Abstract

The capacity of photosensitizing chemicals with ultraviolet A light (UVA) to induce apoptosis is one of the methods to assess their phototoxic and potentially photoallergic properties, since apoptotic cells may be easily presented by antigen-presenting cells. We examined the photoaggravated ability to induce keratinocyte apoptosis of various chemicals that are known as causative agents of photocontact dermatitis and drug photosensitivity involving photoallergic and/or phototoxic machanisms. HaCaT keratinocytes were incubated with 3, 3', 4', 5-tetrachlorosalicylanilide (TCSA), bithionol, diphenhydramine, chlorpromazine, 6-methylcoumarin, sparfloxacin, and enoxacin at 10^{-7} to 10^{-4} M and irradiated with UVA at 4 J/cm^2. Apoptosis and necrosis were evaluated by flow cytometric enumeration of annexin V$^+$ 7-AAD$^-$ and annexin V$^+$ 7-AAD$^+$ cells, respectively. The expression of apoptosis-related molecules, caspase-3 and poly (ADP-ribose) polymerase (PARP), was tested by flow cytometric and western blotting analyses. When compared to non-irradiated cells, significant apoptosis was found in TCSA, bithionol, chlorpromazine, sparfloxacin and enoxacin at 10^{-4} or 10^{-5} M, while necrosis occurred in most of these chemicals at 10^{-4} M. Neither apoptosis nor necrosis was seen in diphenhydramine or 6-methylcoumarin. Caspase-3 and PARP were activated in HaCaT cells phototreated with TCSA or chlorpromazine. We suggest that our method is useful for *in vitro* asessment of not only phototoxicity but also photoallergenicity of chemicals.

1. Introduction

Among solar light-induced disorders, photosensitivity to exogenous agents is the most highly incident pathological condition and includes two diseases, photocontact dermatitis and drug photosensitivity [1]. The former disease is induced by skin application of a contactant and following exposure to ultraviolet light (UV) [2-5], while the latter is evoked by oral administration of a photosensitizing drug and subsequent exposure of the skin to UV [6-10]. In both diseases, the action spectrum is UVA in most cases, and UVB can exceptionally evoke the photosensitivity dermatitis [1,5,6].

Various chemicals have been reported to cause photocontact dermatitis. Historically, the use of halogenated salicylanilide and related compounds, especially 3,3',4',5-tetrachlorosalicylanilide (TCSA) [2] and bithionol [3], resulted in a large number of patients with this skin disease. More recent causative agents are cosmetic or sunscreen products such as 6-methylcoumarin, musk ambtette, benzophenone, and non-steroidal anti-inflammatory drugs [4]. Likewise, there have been various culprit drugs to evoke photosensitivity, including chlorpromazine, quinolones, such as sparfloxacin (SPFX) and enoxacin (ENX), piroxicum, afloqualone, and others [1,6,7].

Both phototoxic and photoallergic mechanisms exist in the pathophysiology of photosensitivity to exogenous agents. The phototoxicity is mediated by reactive oxygens, in particular singlet oxygen [11], while the photoallergenicity occurs as a consequence of immunological events, where the UV-induced formation of photoantigen triggers T cell responses [8-10]. Recently, the incidence of the photoallergic type is higher than that of the phototoxic one [1,6,7]. Our studies have suggested that the vast majority of clinically photoallergic chemicals are photohaptens that binds covalently to protein under exposure to UV [1,6,7,9].

In the photoallergic type of photocontact dermatitis and drug photosensitivity, culprit chemicals reach the epidermis directly from the outside [2-5] or indirectly from the inside by diffusion [7-10]. UVA irradiation induces photoconjugation of epidermal cells with photosensitive chemicals, and photoantigen-bearing epidermal Langerhans cells sensitize T cells in the draining lymph nodes [9-13]. When a given chemical has a strong phototoxicity, however, epidermal cells photomodified with the chemical lose viability, and the formed photoantigen on the damaged keratinocytes or Langerhans cells may be presented by other antigen-presenting cells, such as dermal macrophages or dendritic cells [13]. Since apoptotic cells are easily presented by dendritic cells

[14,15], the capacity of chemicals to evoke apoptosis may determine the feasibility of the sensitivity response. Thus, the apoptosis-inducing property of photosensitizing agents may be useful for evaluation of not only phototoxicity but also photoallergenicity.

In this study, we examined the ability to induce keratinocyte apoptosis of various chemicals that are known as causative agents of photocontact dermatitis or drug photosensitivity. Results suggest that highly photoallergic chemicals have an apoptosis-inducing activity in keratinocytes.

2. Materials and methods

2.1. Cell culture and chemicals

The immortalized human keratinocyte cell line HaCaT cells [16] were grown in Dulbecco's modified Eagles's medium (DMED; Gibco BRL Life Technology, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, 1% $_L$-glutamine (all from Gibco BRL) in an atmosphere of 5% CO_2 and air at 37 °C. Table 1 summarizes photosensitive chemicals used in this study.

