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Analysis of an RNA adduct formed from aminophenylnorharman

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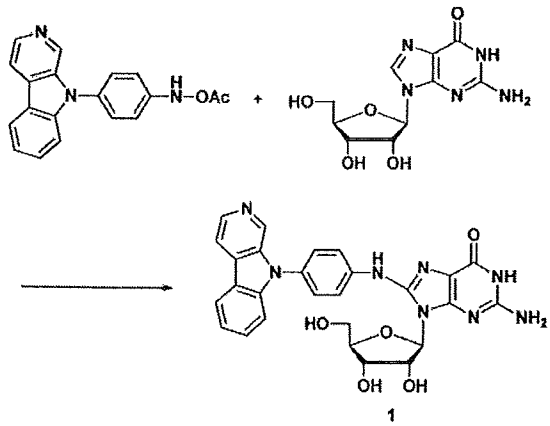
ABSTRACT

The endogenous mutagenic/carcinogenic 9- (4'-aminophenyl) -9*H*- pyrido [3,4-*b*] indole (aminophenylnorharman, APNH) is formed from norharman and aniline in the presence of cytochrome P-450s. The major APNH-DNA adduct has been reported to be 2'-deoxyguanosin-8-yl-aminophenylnorhaman (dG-C8-APNH). In addition, demonstrated formation of APNH-RNA adduct and conducted a structural analysis using various spectrometric approaches. The compound produced from guanosine (Guo) and *N*-acetoxy-APNH, an ultimate mutagenic form of APNH, was concluded to be guanosin-8-yl-APNH (Guo-C8-APNH) on the basis of various spectroscopic analysis. The same adduct was found in the livers of rats administered APNH. The total adduct levels of APNH-RNA were six times higher than total APNH-DNA adducts in the same rat liver samples.



Scheme 1 A reaction mixture of *N*-acetoxy-APNH and guanosine

INTRODUCTION

Aminophenylnorharman (APNH) a product of the enzymatic reaction of norharman with aniline in the presence of S9 mix, has already been reported to be a strong mutagen/carcinogen[1-4]. Norharman and aniline abundantly exist in cigarette smoke, cooked foods and some kinds of vegetables[5-6]. Therefore, humans are exposed to both of these compounds chronically, and APNH is expected to be produced in our body. In a long term carcinogenicity experiment using experimental animals, APNH induced tumors in various tissues, including the liver and colon[3]. APNH is thought to be metabolically activated by CYP1A2 and acetyltransferase to form adducts with 2'-deoxyguanosine[7], and chemical structure of the major DNA adduct has already been reported as dG-C8-APNH, detectable in various tissues of rats and mice after a single administration of APNH[8]. In recent years, some studies have focused on RNA as a biological markers[9-11]. Similar to DNA, RNA consists of nucleobases including guanine, and would be expected to give rise to similar mutagen/carcinogen-RNA adducts. In contrast to DNA modifications by mutagens/carcinogens, which can lead to mutations, RNA modifications have generally been considered biologically meaningless. However, RNA is present in both the cytoplasmic and nuclear compartments, so it has a greater chance of reacting with the exogenous/endogenous carcinogens. Futhermore, RNA exists in a variety of forms, including tRNA, mRNA, rRNA and microRNAs, so that RNA adducts may be unique biological significance in carcinogenesis. Thus, they might offer a sensitive biomaker for exposure analysis. In the present study, we demonstrated the formation of a major APNH-RNA adduct and analyzed its chemical structure using various spectrometric approaches. In addition, we also report the generation of total APNH-RNA adducts at higher levels than total APNH-DNA lesions in the livers of rats administered APNH, as assessed by ^{32}P-postlabeling analysis[12].

RESULTS AND DISCUSSION

We first analyzed a reaction mixture of guanosine (Guo) and *N*-acetoxy-APNH (Scheme 1), an ultimate mutagenic form of APNH, by LC-ESI/MS analysis. As a result, a compound exhibiting molecular ion peak *m/z* 541 along with a fragment ion peak at *m/z* 409, consistent with loss of a ribose moiety, was found to be formed. From ^{1}H-NMR spectroscopy, its chemical structure was concluded to be guanosin-8-yl-APNH (Guo-C8-APNH) (Scheme 1). To comfirm its chemical structure, we synthesized Guo-C8-APNH via the Buchwald-Hartwig coupling reaction[13]

(Scheme 2). This product was shown to be identical to compound 1 in scheme 1 by comparison of their spectroscopic data.

Total RNA obtained from the livers of F344 rats with or without treatment of APNH was analyzed by ^{32}P-postlabeling method under adduct-intensification conditions. Adduct spot corresponding to Guo-C8-APNH were observed in the APNH-treated animals, but not in the control animals. Total adduct levels of APNH-RNA were 28±13.3 (mean±SD) adducts per 10^6 nucleotides. APNH-DNA adducts in DNA samples obtained from the same liver samples were analyzed by ^{32}P-postlabeling method under modified adduct intensification conditions. The TLC pattern was different from the case of total RNA, and their total APNH-DNA levels were 4.5±2.0 (mean±SD) adducts per 10^6 nucleotides. From these observations, it is suggested that APNH binds to both DNA and RNA *in vivo*. APNH-RNA levels were about 6 times higher than those of DNA.

CONCLUSION

We identified an APNH-RNA adduct formed by the reaction of *N*-acetoxy-APNH with Guo. The chemical structure was concluded to be Guo-C8-APNH, similar to that of dGuo-C8-APNH. Guo-C8-APNH could also be detected in rat liver after administration of APNH, suggesting that APNH can damage RNA in a manner similar to DNA *in vivo*.

We are now analyzing the function of APNH-RNA adduct using the synthesis of a Guo-C8-APNH phosphoramidite and the RNA oligonucleotide containing Guo-C8-APNH.

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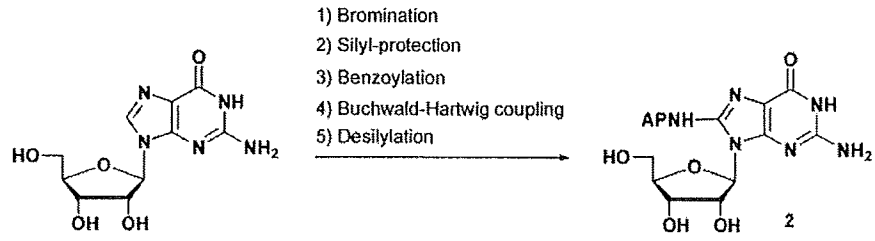

1) Br_2, water/CH_3CN; 2) $DTBS(OTf)_2$, DMF, 0 ℃ to r.t. 3) BnOH, PPh_3, DEAD/Dioxane; 4) APNH, Pd_2dba_3, xantphos, Cs_2CO_3, DMSO; 5) TBAF, THF

Scheme 2 Synthesis of Guo-C8-APNH

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Induction of SCEs in CHL cells by dichlorobiphenyl derivative water pollutants, 2-phenylbenzotriazole (PBTA) congeners and river water concentrates

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PBTA-1
PBTA-2
PBTA-6

ABSTRACT

We recently identified dichlorobiphenyl (DCB) derivatives and 2-phenylbenzotriazole (PBTA) congeners as major mutagenic constituents of the waters of the Waka River and the Yodo River system in Japan, respectively. In this study we examined sister chromatid exchange (SCE) induction by two dichlorobiphenyl derivatives, 3,3′-dichlorobenzidine (DCB, 4,4′-diamino-3,3′-dichlorobiphenyl) and 4,4′-diamino-3,3′-dichloro-5-nitrobiphenyl (5-nitro-DCB); three PBTA congeners, 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1), 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), and 2-[2-(acetylamino)amino]-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-6); and water concentrates from the Waka River in Chinese hamster lung (CHL) cells. Concentration-dependent induction of SCE was found for all DCBs and PBTAs examined in the presence of S9 mix, and statistically significant increases of SCEs were detected at 2 μg per ml of medium or higher concentrations. SCE induction of MeIQx was examined to compare genotoxic activities of these water pollutants. According to the results, a ranking of the SCE-inducing potency of these compounds is the following: 5-nitro-DCB ≈ MeIQx > PBTA6 > PBTA-1 ≈ PBTA-2 > DCB.

Water samples collected at a site at the Waka River showed concentration-related increases in SCEs at 6.25–18.75 ml-equivalent of river water per ml of medium with S9 mix. The concentrations of 5-nitro-DCB and DCB in the river water samples were from 2.5 to 19.4 ng/l and from 4100 to 18,900 ng/l, respectively. However, these chemicals showed only small contribution to SCE induction by the Waka River water.

1. Introduction

Genotoxic compounds are detected in many surface waters in the world. These compounds are often released directly from industrial discharges as a result of insufficient treatment of wastewater or unintentional formation in the environment after discharge of effluents [1–5]. In the previous studies, we found two novel chemical classes, dichlorobiphenyl derivatives and 2-phenylbenzotriazole (PBTA) congeners, as major mutagenic constituents in the water of rivers flowing through several industrial areas in Japan [6–20].

Among dichlorobiphenyl derivatives, 3,3′-dichlorobenzidine (DCB, 4,4′-diamino-3,3′-dichlorobiphenyl), 4,4′-diamino-3,3′-dichloro-5-nitrobiphenyl (5-nitro-DCB), and so forth were identified as major mutagens in the water of the Waka River flowing through an industrial area in Wakayama, where a number of large chemical plants are found [6–9]. 5-Nitro-DCB is a novel chemical and is presumed to be formed unintentionally by the process of wastewater treatment of drainage water containing DCB discharged from chemical plants [6]. DCB is a raw material in the manufacture of polymers and dye intermediates, and there are large-scale chemical plants producing DCB in this industrial area. 5-Nitro-DCB is highly mutagenic in the Ames assay using *Salmonella typhimurium* YG1024, which is an *O*-acetyltransferase-overproducing derivative of TA98, with S9 mix, and its activity was ~7 times higher than that of DCB.

5-Nitro-DCB was detected in river water concentrates at the maximum level of 6.9 μg/g of blue rayon. DCB was also detected in the concentrates at 13.2–104 μg/g of blue rayon. The percent contributions of 5-nitro-DCB and DCB to the mutagenicity of the water concentrates in YG1024 with S9 mix were 11% and 28%, respectively,

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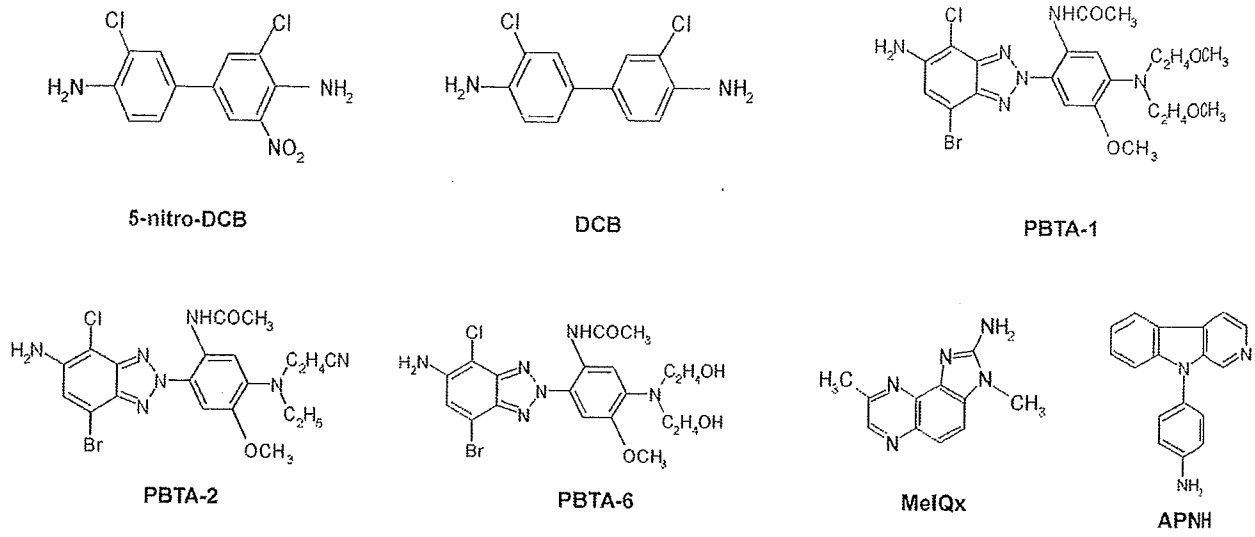

Fig. 1. Chemical structures used in the present study.

on average. 5-Nitro-DCB is a new chemical, and it has no biological activity data except for mutagenicity in the Ames assay.

PBTA congeners were identified as major indirect-acting river water mutagens, and seven kinds of such compounds were detected in highly mutagenic river waters, e.g., the Yodo River system, the Asuwa River, the Nikko River and so on, flowing through areas of textile dyeing industries [10–20]. PBTA congeners were suggested to be formed from corresponding dinitrophenylazo dyes via reduction with sodium hydrosulfide during the industrial dyeing process and following chlorination in the disinfection process at sewage plants. PBTA congeners show potent mutagenicity in *S. typhimurium* YG1024 in the presence of S9 mix. 2-[2-(Acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1), 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), and 2-[2-(acetylamino)amino]-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzo-triazole (PBTA-6) were detected in many river water concentrates [10–15]. However, there are few reports on the genotoxicity of PBTA congeners in mammalian cells.

In this study, we investigated the induction of sister chromatid exchanges (SCEs) by DCB, 5-nitro-DCB, PBTA-1, PBTA-2, PBTA-6, and water concentrates from the Waka River in Chinese hamster lung (CHL) cells to evaluate the genotoxic effect of water pollutants and environmental samples contaminated with DCB and 5-nitro-DCBs. DCB and 5-nitro-DCB in the river water concentrates were quantitatively analyzed, the mutagenicity of the water concentrates were evaluated in YG1024, and the contribution of 5-nitro-DCB and DCB to the mutagenicity of the river water concentrates are also estimated.

