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研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

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研究テーマ　環境化学物質における遺伝毒性の解析

2．本年度の研究業績

（1）　学会・研究会等においての口頭発表　有・(無)　（学会名・内容）

なし

（2）　学会誌等に発表した論文 (有)・無　（雑誌名・論文名）

雑誌名：Environmental　Health　Perspective

論文名：Genotoxic effects of α-endosulfan and β-endosulfan on human Hep G2 cells

3. 今後の研究計画

1） エンドスルファンのα異性体とβ異性体の遺伝毒性のメカニズムを解明する。

2） エンドスルファンは内分泌攪乱物質であるが、そのα異性体とβ異性体の内分泌攪乱作用を比較する。

3） catechin は抗酸化また抗変異原物質であるが、その予防的な作用を解明する。

4. 研究指導者の意見

エンドスルファンは有機塩素類農薬である。エンドスルファンのα異性体とβ異性体の遺伝毒性に関する報告は見られなかった。この研究は姉妹染色分体交換、小核及び SCG の三つのスタンダードな方法で初めてエンドスルファンのα異性体とβ異性体の遺伝毒性及び相違点を in vitro で解明して、非常に有意義な知見をもたらした。今後、この二つの異性体の遺伝毒性、内分泌攪乱作用等のメカニズムの解明、さらに、予防医学への応用などの研究の発展が期待される。

研究指導者氏名 森本兼曩 印

5. 研究報告

別紙形式を参考に、報告本文 4000 字以上で報告して下さい（枚数自由・ワープロ使用）タイトル・要旨等は日本語で、KEY WORDS 以下は日本語或いは英語で記入して下さい。研究成果の発表予定がある場合は原稿・抄録集等を添付して下さい。論文発表に当っては、日中医学協会―日本財団補助金による旨を明記して下さい。

α—エンドスルファンとβ—エンドスルファンのHep G2細胞における遺伝毒性

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論文要旨

「目的及び方法」エンドスルファン（endosulfan）は世界範囲でよく使われ、大きな毒性を持つ有機塩素類農薬である。エンドスルファンの遺伝毒性に関する報告は少ない一方、ほとんど異性体混合物の研究である。エンドスルファンの二つの異性体—α-エンドスルファン（α-endosulfan）とβ-エンドスルファン（β-endosulfan）のそれぞれの遺伝毒性について、今まで報告はなっかた。この二つの異性体の遺伝毒性を解明するため、ヒト肝癌細胞であるHep G2 細胞を使って、姉妹染色体交換 (sister chromatid exchanges, SCE)、小核（micronuclei, MN）及び単細胞電気泳動（single cell gel electrophoresis, SCG; 或いはComet assay）三つの方法で 1×10^{-12} M～1×10^{-3} M までの濃度範囲でα-endosulfan とβ-endosulfanの遺伝毒性を検討した。「結果」β-endosulfanは48時間Hep G2 細胞を暴露した後、SCE（1×10^{-7} M～1×10^{-5} M）及び小核（5×10^{-5} M～1×10^{-3} M）の頻度は有意に増加した。しかし、同じの実験条件で、α-endosulfanはSCE頻度に影響は見られなっかた。小核の頻度に影響はあったが、有意差はなかった。二つの異性体をHep G2 細胞をそれぞれに1時間で暴露した後、SCG法でDNAの損傷を測定した結果、β-endosulfanは 1×10^{-3} Mの濃度でDNAを有意に損傷したが、α-endosulfanは 2×10^{-4} Mの濃度でDNAに有意に損傷した。「総括」以上の結果から見ると、α-endosulfan及びβ-endosulfanはHep G2 細胞に対する遺伝毒性を持っているが、遺伝毒性の特徴と作用濃度は大きく違う一方、α-endosulfanより、β-endosulfanのほうがエンドスルファンの遺伝毒性に大きな影響を占めることを示唆された。

Keywords:

α-endosulfan; β-endosulfan; Hep G2; Sister chromatid exchanges (SCE); Micronuclei (MN); Single-cell gel electrophoresis (SCG); Genotoxicity

1. 目的：

Endosulfan is a synthetic chlorinated cyclodiene, which has been proved to be an environmental endocrine disrupter { ADDIN ENRfu }. It was introduced into the earth's environment in 1956 as a general use insecticide, being primarily used to protect food crops such as tea, fruits, vegetables, and grains as well as wood from a wide

two isomers— α -endosulfan and β -endosulfan.

