CYTOTOXICITY OF CHLOROPEPTIDE IN ISOLATED HEPATOCYTES AND ITS INACTIVATION BY MICROSOMES¹⁾

Yoshio UENO²⁾, Teruo ITO, Hiroko YOSHIZAWA-NAKAKI,
Yoshiaki OMATA and Kohko ICHIMAN

Department of Toxicology and Microbial Chemistry,
Faculty of Pharmaceutical Sciences, Tokyo University of
Science, Ichigaya, Tokyo 162, Japan

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Abstract.....Chloropeptide, a hepatotoxic mycotoxin of *Penicillium islandicum* Sopp, induces gross changes in cell morphology including blebbing and ballooning of the cytoplasmic membrane in the isolated hepatocytes, and this hepatotoxic potential of chloropeptide is abolished by microsomal cytochrome P-450 system.

Key words: chlorine-containing peptide (chloropeptide or cyclochlorotine), hepatotoxin, mycotoxin, hepatocytes, blebbing, cytochrome P-450

INTRODUCTION

Penicillium islandicum Sopp, one of the causal fungi of "Yellow Rice Toxicosis" encountered in Japan just after the War II, produces many toxic secondary metabolites in the mycelia as well as in the culture filtrate. Uraguchi et al. (1961) isolated from the fungal mat a yellow bis-anthraquinone, named luteoskyrin. This compound is hepatotoxic and induces cirrhosis and hepatocellular carcinoma in mice (Uraguchi et al., 1972; Ueno, I. et al., 1973). Tatsuno et al. (1955) isolated from the culture filtrate a cyclic pentapeptide containing two chlorine atoms which they originally named "chlorine-containing

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2) To whom communications should be addressed.

Abbreviations: CP, chloropeptide (chlorine-containing peptide or cyclochlorotine); MS, microsomes; PCB, polychlorobiphenyl; PB, phenobarbital; MC, 3-methylcholanthrene; SGOT, serum glutamic-oxaloacetic transaminase; DMSO, dimethylsulfoxide.

連絡先: 〒162 新宿区市ヶ谷船河町12 東京理科大学 薬学部 毒性・微生物化学教室 上野 芳夫

peptide" (Abbreviated as chloropeptide or CP) (Uraguchi et al., 1972). This toxic peptide is composed of α -amino-n-butyric acid, L- β -amino- β -phenylpropionic acid, L-serine and proline substituted with two chlorine atoms in a molar ratio of 1 : 1 : 2 : 1. Synthetic and X-ray studies elucidated its structure as presented in Fig. 1. The conformation of the chlorine atoms is of *gauche* type (Yoshioka et al., 1973).

During the past ten years, we have investigated the toxicological and biochemical properties of CP. CP is toxic to several experimental animals such as the mouse, rat, guinea-pig and other species (Ueno et al., 1977a) ; it exhibits a high affinity for the liver (Ueno et al., 1977b) ; it alters hepatic drug and glycogen metabolism (Ueno et al., 1978) ; and it accelerates leakage of some hepatocellular enzymes into serum in intoxicated animals (Ito et al., 1981). These data suggest that the high affinity to the hepatic cell is responsible for the severe functional damages.

With an aim to clarify the mechanism of its hepatotoxic nature, we investigated the cytotoxic effect of CP on the isolated hepatocyte *in vitro*. Enzymatic detoxication of CP was also examined by employing microsomal preparations of liver of rats pretreated with various inducers of drug metabolizing enzymes.

Fig. 1. Chemical struture of chloropeptide.

MATERIALS AND METHODS

Chloropeptide: CP was isolated from the culture filtrate of *P. islandicum* Sopp according to the method previously reported (Ishii and Ueno, 1973) and recrystallized from hot methanol before used. The purity (over 98%) was estimated by melting point and UV absorption spectrum. Small aliquots of CP dissolved in distilled water were added brown test tubes (0.5 ml capacity) and the solution was freeze-evaporated dried under reduced pressure. The test tubes thus obtained, each containing 5, 10 or 100 μg of CP, were stored at -20°C until used. CP was dissolved in 0.9% NaCl or the cell suspension medium immediately before used.