2. Materials and methods

2.1. Materials

5-Nitro-DCB (CAS 1073239-90-3) was synthesized according to the method reported previously [6]. Dichlorobenzidine dihydrochloride (CAS 612-83-9) was purchased from Sigma–Aldrich Co. Ltd. (MO, USA). PBTA-1 (CAS 194590-84-6), PBTA-2 (CAS 215245-16-2), and PBTA-6 (CAS 392274-07-6) were synthesized as described previously [11,13,16]. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx, CAS 77500-04-0) was obtained from Wako Pure Chemical Industries (Osaka, Japan). The chemical structures of six compounds used in the present study are shown in Fig. 1. All other chemicals and reagents were of analytical grade.

2.2. Preparation of river water concentrates and analysis of DCBs

Each 10 l water sample was collected at a site where wastewater was discharged from chemical plants and a sewage treatment plant into the Waka River in Wakayama, Japan from September 2006 to March 2007. Collected water samples were passed through Supelpak2 columns (SUPELCO, PA, USA, 20 mm i.d. × 800 mm), and adsorbed materials were then extracted with methanol (300 ml) according to our previous paper [8]. Each extract was used for SCE assay, the Ames assay, and quantification of 5-nitro-DCB and DCB by HPLC. Quantification of 5-nitro-DCB and DCB was performed according to the method reported in the previous paper [6].

2.3. Chemical treatment

All chemicals tested for SCE assay were dissolved in dimethyl sulfoxide(DMSO), and freshly prepared solutions were added to cultures in appropriate final concentrations. The final concentration of DMSO in all cultures was 0.5% (v/v). Aminophenyl-norharman (APNH, Fig. 1) was used as the positive control [22] and was dissolved in DMSO before use.

2.4. SCE assay

CHL cells, obtained from Health Science Research Bank (HSRRB), Japan, were subcultured at a cell density of 1.5×10^5 cells per 60-mm dish and cultivated in 4 ml of Eagle's minimum medium (MEM, Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Trace Scientific Ltd., Melbourne, Australia). In the system with metabolic activation, cells were treated with each chemical for 6 h in the presence of S9 mix. The S9 mix was prepared immediately before use by mixing 1 ml of phenobarbital- and 5,6-benzoflavone-induced rat liver S9 (Oriental Yeast Co. Ltd., Tokyo, Japan) with 0.1 ml of 0.5 M glucose-6-phosphate, 0.4 ml of 0.1 M NADP, 0.2 ml of 1.65 M KCl solution, 0.2 ml of 0.4 M $MgCl_2$ solution, 4 ml of 0.25 M phosphate buffer (pH 7.4), and 4.1 ml of distilled water. The final concentration of S9 used was 1.25% (v/v). After treatment, cells were washed with phosphate buffered saline (PBS) and cultured in fresh medium for a recovery period of 24 h. In an experiment designed to detect SCEs, 5-bromodeoxyuridine (BrdU; final concentration 5 μg/ml) was added to the cultures just after addition of the test chemical.

Colcemid (final concentration 0.2 μg/ml; Gibco) was added to each culture 2 h prior to harvest. Harvested cells were then treated with a hypotonic solution of 75 mM KCl and fixed with cold methanol:acetic acid (3:1, v/v). Solvent-treated cells served as the negative control. Air-dried chromosome preparations prepared for SCEs were stained with the fluorescence plus Giemsa method described by Perry and Wolff [23]. SCEs were scored in 25 well-spread metaphases for each treatment. The results were expressed as the frequency of SCEs per metaphase. The significance between mean SCE in treated versus control groups were determined using the Student's *t*-test.

2.5. Ames assay

Ames assay was performed for DMSO solutions of chemicals and water concentrates described above using *S. typhimurium* YG1024 according to the method reported previously [24–26]. *S. typhimurium* YG1024 were kindly provided by Dr. T. Nohmi from the National Institute of Health Sciences, Tokyo. The S9 mix contained

Table 1
SCEs induced by 5-nitro-DCB, DCB, PBTA-1, PBTA-2 and PBTA-6 in CHL cells in the presence of S9 mix.

Sample	Dose (μg/ml)	SCEs per metaphase		
		MI (%)[c]	Mean ± S.D.	Range
5-Nitro-DCB[a]	1.25	2.0	13.32 ± 6.27	4–29
	2.5	2.5	15.92 ± 7.43	6–31
	5	2.1	18.58 ± 6.01	7–29
	10	2.2	25.04 ± 9.50	11–48
DCB[a]	1	2.9	11.16 ± 4.14	4–20
	2	2.2	14.76 ± 4.35	7–23
	10	2.9	16.96 ± 5.95	8–35
	20	2.5	19.16 ± 7.69	7–37
PBTA-1[b]	1.25	2.6	17.88 ± 5.21	11–29
	2.5	2.6	18.60 ± 8.29	9–34
	5	2.0	19.06 ± 6.27	7–29
	10	1.7	20.04 ± 7.37	10–43
PBTA-2[b]	1.25	2.8	14.28 ± 3.60	8–23
	2.5	2.9	14.56 ± 6.58	6–38
	5	2.7	17.84 ± 5.74	8–28
	10	2.0	20.96 ± 7.81	9–34
PBTA-6[b]	1.25	2.9	15.08 ± 4.95	8–26
	2.5	2.7	16.28 ± 6.13	8–26
	5	2.9	19.40 ± 5.22	12–31
	10	1.8	22.76 ± 7.04	10–36
MeIQx[b]	1.25	3.5	15.97 ± 8.20	6–39
	2.5	3.9	18.20 ± 5.12	9–35
	5	3.7	23.96 ± 9.20	9–43
	10	2.7	26.68 ± 9.02	13–53

SCE frequency for positive control (APNH, 0.005 μg/ml) was 21.44 ± 5.04 (mean ± S.D.).

[a] SCE frequency for Control (DMSO) was 8.68 ± 4.28 (mean ± S.D.).

[b] SCE frequency for Control (DMSO) was 10.16 ± 2.65 (mean ± S.D.).

[c] MI; mitotic index. MI (%) was calculated by counting the number of mitotic cells among 1000 round nuclei.

* Significantly different from control, $p < 0.05$.

** Significantly different from control, $p < 0.01$.

25 μl of S9 (25 mg of protein/ml) at a total volume of 500 μl. Mutagenic activities of test samples were calculated from the linear portions of the dose-response curves obtained with four doses with duplicate plates in two independent experiments, and the results were the average of two independent experiments. The positive controls were 2-aminoanthracene (0.1 μg/plate) and Trp-P-1 (0.01 μg/plate) with S9 mix. The mutagenic potencies were expressed as revertants/l of river water.

3. Results

3.1. SCE induction by DCBs and PBTAs

We evaluated the genotoxic effect of 5-nitro-DCB, DCB, three PBTA congeners, and MeIQx using SCEs in cultured CHL cells in the presence of S9 mix. These chemicals are indirect-acting mutagens for bacteria and show potent mutagenicity with S9 mix [6,7,10,11,13,27]. As shown in Table 1, statistically significant increases in SCEs were found for DCBs and PBTAs at doses used in this study (up to 10 or 20 μg/ml). Among DCBs and PBTAs, the highest SCE frequency, 25.04 ± 9.50 (mean ± S.D.), was detected for 5-nitro-DCB at the dose of 10 μg/ml. MeIQx is a well-known mutagenic and carcinogenic heterocyclic amine [28], and it shares structural features common to those of DCBs and PBTAs. Dose-related increases in the frequencies of SCEs were found for MeIQx between the concentration of 1.25 and 10 μg/ml. The highest SCE frequency, 26.68 ± 9.02, was detected at 10 μg/ml. Concentrations of DCBs, PBTAs, and MeIQx leading to two-fold increases of SCE frequencies relative to that of control were shown as SCE-inducing activity in Table 2, with mutagenicity data by Ames test. SCE-inducing activities of DCBs and PBTAs were from 4.5 to 13.9 μg/ml. The SCE-inducing activity of 5-nitro-DCB, 4.5 μg/ml was as high as that of MeIQx, 4.6 μg/ml. APNH, which was used as a positive control, significantly increased SCE induction at a dose of 0.005 μg/ml in the presence of S9 mix, and this result was consistent with that of our previous report [22].

Table 2
SCE-inducing activity and mutagenicity data of 5-nitro-DCB, DCB, PBTA-1, PBTA-2, PBTA-6 and MeIQx in the presence of S9 mix.

Sample	SCE-inducing activity (μg/ml)[a]	Mutagenicity (revertants/μg)[b]	
		TA98	YG1024
5-Nitro-DCB	4.5	8,700	24,200
DCB	13.9	100	3,400
PBTA-1	8.4	88,000	3,000,000
PBTA-2	8.4	93,000	3,200,000
PBTA-6	6.9	17,900	485,000
MeIQx	4.6	117,000	1,400,000

[a] Concentration leading to a two-fold increase relative to control level.

[b] Data from reference [6,9,12,17,38].

3.2. SCE induction by the water concentrates from the Waka River

The dose-response effects of SCE induction for three water concentrates obtained from the Waka River are shown in Table 3. For all water concentrates, dose-related increases in the frequencies were found between the concentration of 6.25 and 18.75 ml eq/ml of medium in the presence of S9 mix. The highest SCE frequency, 20.84 ± 5.08, was detected for river water concentrate No. 2 at the dose of 18.75 ml/ml, but this concentrate showed toxicity at a higher dose.

3.3. Concentrations of DCBs in the river water and mutagenicity in Salmonella

Table 4 shows amounts of 5-nitro-DCB and DCB in the three water samples from the Waka River and mutagenicity of water concentrates toward *S. typhimurium* YG1024 in the presence of S9 mix. 5-Nitro-DCB and DCB were detected in all the water samples. The concentrations of 5-nitro-DCB and DCB in the river water samples were from 2.5 to 19.4 ng/l and from 4100 to 18,900 ng/l, respectively. The three water concentrates showed potent mutagenicity in YG1024, and the highest activities was detected for the water concentrate No.2. The percent contributions of DCB to the mutagenicity of the river water concentrates were from 8% to 26%, but those of 5-nitro-DCB were less than 1%.

4. Discussion

In the present study, we evaluated the genotoxic effect of the water pollutants 5-nitro-DCB, DCB, PBTA-1, PBTA-2, and PBTA-6, and river water samples, which included 5-nitro-DCB and DCB as constituents, using SCEs in CHL cells. In addition, SCE induction of MeIQx was examined to compare genotoxic activities of these water pollutants. MeIQx was deduced to be possibly carcinogenic to human (Group 2B) by the International Agency for Research on Cancer (IARC) [28], and it has the structural features of an aromatic amine similar to those of DCBs and PBTAs. MeIQx was reported to induce SCEs in human lymphocyte cultures and to show mutagenicity in CHL cells for diphtheria toxin resistance in the presence of S9 mix [29,30]. As shown in Table 1, the dose-response effects of SCE induction were detected for DCBs, PBTAs, and MeIQx at doses from 1.25 to 10 μg/ml or from 1 to 20 μg/ml with S9 mix, and the increases of SCEs were statistically significant at almost all doses tested. All chemicals tested in the present study induced SCE induction in cultured mammalian CHL cells in the presence of S9 mix. Among the composites tested, 5-nitro-DCB was found to have

Table 3
SCEs induced by river water concentrates in CHL cells in the presence of S9 mix.

Sample	Sampling date	Dose (ml/ml)[a]	SCEs per metaphase		
			MI (%)[b]	Mean ± S.D.	Range
Concentrate No. 1	14 September 2006	6.25	2.4	10.12 ± 3.50	3–18
		12.5	2.9	12.12 ± 3.94	5–22
		18.75	2.5	16.16 ± 6.08	6–28
		25	1.2	16.12 ± 6.35	4–31
Concentrate No. 2	19 December 2006	6.25	3.2	12.44 ± 5.08	5–25
		12.5	2.2	18.72 ± 5.95	9–35
		18.75	1.5	20.84 ± 5.08	13–37
		25	0	Toxic	Toxic
Concentrate No. 3	29 March 2007	6.25	2.8	10.64 ± 2.90	6–16
		12.5	2.4	14.00 ± 5.42	6–28
		18.75	2.6	17.44 ± 6.87	9–38
		25	2.1	16.36 ± 5.18	10–29

SCE frequency for Control (DMSO) was 8.68 ± 4.28 (mean ± S.D.). SCE frequency for positive control: APNH (0.005 μg/ml) 21.44 ± 5.04 (mean ± S.D.).
[a] Dose is expressed as ml eq of river water per 1 ml of medium.
[b] MI; mitotic index. MI (%) was calculated by counting the number of mitotic cells among 1000 round nuclei.
** Significantly different from control, $p < 0.01$.

the most pronounced frequency of SCEs, showing the same level of MeIQx, although mutagenicity with *Salmonella* TA 98 and YG1024 of 5-nitro-DCB was relatively low compared with those of PBTA congeners and MeIQx as shown in Table 2. Moreover, SCE-inducing activity of 5-nitro-DCB was 1.5–2 times higher than those of PBTA congeners and was 3 times higher than that of DCB. A ranking of the SCE-inducing potency of these compounds is the following: 5-nitro-DCB ≈ MeIQx > PBTA6 > PBTA-1 ≈ PBTA-2 > DCB.

