RESULTS

Demographic data for the 67 448 patients

Among the 67 448 patients, 32 062 (47.54%) cases were recruited from university hospitals, 12 709 (18.84%) from district-based hospital and 22 677 (33.62%) from private clinics (Table 1). More patients were enrolled in August 2007 (18 103) than in February 2008 (15 467) (Table 1). With regards to the age distribution, the group aged 71–75 years (6157; 9.13%) was the biggest, followed by groups aged 66–70 (5629; 8.35%), 56–60 (5543; 8.22%) and 61–65 (5413; 8.03%) (Table 2). For patients aged under 20 years, the group aged 0–5 years formed the biggest population (4192; 6.22%). Among the 67 448 patients, there were 30 899 (46.1%) males and 36 125 (53.9%) females; the sex of 424 patients was not described. Female patients aged between 16 and 60 years tended to visit dermatology clinics more frequently than their male counterparts (Table 2).

Prevalence of skin disorders

We classified skin diseases into 85 categories, as listed in Table 3, and determined the prevalence of each. The 20 most common diseases were miscellaneous eczema (12 590; 18.67%) followed, in order, by atopic dermatitis (6733; 9.98%), tinea pedis (4379; 6.49%), urticaria/angioedema (3369; 4.99%), tinea unguium (3231; 4.79%), viral warts (3028; 4.49%), psoriasis (2985; 4.43%), contact dermatitis (2643, 3.92%), acne (2430; 3.6%), seborrheic dermatitis (2213; 3.28%), hand eczema (2024; 3%), miscellaneous benign skin tumors (1666; 2.47%), alopecia areata (1653; 2.45%), herpes zoster/zoster-associated