Dehalogenated CP was prepared as follows : 5 mg of CP was dissolved in 5 ml of 2N NH_4OH -methanol, and the solution was kept at 37°C overnight. After evaporation of the solvent under reduced pressure at 50°C, the residue was used as the dechloropeptide. The purity and amount were estimated by UV spectrometry (Ishikawa et al., 1970).

Preparation of hepatocytes: Hepatocytes were prepared from nonfasted male Sprague-Dawley rats (250–300 g body weight) by the method of Seglen (1973) with modifications as follows. The liver was first perfused with the Ca^{++} -free perfusion medium *in situ* through the portal vein and then with the collagenase buffer (100 ml, collagenase 220 U/ml, type 1, Sigma). The latter solution was recirculated through the liver for 20 min at 37°C at a flow rate of 15 ml/min. The liver was isolated, weighed, and transferred to a plastic vessel. The cells were liberated from the connective tissues in 75 ml of the suspension buffer, and the cell suspension obtained was filtrated over two layers of gauze and incubated at 37°C for 30 min with occasional shaking. The temperature of the cell suspension was slowly lowered in ice-cold water, filtrated over two layers of gauze and a nylon filter (92 μm mesh), and centrifuged at 200 rev/min for 5 min in the cold room (2–4°C). The precipitated cells were suspended with 40 ml of an ice-cold washing buffer and rinsed three more times. Finally, the cells were suspended in 20–30 ml of the suspension buffer, filtered over a nylon filter (62 μm mesh), and the number of cells was counted in a hemocytometer. The number of cells was adjusted to $1\text{--}4 \times 10^6/\text{ml}$ by addition of the suspension buffer. The cell suspension thus obtained was kept in an ice-water bath until used. A yield of $1\text{--}2 \times 10^8$ cells per liver with a 80–90% viability was generally obtained.

Cell viability was assayed by trypan blue exclusion. Trypan blue (0.6% in 0.48% NaCl solution, Gibco; filtered over a 0.22 μm Millipore filter) was added to the cell suspension in 1 : 1 ratio (v/v). Within 10 min the number of cells that excluded the dye was counted, using a 10-mm² eyepiece grid in an inverted microscope at $\times 400$ magnification. Cell suspensions with a cell viability over 80% were used for the experiments. When the cells were kept at 0–4°C, the isolated hepatocytes are usable for experiments for at least 5 hr after the isolation.

Assay of cytotoxicity: A cell suspension ($1\text{--}4 \times 10^6/\text{ml}$, 0.9 ml) was transferred to a plastic tube (1.5 by 10 cm) with a plastic pipette, and 0.1 ml of CP dissolved in the cell suspension medium or 0.1 ml of the enzyme solution described below was added, and the mixture was incubated at 37°C for the desired times under an atmosphere of 95% O_2 and 5% CO_2 with shaking (100 cycle/min). The cytotoxicity of CP was estimated either from the decrease of cell viability or from morphological changes, especially bleb formation, as noted below.

Preparation of microsomes and cytosol: Male Sprague-Dawley rats (6 week-old) were either untreated or injected with PB, MC or PCB (Kanechlore 500) as follows (Yoshizawa et al., 1981): MC dissolved in olive oil or PB dissolved in water was injected intraperitoneally (25 mg/kg or 75 mg/kg, respectively) for 4 consecutive days in the morning, and the rats were killed on the morning of the fifth day. PCB was dissolved in olive oil and injected intraperitoneally (500 mg/kg) and the rats were killed on the sixth morning. The rats were fasted overnight before they were killed.

The livers were perfused with an ice-cold 1.15% KCl solution. The perfused livers were weighed, minced and homogenated in three up-and-down strokes in 4 vol. of 1.15% KCl in a Teflon homogenizer. The homogenate was centrifuged at $9,000 \times g$ for 15 min.