All water concentrates from the Waka River also showed dose-related increases in SCEs between the concentration of 6.25 and 18.75 ml eq/ml of medium in CHL cells with S9 mix (Table 3). SCE-inducing activities of water concentrates were from 13 to 24 ml eq/ml and a ranking of the water concentrates for SCE induction was concentrate No. 2 > 3 > 1. Both 5-nitro-DCB and DCB were detected in all water concentrates, but amounts of DCB in the water samples were about 1000-fold or higher than those of 5-nitro-DCB. Since DCB and 5-nitro-DCB showed similar SCE-inducing activity, and the amounts of DCB in the water samples were much higher than those of 5-nitro-DCB, the contribution of DCB to SCE-induction of river water concentrates may be larger than that of 5-nitro-DCB. However, contribution ratios of SCE activities based on the concentration of DCB and 5-nitro-DCB, respectively, to the total SCE activities by the river water concentrates were <3% and <0.01%, respectively. Some unknown compounds may be affecting SCE induction of the river water.

Mutagenicity of these river water concentrates was also examined by the Ames assay using YG1024 with S9 mix (Table 4). These concentrate showed potent activities, and percent contributions of DCB, i.e., 8–20%, was much higher than those of 5-nitro-DCB, which was <1%. These high percent contributions of DCB were caused by an abundance of DCB in the river water examined in this study. In a previous study, we quantified 5-nitro-DCB and DCB in blue rayon extracts from the Waka River water and detected relatively high amounts of 5-nitro-DCB [6]. The concentration of 5-nitro-DCB in the river water likely differs on sampling days. More quantitative investigations are necessary to estimate the effect of DCB and 5-nitro-DCB to the genotoxicity of the Waka River water.

In a previous study, we reported that blue rayon extracts from the water of the Yodo River system, Japan, collected in October and December, 1991, showed SCE induction in CHL cells with S9 mix [21]. PBTA-1, PBTA-2, and PBTA-6 were detected in the water samples collected from the same river system in 1994, 1995, and 1999, respectively [10,11,13]. Besides these three PBTAs, other PBTA congeners were continually detected in the water samples collected from this river system from 1994 to 2005 [15]. From synthesis studies, PBTAs are thought to be formed from corresponding dinitrophenylazo dyes used in textile dyeing factories and released into the river system. The SCE induction by the blue rayon extracts from the Yodo River system might be due to PBTA congers.

Table 4
Mutagenicity of water concentrates from the Waka River and amounts of 5-nitro-DCB and DCB.

Sample	Mutagenicity (revertants/l)[a]	Amount (ng/l)		Contributionratio (%)[b]	
		5-Nitro-DCB	DCB	5-Nitro-DCB	DCB
Concentrate No. 1	246,200	4.8	18,900	<1	26.1
Concentrate No. 2	374,800	19.4	18,200	<1	16.5
Concentrate No. 3	179,300	2.5	4,100	<1	7.8

[a] Mutagenicity was examined in *S.typhimurium* YG1024 with S9 mix.
[b] The mutagenic potencies of 5-nitro-DCB and DCB used to calculate the contribution ratios were 24,200 and 3400 revertants/μg, respectively [6,7].

Besides 5-nitro-DCB and DCB, three dichlorobiphenyl derivatives, i.e., 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl (ADDB), 3,3′-dichloro-4,4′-dinitrobiphenyl, and 4-amino-3,3′-dichloro-4′-nitrobiphenyl, which were mutagenic in YG1024, were detected in water samples collected from the Waka River in 2003–2004 [8,9]. These DCB derivatives are thought to be formed from DCB, like 5-nitro-DCB. DCB was positive in some *in vivo* genotoxicity assays, e.g., the chromosomal aberration test [31], the micronucleus assay [32], and the alkaline single cell gel electrophoresis assay (comet assay) [33]. Moreover, DCB is carcinogenic in mice, rats, hamsters, and dogs [34], and it has been designated a probable human carcinogen (Group 2B) by IARC [35]. PBTA-1 and PBTA-2 induced micronuclei in Chinese hamster cell line V79-NZ [36]. PBTA-6 and ADDB induced micronuclei in gill cells by i.p. injection into goldfish [37]. Furthermore, DNA damaging activity was detected for PBTA-6 and ADDB in peripheral erythrocytes of goldfish *in vivo* by the comet assay [37].

Our results indicate that various dichlorobiphenyl derivatives and PBTA congeners were detected in the water of the Waka River and the Yodo River system, respectively. Except for DCB, biological activities of these water pollutants have been evaluated mostly by the Ames assay, and data on biological effects of these compounds, including genotoxicity in mammalian cells and *in vivo*, are quite limited. To estimate risks of these compounds to aquatic biota and human health, further investigations on their biological activities to aquatic organisms and experimental animals are necessary. In addition, quantitative studies on these compounds in these rivers are important, and exposure levels of aquatic organisms and human

to those compounds need to be determined. Because aquatic organisms inhabiting in these rivers may be exposed chronically to these genotoxic chemicals, ecological studies, including the incidence of cancer in fish and aquatic animals, are also needed.

Conflicts of interest

None.

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Molecular cloning of apoptosis-inducing Pierisin-like proteins, from two species of white butterfly, *Pieris melete* and *Aporia crataegi*☆

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ABSTRACT

Pierisin-1, present in cabbage butterfly, *Pieris rapae*, induces apoptosis against various kinds of cancer cell lines. Another cabbage butterfly, *Pieris brassicae*, also has an apoptosis-inducing protein, Pierisin-2. These proteins exhibit DNA ADP-ribosylating activity. Pierisin-like proteins are found to be distributed in subtribes Pierina, Aporiina and Appiadina. In this study, we performed the cDNA cloning of Pierisin-like proteins designated Pierisin-3 from gray-veined white, *Pieris melete*, and Pierisin-4 from black-veined white, *Aporia crataegi*. The nucleotide sequences of Pierisin-3 and -4 encode an 850 and an 858 amino acid protein, respectively. The partial peptide sequences of Pierisin-3 and -4 purified from pupae were identical to the deduced amino acid sequence of ORF. The deduced amino acid sequence revealed that Pierisin-3 is 93% similar to Pierisin-1 and Pierisin-4 is 64%. Pierisin-3 and -4 synthesized *in vitro* with the rabbit reticulocyte lysate exhibited apoptosis-inducing activity against human cervical carcinoma HeLa and human gastric carcinoma TMK-1 cells. Site-directed mutagenesis at a glutamic acid residue comprising the NAD-binding site resulted in a significant decrease in cytotoxicity of both proteins. Moreover, the proteins incubated with calf thymus DNA and β-NAD resulted in the formation of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine, as in the case of Pierisin-1 and -2. These findings could provide useful information for understanding the importance of apoptosis-inducing ability and molecular evolution of Pierisin-like proteins in family Pieridae.

1. Introduction

Pierisin-1, an apoptosis-inducing protein, has been identified from the cabbage butterfly, *Pieris rapae* (Koyama et al., 1996; Watanabe et al., 1998). Pierisin-1 has potent cytotoxicity against various human cancer cell lines, with 50% inhibitory concentration values ranging from 0.043–150 ng/ml (Kono et al., 1999). The amino acid sequence of this protein deduced from the cDNA contains 850 amino acids with a calculated molecular weight of 98,000. Amino acid alignment indicates that Pierisin-1 is 32% similar to the mosquitocidal toxin (MTX), a kind of ADP-ribosyltransferase from *Bacillus sphaericus* SSII-1 (Watanabe et al., 1999; Carpusca et al., 2006).

ADP-ribosylating toxins, such as pertussis and cholera toxins, target α-subunits of G-proteins (Zhang et al., 1995; Locht and Keith, 1986) while diphtheria toxin ADP-ribosylates a diphthamide residue on elongation factor 2 (Bell and Eisenberg, 1996). MTX ADP-ribosylates proteins with molecular masses of 42 and 38 kDa in lysates of mosquito *Culex quinquefasciatus* G7 cells (Thanabalu et al., 1993) and *Escherichia coli* elongation factor Tu (Schirmer et al., 2002). ADP-ribosyltransferases also exist in mammals. For example, mammalian ectoenzyme ART2-catalyzed ADP-ribosylation of cell membrane proteins induces formation of cytolytic membrane pores by activating the P2X7 purinoceptor (Koch-Nolte et al., 2008). On the other hand, Pierisin-1 modifies N^2 amino groups of guanine residues in DNA to yield N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (Takamura-Enya et al., 2001). The 27-kDa N-terminal region of Pierisin-1 has DNA ADP-ribosylating activity, and the 71-kDa C-terminal region binds to glycosphingolipid receptors such as globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4) on the surfaces of mammalian cells and incorporates Pierisin-1 into cells (Kanazawa et al., 2001; Matsushima-Hibiya et al., 2003). The mRNA of pierisin-1 is highly expressed in the fifth instar larvae, and the protein is accumulated in the fat bodies during the fifth instar larvae to early pupae (Watanabe et al., 2004). Therefore, Pierisin-1 may act to remove the unnecessary cells of larval tissues in the pupation. Moreover, Pierisin-1 may have the ability to protect from natural enemies, such as parasitic wasps, by exhibiting potent cytotoxicity in *P. rapae*.

The other cabbage butterfly, *Pieris brassicae*, also contains an apoptosis-inducing protein named Pierisin-2. cDNA of Pierisin-2 encodes 850 amino acids. The amino acid sequence deduced from the cDNA shows that Pierisin-2 is 91% similar to Pierisin-1. Pierisin-2 exhibits cytotoxicity similar to Pierisin-1 (Matsushima-Hibiya et al., 2000). Pierisin-2 catalyzes

Abbreviations: Gb3, globotriaosylceramide; Gb4, globotetraosylceramide.

☆ Database: The nucleotide sequences of pierisin-3 from *Pieris melete* and pierisin-4 from *Aporia crataegi* have been submitted to the DDBJ/EMBL/GenBank database under the accession numbers AB477051 (pierisin-3) and AB477052 (pierisin-4).

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ADP-ribosylation of dG in DNA to give the same reaction product as demonstrated for Pierisin-1 (Takamura-Enya et al., 2004).

Recently, we reported the distribution of Pierisin-like proteins in the subfamily Pierinae, family Pieridae (Matsumoto et al., 2008). Protein extracts from 13 species of butterflies in this subfamily exhibited DNA ADP-ribosylating activity and cytotoxicity against the human cervical carcinoma HeLa and gastric carcinoma TMK-1 cells. All of these extracts contained substances recognized by anti-Pierisin-1 antibodies. Moreover, sequences containing NAD-binding sites, conserved in ADP-ribosyltransferases, were amplified from genomic DNA from 13 species of butterflies by PCR. However, three species of butterflies in the subfamily Pierinae and four species of butterflies in the subfamily Coliadinae, the family Pieridae showed neither cytotoxicity nor ADP-ribosylating activity, and did not contain substances recognized by anti-Pierisin-1 antibodies. Sequences containing NAD-binding sites were not amplified from the genomic DNA from these seven species (Matsumoto et al., 2008). Thus, Pierisin-like proteins are distributed in butterflies not only of the subtribe Pierina, including the genus *Pieris*, but also of the subtribes Aporiina and Appiadina. Interestingly, the subfamily Pierinae is divided into two types by the morphology of the pupal stage (Braby et al., 2006), and this classification of pupal morphology corresponds with the distribution of Pierisin-like protein, except for that in *Appias lyncida* (Matsumoto et al., 2008).

In this study, we report the cDNA cloning of apoptosis-inducing proteins named Pierisin-3 from the gray-veined white, *Pieris melete*, and named Pierisin-4 from the black-veined white, *Aporia crataegi*. These two species belong to subtribes Pierina and Aporiina respectively, and these extracts had been shown to have Pierisin-like activity (Matsumoto et al., 2008). The deduced amino acid sequence from the cDNA indicated that Pierisin-3 and -4 have a close amino acid similarity to Pierisin-1 and -2. Both *in vitro* expressed proteins exhibited cytotoxicity and DNA ADP-ribosylating activity similar to Pierisin-1 and -2. The evolutionary conservation for amino acid sequences of Pierisin-3 and -4 to compare with Pierisin-1 and -2 is discussed.

2. Materials and methods

2.1. Insects and RNA isolation

The fifth instar larvae of *P. melete* were collected in the Tochigi Prefecture, Japan, and were reared on natural host plants, cabbage, *Brassica oleracea*, at room temperature until pupation in our laboratory and stored at −80 °C until use. The fifth instar larvae of *A. crataegi* were purchased from the Eikoh Science Corp. (Osaka, Japan), and were reared on natural host plants, Yoshino cherry, *Prunus yedoensis*, at room temperature until pupation in our laboratory and stored at −80 °C until use. Some fifth instar larvae of *P. melete* and *A. crataegi* were frozen at day 1 and stored at −80 °C for the preparation of total RNA. Total RNA was prepared from the whole body of a day 1 fifth instar larvae by using Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The 5′ RACE and 3′ RACE were performed by using GeneRacer™ Kit (Invitrogen) according to the manufacturer's instructions and 1 μg of total RNA as starting material. The 5′- and 3′- phosphate groups of RNA were dephosphorylated, the 5′-cap structures of full length mRNA were replaced by GeneRacer™ RNA Oligo by oligo-capping method (Maruyama and Sugano, 1994). Then, first-strand cDNA synthesis was carried out with SuperScript® III Reverse Transcriptase using GeneRacer™ Oligo dT Primer.

2.2. cDNA cloning of Pierisin-3 and -4

For cDNA cloning of Pierisin-3 and Pierisin-4 from *P. melete* and *A. crataegi*, respectively, the sequences containing NAD-binding sites, which are conserved in ADP-ribosyltransferase were cloned. Then, the full length of cDNAs was obtained by PCR, as described below.