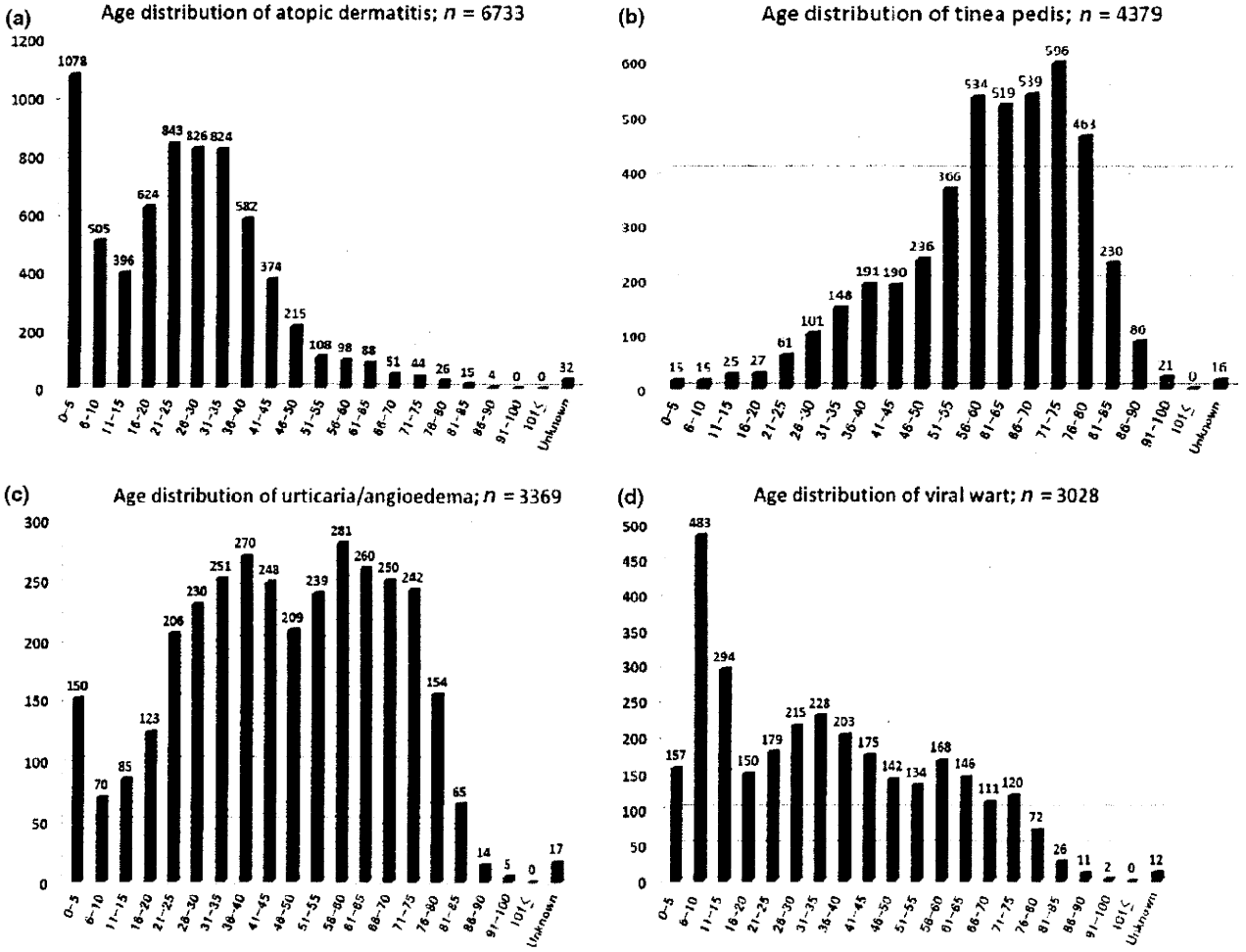

Figure 1. Age distribution of atopic dermatitis, tinea pedis, urticaria/angioedema and viral wart.

pain (1609; 2.39%), skin ulcers (non-diabetic) (1334; 1.98%), prurigo (1229; 1.82%), epidermal cysts (1194; 1.77%), vitiligo vulgaris (1134; 1.68%), seborrheic keratosis (1095; 1.62%) and drug eruption/toxicoderma (1018; 1.51%). These top 20 categories covered 57 577 (85.34%) of the 67 448 patients (Table 3).

Age distributions of common diseases

The age distribution of atopic dermatitis was biphasic, peaking at 0–5 and 21–35 years of age (Fig. 1a). Tinea pedis peaked at 56–75 years of age (Fig. 1b). Tinea unguium showed a similar pattern (data not shown). Urticaria/angioedema showed a triphasic distribution pattern (Fig. 1c), whereas viral warts peaked at 6–15 years of age (Fig. 1d). Psoriasis peaked at 56–65 years of age (Fig. 2a). The age distribution for contact dermatitis was somewhat evenly dispersed (Fig. 2b). The peak age for acne was 16–25 years (Fig. 2c), whereas that for seborrheic dermatitis was 71–75 (Fig. 2d). Hand eczema was distributed evenly in adults (Fig. 3a). The peak age for alopecia areata was 31–35 years (Fig. 3b). Herpes zoster/zoster-associated pain and prurigo were prominent in elderly patients (Fig. 3c,d). Epidermal cysts occurred in adults of all ages (Fig. 4a). Vitiligo vulgaris and drug eruption/toxicoderma were preponderant in elderly people (Fig. 4b,c). Notably, the age distribution for burns peaked in the group aged 0–5 years (Fig. 4d).

In Tables 4 and 5, we list the top five skin disorders for each age group. Miscellaneous eczema appeared in every age group, whereas atopic dermatitis was among the top five diseases for age groups under 50 years. The disease encountered most frequently in groups aged 6–40 years was atopic dermatitis.