Endosulfan is toxic not only to insects, but also to fishes, animals and humans (2, 3). Autopsy examinations have revealed its damage to liver, lung and brain (3). However, the data regarding its genotoxicity (4), especially that of its two isomers, is very limited. Perhaps because its carcinogenicity and genotoxicity have not been confirmed, endosulfan is still widely used and continues to pollute the human environment not only in the developing countries but in the developed countries as well (5).

In this study, we observed the influence of α - endosulfan and β -endosulfan on the frequency of SCE, MN and the DNA damage assessed by SCG in Hep G2, a target cell line that expresses estrogen receptors (6) and is able to metabolize xenobiotics like S9 in vitro or in vivo (7).

2. 方法

2.1. Materials

α -endosulfan ([α -1,4,5,6,7,7-Hexachlorbicyclo-[2.2.1]-5-hepten-2,3-bis (methylen) sulfit] $C_9H_6Cl_6O_3S$) was from Wako Pure Chemical Industries, Japan. β -endosulfan ([β -1,4,5,6,7,7-Hexachlorbicyclo-[2.2.1]-5-hepten-2,3-bis (methylen) sulfit] $C_9H_6Cl_6O_3S$) was from Riedel-deHaen, Germany. For use, they were dissolved in dimethyl sulfoxide (DMSO, E. Merck, Darmstadt, Germany) to prepare a 0.5 M stock solution.

2.2. Cell culture

Hep G2 is from the cell bank of the Institute of Physical and Chemical Research of Japan and was cultured in Dulbecco's Modified Eagle's Medium (Sigma) containing 10% fetal bovine serum (Biosciences PTY Ltd., Australia) and 1% penicillin-streptomycin (Life Technologies™). Before use, Hep G2 cells were taken from fluid nitrogen and washed two times with fresh culture medium. After culturing for 2 passages in 37℃ and 5% CO_2, Hep G2 cells were prepared for use in the experiments.

2.3. Sister Chromatid Exchanges (SCE) and Micronuclei (MN)

2×10^5 Hep G2 cells were divided into 5 ml of culture medium and cultured for 48 h. After discarding 2.5 ml of supernatant, 2.5 ml of new culture medium containing different concentrations of either α -endosulfan or β -endosulfan were added for both the SCE and MN assays. 5-Bromo-2'-deoxy uridine (Sigma) was added in the SCE test (final concentration 40 μ M). The cells were cultured for another 48 h. Six hours before collection, colcemid (Sigma) was added to the SCE assay (final concentration 2×10^{-7} M). Vehicle controls were 0.2% DMSO. Mitomycin C (Sigma) was used as a positive control.

After 48 h of treatment in both SCE and MN assays, the Hep G2 cells were collected with 0.25% trypsin-EDTA (Life Technologies^{TM}). The cells were then treated with 0.075 M KCl for 10 min and fixed with Carnoy's solution (methanol:acetic acid = 3:1, v/v) for 30 min. The cells were washed twice with the Carnoy's solution and stored in methanol containing 1% (v/v) acetic acid.

For the measurement of SCE, the slides were stained using the florescence-plus-Giemsa (FPG) differential staining method. SCE in 30 cells was blindly evaluated per concentration for each experiment. The final result was the combination of two independent experiments (n=60 metaphases). For the measurement of MN, slides were stained with acridine orange (40 micrograms/ml, Sigma) solution, and the frequency of the micronucleated cells per 1000 Hep G2 cells was taken as the endpoint.

The judgement of MN: we judged the micronuclei according to the following criteria: 1) size: ϕ 1/10-1/2 of the main nucleus size. 2) number: $\leqq$ 3 MN/cell. 3) only one main nucleus in the cells counted. 4) the micronuclei must be round and have a clear boundary. 5) the micronuclei must have the same color and staining degree as the main nucleus. 6) the micronuclei must be clearly separated from the main nucleus. 7) the micronuclei must be in the same cell plasma with the main nucleus.