The sequences containing NAD-binding sites were amplified using degenerate primers PierisinDP_NF1 and PierisinDP_NR1 deduced from the Pierisin-1 and -2 amino acid sequences (DDBJ accession numbers AB030305 and AB037676) (Watanabe et al., 1999; Matsushima-Hibiya et al., 2000). The PCR was performed for 2 min at 98 °C followed by 35 cycles of 10 s at 98 °C, 20 s at 58 °C, 1 min at 72 °C, and a final extension for 7 min at 72 °C by using Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) in iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The purified PCR products were cloned into pCR®-Blunt II-TOPO® vector (Invitrogen), and were then sequenced in both directions, using ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Amersham Place, England).

In Pierisin-3, Pm_F5 primer and GeneRacer™ 3′ Nested Primer were used for 3′ RACE and Pm_R2 primer and GeneRacer™ 5′ Nested Primer were used for 5′ RACE. The PCR was performed for 2 min at 98 °C followed by 35 cycles of 30 s at 98 °C, 30 s at 58 °C, 1–4 min at 72 °C, and a final extension for 7 min at 72 °C. The PCR products were excised from the agarose gel and purified by using Wizard® SV Gel and the PCR Clean-Up System, and then the PCR fragments were cloned into the pCR®-Blunt II-TOPO® vector or StrataClone™ PCR Cloning Vector pSC-B (STRATAGENE, La Jolla, CA, USA) and sequenced. The full length of cDNA was obtained by PCR using forward primer PmF_F and reverse primer PmF_R. The amplified PCR product was purified by Wizard® SV Gel and PCR Clean-Up System and subjected to the PCR direct sequencing to determine the full length cDNA sequence.

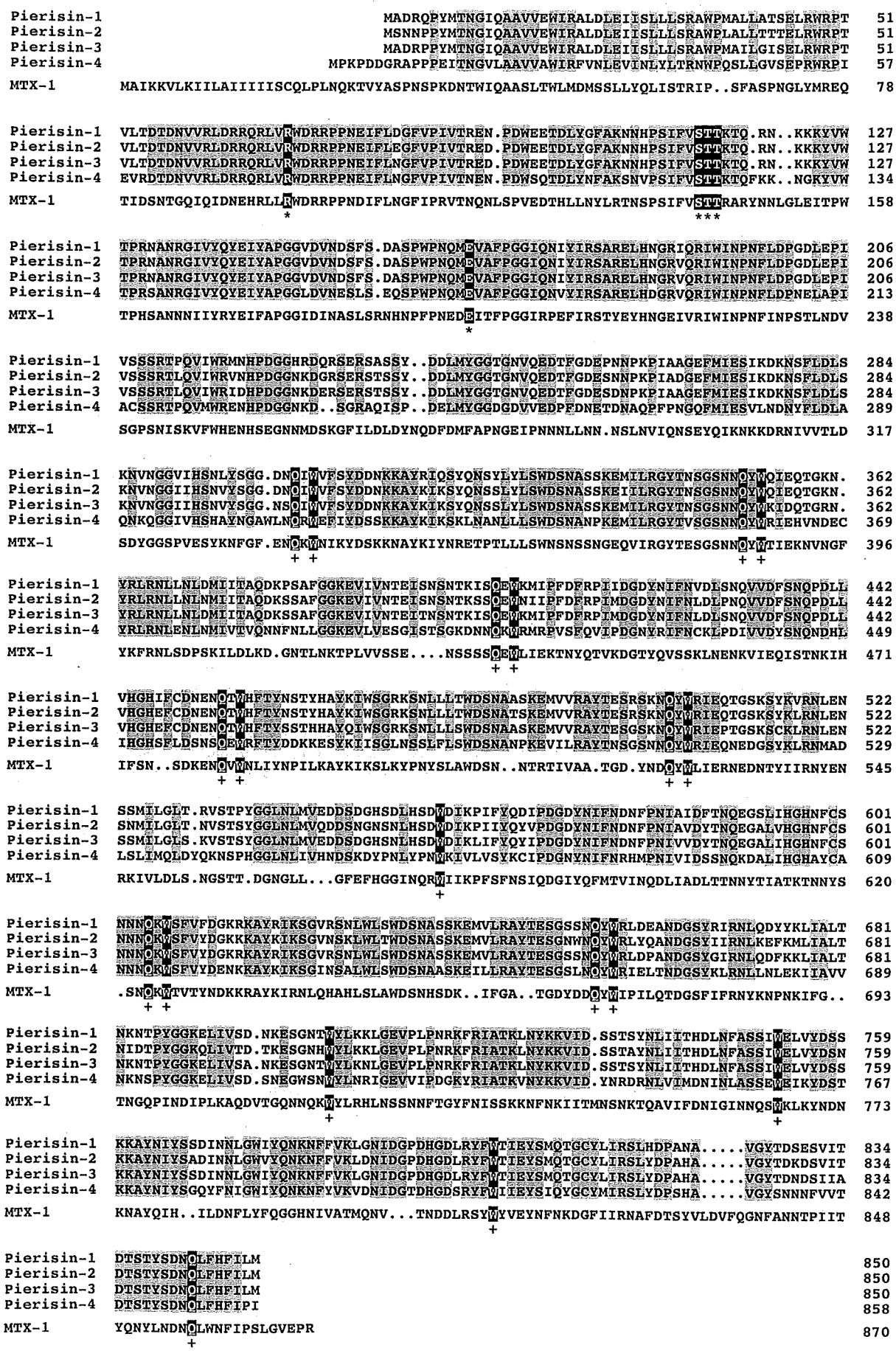
In the case of Pierisin-4, Ac_F1 primer and GeneRacer™ 3′ Primer were used for 3′ RACE and Ac_R3 primer and GeneRacer™ 5′ Nested Primer for 5′ RACE were used. Moreover, the full length of cDNA was obtained by the PCR using forward primer AcF_F and reverse primer AcF_R. The amplified PCR product was purified and sequenced in both directions as in Pierisin-3.

2.3. Construction of clones of the coding region of the Pierisin-3 and -4 genes

The coding region of the *Pierisin-3* gene, with 442 bp of 3′-flanking region, was amplified, and *Sal*I and *Eco*RI restriction sites were introduced into the 5′ and 3′ ends, using the primer pair Pm_Sal and Pm_Eco. The amplified fragment was digested with *Sal*I and *Eco*RI and inserted into the *Sal*I-*Eco*RI site of pMAL-p2x (New England Biolabs, Ipswich, MA, USA) in the opposite direction to the *tac* promoter. In the same way, the coding region of the *Pierisin-4* gene, with 355 bp of 3′-flanking regions, was amplified, and *Nhe*I and *Sac*I restriction sites were introduced into the 5′ and 3′ ends, using the primer pair Ac_Nhe and Ac_Sac. These clones were confirmed by sequencing the insert in both directions.

2.4. Purification and amino acid sequence determination of Pierisin-3 and -4

Because the chromatographic characteristics of *Pierisin-3* from *P. melete* were expected to be similar to *Pierisin-1*, purification of *Pierisin-3* from pupae of *P. melete* was carried out using the same method for *Pierisin-1* (Watanabe et al., 1998, 1999). The crude extracts from 112 pupae were added with ammonium sulfate to give 35% saturation and then centrifuged. The precipitates were dialyzed and applied to an anion-exchange DEAE-cellulose DE52 column (2.5×7 cm; Whatman, Kent, England). The fractions containing the cytotoxicity were separated by SDS-PAGE. About 98-kDa bands of these fractions were excised and digested in gel with trypsin. The digested peptides were separated by reverse-phase-HPLC with a Symmetry® C18 3.5 μm column (1.0×150 mm; Waters, Milford, MA, USA). Amino acid sequences of the isolated peptides were determined by a Procise 494 cLC Protein Sequencing System (Applied Biosystems).


Pierisin-1 MADRQPYMTNGIQAAVVEWIRALDLEIISLLLSRAWPMALLATSELRWRPT 51
Pierisin-2 MSNNPPYMTNGIQAAVVEWIRALDLEIISLLLSRAWPLALLTTTELRWRPT 51
Pierisin-3 MADRPPYMTNGIQAAVVEWIRALDLEIISLLLSRAWPMAILGISELRWRPT 51
Pierisin-4 MPKPDDGRAPPPEITNGVLAAVVAWIRFVNLEVINLYLTRNWPQSLLGVSEPRWRPI 57
MTX-1 MAIKKVLKIILAIIIIISCQLPLNQKTVYASPNSPKDNTWIQAASLTWLMDMSSLLYQLISTRIP..SFASPNGLYMREQ 78
Pierisin-1 VLTDTDNVVRLDRRQRLVRWDRRPPNEIFLDGFVPIVTREN.PDWEETDLYGFAKNNHPSIFVSTTKTQ.RN..KKKYVW 127
Pierisin-2 VLTDTDNVVRLDRRQRLVRWDRRPPNEIFLEGFVPIVTRED.PDWEETDLYGFAKNNHPSIFVSTTKTQ.RN..KKKYVW 127
Pierisin-3 VLTDTDNVVRLDRRQRLVRWDRRPPNEIFLNGFVPIVTRED.PDWEETDLYGFAKNNHPSIFVSTTKTQ.RN..KKKYVW 127
Pierisin-4 EVRDTDNVVRLDRRQRLVRWDRRPPNEIFLNGFVPIVTNEN.PDWSQTDLYNFAKSNVPSIFVSTTKTQFKK..NGKYVW 134
MTX-1 TIDSNTGQIQIDNEHRLLRWDRRPPNDIFLNGFIPRVTNQNLSPVEDTHLLNYLRTNSPSIFVSTTRARYNNLGLEITPW 158
Pierisin-1 TPRNANRGIVYQYEIYAPGGVDVNDSFS.DASPWPNQMEVAFPGGIQNIYIRSARELHNGRIQRIWINPNFLDPGDLEPI 206
Pierisin-2 TPRNANRGIVYQYEIYAPGGVDVNDSFS.DASPWPNQMEVAFPGGIQNIYIRSARELHNGRVQRIWINPNFLDPGDLEPI 206
Pierisin-3 TPRNANRGIVYQYEIYAPGGVDVNDSFS.DASPWPNQMEVAFPGGIQNIYIRSARELHNGRVQRIWINPNFLDPGDLEPI 206
Pierisin-4 TPRSANRGIVYQYEIYAPGGLDVNESLS.EQSPWPNQMEVAFPGGIQNVYIRSARELHDGRVQRIWINPNFLDPNELAPI 213
MTX-1 TPHSANNNIIYRYEIFAPGGIDINASLSRNHNPFPNEDEITFPGGIRPEFIRSTYEYHNGEIVRIWINPNFINPSTLNDV 238
Pierisin-1 VSSSRTPQVIWRMNHPDGGHRDQRSERSASSY..DDLMYGGTGNVQEDTFGDEPNNPKPIAAGEFMIESIKDKNSFLDLS 284
Pierisin-2 VSSSRTLQVIWRVNHPDGGNKDGRSERSTSSY..DDLMYGGTGNVQEDTFGDESNNPKPIADGEFMIESIKDKNSFLDLS 284
Pierisin-3 VSSSRTLQVIWRIDHPDGGNKDERSERSTSSY..DDLMYGGTGNVQEDTFGDESDNPKPIAAGEFMIESIKDKNSFLDLS 284
Pierisin-4 ACSSRTPQVMWRENHPDGGNKD..SGRAQISP..DELMYGGDGDVVEDPFDNETDNAQPFPNGQFMIESVLNDNYFLDLA 289
MTX-1 SGPSNISKVFWHENHSEGNNMDSKGFILDLDYNQDFDMFAPNGEIPNNNLLNN.NSLNVIQNSEYQIKNKKDRNIVVTLD 317
Pierisin-1 KNVNGGVIHSNLYSGG.DNQIWVFSYDDNKKAYRIQSYQNSYLYLSWDSNASSKEMILRGYTNSGSNNQYWQIEQTGKN. 362
Pierisin-2 KNVNGGIIHSNVYSGG.DNQIWVFSYDDNKKAYKIKSYQNSSLYLSWDSNASSKEIILRGYTNSGSNNQYWQIEQTGKN. 362
Pierisin-3 KNVNGGIIHSNVYSGG.NSQIWVFSYDDNKKAYKIKSYQNSSLYLSWDSNASSKEMILRGYTNSGSNNQYWKIDQTGRN. 362
Pierisin-4 QNKQGGIVHSHAYNGAWLNQRWEFIYDSSKKAYKIKSKLNANLLLSWDSNANPKEMILRGYTVSGSNNQYWRIEHVNDEC 369
MTX-1 SDYGGSPVESYKNFGF.ENQKWNIKYDSKKNAYKIYNRETPTLLLSWNSNSSNGEQVIRGYTESGSNNQYWTIEKNVNGF 396
Pierisin-1 YRLRNLLNLDMIITAQDKPSAFGGKEVIVNTEISNSNTKISQEWKMIPFDFRPIIDGDYNIFNVDLSNQVVDFSNQPDLL 442
Pierisin-2 YRLRNLLNLNMIITAQDKSSAFGGKEVIVNTEISNSNTKSSQEWNIIPFDFRPIMDGDYNIFNLDLPNQVVDFSNQPDLL 442
Pierisin-3 YRLRNLLNLDMIITAQDKSSAFGGKEVIVNTEITNSNTKISQEWKMIPFDFRPIMDGDYNIFNLDLSNQVVDFSNQPDLL 442
Pierisin-4 YRLRNLENLNMIVTVQNNFNLLGGKEVLVESGISTSGKDNNQKWRMRPVSFQVIPDGNYRIFNCKLPDIVVDYSNQNDHL 449
MTX-1 YKFRNLSDPSKILDLKD.GNTLNKTPLVVSSE....NSSSSQEWLIEKTNYQTVKDGTYQVSSKLNENKVIEQISTNKIH 471
Pierisin-1 VHGHIFCDNENQTWHFTYNSTYHAYKIWSGRKSNLLLTWDSNAASKEMVVRAYTESRSKNQYWRIEQTGSKSYKVRNLEN 522
Pierisin-2 VHGHEFCDNENQTWHFTYNSTYHAYKIWSGRKSNLLLTWDSNATSKEMVVRAYTESRSKNQYWRIEQTGSKSYKLRNLEN 522
Pierisin-3 VHGHEFCDNENQTWHFTYSSTHHAYQIWSGRKSNLLLSWDSNAASKEMVVRAYTESGSKNQYWRIEPTGSKSCKLRNLEN 522
Pierisin-4 IHGHSFLDSNSQEWRFTYDDKKESYKIISGLNSSLFLSWDSNANPKEVILRAYTNSGSNNQYWRIEQNEDGSYKLRNMAD 529
MTX-1 IFSN..SDKENQVWNLIYNPILKAYKIKSLKYPNYSLAWDSN..NTRTIVAA.TGD.YNDQYWLIERNEDNTYIIRNYEN 545
Pierisin-1 SSMILGLT.RVSTPYGGLNLMVEDDSDGHSDLHSDWDIKPIFYQDIPDGDYNIFNDNFPNIAIDFTNQEGSLIHGHNFCS 601
Pierisin-2 SNMILGLT.NVSTSYGGLNLMVQDDSNGNSNLHSDWDIKPIIYQYVPDGDYNIFNDNFPNIAVDYTNQEGALVHGHNFCS 601
Pierisin-3 SSMILGLS.KVSTSYGGLNLMVEDDSDGHSNLHSDWDIKLIFYQYIPDGDYNIFNDNFPNIVVDYTNQEGALIHGHNFCS 601
Pierisin-4 LSLIMQLDYQKNSPHGGLNLIVHNDSKDYPNLYPNWKIVLVSYKCIPDGNYNIFNRHMPNIVIDSSNQKDALIHGHAYCA 609
MTX-1 RKIVLDLS.NGSTT.DGNGLL...GFEFHGGINQRWIIKPFSFNSIQDGIYQFMTVINQDLIADLTTNNYTIATKTNNYS 620
Pierisin-1 NNNQKWSFVFDGKRKAYRIKSGVRSNLWLSWDSNASSKEMVLRAYTESGSSNQYWRLDEANDGSYRIRNLQDYYKLIALT 681
Pierisin-2 NNNQKWSFVYDGKKKAYKIKSGVNSKLWLTWDSNASSKEMVLRAYTESGNWNQYWRLYQANDGSYIIRNLKEFKMLIALT 681
Pierisin-3 NNNQKWSFVYDGKRKAYRIKSGVRSNLWLSWDSNASSKEMVLRAYTESGSSNQYWRLDPANDGSYGIRNLQDFKKLIALT 681
Pierisin-4 NNNQKWSFVYDENKKAYKIKSGINSALWLSWDSNAASKEILLRAYTESGSLNQYWRIELTNDGSYKLRNLLNLEKIIAVV 689
MTX-1 .SNQKWTVTYNDKKRAYKIRNLQHAHLSLAWDSNHSDK..IFGA..TGDYDDQYWIPILQTDGSFIFRNYKNPNKIFG.. 693
Pierisin-1 NKNTPYGGKELIVSD.NKESGNTWYLKKLGEVPLPNRKFRIATKLNYKKVID.SSTSYNLITTHDLNFASSIWELVYDSS 759
Pierisin-2 NIDTPYGGKQLIVTD.TKESGNHWYLKKLGEVPLPNRKFRIATKLNYKKVID.SSTAYNLITTHDLNFASSIWELVYDSN 759
Pierisin-3 NKNTPYGGKELIVSA.NKESGNTWYLKNLGEVPLPNRKFRIATKLNYKKVID.SSTSYNLITTHDLNFASSIWELVYDSS 759
Pierisin-4 NKNSPYGGKELIVSD.SNEGWSNWYLNRIGEVVIPDGKYRIATKVNYKKVID.YNRDRNLVIMDNINLASSEWEIKYDST 767
MTX-1 TNGQPINDIPLKAQDVTGQNNQKWYLRHLNSSNNFTGYFNISSKKNFNKIITMNSNKTQAVIFDNIGINNQSWKLKYNDN 773
Pierisin-1 KKAYNIYSSDINNLGWIYQNKNFFVKLGNIDGPDHGDLRYFWTIEYSMQTGCYLIRSLHDPANA.....VGYTDSESVIT 834
Pierisin-2 KKAYNIVSADINNLGWVYQNKNFFVKLDNIDGPDHGDLRYFWTIEYSMQTGCYLIRSLYDPAHA.....VGYTDKDSVIT 834
Pierisin-3 KKAYNIYSSDINNLGWIYQNKNFFVKLGNIDGPDHGDLRYFWTIEYSMQTGCYLIRSLYDPAHA.....VGYTDNDSIIA 834
Pierisin-4 KKAYNIYSGQYFNIGWIYQNKNFYVKVDNIDGTDHGDSRYFWIIEYSIQYGCYMIRSLYDPSHA.....VGYSNNNFVVT 842
MTX-1 KNAYQIH..ILDNFLYFQGGHNIVATMQNV...TNDDLRSYWYVEYNFNKDGFIIRNAFDTSYVLDVFQGNFANNTPIIT 848
Pierisin-1 DTSTYSDNQLFHFILM 850
Pierisin-2 DTSTYSDNQLFHFILM 850
Pierisin-3 DTSTYSDNQLFHFILM 850
Pierisin-4 DTSTYSDNQLFHFIPI 858
MTX-1 YQNYLNDNQLWNFIPSLGVEPR 870