2.2. UV light source

As UVA source, black light (FL20SBLB, Toshiba Electric Co., Tokyo, Japan) emitting UVA ranging from 320 to 400 nm with a peak emission at 365 nm was used. With a UV radiometer (Topcom Co., Tokyo, Japan), the energy output of three 20-W tubes of black light at a distance of 20 cm was 2.7 mW/cm^2 at 365 nm and 0.17 mW/cm^2 at 305 nm. Through a culture plastic dish, UVB was cut as low as a negligible level. As a control of apoptosis-inducing irradiation, sunlamp (FL-20 bulbs, Toshiba Electric Co.) emitting broadband UVB ranging 280-340 nm with a peak emission at 305 nm was used. Through a culture dish, the irradiance by sunlamp corresponded to 0.33 mW/cm^2 at 305 nm.

2.3. Preparation of UVA-photomodified cells with chemicals and UVB-irradiated cells

Chemicals were dissolved in 100 % ethanol and diluted into phosphate-buffered saline (PBS; pH 7.4). The final concentration of ethanol in culture was less than 1%. Semiconfluent HaCaT cells were obtained in 90 mm dishes (Corning Glass Works,

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Corning, NY) by seeding the same number of cells in each experiment. After washing twice with 2 ml of PBS, they were incubated with 2 ml of PBS including 10^{-7}-10^{-4} M chemicals or PBS alone at 37 °C for 1 hr. After incubation, the cells were irradiated with UVA source with the dish laid on. UVA at 4 J/cm^2 had no effect on the viability of HaCaT cells. Control samples were kept in the dark under the same conditions. HaCaT cells irradiated with UVB at 60 mJ/cm^2 were used as positive control of apoptotic cells. After UVA or UVB irradiation, supernatants were removed and fresh medium was added, and the cells were cultured in a CO$_2$ incubator.

2.4. Flow cytometric analyses of apoptotic and necrotic cells, and activation of caspase-3

The phototreated or control cells were cultured for 12 hr, harvested by trypsinization, and collected by centrifugation at 300 g for 5 min at room temperature. Cells were washed with cold PBS and stained with annexin V-FITC apoptosis detection kit I (BD Biosciences, Pharmingen, San Diego, CA) according to the manufacturer's instructions. Cells positive for 7-amino-actinomycin D (7-AAD), annexin V-fluorescein isothiocyanate (FITC) or both were quantified by flow cytometry using a Becton Dickinson FACSCalibur (Becton Dickinson, Mountain View, CA).

Because casepase-3 is a key protease that is activated during the early stage of apoptosis [17], we also examined the positivity of active caspase-3 in the photomodified HaCaT cells by flow cytometry. As a positive control, HaCaT cells were also exposed to UVB at 60 mJ/cm^2, and incubated for 12 hr. After washing with cold PBS, the treated or control cells were permeabilized, fixed, and stained with phycoerythrin (PE)-labelled active caspase-3 according to the manufacturer's instructions (BD Biosciences, Pharmingen). The positivity for active caspase-3 was analyzed by FACSCalibur.

2.5. Western blotting

Cells were lysed in a sample buffer (6.25 mM Tris HCl, pH 6.8, 5 % 2-mercaptoethanol, 2 % SDS, 20 % gliceol, and 0.005 % BPB) and incubated in boiling water at 90°C for 5 min. After incubation for 5 min on ice and centrifugation at 10,000 rpm, supernatants were applied to 7.5 % or 15 % SDS-PAGE at 35 V for 16 or 21 min, blotted to PVDF membranes and incubated with an antibody directed against intact/cleaved form poly (ADP-ribose) polymerase (PARP) (all from BD Pharmingen)

for 1 hr at room temperature. Signals were detected by the use of protein detector Western blot kit BCIP/NBT System (KPL, Inc., Gaithersburg, USA). Loading of proteins to verify equivalent distribution of proteins in each well was confirmed by Coomassie brilliant blue staining (BIO-RAD Laboratories, Hercules, CA).

2.6. Statistical analyses

Welch's *t*-test or Student's *t*-test was employed to determine statistical differences between means. *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Photoconjugation of HaCaT cells with TCSA

Fluorescent microscopy was employed to test whether TCSA was coupled to HaCaT cells after UVA irradiation, because TCSA has a fluorescent property and its presence is easily detectable under long-wave UV [12]. HaCaT cells, cultured in slide chambers, were incubated with in a TCSA solution at 10^{-4} or 10^{-5} M, and exposed to 4 J/cm^2 UVA. After extensive washing, the cells were observed in a fluorescent microscopy under an exciter filter for FITC. The coupling of TCSA to HaCaT cells phototreated with 10^{-4} M TCSA was ascertained by the presence of membrane and cytoplasmic fluorescence (Fig. 1A). The fluorescence was barely perceptible in the cells phototreated with 10^{-5} M Fig. 1B). Cells that were incubated with TCSA without irradiation had virtually no fluorescence (Fig. 1C).

3.2. Induction of apoptosis and necrosis by photosensitizing chemicals and UVA

HaCaT cells were exposed to UVA at 4 J/cm^2 in the presence or absence of each of seven photosensitizing chemicals. The induction of apoptosis and necrosis was measured 12 hr after treatment by flow cytometry. In the dot plot histogram, the lower right quadrant represents early apoptotic cells that are positive for only annexin V, and the upper right quadrant positive for both 7-AAD and annexin V shows the latest stage of apoptosis. The upper quadrants shows necrotic cells positive for 7-AAD. As represented by Fig. 2, HaCaT cells that were phototreated with TCSA at 10^{-5} M underwent apoptosis, and ells phototreated with TCSA at a higher concentration of 10^{-4} M exhibted necrosis.