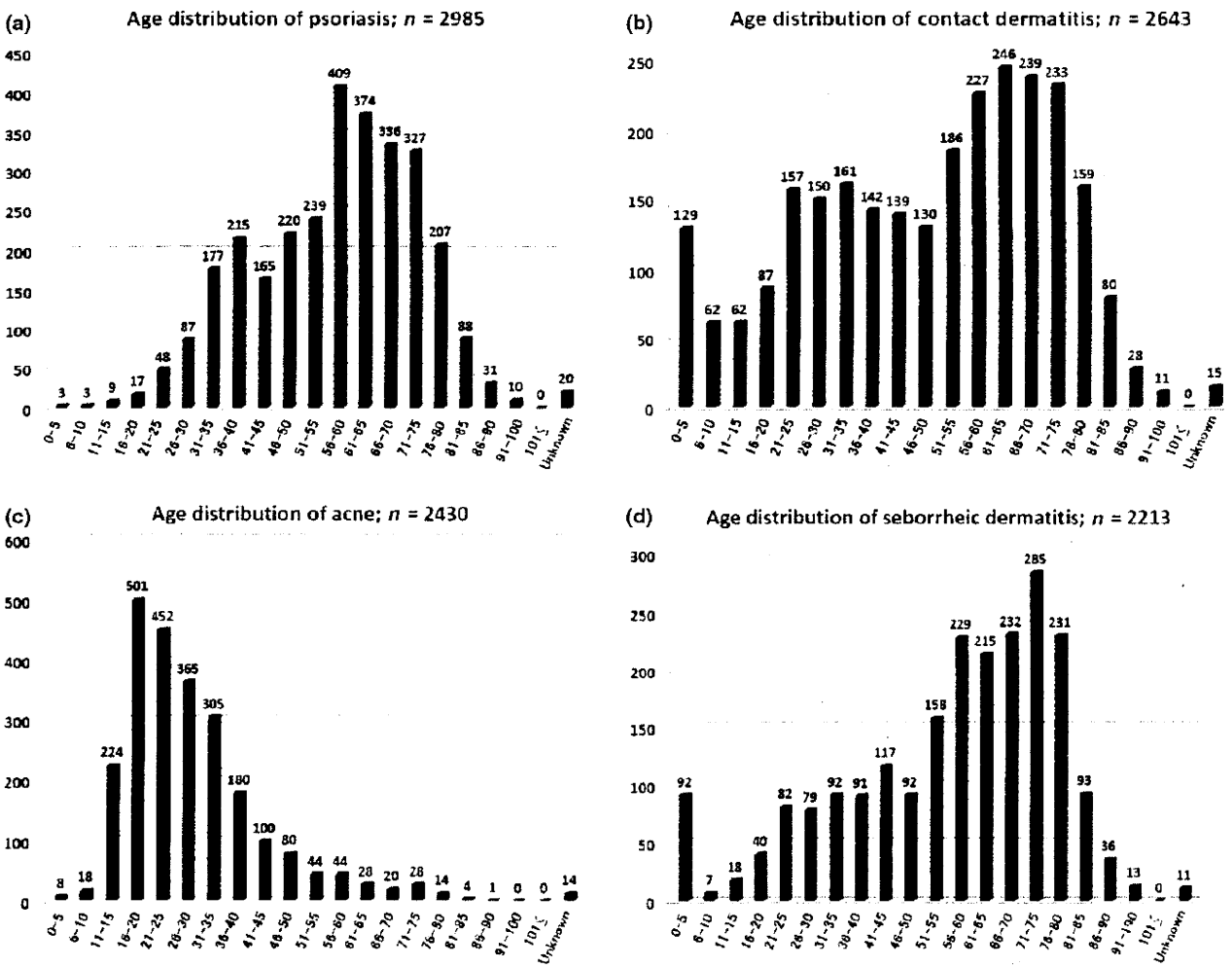

Figure 2. Age distribution of psoriasis, contact dermatitis, acne and seborrheic dermatitis.