Pierisin-4 was isolated from the 19 pupae of *A. crataegi* using a method similar to that for *Pierisin-3*, except for the purification with Phenyl–Sepharose CL-4B hydrophobic interaction column (1.5×6 cm; GE Healthcare) after purification with DEAE-cellulose DE52 column.

2.5. In vitro transcription and translation of Pierisin-3 and -4

To express *Pierisin-3* and *-4* genes, *in vitro* transcription and translation were carried out as previously described (Watanabe et al., 1999). To obtain cDNA fragments containing a T7 promoter sequence at the 5′ end of the coding region, the primer pairs Pm_T7 and Pm_R3 for *Pierisin-3*, Ac_T7 and Ac_R2 for *Pierisin-4*, were used for the PCR. The amplified PCR products were transcribed using MEGAscript® T7 Kit (Ambion, Austin, TX, USA) and translated using rabbit reticulocyte lysate (Retic Lysate IVT™, Ambion). The translation efficiencies were confirmed by autoradiography of SDS-PAGE gels of [^{35}S]methionine incorporated products.

2.6. Site-directed mutagenesis

A DNA fragment containing a sequence alteration at the desired position was amplified from an intact cDNA subclone of Pierisin-3 and -4 with overlap PCR technique described previously (Matsushima-Hibiya et al., 2003; Nakano et al., 2006) To obtain overlapped 5′- and 3′- fragments of Pierisin-3, two separate PCR reactions were carried out using a 5′ primer Pm_T7 and 3′ primers PmD_R or PmQ_R for 5′-fragment, and 5′ primers PmD_F or PmQ_F and 3′ primer Pm_R3 for 3′-fragment. These mutations are that the glutamic acid residue at position 165 was replaced with aspartic acid or glutamine. The 5′ and 3′ fragments were mixed together and used as the template for a second round PCR to obtain full length mutated DNA fragments.

In the case of Pierisin-4, two separate PCR reactions were carried out using a 5′ primer Ac_T7 and 3′ primers AcD_R or AcQ_R for 5′-fragment, and 5′ primers AcD_F or AcQ_F and 3′ primer Ac_R2 for 3′-fragment. These mutations are that the glutamic acid residue at position 172 was replaced with aspartic acid or glutamine. The 5′ and 3′ fragments were mixed together and used as the template for a second round PCR to obtain full length mutated DNA fragments. The appropriate recombination was confirmed by DNA sequencing, and the resultant DNA was used as the template for the *in vitro* expression system described here.

2.7. Analysis of cytotoxicity and DNA ADP-ribosylating activity of in vitro expressed proteins

The cytotoxicity of the *in vitro* expressed proteins against HeLa cells and TMK-1 cells was examined by using WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (Dojindo Laboratory, Kumamoto, Japan)] cell proliferation assay as described previously (Watanabe et al., 1998; Kono et al., 1999). Apoptotic cells were stained with TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay using *in situ* Apoptosis Detection Kit (Takara Bio, Otsu, Japan) and Hoechst 33342 after the formalin fixation, and analyzed with a fluorescence microscope. DNA ADP-ribosylating activity was determined by HPLC analysis as previously described (Matsumoto et al., 2008; Nakano et al., 2006).

To confirm the structure of the ADP-ribosylated DNA, the reaction of the *in vitro* expressed proteins, DNA and β-NAD was performed and the detection of the reaction products was carried out as described previously

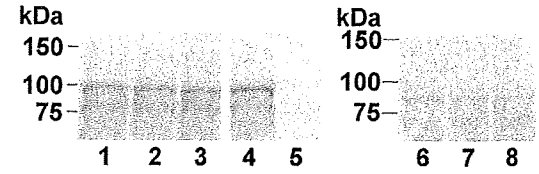


Fig. 2. Detection of *in vitro* expressed [^{35}S]methionine-labeled Pierisin-3 and -4, and their mutated proteins by SDS-PAGE. Five-microliters each of *in vitro* expressed lysates were loaded; lane 1, Pierisin-3 wild type; lane 2, Pierisin-3 E165D; lane 3, Pierisin-3 E165Q; lane 4, Pierisin-1 wild type; lane 5, lysate without template RNA; lane 6, Pierisin-4 wild type; lane 7, Pierisin-4 E172D; lane 8, Pierisin-4 E172Q.

(Nakano et al., 2006). Fifty microliters of the *in vitro* expressed proteins were incubated with 0.1 mg of calf thymus DNA, 2 μmol β-NAD in 1 ml of reaction buffer for 2 h at 37 °C. The DNA was recovered, digested by treatment with micrococcal nuclease and phosphodiesterase II, and treated with bacterial alkaline phosphatase. The reaction products were analyzed by HPLC with a LC-10A system (Shimadzu, Kyoto, Japan) armed with a SPD 10Avp photodiode array detector (Shimadzu) and a Develosil RPAQUEOUS column (4.6×250 mm; Nomura Chemical, Seto, Japan). These products were also analyzed by electrospray ionization-mass spectrometry using ZQ 2000 instrument (Micromass, Manchester, England) armed with an HP 1000 HPLC system (Hewlett-Packard, Palo Alto, USA).

3. Results

3.1. Identification of Pierisin-3 in P. melete

To obtain partial cDNA fragments of Pierisin-3, degenerate primers were designed from the conserved catalytic site motif of ADP-ribosylating protein. Total RNA was extracted from the fifth instar larva of *P. melete*. About 300-bp PCR products were cloned and sequenced to design the primer for 5′ and 3′ RACE. In addition, the PCR amplified Pierisin-3 coding sequence was inserted into the pMAL-p2x vector. The basal-level expression of the *Pierisin-3* gene would be highly toxic to *E. coli*, as expected from our studies of Pierisin-1 and -2, and we inserted the PCR product in the vector in an opposite direction to the *tac* promoter. The positive clones were sequenced and confirmed that the two of three clones had no nonsynonymous substitution. One of these two clones had less synonymous substitutions (G1516A, T2137C, T2176A). Thus, we concluded that the clone is an intact clone at the amino acid level, and it was employed for the Pierisin-3 expression *in vitro*.

The complete cDNA sequence of Pierisin-3 consists of 3347 bp associated with a putative initiator codon ATG at position 95–97, a stop codon at position 2645–2647 and a polyadenylational signal at position 3328–3333 close to the poly A sequence (Suppl. Fig. 1A). The ORF encodes 850 amino acids with a calculated molecular weight of 97,598. From the deduced amino acid sequence, Pierisin-3 is 93% similar to Pierisin-1 and 91% to Pierisin-2 (Fig. 1). Furthermore, the partial peptide sequences of Pierisin-3 purified from pupae in the same process as Pierisin-1 were identical to the deduced amino acid sequence of ORF. The identified three internal amino acid sequences, Glu91-Gly102, Gly135-Ala144 and Ser816-Gly825, are shown in Suppl. Fig. 1A (bold type with underlines). The essential regions for ADP-ribosyltransferase activity (Masignani et al., 2000; Domenighini

Fig. 1. Alignment of the deduced amino acid sequences of Pierisin-3 and -4. These sequences were aligned together with that of Pierisin-1 (DDBJ accession number AB030305), Pierisin-2 (DDBJ accession number AB037676) and MTX (DDBJ accession number M60446). Identical amino acid residues in Pierisins are highlighted in gray. The conserved arginine (Region I), Ser–Thr–Thr motif (Region II), and glutamic acid residues (Region III) for ADP-ribosylating activity and the QXW motifs for receptor binding are boxed in black with asterisks (*) or plus signs (+). Sequence alignment between Pierisins and MTX was decided referring to Watanabe et al. (1999) and Carpusca et al. (2006).