Data obtained in the seven chemicals are summarized in Fig. 3. In the absence of

UVA, no substantial number of apoptotic cells was observed in any of chemicals (Fig. 3A, C, E, G, I, K and M). Necrosis was induced at ~30% with TCSA or bithionol at 10^{-4} M without UVA (Fig. 3B and D), suggesting that they are slightly cytotoxic to keratinocytes at this concentration. Chlorpromazine, diphenylhydramine, 6-methylcoumarin, SPFX, and ENX had no cytotoxic effect on the cells (Fig. 3F, H, J, L and N).

UVA irradiation greatly enhanced the number of early apoptotic cells as compared to non-irradiated cells in TCSA, bithionol and chlorpromazine at 10^{-5} M, and SPFX and ENX at 10^{-4} M (Fig. 3A, C, E, K and M). TCSA, bithionol, and chlorpromazine at 10^{-4} M plus UVA induced necrosis rather than apoptosis (Fig. 3B, D and F), while the quinolone derivatives, SPFX and ENX, minimally induced necrosis even at 10^{-4} M with UVA (Fig. 3L and N). Diphenylhydramine and 6-methylcoumarin at 10^{-4} M had no photoexciting effect on apoptosis or necrosis (Fig. 3G, H, I and J).

3.3. Induction of apoptosis signalings by photosensitizing chemicals and UVA

Caspase activation plays a key role in the process of apoptosis. In particular, caspase-3 is activated during early apoptosis, and its active form is considered to be an excellent marker of cells undergoing apoptosis [18,19]. HaCaT cells were cultured with TCSA, bithionol or chlorpromazine at 10^{-5} M, or SPFX or ENX at 10^{-4} M, with or without UVA exposure. As shown in Fig. 4, without UVA, caspase-3 was not activated in any of the five photosensitizing chemicals. In all chemicals, however, caspase-3 was activated by UVA irradiation at various degrees. Since UVB radiation is well known to activate both the membrane death receptor and the intrinsic or mitochondrial apoptotic signaling pathways in epidermal keratinocytes [20], we compared the apoptotic effect of the chemical/UVA with that of UVB at 60mJ/cm². TCSA and chlorpromazine with the help of UVA yielded caspase-3 activation at comparable levels to UVB.

PARP is a substrate for active caspase-3 [19,20]. To further confirm the apoptosis via caspase-3, PARP was examined by Western blotting. HaCaT cells were cultured with TCSA, bithionol, or chlorpromazine at 10^{-5} M with or without UVA exposure. As shown in Fig. 5, the treatment of cells with TCSA or chlorpromazine under UVA produced an 85-kDa fragment of cleaved PARP, indicating activation of this molecule.

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4. Discussion

In general, photoallergic chemicals have various extents of phototoxic potential, as represented by TCSA [12,13]. On the other hand, there exist some chemicals possessing an exclusively phototoxic moiety, such as psoralen and coal tar [21]. Therefore, photosensitizing chemicals have phototoxic or both phototoxic and photoallergic potentials. Apoptosis is usually considered to be one of the phototoxic outcome [22]. However, given that apoptotic cells bearing certain antigenic chemicals are easily captured and their antigenic determinants are presented by dendritic cells [14,15], apoptosis also seems to be closely related to the immunological response where T cells are primed. Thus, apoptosis induced by photosensitizing agents plus UVA may at least partly reflect photoallergy to a given reagent.

The present study demonstrated that apoptosis of keratinocytes is induced by some of the photosensitizing chemicals that cause photocontact dermatitis and drug photoallergy. Among the chemicals examined here, TCSA has been considered to be one of the strongest photohaptens, as TCSA yields a higher magnitude of murine photocontact dermatitis than bithionol, tribromsalen, musk ambrette, 6-methylcoumarin, benzocaine, hexachlorophene, and sulfanilamide [5]. Accordingly, the ability of TCSA to evoke apoptosis was the highest in our assessment. When determined by the murine contact photosensitivity, chlorpromazine is another drug to possess a strong photosensitizing ability, and bithionol has a moderate potency [5]. We also found that discernible levels of apoptosis were induced by these chemicals. Thus, it is likely that the photoallergic capacity is associated with the apoptosis-inducing ability of photosensitizing chemicals.

We found that caspase-3 activation and PARP expression were induced in HaCaT keratinocytes by treatment with the photosensitizing chemicals plus UVA. In general, apoptosis is induced by a couple of different pathways in which mitochondrial events and Fas/Fas-L stimulation are involved [23]. Although the signaling pathway of apoptosis induced by photosensitizing chemicals remains unfully elucidated, there have been several studies using fluoroquinolones, which are representative chemicals causative for both phototoxic and photoallergic dermatitis [7-10]. Apoptosis by lomefloxacin, a representative fluoroquinolone, is caused by caspase-3 activation and Fas-L induction [24]. In addition, photosensitized lomefloxacin alters mitochondrial membrane [25]. These findings suggest that both pathways involving the Fas/Fas-L and

mitochondrial changes mediate apoptosis by photosensitizing chemicals. Notably, the fluoroquinolones to induce such apoptotic events are highly photoallergic as well [7-10].

