobenzene(o-DCB), p-chloroaniline(pCA), 3,4-dichloroaniline(DCA), tris (2-chloroethyl) phosphate(TCEF), triclosan(TS), triclocarban(TC), 2,5-dichlorophenol(pDCP), and 2,5-dichloroanisole(pDCA) , for extrapolation to assess the risk of effects on human. This study shows that the cytotoxicity of the nine compounds as determined by the use of 50% inhibitory concentration (IC_{50}) came in the order as follows: TC> TS> DCA > pDCP> pDCA > pDCB > pCA > oDCB =TCEF. IC_{50} for the cytotoxicity and non-observed adverse effect level (NOAEL) of the chlorinated organic compounds were found to be correlated. This indicates that cytotoxicity may be an effective indicator for estimating the NOAEL of a chemical substance.

Introduction

Chlorinated organic compounds are detected a large varied molecules at low concentration levels in the environment. It is therefore necessary to use an approach appropriate for the actual exposure level to assess the risk of their effects on human health. The immortalized hepatocytes used in this study are cultured cells that have been established as a cell line that maintains the characteristics of normal liver cells such as its albumin production ability.

There have been a variety of study reports on the toxicity assessment of chlorinated organic compounds using animal cell culture. The number of studies, however, is very small when it comes to ones that verify the extent to which the results of toxicity assessment obtained by using *in vitro* cultures can be extrapolated to the toxicity assessment of *in vivo* animals and humans. In this study, we analyzed the correlation between IC_{50}, the concentration level that triggers 50% cytotoxicity, and NOAEL (liver), the chronic toxicity index, *in vitro* experiment of the effects of chlorinated organic compounds on immortalized hepatocytes. Based on this analysis, we examined whether the findings of toxicity evaluation using cultured immortalized hepatocytes conformed to

the *in vivo* toxicity data collected from literature.

We investigated the cytotoxic and DNA synthesis inhibition of nine chlorinated organic compounds detected in industry wastewater and rivers (pDCB, oDCB, pCA, DCA, TCEF, TS, pDCA, and pDCP) for extrapolation to assess the risk of effects on humans[1].

Materials and Methods

Cell Culture : LEA-RA9 cells that were immortalized using adenovirus-V recombinant SV40 were used as the immortalized rat hepatocytes[2]. After the immortalized rat hepatocytes were seeded at 5 x 10^4 cells/well in 24 well plates containing 10% FBS Williams' E culture medium, they were cultured for 48 hours. The chlorinated organic compounds were added to the culture medium, after which, 18 hours later, it was replaced with Earle's culture solution containing 10% alamar Blue and cultured an additional 2 hours.

Cytotoxicity Test : The inhibition rate of living cells by chlorinated organic compounds was determined by the alamar blue assay (Biosource CA, USA) , which is performed with a fluorimetric method. The potency of each chlorinated organic compound was calculated by creating a concentration-viability curve for each compound and mixture and calculating the concentration of the substance that indicated IC_{50}(μM).

S-period DNA synthesis : Immortalized rat hepatocytes were seeded at 1x10^4 cells/well in 24 well plates containing 10% FBS Williams' E culture medium. After culturing 24 hours, chlorinated organic compounds were added in the manner of cytotoxicity test, and ^{3}H-thymidine was added. At this time, 24 hours after chlorinated compounds were added; the cells were treated with 5% TCA, and measured for the amount of ^{3}H-thymidine contained in the acid-insoluble fraction. The sample value was deemed as the uptake quantity of ^{3}H-thymidine in the S-period DNA synthesis

CYP1A1 protein content : In the same manner as cytotoxicity test, after immortalized rat hepatocytes were cultured for 48 hours at 5 x 10^5 cells in a 10cm culture dish, the culture medium was replaced with serum-free Williams' E culture medium, and the cell were cultured for 90 hours after the three chlorinated organic compounds, pCA, pDCB, TS, as well as 3-methylcholanthrene (3-MC), which is a CYP1A1 inducing agent, were added. Then it was solubilized with 20% glycerol/0.1M calcium PBS, treated in an ultracentrifugation, after which the sediments were collected as microsome fractions. The CYP1A1 contained in the microsome fractions were measured using the Amersham Cytochrome p450 ELISA system.

Statistical analysis using toxicological information: After the nine chlorinated organic compounds were studied for cytotoxicity, data on cytotoxicity regarding test animals were collected and organized. Data were obtained from RTECS (Registry of Toxic Effects of Chemical Substances) at Chembank (SilverPlatter), HSBN (Hazardous Substances Data Bank), IRIS (Integrated Risk Information System) and literature search on Medline.