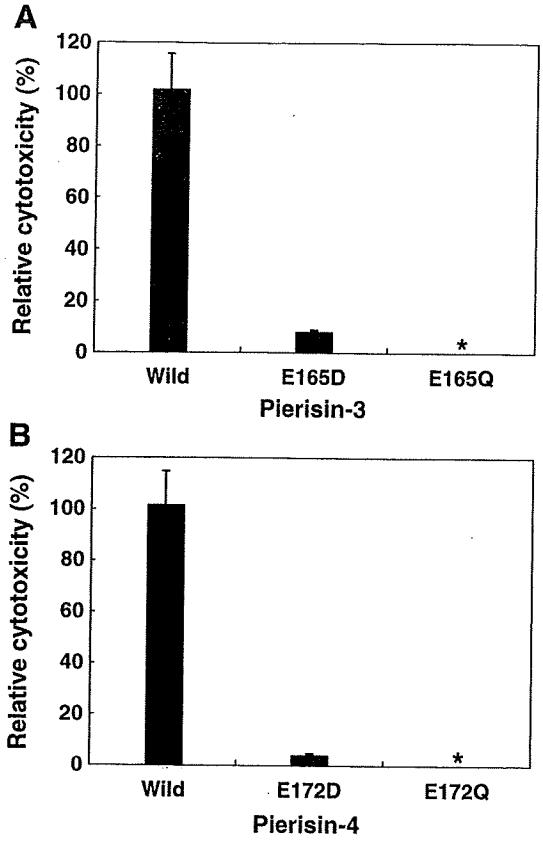


Fig. 3. Cytotoxicities of wild type and mutated Pierisin-3 or -4 against HeLa cells. Cells were incubated with a final concentration of 1% of rabbit reticulocyte lysate containing *in vitro* expressed Pierisin-3 (A) and Pierisin-4 (B) for 48 h at 37 °C and subjected to WST-1 cell proliferation assay. The cytotoxicity of mutated proteins relative to the wild type proteins is indicated. Asterisks show cytotoxicity not detected in this assay. Data are means of three independent experiments. Error bars represent standard deviations.

and Rappuoli, 1996), and ricin B chain-like QXW motif that binds glycosphingolipid receptors, such as Gb3 and Gb4, are conserved, as shown in Fig. 1 (Matsushima-Hibiya et al., 2003).

3.2. Identification of Pierisin-4 in A. crataegi

cDNA sequence and subcloning of Pierisin-4 were determined using the same method as that for the Pierisin-3. Three positive clones were sequenced and confirmed that all the clones had no synonymous nor nonsynonymous substitution. Thus, we concluded that these clones are intact clones, and one of the clones was employed for the Pierisin-4 expression *in vitro*. Pierisin-4 consists of 3319 bp associated with a putative initiator codon at position 77–79, a stop codon at position 2651–2653 and a polyadenylational signal at position 3293–3298 close to the poly A sequence l (Suppl. Fig. 1B). The ORF encodes 858 amino acids with a calculated molecular weight of 99,204. Amino acid alignment showed that Pierisin-4 is 64% similar to Pierisin-1, -2 and -3 (Fig. 1). Deduced amino acid sequence entirely encodes the partial peptide sequences of purified Pierisin-4 from pupae of *A. crataegi*. The essential regions for ADP-ribosyltransferase activity (Masignani et al., 2000; Domenighini and Rappuoli, 1996), and ricin B chain-like QXW motif are also conserved, as shown in Fig. 1 (Domenighini and Rappuoli, 1996).

3.3. Cytotoxicity and apoptosis-inducing activity of in vitro expressed Pierisin-3 and -4

Pierisin-3 and -4 were expressed by rabbit reticulocyte lysate, since it is impossible to express Pierisin-3 and -4 in *E. coli*, probably due to the high toxicity of the Pierisins. The translation efficiency and molecular mass of the *in vitro* expressed Pierisin-3 and their mutated products were confirmed by SDS-PAGE of [^{35}S]methionine-labeled proteins, and those of the *in vitro* expressed Pierisin-4 and their mutated products were also confirmed in the similar way (Fig. 2). To examine whether Pierisin-3 and -4 have cytotoxicity and apoptosis-inducing activity against mammalian cells, HeLa and TMK-1 cells were treated with *in vitro* expressed Pierisin-3 and -4. Both expressed proteins showed cytotoxicity (Fig. 3), and similar cytotoxicity of both expressed proteins was observed in TMK-1 cells (data not shown). Furthermore, the cytotoxicity levels of the *in vitro* expressed Pierisin-3 and -4 were almost equivalent to the *in vitro* expressed Pierisin-1. Both expressed proteins induced chromatin condensation and nuclear fragmentation indicating apoptotic cell death, in HeLa cells, as observed by fluorescence microscopy (Fig. 4). Glutamic acid residues, Glu165 in Pierisin-3 and Glu172 in Pierisin-4 (Fig. 1, boxed in black),

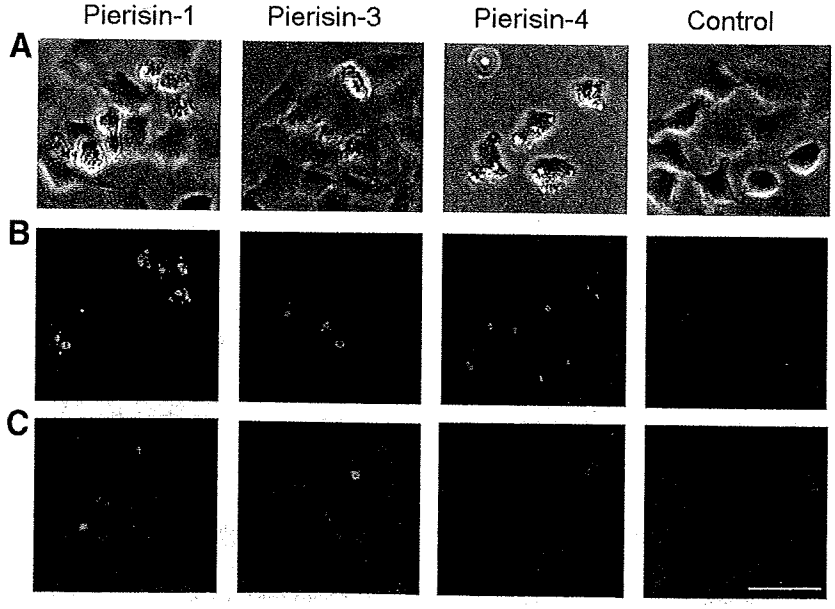


Fig. 4. Morphological analysis of HeLa cells treated with the *in vitro* expressed Pierisin-3 and -4 proteins. The cells were treated for 24 h with 1% of *in vitro* expressed proteins or 1 ng/ml purified Pierisin-1. (A) Phase-contrast micrographs for morphological changes analysis. (B) Fluorescence micrograph of TUNEL assays for detection of DNA fragmentation undergoing apoptotic cells. The TUNEL-positive cells were labeled by FITC. (C) Fluorescence micrograph of Hoechst 33342-stained cells. Bar = 50 μm.

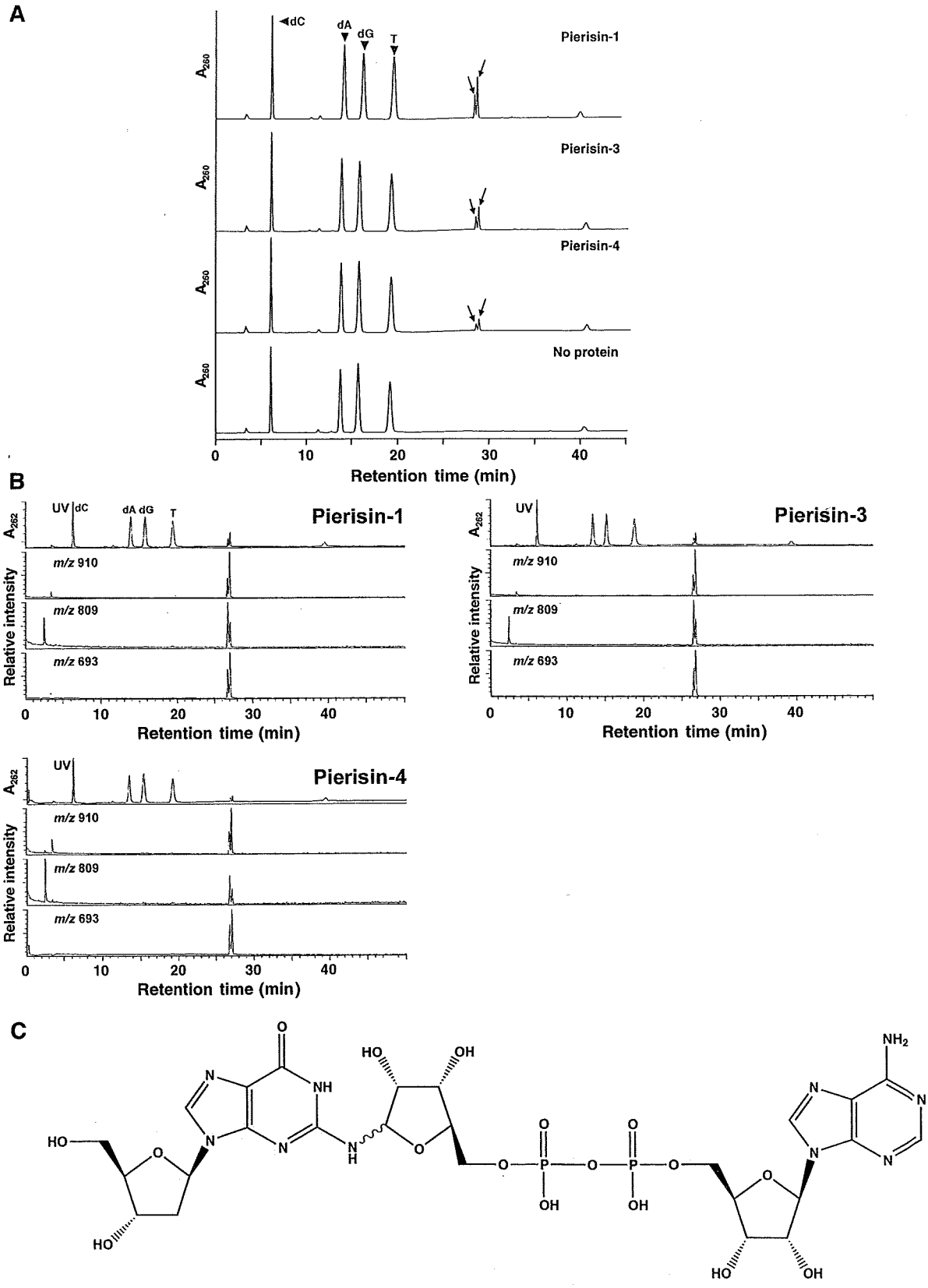


Fig. 5. Analysis of reaction products formed from DNA treated with Pierisins and β-NAD. HPLC elution patterns of hydrolysate of DNA incubated with purified Pierisin-1, *in vitro* expressed Pierisin-3, -4, or no protein. These DNA samples were enzymatically hydrolyzed to deoxyribonucleosides (dA, dC, dG and T) and injected into a Develosil RPAQUEOUS column. The UV absorbance of the eluate was monitored at 260 nm. Arrows indicate the peaks coincident with N^2-(ADP-ribos-1-yl)-2'-deoxyguanosine. Arrowheads indicate deoxycytidine (dC), deoxyadenosine (dA), deoxyguanosine (dG) and thymidine (T). (B) Liquid chromatography-electrospray ionization-MS analysis of reaction products formed from DNA and Pierisins. The HPLC profiles (UV) and the ion chromatograms (*m/z* 910, 809 and 693) of DNA hydrolysate are shown. (C) Chemical structure of N^2-(ADP-ribos-1-yl)-2'-deoxyguanosine.

were predicted to be the catalytic center for the ADP-ribosylating activity. As in the cases of Pierisin-1 and -2, site-directed mutation of Pierisin-3 and -4 by replacements of these glutamic acid by aspartic acid and glutamine resulted in markedly reduced or entirely lost cytotoxicity to HeLa cells, respectively (Fig. 3). Induction of nuclear condensation and chromatin fragmentation by mutated proteins were also reduced concurrently (data not shown).

3.4. Formation of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine by in vitro expressed Pierisin-3 and -4

In order to investigate DNA ADP-ribosylating activity of the *in vitro* expressed Pierisin-3, this protein was incubated with calf thymus DNA and β-NAD, then the DNA was enzymatically digested, and the formation of the ADP-ribosylated DNA adducts was analyzed by HPLC. The chromatograms for the Pierisin-3 showed that the area of the dG peak decreased approximately 10%, while the area of dC, dA, and T peaks remained unchanged. In addition, two peaks corresponding to the ADP-ribosylated dG were newly detected at retention times of 28.5 and 29.0 min, as in Pierisin-1 (Fig. 5A). The λ_{max} of UV spectra of the newly detected peak fractions were 256 nm, as in the case for Pierisin-1. The UV spectra of these two peaks were exactly the same as those for the N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (data not shown). The area of these two peaks was reduced with site-directed mutation of Pierisin-3 (data not shown). In addition, the LC-ESI-MS analysis showed the reaction products in this peak fraction to have a molecular ion peak at *m/z* 809, an ion peak at *m/z* 693 arising from the loss of a deoxyribose moiety, and an ion peak at *m/z* 910 corresponding to a triethylamine addition, derived from HPLC eluent, to the parent mass at *m/z* 809 (Fig. 5B,C). The results of these analyses showed that N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine was the reaction product generated through the reaction of the calf thymus DNA and the β-NAD with *in vitro* expressed Pierisin-3. Similarly, in the case of the Pierisin-4, the area of the dG peak decreased approximately 8%, and the newly detected two peaks at retention times of 28.5 and 29.0 min were shown to be N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine by the UV spectra and the LC-ESI-MS analysis. (Fig. 5A,B).

4. Discussion

The protein extract from *P. melete* and *A. crataegi* exhibited cytotoxicity against HeLa cells and DNA ADP-ribosylating activity (Matsumoto et al., 2008). In the present study, we cloned the cDNA of apoptosis-inducing protein from *P. melete*, named Pierisin-3, and *A. crataegi*, named Pierisin-4. Deduced amino acid sequence of Pierisin-3 encodes 850 amino acids, and the *in vitro* expressed Pierisin-3 exhibited cytotoxicity against HeLa and TMK-1 cells. In addition, Pierisin-3 catalyzed ADP-ribosylation of 2′-deoxyguanosine residue in DNA to form N^2-(ADP-ribos-1-ly)-2′-deoxyguanosine. Pierisin-4 encodes an 858 amino acid, and Pierisin-4 protein also exhibited cytotoxicity and DNA ADP-ribosylating activity similar to the Pierisin-3 from *P. melete*.