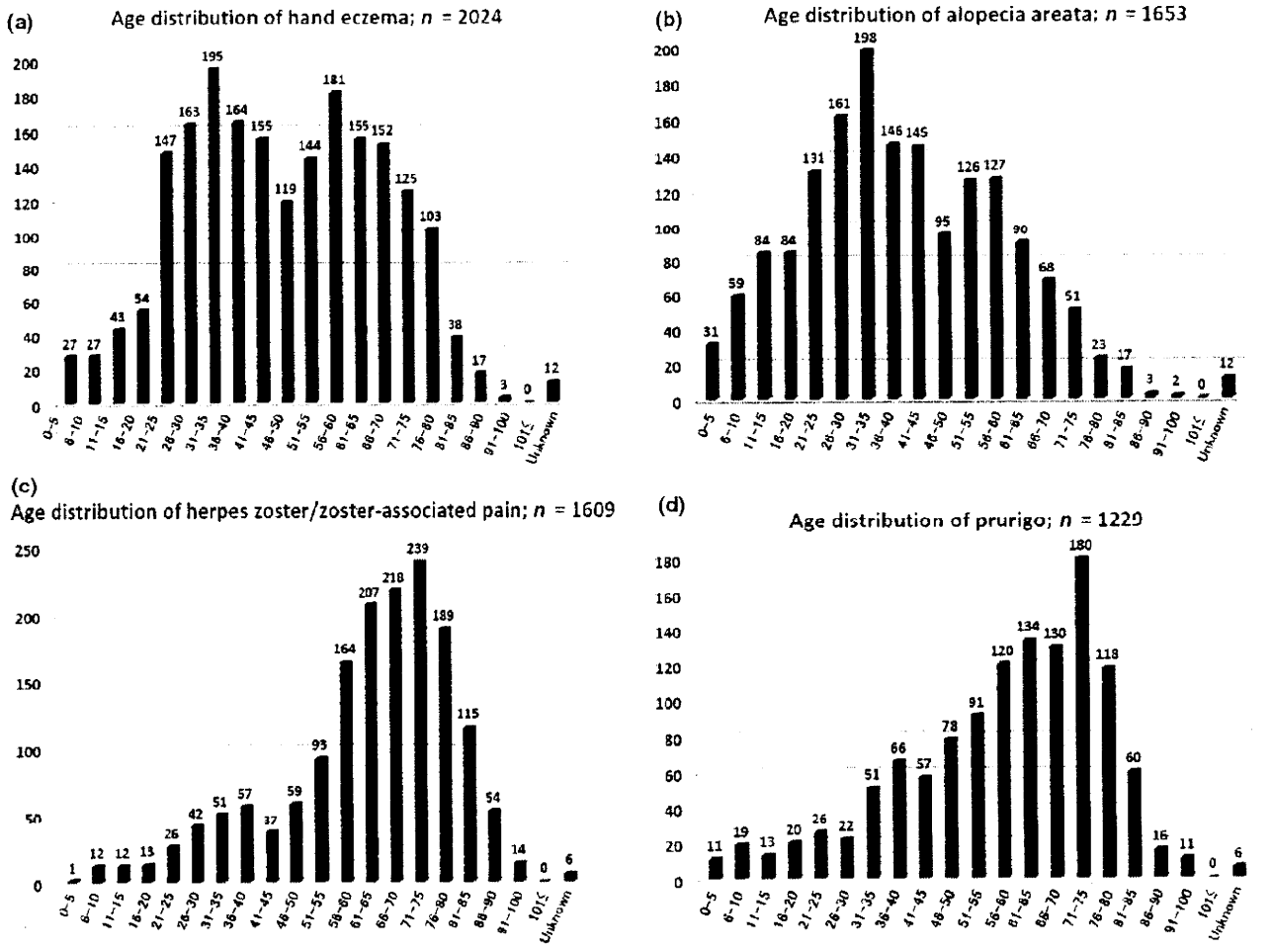

Figure 3. Age distribution of hand eczema, alopecia areata, herpes zoster/zoster-associated pain and prurigo.

Molluscum and impetigo were common in patients aged 0–10 years. Viral warts were among the top five diseases for groups aged 6–45 years. Acne was common in groups aged 11–35 years. Urticaria/angioedema was among the top five diseases for a wide range of age groups from 11–70 years old. Tinea pedis was common in groups aged above 41 years old. Psoriasis appeared in the top five diseases in middle-aged and older people with ages ranging 46–80 years old.

Sex differences

Difference in the incidence of skin disorders between the sexes are shown in Table 6. The prevalence of diabetic dermatoses, psoriasis, androgenic alopecia, syphilis and erythroderma in males was more than twice that in females, whereas the prevalence of hand eczema, systemic sclerosis, systemic lupus erythematosus, dermatomyositis, reticular/racemous livedo, pigmented nevus, chloasma/senile freckle, erythema nodosum and rosacea/rosacea-like dermatitis was more than twice as high in females than males (Table 6).

Correlation between patient numbers and the average low temperature, average high temperature and average humidity in the months of clinic visits

Because this study was a nationwide survey for Japan, a wide variation of climates had to be considered. We therefore searched for correlations between patient numbers and average low temperature, average high temperature and average humidity of the month in which patients visited clinics. The numbers of visiting patients diagnosed with urticaria/angioedema (Fig. 5), insect bites (Fig. 5), tinea pedis (Fig. 6)