Keratinocytes are physiologically exposed to sunlight and capable of responding to UV light to produce cytokines, chemokines, and prostaglandins [26]. Furthermore, the UV effect on keratinocyte apoptosis has been considerably studied [19,20,27]. As demonstrated in this study, TCSA, bithionol, and chlorpromazine, SPFX, and ENX were identified as apoptosis-inducing photosensitizers for keratinocytes. We suggest that this *in vitro* system is one of the useful screening methods to predict both phototoxic and photoallergic potentials of chemicals.

Acknowledgements

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Figure legends

Fig. 1. Fluorescence of TCSA in the membrane and cytoplasm of TCSA-photoconjugated HaCaT cells. HaCaT cells were incubated with TCSA at the indicated concentration or in PBS alone irradiated with UVA at 4 J/cm^2, and observed in a fluorescence microscopy.

Fig. 2. Representative dot-charts of annexin-V/7-AAD bivariate flow cytometry. HaCaT cells were treated with TCSA at the indicated concentration with or without UVA exposure (4 J/cm^2). The lower left quadrant (annexin-/7-AAD -) contains viable cells. The lower right quadrant (annexin+/7-AAD-) represents apoptotic cells. The upper both left and right quadrant (7-AAD+) shows necrotic cells.

Fig. 3. Apoptosis and necrosis in HaCaT cells treated with each chemical plus UVA. HaCaT cells were treated with the indicated chemical and/or UVA. Results were expressed as the mean percentage ± SD of apoptotic and necrotic cells from three independent experiments. *$P<0.05$, compared with non-irradiated control.

Fig. 4. Activation of caspase-3 in HaCaT cells treated with each chemical plus UVA. Data represent the percentage (mean±SD, n=3) of active caspase-3-positive cells induced by TCSA, bithionol or chlorpromazine at 10^{-5} M, or SPFX or ENX at 10^{-4} M with or without UVA (4 J/cm^2). *$P<0.05$, **$P<0.01$, compared with non-irradiated control cells. As a positive control, HaCaT cells were irradiated with UVB (60 mJ/cm^2) (n=3, mean±SD).

Fig. 5. Expression of PARP in HaCaT cells treated with each chemical plus UVA. HaCaT cells were treated with TCSA, bithionol, or chlorpromazine at 10^{-5} M with or without UVA at 4 J/cm^2. As positive and negative control, UVB (60 mJ/cm2) irradiated or non-treated HaCaT cells were used. Cell lysates were collected 12 hr after irradiation.

Table 1
Chemical materials used in this study

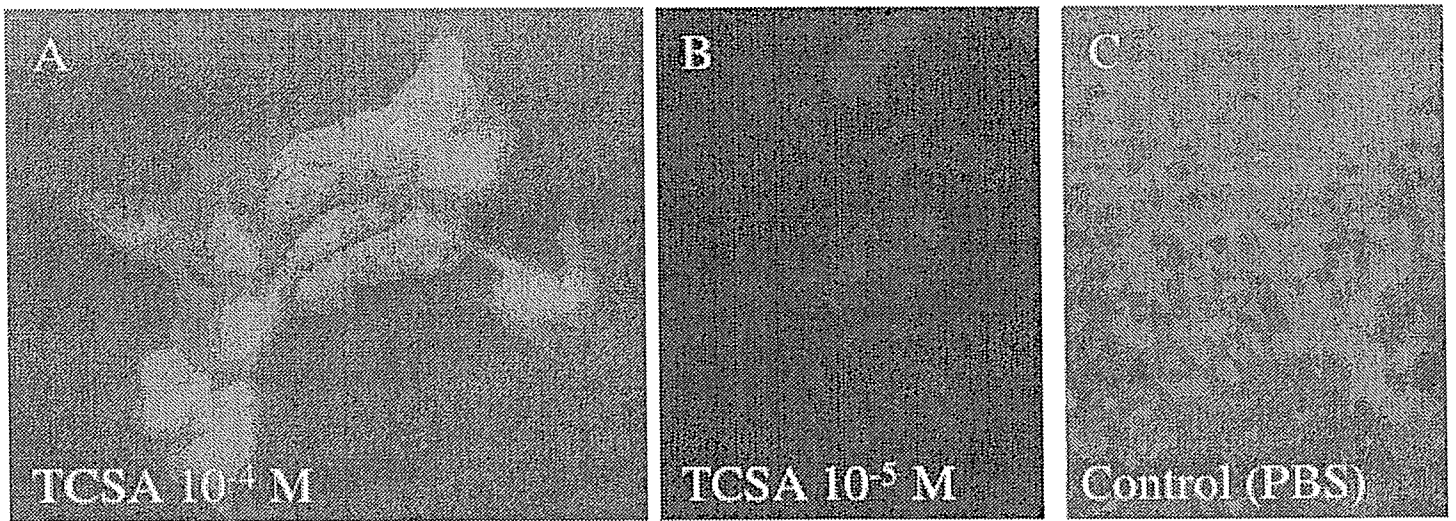| Chemicals	Source	Structure	Usage
3, 3', 4' 5-tetrachlorosalicylanilide (TCSA)	Eastman Kodak, Rochester, N.Y.	Cl2C6H2(OH)CONHC6H3Cl2	Anti-microbial agent
Bithionol	Sigma-Aldrich. Milano, Italy	C12H6Cl4O2S	Anti-microbial agent
6-methlcoumarin	Wako Chemical Co.. Tokyo. Japan	CH3C6H3OCOCH:CH	Perfume
Diphenhydramine	Sigma-Aldrich, Milano, Italy	C17H21NO·HCl	Anti-histamic drug
Chlorpromazine	Sigma-Aldrich. Milano, Italy	C17H21NO·HCl	Major tranquilizer
Spafloxacin (SPFX)	Dainippon Sumitomo Pharma Co.. Osaka, Japan	C19H22F2N4O3	Anti-microbacterial quinolone
Enoxacin (ENX)	Dainippon Sumitomo Pharma Co., Osaka, Japan	C15H17FN4O3·1 1/2H2O	Anti-microbacterial quinolone