In this study, the *in vitro* expressed Pierisin-3 and -4 were shown to have cytotoxicity and DNA ADP-ribosylating activity similar to Pierisin-1. The three highly conserved regions in the ADP-ribosyltranferase (Masignani et al., 2000; Domenighini and Rappuoli, 1996) and their surrounding amino acid sequence hold high homology in Pierisin-3 and -4 (Fig. 1). The arginine residue (Region I) is thought to maintain the structure of the reaction pocket, the Ser–Thr–Thr/Ser motif (Region II) is considered to construct a β-strand-α-helix structure, and the glutamic acid residue (Region III) serves as NAD-binding site.

Moreover, the C-terminal regions of Pierisin-3 and -4 consist of a ricin B chain-like domains including QXW motif (Fig. 1). These domains possess receptor-binding ability, responsible for incorporating Pierisin-1 into cells (Matsushima-Hibiya et al., 2003). Thus, Pierisin-3 and -4 would have the same abilities of receptor recognition and incorporation in mammalian cancer cell lines.

Pierisin-1, -2, -3 and -4 have similar cytotoxicity and DNA ADP-ribosylating activity. However, Pierisin-1, -2 and -3 slightly differ from Pierisin-4 in their amino acid sequence. Pierisin-1, -2 and -3 have 91% to 93% amino acid identity, while Pierisin-4 shares 64% amino acid identity not only with Pierisin-1 but also with Pierisin-2 and -3. Differences are also observed in chromatographic patterns of these proteins. On the DEAE-cellulose anion-exchange column chromatography, Pierisin-1, -2 and -3 were eluted with 40–70 mM NaCl, while Pierisin-4 was eluted with 12-35 mM NaCl (data not shown). Furthermore, on the Phenyl–Sepharose hydrophobic interaction column chromatography, Pierisin-4 was eluted with higher concentrations of ammonium sulfate (9–10%) compared to Pierisin-1 and -2 (≤1%). These results suggested that the isoelectric point and hydrophobicity of Pierisin-4 are different to some extent from those of Pierisin-1, -2 and -3, and these differences may reflect the genetic distance between the genera *Pieris* and the *Aporia*. Interestingly, MTX shares 32% amino acid identity not only with Pierisin-1, -2 and -3, but also with Pierisin-4. This suggests that the conserved regions among MTX and those four Pierisins should be the most important regions for the ADP-ribosylation/receptor binding, such as the ADP-ribosyltransferase to construct the NAD-binding core, and ricin B chain-like QXW motif for the receptor binding (Fig. 1, boxed in black). The regions with low homology between the Pierisins and the MTX might contain motifs for the recognition and targeting of DNA or protein.

In this study, we cloned cDNAs of Pierisin-3 and -4 from the total RNA of a day 1 fifth instar larva in *P. melete* and *A. crataegi*, respectively, because the mRNA of Pierisin-1 is highly expressed in *P. rapae* at this stage. Since the amounts of Pierisin-1 are at maximum from the fifth instar larva to early pupa, Pierisin-1 might play an important role in pupal metamorphosis (Watanabe et al., 2004). Pierisin-2, -3 and -4 have similar properties to Pierisin-1, and these proteins are also considered to play some role in the developmental stages, because of the larvae and the pupae of *P. brassicae*, *P. melete* and *A. crataegi* exhibiting higher cytotoxicity than the adults (Matsumoto et al., 2008). It is also possible that these proteins are protective against invading organisms, such as the parasitic wasps. Understanding the role of the Pierisins and investigation of the origin of these genes should provide information about the biological significance of the Pierisins in Pierina butterflies.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbpb.2009.07.007.

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RESEARCH ARTICLE

Effect of polymerized toner on rat lung in chronic inhalation study

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Abstract

In order to evaluate the chronic effect of polymerized toner particles on the lung, inflammation- and fibrosis-related genes were analyzed and 8-hydroxydeoxyguanosine (8-OHdG) was examined by using the lung tissue of rats subjected to 24 months of toner inhalation exposure. Wistar female rats were divided into four groups (5 weeks old, 30 rats in each): the high concentration exposure group (16.3 ± 0.6 mg/m^3), the medium concentration exposure group (4.4 ± 0.3 mg/m^3), the low concentration exposure group (1.6 ± 0.2 mg/m^3), and the control group (clean air). The material used was black toner, and its aerodynamic diameter in the exposure chamber was 3.0 μm. The rats were exposed to the material for 24 months (6 hours/day, 5 days/week) and dissected after the exposure period. RNA was extracted from one lung and the gene expression related to inflammation and fibrosis. Matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-2 (TIMP-2), and type I collagen were analyzed according to the ratio of each gene/β-actin. Also, 8-OHdG level in the lung tissue was measured by HPLC with an electrochemical detector. Small fibrotic foci were found in the toner exposed groups; however, progressive or irreversible fibrosis was not found. The incidence of small fibrotic foci and cell aggregation increased in a dose-dependent manner. There were no significant differences of expression of MMP-2, TIMP-2, and type I collagen between the control group and each exposed group. Lung tumors did not develop in each group. A significant production of 8-OHdG was not observed in the toner exposed groups. In conclusion, toner produced by polymerization was not associated with evidence of carcinogenesis in this experiment.

Keywords: *Polymerised toner; inhalation; rat; lung; fibrosis*

Introduction

Since toner for copiers and printers is widely used in many countries, occupational exposure may occur not only in production facilities but also in offices. Case reports on pneumoconiosis and hypersensitivity pneumonitis caused by toner have been published (Armbruster et al., 1996; Gallardo et al., 1994), and recently, fine particle generation by printers and allergy symptoms in printer users have been reported (Crijins et al., 1987). Thus, the biological effect of toner has become a concern. However, the causal relationship between toner and its effect on health is unknown.

There have been developments within toner manufacturing, and in addition to the conventional grinding process, the polymerization process has recently been used more widely, and its demand is increasing (Nanya et al., 2004). Differences in the toner manufacturing process may lead to differences in physicochemical properties of toner particles, thereby bringing about different biological effects. It is reported that the toner particle produced by polymerization has a smaller diameter, a narrower size range, and a smoother surface than those of the toner particle produced by grinding. Studies have reported that a finer particle size enhances biological response (Oberdorster et al., 2005) and toner surface properties influence its surface activity; thus, the biological effect is assumed to be different between ground and polymerized particles.

Pulmonary fibrosis and cancer are major pathologies caused by a chronic effect of particle exposure, and are typical pathological endpoints for hazard evaluation (Morimoto et al., 2005a, 2005b). In fibrosis, a major prerequisite lesion

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is an excess deposition of matrix, i.e. collagen, in association with an imbalance between the production and degradation systems of collagen (Seyer et al., 1976; Woessner, 1991), and the increased imbalance is assumed to lead to fibrosis. Matrix metalloproteinases (MMPs) for collagen production are associated with the former, and the balance between MMPs for collagen metabolism and tissue inhibitors of metalloproteinase (TIMPs) to suppress collagen metabolism is associated with the latter. In regard to cancer, DNA damage caused by oxidative stress may result in gene mutation and eventually in carcinogenesis. Studies have reported that a marker of typical oxidative damage of DNA, 8-hydroxydeoxyguanosine (8-OHdG), is associated with carcinogenesis of various organs including the lung, and is a biomarker of lung cancer (Farinati et al., 1998; Kasai & Nishimura, 1984).

In order to evaluate the biological effect of polymerized toner, we examined the matrix system and production of 8-OHdG in the lung tissue of rats exposed to air containing polymerized toner for 24 months.

Materials and methods

Materials

The polymerized toner used in the present study was Imagio Toner Type 26 Black (Ricoh Company, Ltd.). The composition of this toner is >80% polyester resin, 1–10% wax, 1–10% silica, and 1–10% carbon black, as listed by the manufacturer. Scanning electron micrographs of toner particles in an exposure chamber are shown in Figure 1. The particle size distribution was determined by an Andersen cascade impactor (AN-200; Sibata Sci. Tech. Co., Japan). The mass median aerodynamic diameter and geometric standard deviation were 3.0 ± 1.7 μm.

Inhalation system (Figure 2)

The inhalation system consisted of a dust generator, exposure chambers (volume 0.57 m^3), and gas–liquid–solid separators, as described by Tanaka et al. (1983). A continuous fluidized bed was used as the dust generator. Toner was fed from the dust generator into the exposure chamber with the feed rate being controlled by the rotating speed of the screw feeder and the flow rate for fluidization. The toner aerosol concentration in the exposure chamber was measured daily by the isokinetic suction of air though a glass fiber filter beside the chamber. The glass fiber filter was weighed before and after the measurements in accordance with the standard method in JIS-Z8808, and the measured daily exposure weight concentration was determined as mg/m^3.

During exposure rats were kept in this chamber, and it was cleaned at the end of each exposure day. The rats were fed pellet-type food, and their weight was measured once a week.

Animals

One hundred twenty specific pathogen-free female Wistar rats, 5 weeks of age, were purchased from Kyudo (Kumamoto, Japan). These rats were divided into four groups: high exposure, medium exposure, low exposure, and a control group. Each group totaled 30 rats, which were then divided into 10 and 20 rats for the 12 and 24 months inhalation groups, respectively. The exposure groups were subjected to inhalation exposure for 6 hours a day, 5 days a week for up to 2 years (Tanaka et al., 1983). Daily average exposure concentrations and standard deviations in the low, medium, and high exposure groups were 1.6 + 0.2, 4.4 + 0.3, and 16.3 + 0.6 mg/m^3, respectively. The control rats were exposed to only clean air in a same-sized chamber located in the same air-conditioned room.

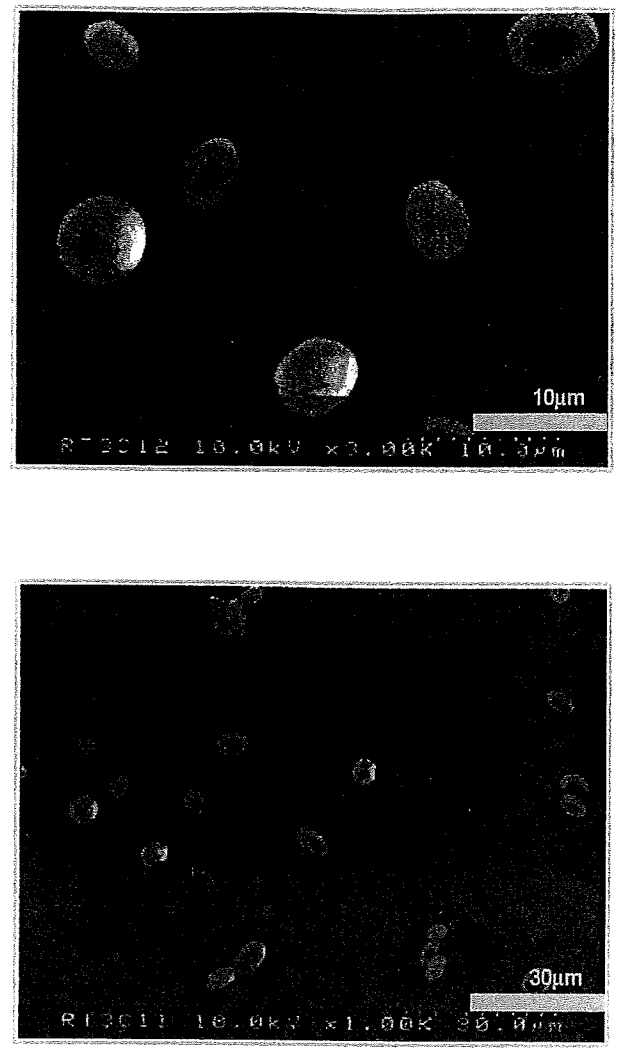

Figure 1. Scanning electron micrographs of toner particles in exposure chamber.

After 1 or 2 years of inhalation exposure, the rats were injected intraperitoneally with a fatal overdose of phenobarbital. The rats were handled according to the guidelines described in the Japanese *Guide for the care and use of laboratory animals* as approved by the Animal Care and Use Committee, University of Occupational and Environmental Health, Japan.