The resulting $9,000\times g$ supernatant fluid is referred to as S-9. The S-9 was further centrifuged at $100,000\times g$ for 60 min, and the resulting microsomes were suspended in 1.15% KCl, and the suspension was centrifuged as above. The pellet was suspended in distilled water and stored at -80°C . The $100,000\times g$ supernatant fraction was also used for experiments as cytosol. Protein was determined by the method of Lowry et al. (1951).

Enzyme treatments of chloropeptide: The reaction mixture contained, in a final volume of 1.0 ml, 120 mM potassium phosphate buffer (pH 7.4), 10 mM MgCl_2 , 0.6 mM NADP, 20 mM G-6-P, 0.2 unit/ml G-6-P dehydrogenase, $5.0\text{ }\mu\text{g/ml}$ CP, and microsomes or cytosol. The mixture was incubated for 1 hr at 37°C under shaking, and then cooled in ice-cold water. A portion (0.1 ml, $0.5\text{ }\mu\text{g}$ CP) of the reaction mixture was assayed for the formation of blebs as described above. The inhibitors of the monooxygenase system such as SKF 525A and metyrapone were dissolved in DMSO, and added to the above reaction mixture in a final concentration of 0.1 mM. The final concentration of DMSO was 1%. Preliminary experiments revealed neither cytotoxicity nor bleb formation by this concentration of DMSO.

Assay of serum transaminase activity: SGOT activity was estimated with S. TA-test (Wako Pure Chemical Industries, Ltd., Osaka).

Scanning electron microscopy: Scanning electron microscopy was performed in a JSM T20 microscope. Samples were fixed in 2% glutaraldehyde, treated with 2% osmium tetroxide, and dehydrated in ethanol. Portions of the plastic surface were dried by the critical point drying technique, mounted on studs and then coated with gold.

RESULTS

Effects of chloropeptide on the cell viability

The hepatocytes were incubated at 0°C or 37°C in the presence or absence of CP for 60 and 120 min, and the cell viability was counted to give the results shown in Fig. 2.

Fig. 2. Effect of chloropeptide on the viability of hepatocytes.

Each point in the figure represented an average of three separate experiments, in which about 200 cells were counted for each experiment. When the cells were incubated at 0°C, no significant decrease in the cell viability was observed either in the presence or absence of 100 µg/ml of CP. When the cells were incubated at 37°C, the initial viability of 86% decreased to 80 and 76% after 60 and 120 min of incubation, respectively, in the absence of CP, and this decrease was accelerated by the addition of CP: the viabilities in the presence of 10 and 100 µg/ml of CP were 78 and 76% at 60 min and 67 and 55% at 120 min, respectively. These data indicated that CP did not exhibit cytotoxicity when the cells were incubated at a low temperature, and the toxin caused a decrease in cell viability only when the cells were exposed for a rather high dose (100 µg/ml) of CP.

Bleb formation on the cytoplasmic membranes

During the course of observation of the cell, an alteration of the outer membrane of the hepatocytes and a subsequent aggregation into cell clusters were observed under the microscope. When the hepatocytes were incubated with a low concentration (1–2 µg/ml) of CP, occasional blebs of the cytoplasmic membrane increased in size and number upon prolonged incubation with CP.

The morphological changes are shown in Photo 1. In the control cells, such small bleb formation was not observed or was very rare (below 10%); no blebs were observed when the cells were incubated with CP at 0°C. The cells with blebs did not stained with trypan blue and therefore were counted as positive in the cell viability test. However, they formed a cluster when incubated for longer times (2 hr or more).

In order to observe the morphological changes in more detail, the cell surface of the hepatocytes was observed under a scanning electron microscope. As shown in Photo 2, the control cells exhibit regularly arranged microvilli on the cell membrane. However, the hepatocytes which were exposed to a low concentration of CP (0.1–2 µg/ml) possessed many blebs on the surface, which could be likened to “balloons”.

Dose- and time-dependent formation of blebs is summarized in Fig. 3. The formation of blebs was proportional to the time of treatment of the cells with CP, and the percentage of the hepatocytes with blebs or balloons increased to 35, 65 and 100% in the

Photo 1. Light microscopic pictures from normal and poisoned hepatocytes.