A — TCSA 10⁻⁴ M
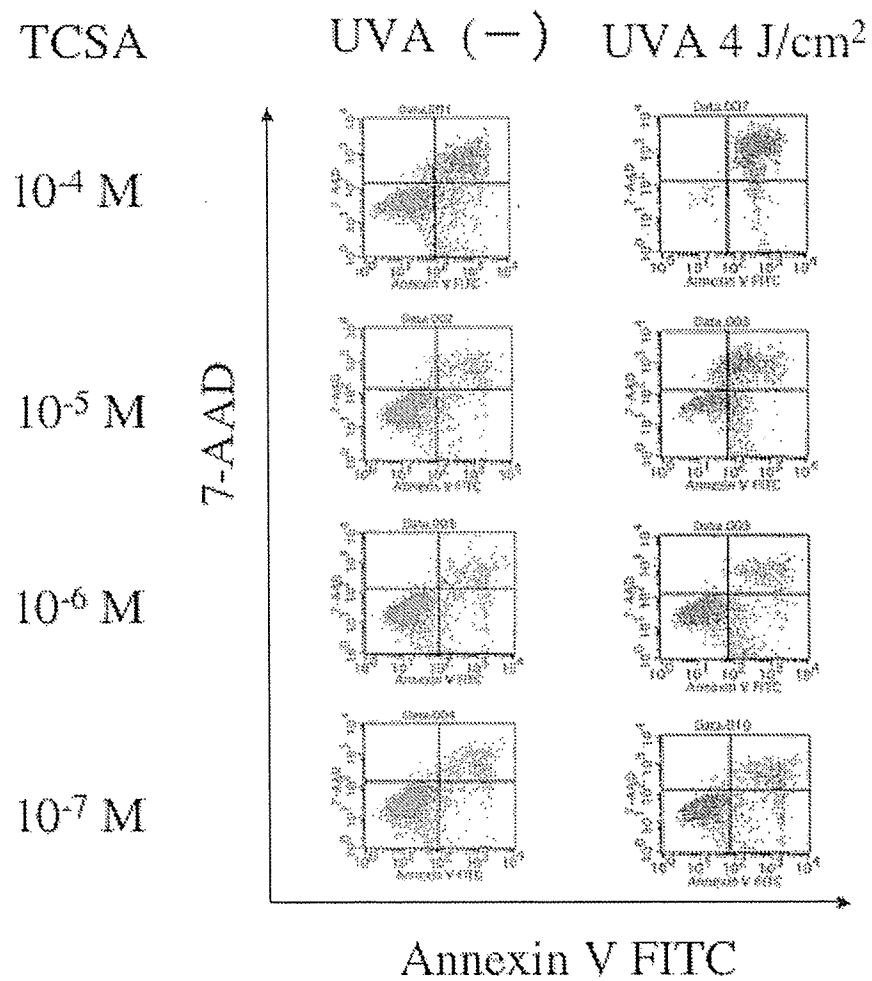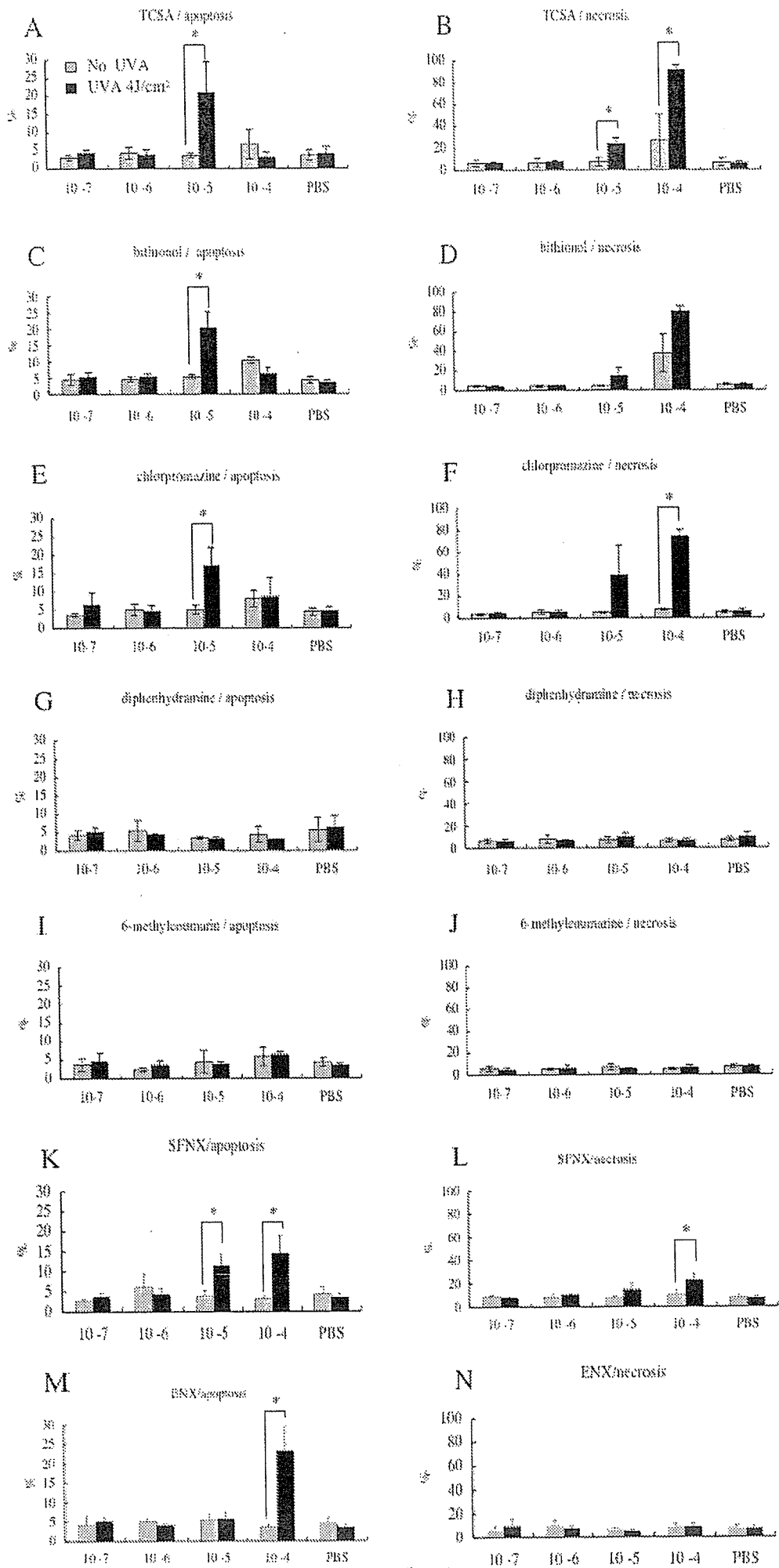
B — TCSA 10⁻⁵ M