To assess the effects of α-endosulfan and β-endosulfan on the cell cycle kinetics of the cells, we scored the proliferation index (PI) on the same slides used for counting SCE. Cell cycle kinetics were evaluated by the proportion of the first (X_1), second (X_2), and third (X_3) division cells in 100 consecutive metaphases for each independent experiment. The proliferation index (PI) was calculated according to the following formula: $PI = (1 \times X_1 + 2 \times X_2 + 3 \times X_3)$.

2.4. Single-Cell Gel Electrophoresis (SCG) (8)

2×10^5 Hep G2 cells were divided into 5 ml of culture medium and cultured for 48 h. After 2.5 ml supernatant was discarded, 2.5 ml of a new culture medium containing different concentrations of either α-endosulfan or β-endosulfan was added. The vehicle control was 2% DMSO. After 1 h of treatment, the cells were collected with 0.25% trypsin-EDTA and adjusted to 1×10^6 cells/ml cell suspension after thorough mixing. 25 μl of the cell suspension was taken and mixed with 75 μl of 0.75% low-melting agarose (Nusieve GTG, FMC BioProducts) and then placed on pre-cleaned frosted micro slides (Matsunami Glass Ind., LTD, Japan) which were first covered with 80 μl of 0.5% normal-melting agarose (Sigma) (to make this layer adhere better to the slide, we used 20 μl of 0.5% normal melting agarose to cover the slide and dried it beforehand). The mixed-cell suspension were immediately covered with a coverglass,

and the slides were then kept at 4℃ for 10 min to allow solidification of the agarose. After gently removing the coverglass, the slides were covered with a third layer of low-melting agarose using a coverglass, and then kept at 4℃ for another 10 min to allow solidification of the agarose. After gently removing the coverglass, the slides were immersed in a lysing solution for 1 h and then moved to the electrophoretic buffer to allow 20 min for the unwinding of DNA strands as described by Singh et al. (8). The electrophoresis time was 20 min under 25 V and 300 mA using an electrophoresis compact power supply (ATTO Corporation, Japan). After staining with 20 μg/ml ethidium bromide (Sigma), DNA strand breaks were measured under a fluorescent microscope using a DNA SCG test system (Keio Electronic Ind., Co., Ltd, Japan). All the slides were examined 5 h after staining, and only the cells in the central part of the slides were detected. In the present study, the cell tail length was used to represent the degree of DNA damage to the Hep G2 cells.

2.5. Statistics: Dunnett's test in SPSS statistical software was used for the SCE and SCG assays. Chi-square test in SPSS statistical software was used for the MN assay. For all three assays, the results of two independent experiments were combined for the analysis.

3. 結果

3.1. Influence of α-endosulfan and β-endosulfan on frequency of sister chromatid exchanges (SCE) in Hep G2 cells

In this study, we treated Hep G2 cells with concentrations of α-endosulfan and β-endosulfan ranging from 1×10^{-12} M through 1×10^{-5} M for 48 h. Because Hep G2 is an aneuploid cell line (modal number = 55 chromosomes; range = 50-60 chromosomes), we calculated SCE per chromosome. As shown in Table 1, β-endosulfan caused a significant increase in SCE from 1×10^{-7} M through 1×10^{-5} M. In contrast, α-endosulfan failed to show any significant effect. SCE frequency was about 0.19/chromosome in 0.2% DMSO (vehicle control) against 0.27/chromosome in the highest concentration (1×10^{-5} M) of β-endosulfan. Positive controls treated with 1×10^{-7} M of mitomycin C resulted in 0.484/chromosome ($P < 0.01$).

In these experiments, the addition of α-endosulfan and β-endosulfan did not cause any significant change in the proliferation index compared to the cultures with the control of 0.25% DMSO.