(A) Control: normal rat hepatocytes (×400)

(B) Isolated hepatocytes 60 min after poisoning with 0.5 µg/ml of chloropeptide (×400)

Photo 2. Scanning electron micrographs from normal and poisoned hepatocytes.
 (A) Control: normal rat hepatocyte
 (B) Isolated hepatocyte 60 min after poisoning with 0.5 µg/ml of chloropeptide

Fig. 3. Dose and time-dependent blebbing of the hepatocytes.

presence of 0.1, 0.2 and 0.5 µg/ml of CP, respectively, when estimated 90 min after incubation.

On the other hand, no bleb formation was observed with the dehalogenated peptide even at a concentration of 100 µg/ml (data not presented).

Inactivation of chloropeptide by microsomes

With an aim to determine whether CP directly attacked the hepatocytes or some active metabolite(s) induced the alteration of cell membranes mentioned above, we investigated the toxicity of CP after incubation with the drug-metabolizing enzyme system of the liver of rats, which had been either untreated or treated with inducers such as MC, PB and PCB.

Preliminary experiments with S-9 of the livers of PCB-treated rats revealed that 0.5 µg/ml of CP caused the blebbing in all hepatocytes when incubated for 60 min but no

blebs were detected when CP was preincubated with the S-9 and the NADPH-generating system (data not presented). Further experiments with microsomes and cytosol demonstrated that the microsomes derived from the livers of PCB-treated rats weakened the potency of CP to form blebs in proportion to the amount of microsomal protein added (Table 1). The microsomes from PB-pretreated rats were also effective for the inactivation of CP. On the other hand, the microsomes derived from MC-treated rats were less effective, and the microsomes derived from untreated rats and the cytosol fraction did not inactivate the cytotoxicity of CP. This inactivation process was an NADPH-dependent enzyme reaction, since heat-denaturation of the microsomes or depletion of NADPH from the reaction mixture allowed the blebbing. It was interesting finding that SKF 525A as well as metyrapone inhibited the microsomal inactivation of CP (Table 2).

Hepatotoxicity of microsome-treated chloro peptide

Since it was demonstrated that CP lost the ability to induce blebbing upon treatment with the PCB- and PB-microsomes, we compared the hepatotoxicity of untreated- and microsome-treated CP by estimating the elevation of serum transaminase in mice.

CP (25 $\mu\text{g/ml}$) was preincubated at 37°C for 60 min in the presence or absence of the PCB-microsomes and injected subcutaneously into mice in a dose of 0.25 mg/kg, and the activity of SGOT was estimated 3 h after the injection. The dose and duration of time were selected based on data previously reported (Ito et al., 1981). In the mice injected with untreated CP, a marked elevation of SGOT was observed (Table 3). On the other hand, no significant increase of SGOT was observed in mice injected with CP pretreated with the PCB-microsomes, and omission of NADPH from the incubation mixture allowed the elevation of SGOT activity.

Table 1. Microsomal inactivation of chloro peptide.

Enzyme System	Protein ($\mu\text{g/ml}$)	Chloro peptide (0.5 $\mu\text{g/ml}$)	Bleb formation ^a (%)
—	—	—	0—5
—	—	+	95—100
Control-MS	320	+	85—100
PCB-MS	17	+	90—100
	68	+	40—50
	170	+	20—50
	340	+	0—10
PCB-MS (denatured) ^b	340	+	90—100
PCB-MS (NADPH omitted) ^c	340	+	75—100
MC-MS	360	+	60—85
PB-MS	440	+	0—5
PB-cytosol	150	+	95—100

a: Average range of three separate experiments.

b: Heated at 100°C for 5 min.

c: NADPH-generating system was omitted from the reaction mixture.

Table 2. Effects of inhibitors of drug metabolism on the microsomal inactivation of chloropeptide.