C — Control (PBS)

Fig. 1

TCSA   UVA (−)   UVA 4 J/cm²

10⁻⁴ M

10⁻⁵ M

10⁻⁶ M

10⁻⁷ M

7-AAD

Annexin V FITC

Fig. 2

A TCSA / apoptosis
B TCSA / necrosis
C bithionol / apoptosis
D bithionol / necrosis
E chlorpromazine / apoptosis
F chlorpromazine / necrosis
G diphenhydramine / apoptosis
H diphenhydramine / necrosis
I 6-methylcoumarin / apoptosis
J 6-methylcoumarin / necrosis
K SFNX/apoptosis
L SFNX/necrosis
M ENX/apoptosis
N ENX/necrosis

No UVA
UVA 4J/cm²

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Fig. 3

Fig. 4

Fig. 5

M

Control

TCSA

TCSA+UVA

Bithionol

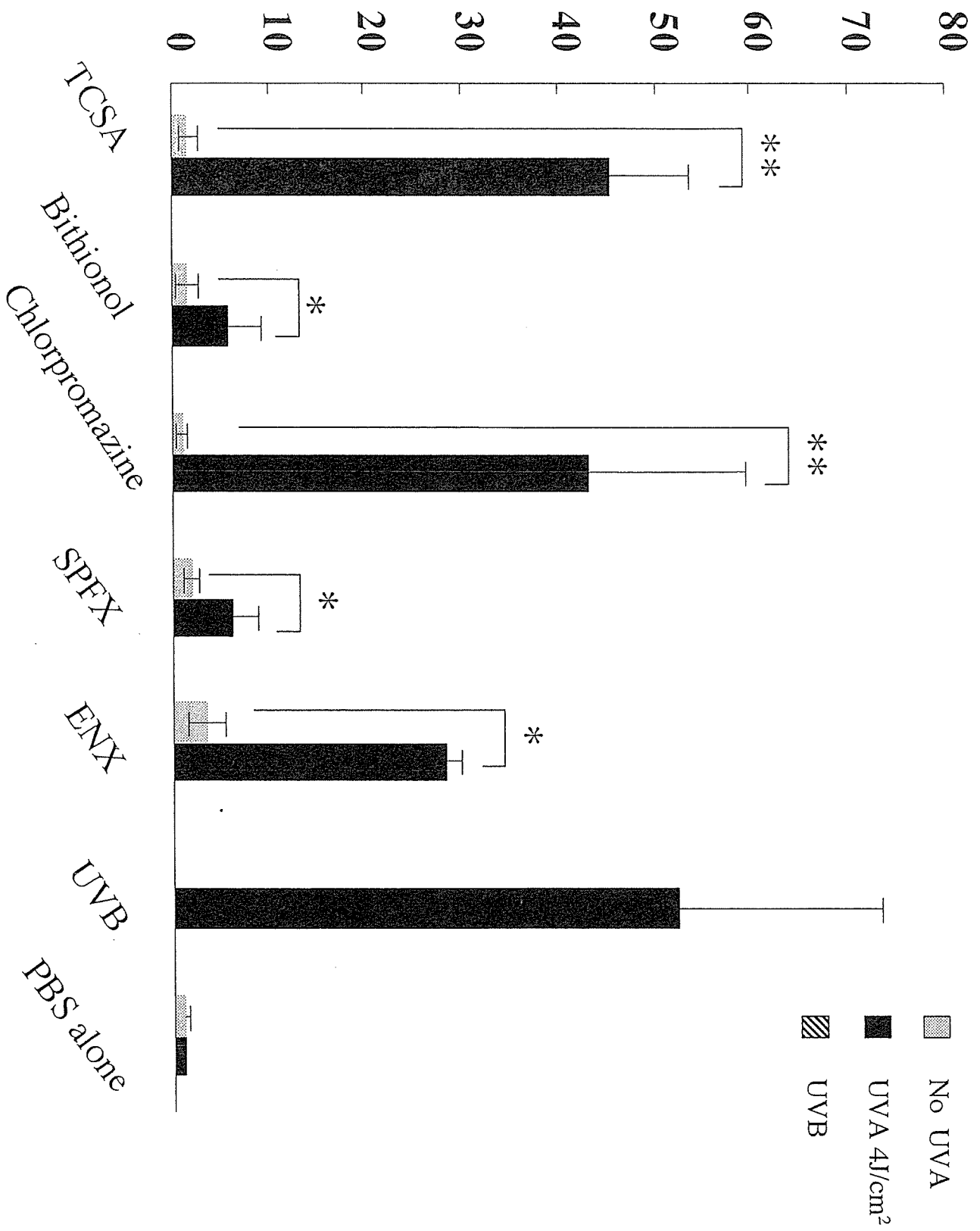Bithionol+UVA

Chlorpromazine

Chlorpromazine+UVA

UVB

PARP

116 kDa

85 kDa
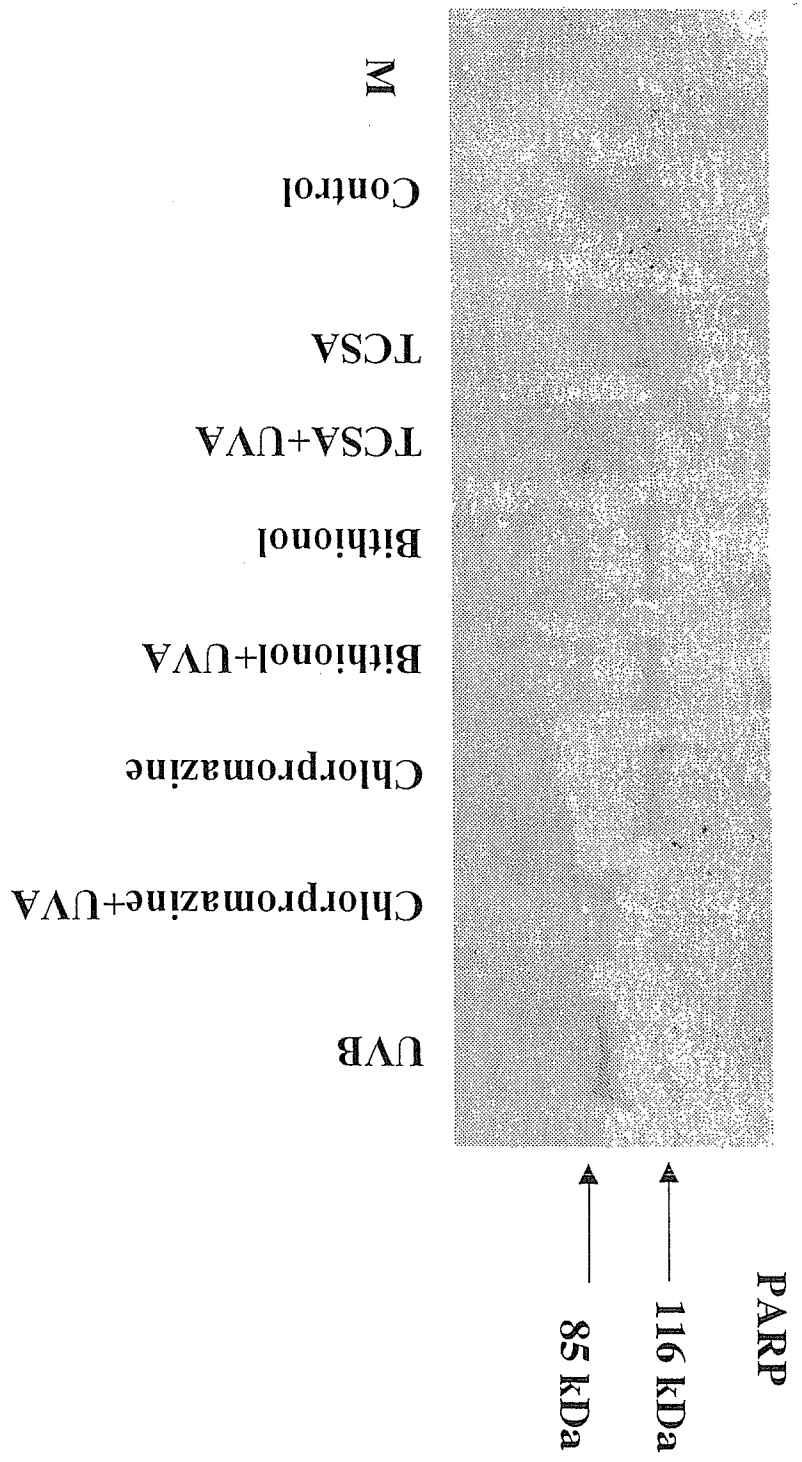
PRESENCE AND ESTROGENICITY OF ANTHRACENE DERIVATIVES IN COASTAL JAPANESE WATERS