3.2. Influence of α-endosulfan and β-endosulfan on frequency of MN in Hep G2 cells

In the present study, we used the frequency of micronucleated Hep G2 cells to represent the effects of α-endosulfan and β-endosulfan on MN induction in the Hep

G2 cells. As shown in Table 2, after treating Hep G2 cells for 48 h with β-endosulfan from 5×10^{-5} M through 1×10^{-3} M, the frequency of micronucleated cells was significantly increased; the frequency at 1×10^{-3} M was about 6 times that of the control. Although we failed to find any significant increase of MN in Hep G2 cells treated with α-endosulfan, slight increases in micronucleated cells were observed at higher concentrations ($5 \times 10^{-5} \sim 1 \times 10^{-3}$ M). In the present study, mitomycin C (1×10^{-6} M) showed a much stronger potency to induce MN (116 micronucleated cells in 2000 cells) than β-endosulfan ($P < 0.01$).

3.3. Influence of α-endosulfan and β-endosulfan on induction of DNA strand breaks as evaluated by SCG assay in Hep G2 cells

In the present study, we measured the tail length of Hep G2 cells treated with different concentrations of α-endosulfan and β-endosulfan. As shown in Table 3, after 1 h of treatment, α-endosulfan induced significant increases in DNA strand breaks from 2×10^{-4} M through 1×10^{-3} M, as did β-endosulfan at 1×10^{-3} M.

4. 考察

Endosulfan is an insecticide with estrogenic activity which is toxic to many fishes and mammals. Some reports suggested that it could accumulate in aquatic animals (9) and cause human fatalities (3). The genotoxicity of its two isomers, however, has not been confirmed.

To study the genotoxicity of endosulfan, we used Hep G2 cells in the present study; firstly because endosulfan is hepatoxic (3), and secondly because the metabolic property of human Hep G2 cells will offer a chance to examine the effects of its metabolites on those cells. The result will thus be more comparable to findings in an in vivo study. Finally, because the Hep G2 cell line is well reported in the genotoxic studies using SCE, MN and SCG assays (10, 11), we believe using, such cells to examine the genotoxicity of endosulfan is reasonable.

Our repeated in vitro experiments showed that both α-endosulfan and β-endosulfan induced DNA strand breaks as detected by SCG assay. Nevertheless, Hep G2 cells seem more sensitive to α-endosulfan than to β-endosulfan as shown by the cell tail length. As we used a dry-layer gel technique on the slides, the three layers of gel were easily prepared. Using trypsin-EDTA to collect the Hep G2 cells kept the individual cells well separated and evenly distributed in the second layer of gel.

For the SCE and MN assays, only β-endosulfan showed significant effects on the cell line, and our present results correspond to the increased frequency of SCE in blood lymphocytes from workers using pesticides including endosulfan (12) and to the increased SCE induced by endosulfan (1×10^{-6} M, mixture of α-endosulfan and β

-endosulfan) in human lymphoid cells (13). The result of MN induced by β -endosulfan is also in agreement with an in vivo study of endosulfan (14).

In our present study, all three endpoints were from two independent experiments, and the results were satisfactorily repeated. Although both α -endosulfan and β -endosulfan showed genotoxicity to Hep G2 cells at different concentrations by different endpoints, neither of them showed any apparent effects on cell cycle kinetics in the cell line.

Of the three biomarkers used in this study, SCE and MN was found to be more sensitive in detecting the genotoxicity of β -endosulfan, but SCG was less sensitive. For α -endosulfan, only SCG showed genotoxicities. The concentration of α -endosulfan needed to induce DNA strand breaks was apparently lower than that of β -endosulfan. Thus, our result suggests that β -endosulfan more readily induced SCE and MN, while α -endosulfan more easily induced DNA strand breaks as detected by SCG in the Hep G2 cells.

We have little data regarding the mechanisms of the genotoxicity of α -endosulfan and β -endosulfan. Clastogenic activity of the two compounds and /or their metabolites may exist, although only sparse data are available so far (15, 16). In addition, as spindle poisoning is reported to relate to MN formations such as bisphenol and diethylstilbestrol (17), whether β -endosulfan actually induced MN through spindle poisoning should also be examined in future studies.