Chloropeptide* (0.5 μ g/ml)	Inhibitors ^a (0.1 mM)	Bleb formation ^b (%)
—	—	0—5
+	—	95—100
—	SKF 525A	0—5
±	SKF 525A	95—100
—	Metirapone	0—1
±	Metirapone	90—100

a: Chloropeptide (5.0 μ g), PCB-microsomes (340 μ g protein) and inhibitors or DMSO, in a total volume of 1.0 ml, were incubated as described in MATERIALS AND METHODS, and 0.1 ml portions (0.5 μ g CP) were assayed for blebbing.

b: Average range of three separate experiments.

Table 3. Effect of pretreatment of chloropeptide with microsomes on the serum transaminase-elevating activity in mice.

Groups of mice ^a (0.25 mg CP/kg)	Serum GOT activity (Karmen units) ^b	
	Exp. 1	Exp. 2
0.9% NaCl solution only	50 $\pm$ 5	59 $\pm$ 10
untreated chloropeptide	1800 $\pm$ 150	1750 $\pm$ 120
PCB-MS only	59 $\pm$ 20	85 $\pm$ 24
PCB-MS treated chloropeptide	70 $\pm$ 15	172 $\pm$ 30
PCB-MS treated chloropeptide (no NADPH-generating system added)	—	675 $\pm$ 80

a: Chloropeptide (25 μ g) was incubated with or without PCB-microsomes (1.7 mg protein), in a total volume of 1.0 ml, as described in MATERIALS AND METHODS, and the reaction mixture was s. c. injected to 3-4 mice in dose of 0.1 ml (2.5 μ g CP) per 10 g of body weight.

b: SGOT activity was estimated 3 hr after the injection. The mean $\pm$ S. D.

In order to confirm the loss of the hepatotoxicity of CP by pretreatment with the PCB-microsomal enzymes, the livers of mice injected with CP were subjected to histopathological observations. Hematoxylin-eosin stained sections of livers of mice injected with untreated CP, revealed a marked centrilobular necrosis and hyaline droplets while these pathological changes were not observed in the mice treated with the CP incubated with the PCB-microsomes.

DISCUSSION

In the earlier papers (Ueno et al., 1977b; Ito et al., 1981), we demonstrated that CP was hepatotoxic and that more than 20% of the s. c. administered [³H] CP accumulated in the liver *in vivo*. Histologically, CP produced centrilobular necrosis, fatty degeneration, fibrin-positive hyaline droplets, and red blood corpuscles in the liver (Uraguchi et al., 1961). To study the hepatotoxic mechanism of CP, we investigated *in vivo* effects of CP on the isolated hepatocytes.

The present paper clearly demonstrates that CP is actually cytotoxic to hepatocytes

as judged by the trypan blue exclusion method (Fig. 2). However, the concentration of CP needed for a decrease in cell viability was high (more than 10–100 $\mu\text{g/ml}$) and may not be relevant to the *in vivo* toxicity of CP.

An interesting as well as important finding was the induction of blebs on the hepatocellular membranes by an extremely low concentration (0.1–0.5 $\mu\text{g/ml}$) of CP (Fig. 3). This morphological change is characteristic and it probably represents the primary effect of CP on the hepatocytes. Formation of innumerable invaginations on the outer membrane of hepatocytes were demonstrated by the light and scanning electron microscopes (Photo 1–2).

Blebbing similar to that observed on hepatocytes exposed to CP has been observed with several hepatotoxic agents such as phalloidin (Frimmer, 1977; Kane et al., 1980), bromobenzene (Thor et al., 1978) and carbon tetrachloride (Chenery et al., 1981). In this respect, the blebbing is considered to be an early and common morphological lesion of the cytoplasmic membrane of hepatocytes exposed to certain hepatotoxic agents.

There are many reports concerning the hepatotoxicity of toxicants in isolated hepatocytes as reviewed by Sirica and Pitot (1980). The calcium dependency of the toxic cell damages aroused great interest in the field of cell biology (Schanne et al., 1979). Actually, according to the experiments with hepatotoxic agents such as amphotericin B, phalloidin and calcium ionophores, the cells are likely to be damaged by processes involving at least two stages; disruption of the integrity of the cytoplasmic membrane and an influx of outer calcium into the inside of cells across the damaged cytoplasmic membranes. This latter step represents, or at least, initiates a toxic injury of the cells (Schanne et al., 1979; Kane et al., 1980).