Ryo Kurihara,† Fujio Shiraishi,‡ Noriho Tanaka,§ and Shinya Hashimoto*†
†Laboratory of Ecological Chemistry, Institute for Environmental Sciences, University of Shizuoka, Shizuoka 422-8526, Japan
‡National Institute for Environmental Studies, Ibaraki 305-8506, Japan
§Hadano Research Institute, Food and Drug Safety Center, Kanagawa 257-8523, Japan

Abstract—An analytical method was developed to measure levels of anthracene (ANT) and its derivative compounds 9,10-anthraquinone (ATQ) and eight hydroxy-anthraquinones (hATQs) in seawater from Tokyo Bay and Suruga Bay, Japan. The hATQs produced through photochemical reaction of ANT are known to be toxic. Seawater samples contained ANT at levels ranging from <0.2 to 4.7 ng/L, ATQ from 3.9 to 200 ng/L, 1-hydroxyanthraquinone (1-hATQ) from <0.9 to 5.3 ng/L, and 2-hATQ from 1.6 to 5.5 ng/L. The yeast two-hybrid system was also used to evaluate the estrogenic activity of these compounds. Estrogen agonist and antagonist tests with or without rat liver S9 were carried out. Some compounds showed estrogenic activity: The strongest (2-hydroxyanthraquinone) was of similar potency to *p*-nonylphenol. Concentrations of some estrogenic derivatives in the samples were higher than those of the parent ANT. Polycyclic aromatic hydrocarbons (PAHs) such as ANT appear able to be transformed into toxic compounds in the environment when they are irradiated by sunlight, so it is important to monitor not only PAHs but also hydroxyl-PAH-quinones in the environment.

Keywords—Anthracene derivatives   Hydroxy-anthraquinones   Photochemical reaction   Marine environment   Estrogenicity

INTRODUCTION

Petroleum is the world's most important energy source. One of the drawbacks of oil spills is marine pollution, a serious environmental problem. If these spills remain in the environment, marine organisms are exposed to the chemical compounds in them, with disastrous effects, particularly after tanker accidents [1,2]. Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants that are present in petroleum and form as by-products of the incomplete combustion of organic compounds. Polycyclic aromatic hydrocarbons can be detected in the atmosphere [3], marine core sediments [4], and living organisms [5] and have spread all over the world. Many reports have been made of intact PAHs in the environment [3–5]. Exposure to light results in photochemical reactions that enhance the toxicity of PAHs [6–9], and they can form photochemical reaction products, such as hydroxyl-PAH-quinones, that have greater toxicity than the parent compounds. For these reasons, it is important to study the environmental existence and toxicity of PAHs and their derivatives.

The present study focused on the PAH anthracene (ANT), some of whose photochemical reaction products have greater toxicity to higher plants than ANT itself [8]. These photochemical reaction products include 9,10-anthraquinone (ATQ), hydroxy-anthraquinones (hATQs), and lower-molecular-weight compounds [10]. The reaction pathways of these compounds have been proposed in previous literature [10]. When ANT is irradiated with simulated solar radiation (SSR), it is converted mainly to ATQ via an intermediate. The intermediate has been proposed to be the 9,10-endoperoxide of ANT [10]. The ATQ is then hydroxylated to hATQs, such as 1-hATQ or

2-hATQ. Derivatives of ATQ are important materials in industrial [11] and medical [12] applications, and their toxicities have been reported in a previous study [13].

To the best of our knowledge, little information exists on the photochemical reaction products of ANT in the environment. The amounts of ANT and ATQ in coastal marine sediments from sites in the northeastern United States have been reported [14], although the environmental existence of 1-hATQ [15] and 2-hATQ [16] has been reported only by preliminary analysis.

Many natural and synthetic compounds, such as 17β-estradiol, estrone, 17-ethinylestradiol, diethylstilbestrol, *p*-nonylphenol, and bisphenol A, are known to have estrogenic activity [17]. Recently, some hydroxylated environmental pollutants have been found to be estrogenic, for example, hydroxylated benzophenones [18], polychlorinated biphenyls [19], and PAHs [20]. The photochemical reaction products of ANT have variable toxicity and carcinogenicity to organisms such as higher plants and August Copenhagen Irish rats [8,13]. Photosensitization and photomodification of PAHs are mechanisms related to the enhancement of toxicity, and it is therefore important to evaluate the estrogenic activity of both the primary compounds and their photochemical reaction products. Although some hydroxyl-PAHs have been reported to interact with estrogen receptors and show weak estrogenic activity [20], the estrogenicity of such ANT derivatives has never been fully investigated.

Receptor-binding assays, a Michigan Cancer Foundation-7 (MCF-7) cell proliferation assay (estrogen screen, E-screen), and reporter gene assays in animal cell lines have recently been developed as in vitro screening techniques to evaluate estrogenic activity [21]. The yeast two-hybrid system is a popular method. A recently developed yeast two-hybrid assay has

* To whom correspondence may be addressed
(hashimos@u-shizuoka-ken.ac.jp).