Periodic tests for microorganisms such as viruses, bacteria, and mycoplasma were carried out using sentinel rats

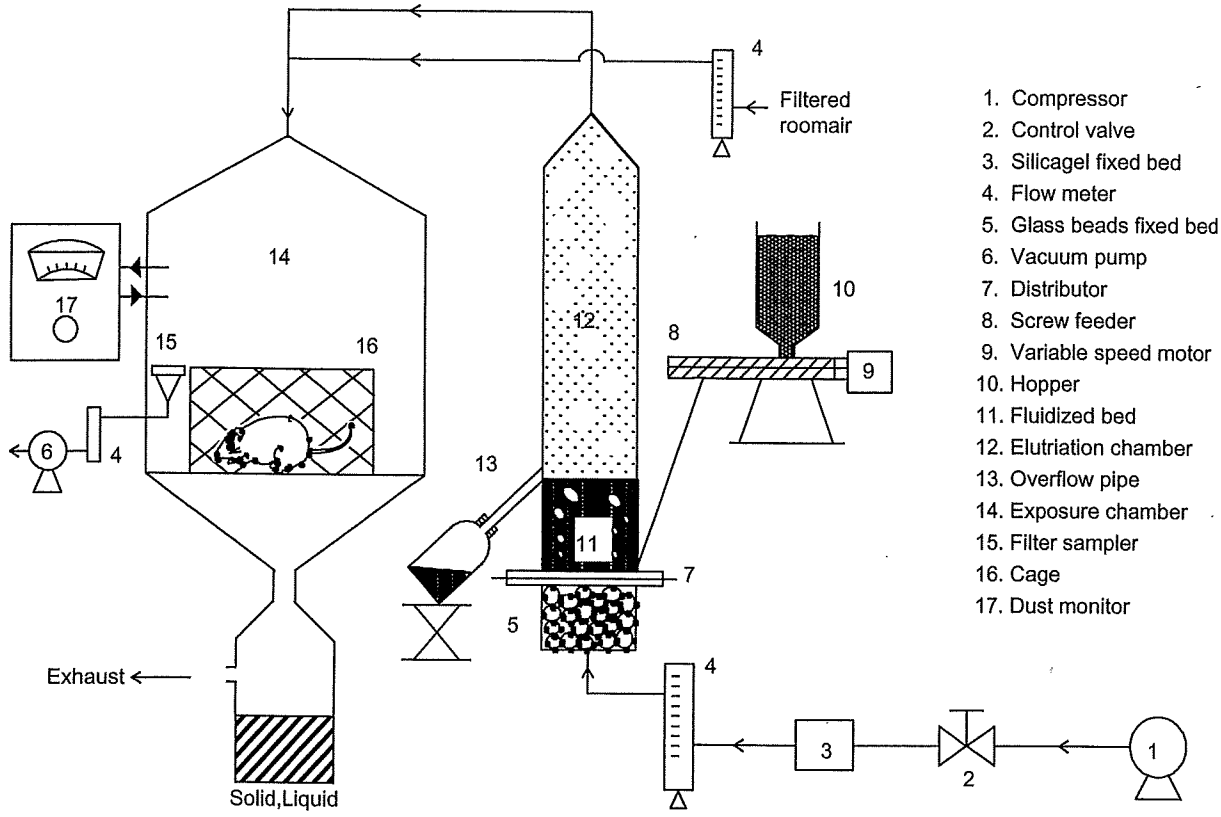

Figure 2. Schematic diagram of dust exposure system.

during the experiment, and pathogens were not observed in the rats.

Preparation of RNA, cDNA synthesis, and polymerase chain reaction

Total RNA from the lung was prepared in the presence of guanidium thiocyanate (Chomczynski & Sacchi, 1987). Single-strand cDNA was synthesized with Moloney murine leukemia virus-derived reverse transcriptase (Perkin Elmer, Norwalk, CT) using 500 ng of total RNA. An equal amount of cDNA from each sample was amplified by specific primers for each gene. Amplification was performed with a thermocycler (Astech, Japan) under the following conditions: denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 2 minutes for the target and β-actin genes.

The fragments amplified by polymerase chain reaction (PCR) were detected by electrophoresis on 2% agarose gel. The PCR products were resolved using gel electrophoresis and visualized by ethidium bromide staining. The gel was photographed with Polaroid type 665 positive/negative film (Polaroid Corp., Cambridge, MA) under ultraviolet light at identical exposure and development times. The bands from the positive film were scanned, and the density of MMP-2, TIMP-2, and type I collagen PCR product was measured using National Institute of Health (NIH) Image 1.55 software (written by Wane Rasband at NIH, Bethesda, MD).

Analysis of 8-OHdG in lung DNA

The 8-OHdG level in the DNA of lungs exposed to the toner was measured according to previously described methods (Kawai et al., 2007). Briefly, cellular DNA from rat lungs (n = 8) was isolated using a DNA extractor WB Kit (Wako, Japan). The isolated DNA was digested with nuclease P1 (Yamasa, Japan) and alkaline phosphatase to obtain 8-OHdG in the nucleoside form. The nucleoside solution was filtered with Ultrafree-Probind (Millipore, Billerica, MA) and was injected into a high-performance liquid chromatography (HPLC) column (Shiseido Capcell Pak C18 MG 4.6 × 250 mm; Tokyo, Japan) equipped with an electrochemical detector (ECD-300; Eicom Co., Kyoto, Japan) with a flow rate of 1.0 ml/min. The mobile phase consisted of 10 mM NaH_2PO_4 containing 8% methanol. 8-OHdG in DNA was calculated as 8-OHdG/10^6 dG.

Statistical analysis

Statistical analysis was carried out using the Mann-Whitney test with differences at $p < 0.05$ considered to be statistically significant.

Tissue preparation for H&E stain

After removal of the lungs, the left lung was inflated and fixed by intratracheal instillation of 4% paraformaldehyde at 25 cm H_2O pressure. The lungs and trachea were resected from the surrounding tissue, and allowed to stand at 4°C for 24 hours. The tissue was washed for 10 minutes

in phosphate-buffered saline, dehydrated by immersion in a graded series of ethanol washes for 1 hour per wash, then maintained in 100% ethanol at 4°C. The lung tissue was embedded in paraffin, and 5 μm-thick sections were cut from the lobe. The samples were then sectioned and stained with hematoxylin and eosin (H&E). The lung pathology was scored 0–5 for fibrotic change, according to the methods described. ESP scores, criteria for fibrosis at the bronchiolar–alveolar junction, were as follows (Bernstein et al., 2006): grade 0, a normal lung; grade 1, minimal, just detectable, very few, very small foci of collagen deposition; grade 2, slight, fairly easily detected, few, small foci of collagen deposition; grade 3, moderate, easily detected foci of collagen deposition in considerably enlarged areas at the bronchiolar–alveolar junction; grade 4, marked, obvious, or extensive foci of collagen deposition extending from the bronchiolar–alveolar junction into the interstitium of more peripheral parts of the lung parenchyma; grade 5, severe, widespread collagen deposition with consolidation at the bronchiolar–alveolar junction, sometimes with interlobular linking. The severity of the fibrotic changes in each lung section was assessed as a mean score of severity from the observed fibrotic fields.

In order to quantify inflammation in H&E-stained lung specimens, the areas of inflammation were determined. Six random digital images were taken per lung section with a digital camera (DS-5M; Nikon Instech Co. Ltd., Kanagawa, Japan) under light microscopy. A 300-point grid was placed over each image on the computer screen and we examined pulmonary inflammation in each using the point counting method. The percentage area of inflammation, excluding air spaces in the lung parenchyma, was calculated (Ogami et al., 2009).

Results

Survival rate

The number of deaths at earlier than 24 months was eight in the control group, 10 in the low concentration exposure group, nine in the medium concentration exposure group, and six in the high concentration exposure group, and there was no difference of survival rate among the four groups. In addition, there was no difference of behavior.

Wet weight of organs

There were no differences in wet weights of the lungs, liver, kidneys, and spleen among the control group and the exposed groups at 12 months and at 24 months.

Pathological findings of the lung

The pathological findings of the lung are shown in Table 1 (at 12 months), Table 2 (at 24 months), and Figure 3.

No tumors, including benign tumors, were found in the exposed group at 12 and 24 months. There was no dysplasia of alveolar and respiratory epithelia in the exposed groups, although hyperplasia was observed in all groups.

Small fibrotic foci and cell aggregation were not found in the control group after 12 months; however, small fibrotic foci, i.e. mild fibrosis, was found in the toner exposed groups. After 24 months, small fibrotic foci or mild fibrosis was observed in the toner exposed groups, and the morbidity of small fibrotic foci was higher in the high concentration exposure group. However, the ESP score showed local mild fibrosis of level 1 or 2, and inhaled toner was localized around cell aggregation in alveoli. Progressive or irreversible fibrosis, which is observed in the case of exposure to crystalline silica and asbestos, was not observed.

We also quantified inflammation in lungs according to the inflammation area (Figure 4). Compared to controls, no significant increase in inflammation score was observed in the exposed groups at either 12 or 24 months.

Type I collagen gene expression

There was no significant difference in gene expression of β-actin between the control and toner-exposed groups. Figure 5 shows type I collagen expression after 12 and 24 months. There was no significant difference of expression between the control group and each exposed group after 12 months. Similarly, no significant difference of expression was

Table 1. Pathological findings in rat lungs at 1 year of toner inhalation.

	Small foci of collagen (n (%))	Accumulation of macrophages (n (%))	Bronchial pneumonia (n (%))	Tumors (n (%))	Pleural thickening (n (%))
Control (n = 10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Low concentration exposure group (n = 10)	2 (20)	3 (30)	0 (0)	0 (0)	0 (0)
Medium concentration exposure group (n = 10)	2 (20)	3 (30)	0 (0)	0 (0)	0 (0)
High concentration exposure group (n = 10)	5 (50)	3 (30)	2 (20)	0 (0)	0 (0)

Table 2. Pathological findings in rat lungs at 2 years of toner inhalation.

	Small foci of collagen (n (%))	Accumulation of macrophages (n (%))	Bronchial pneumonia (n (%))	Tumors (n (%))	Pleural thickening (n (%))
Control (n = 12)	2 (17)	4 (33)	2 (17)	1 (8)	0 (0)
Low concentration exposure group (n = 10)	3 (30)	1 (10)	1 (10)	0 (0)	0 (0)
Medium concentration exposure group (n = 11)	3 (27)	2 (18)	0 (0)	0 (0)	0 (0)
High concentration exposure group (n = 14)	9 (64)	7 (50)	1 (7)	0 (0)	0 (0)

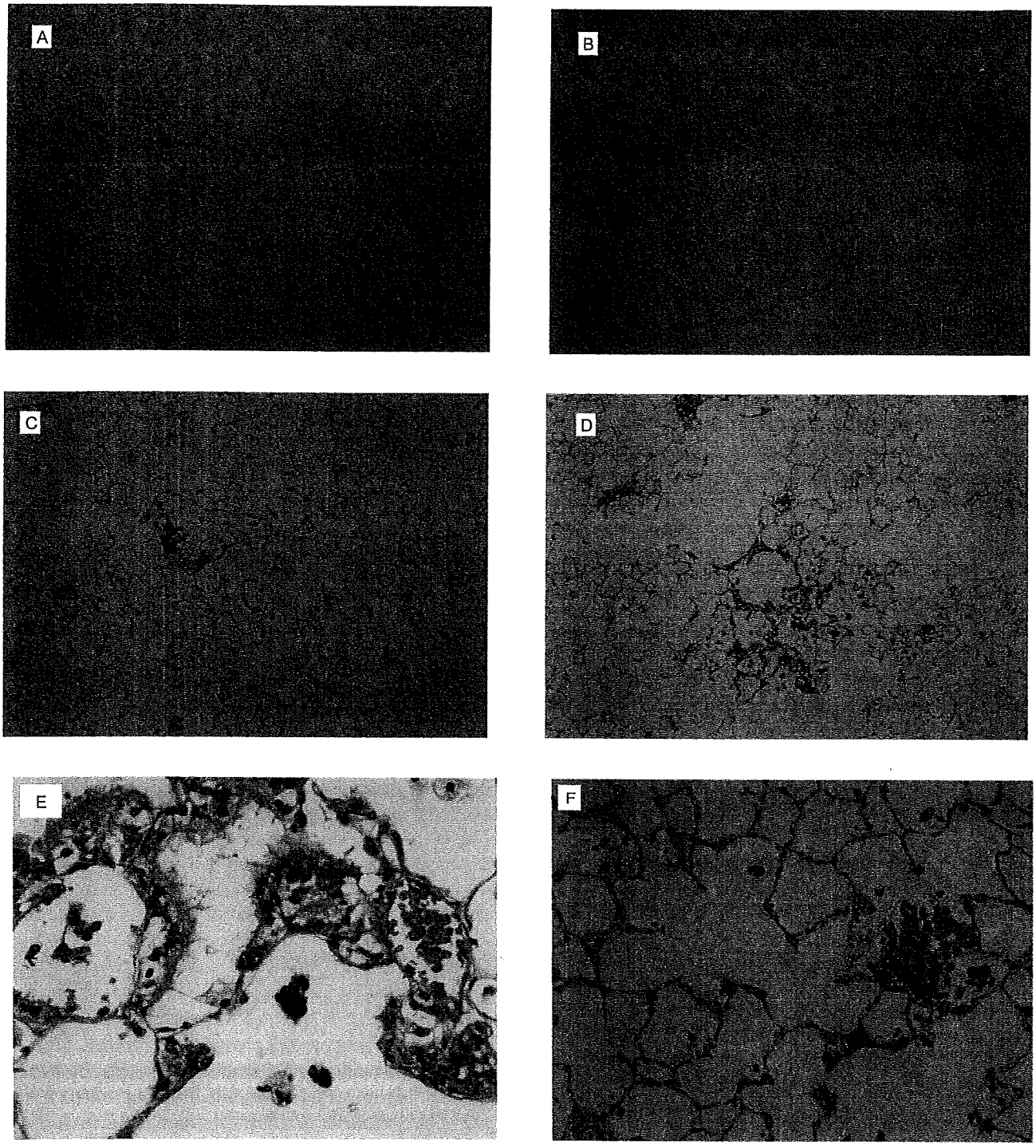

Figure 3. Pathological findings in lung tissue: small fibrotic foci. (A) Control group; (B) low exposure group; (C) medium exposure group; (D) high exposure group; (E) aggregates of alveolar macrophages and alveolar epithelial hyperplasia in high exposure group, ×400; (F) aggregates of alveolar macrophages with inhaled toner in alveoli in high exposure group, ×400.

found between the control group and each exposed group after 24 months.

TIMP-2 gene expression

TIMP-2 gene expression after 12 and 24 months is shown in Figure 6. There was no significant difference of expression in the low, medium, and high concentration exposure groups as compared with the control group after both 12 and 24 months.

MMP-2 gene expression

MMP-2 gene expression after 12 and 24 months is shown in Figure 7. No significant difference of expression was found between the control and each exposed group after 12 months.