At the present, no direct evidence for the mechanism of CP-dependent induction of blebs is available. Morphological studies with electron microscopy, biochemical examinations on the interaction between the cytoplasmic membrane and CP, and physiological studies with calcium ionophores and antagonists are currently in progress in our laboratory.

Another finding was the inactivation of CP by the microsomal monooxygenase system. As shown in Table 1, the cytotoxic potential of CP, estimated by the bleb formation, disappeared when the toxin was preincubated with microsomes from PCB- and PB-, but not MC-, and non-treated animals. These data and the results with the monooxygenase inhibitors (Table 2) would lead to the conclusion that cytochrome P-450 is responsible for the metabolic inactivation of CP. The requirement of NADPH for the microsomal inactivation (Table 1) also supports the monooxygenase involvement.

As shown in Table 1, the inactivation of CP is proportional to the amount of PCB-microsomes, and about 340 μg protein of this microsome preparation is required for the inactivation of 5 μg of CP under the present experimental conditions. This amount of PCB-microsomes corresponds to about 0.1 g of the liver or $1\text{--}4 \times 10^6$ hepatocytes. The content of cytochrome P-450 in the normal microsomes is much less than in those of the induced livers (Yoshizawa et al., 1981). Therefore, it is conceivable that CP (5 μg) added to the cell suspension ($1\text{--}4 \times 10^6$ cells) was not inactivated by the cytochrome

P-450 system of the normal hepatocytes. Actually, the microsomes derived from the control rat liver was not able to inactivate CP (Table 1). This means that the hepatic cytochrome P-450 level in the normal animal is not enough for the detoxication of a lethal dose of CP *in vivo*. This may be one of the reasons why CP induces the hepatocellular damage *in vivo* irrespective of the presence of the microsomal detoxication system.

Another problem is that the property of CP to induce the blebbing *in vitro* is closely related to the *in vivo* hepatotoxicity. The results presented in Table 3 show that the enzyme-treated CP caused neither the elevation of SGOT nor the characteristic hepatocellular damage. Therefore, we concluded that the *in vivo* hepatic injury reflects the *in vitro* cytotoxicity to the isolated hepatocytes.

The chemical nature of the inactivated CP is under investigation. Cytochrome P-450-dependent dehalogenation of CP is one possibility. As reported in the preceding paper (Ishikawa and Ueno, 1970), the dehalogenated CP prepared by ammonolysis of CP is not toxic as far as the lethal toxicity to mice is concerned, and no blebbing was observed with a high concentration (100 $\mu\text{g/ml}$) of this dehalogenated peptide. The data on the microsomal metabolite(s) of CP will be reported elsewhere in detail.

This is the first report which demonstrated the *in vitro* cytotoxicity of CP to the isolated hepatocytes and the cytochrome P-450 dependent detoxication mechanism. These basic approaches are expected to give a key to understanding the relationship between the cytotoxicity, interaction with the cytoplasmic membrane and cytochrome P-450 system of the microsomes.

SUMMARY

Cytotoxic effects of chloropeptide, a hepatotoxic cyclic pentapeptide of *Penicillium islandicum* Sopp, on isolated rat hepatocytes were investigated. Although the hepatotoxin did not markedly reduce the cell viability when monitored by the dye exclusion technique, gross changes in cell morphology including blebbing and ballooning of the cytoplasmic membrane were observed at low concentration (0.1-0.5 $\mu\text{g/ml}$) of the peptide. The hepatotoxic effects of chloropeptide, estimated by the blebbing of the isolated hepatocytes *in vitro*, elevation of serum glutamic-oxaloacetic transaminase and hepatocellular damages *in vivo*, were abolished when chloropeptide was preincubated with polychlorobiphenyl- and phenobarbital-induced microsomes in the presence of NADPH, and this microsomal inactivation of chloropeptide was inhibited by SFK 525A and metyrapone. These findings indicate that the inactivation of chloropeptide is cytochrome P-450-dependent.

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