Results and Discussion

The cytotoxicity of the nine compounds as determined by the use of IC_{50} came in the order as follows: TC> TS> DCA > pDCP> pDCA > pDCB > pCA > oDCB =TCEF. The mixture consisting of equal portions of the nine compounds had stronger cytotoxicity than DCA alone（Table1）. DNA synthesis, due to a process that

addresses the concentration level that triggers cell damage, was inhibited at 50% at 1/2 to 1/5 the concentration level of IC_{50}. DNA synthesis inhibition strength of the six compounds ranged in the order of TC>TS>pDCB>oDCB>pCA>TCEF, which was very similar to IC_{50} for the cytotoxicity（Table 1）.

In seven types of chlorinated organic compounds, a correlation (r = 0.947) was also found between the IC_{50} for the cytotoxicity of immortalized hepatocytes measured using aloma-blue coloring and NOAEL (liver), the chronic toxicity level at which cancer and liver damage are triggered（Fig.1）. From these, it is considered that the *in vitro* assessment of cell damage using cultured immortalized hepatocytes is effective in estimating the carcinogenic risk carried by chlorinated organic compounds, as well as in estimating the NOAEL for liver damage. A comparison of the cytotoxicity potency order with NOAEL, the chronic toxicity index for animals, revealed a high correlation when the conditions were corrected for equal test period and to match the cells' original organs with the target organs.

We investigated the CYP1A1 induction potency of pCA and pDCB, which have low unit risks, and TS, which is know to be mutagenic. When the cells were processed at a non-cytotoxicity inducing, low-concentration level, increase in the amount of CYP1A1 was observed. pCA, pDBC and TS were found to induce 1.5 to 2 times the CYP1A1 as the control within immortalized rat hepatocytes. In particular, TS induced CYP1A1 2.1 times more than the control. 3-MC, a substance known to develop non-specific hepatotoxicity, induced CYP1A1 2.2 times more than the control, thereby inducing a similar amount of CYP1A1 as TS.

In this study, immortalized hepatocytes were used as the culturing system. Immortalized hepatocytes are being used in many fields today due to their ease in subculturing and to their normal cell characteristics being maintained relatively better than those derived from cancer cells. As such, immortalized hepatocytes were considered to be an effective culturing system in studying the effects of environmental samples on humans in which a number of specimens had to be measured.

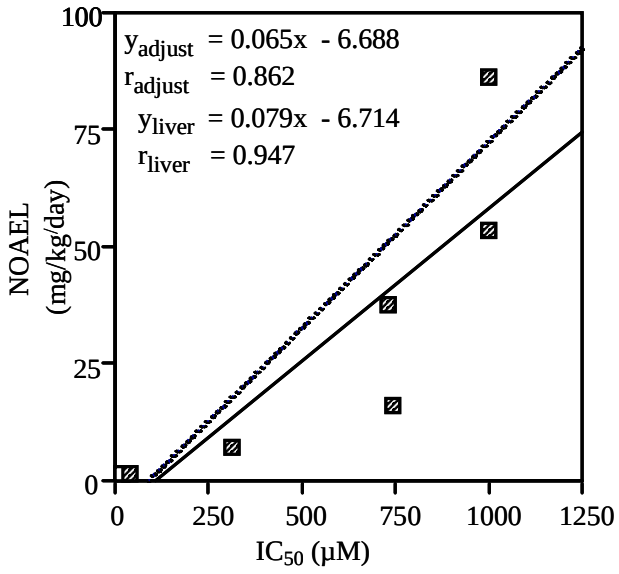

Fig. 1 Relationship between NOAELadjust (–) or NOAELliver (– –) and IC50 among chlorinated organic compounds.
NOAELadjust was adjusted for study duration.
NOAELliver was selected from NOAEL on the liver injury.

Table 1 Cytotoxicity of chlorinated organic compounds in immortalized rat hepatocyte

Chlorinated organic Compounds (abbreviation, CAS No.)	IC50(μM) for the Cytotoxicity	50% of inhibitory concentration for the DNA synthesis
p-chloroaniline (pCA, 106-47-8)	740	300
3,4-dichloroaniline (DCA, 95-76-1)	310	–
p-dichlorobenzene (pDCB, 106-46-7)	730	230
o-Dichlorobenzene (oDCB, 95-50-1)	>1000	220
Tris(2-chloroethyl) phosphate (TCEF, 115-96-8)	>1000	860
Tri chlosan (TS,3380-34-5)	39	27
Triclocalban (TC,101-20-2)	16	7
2,5-Diclorophenol (pDCP, 583-78-8)	390	–
2,5-Dichloroanisole (pDCA,1984-58-3)	600	–
Compounds mixture	82	–

Table 2 Effects of chlorinated organic compounds on CYP1A1 contents in immortalized rat hepatocyte

Control	3-MC (5×10^{-6}M)	pCA (1×10^{-4}M)	pDCB (1×10^{-4}M)	TS (1×10^{-5}M)
O.28±0.02	0.62±0.06	0.45±0.02	0.43±0.00	0.60±0.03

(n=2 , μg / mg microsomal prptein)

Acknowledgements

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