Another possible target of research might be the estrogenic effects of α -endosulfan and β -endosulfan (18), given the existence of estrogen receptors in Hep G2 cells (19). Many environmental estrogenic disrupters, such as diethylstilbestrol (DES), are reported to induce SCE either in vitro or in vivo, and it seems that their effects tend to be restricted to cells with abundant estrogen receptors (20, 21). Endosulfan (a mixture of α -endosulfan and β -endosulfan) is able to combine with estrogen receptors and exert biological effects (22), and although we have no direct evidence, it is difficult to exclude the possibility that α -endosulfan and β -endosulfan bind with the estrogen receptors in Hep G2 cells (19, 23) to induce genotoxicity in these cells.

As α -endosulfan and β -endosulfan can be metabolized by Hep G2 cells, the genotoxicitiy we found in the cell line may include their metabolites. Further research is needed to determine whether α -endosulfan and β -endosulfan or their metabolites are responsible for the observed genotoxicity.

Our findings are based on a study using a human hepatoma Hep G2 cell line so as to extrapolate the results to humans. However, further studies with normal human cells

and human subjects exposed to the same agents are needed.

In conclusion, our present study has shown for the first time that both α-endosulfan and β-endosulfan are genotoxic to Hep G2 cells, and that the genotoxicity of β-endosulfan is more potent than that of α-endosulfan. Although the underlying mechanism is still beyond our knowledge, the clastogenicity and estrogenicity of the two isomers suggest the need for further studies.

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附表

Table 1. The effects of α-endosulfan and β-endosulfanon on the frequency of sister chromatid exchanges (SCE) in Hep G2 cells

Concentrations (M)	n	α-endosulfan		β-endosulfan	
		SCE[a]	PI	SCE[a]	PI
Control	60	0.206±0.062	1.99	0.191±0.085	2.02
1×10^{-12}	60	0.203±0.078	2.00	0.200±0.047	2.08
1×10^{-11}	60	0.216±0.068	2.00	0.204±0.058	2.01
1×10^{-10}	60	0.204±0.071	2.04	0.199±0.113	1.93
1×10^{-9}	60	0.204±0.076	2.00	0.196±0.075	1.99
1×10^{-8}	60	0.206±0.060	2.02	0.220±0.070	2.01
1×10^{-7}	60	0.197±0.065	2.02	0.248±0.080*	2.02
1×10^{-6}	60	0.205±0.053	1.91	0.252±0.072**	1.97
1×10^{-5}	60	0.217±0.059	2.00	0.272±0.065**	1.93

Abbreviations: PI, proliferation index; SCE, sister chromatid exchanges

[a] Data are presented as means±standard deviations.

*Statistically significant difference when compared to control (Dunnett's test, $P < 0.05$).

**Statistically significant difference when compared to control (Dunnett's test, $P < 0.01$).

Table 2. Effects of α-endosulfan and β-endosulfanon on the induction of micronuclei (MN) in Hep G2 cells

Concentration (M)	n	Micronuclei[a]	
		α-endosulfan	β - endosulfan
Control	2000	20	13
1×10^{-7}	2000	18	14
1×10^{-6}	2000	17	13
5×10^{-6}	2000	14	17
1×10^{-5}	2000	16	22
5×10^{-5}	2000	26	39**
1×10^{-4}	2000	26	51**
5×10^{-4}	2000	26	74**
1×10^{-3}	2000	30	82**

[a] Data represent the number of Hep G2 cells with at least one micronucleus.

** Statistically significant difference when compared to control (chi-squire test, $P < 0.01$).

Table 3. The effect of α-endosulfan and β-endosulfanon on DNA strand breaks as detected by single-cell gel electrophoresis (SCG) assay in Hep G2 cells

Concentration (M)	α - endosulfan		β - endosulfan	
	n	Tail length (μm)[a]	n	Tail length (μm)[a]
Control	116	49.28±16.57	123	40.26± 8.26
2×10^{-5}	119	54.73±18.08	99	42.36± 7.31
1×10^{-4}	110	53.37±16.73	99	39.64± 7.34
2×10^{-4}	128	58.32±16.08**	108	43.68± 9.58
5×10^{-4}	118	60.13±15.86**	90	44.33±11.33
1×10^{-3}	108	63.56±32.73**	126	50.27±13.38**

[a]Data are presented as means±standard deviations.

** Statistically significant difference when compared to control (Dunnett's test, $P < 0.01$).