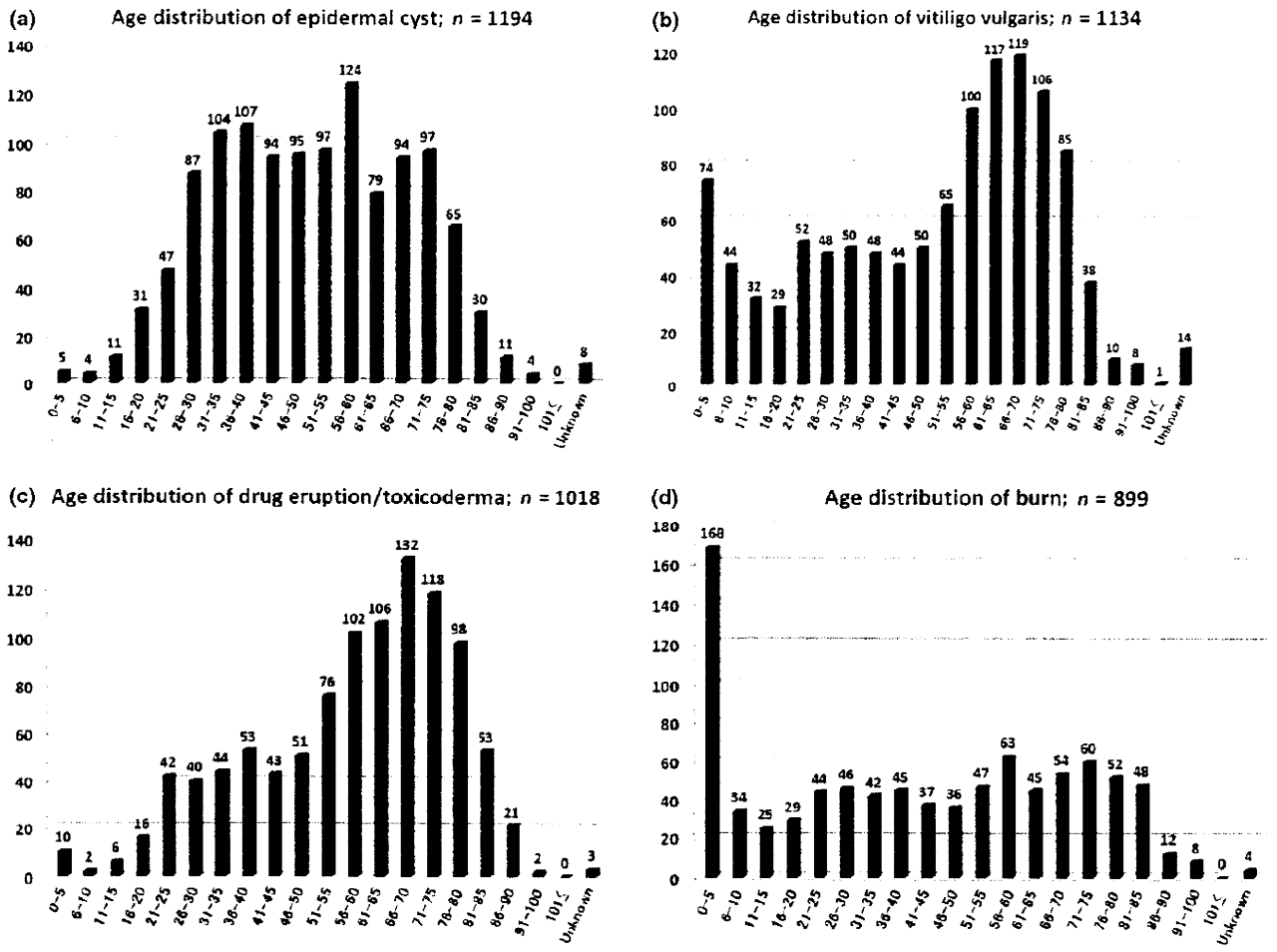

Figure 4. Age distribution of epidermal cyst, vitiligo vulgaris, drug eruption/toxicoderma and burn.

or impetigo (Fig. 6) showed a significant correlation with the average low temperature and with the average high temperature (data not shown). The numbers of visiting patients diagnosed with atopic dermatitis, contact dermatitis or molluscum contagiosum were also positively correlated with the average low temperature and average high temperature (data not shown). The numbers of patients diagnosed with seborrheic dermatitis showed a negative correlation with the average humidity (Fig. 7). The average humidity was also significantly and negatively correlated with atopic dermatitis, hand eczema and prurigo (data not shown).

DISCUSSION

There are a number of limitations and biases in hospital-based prevalence studies, including institutional specificity (university hospital, pivotal local hospital or private clinic), differences in localization, climatic and seasonal differences, and differences in skills in diagnosis.[1,4–6] This study, conducted in fiscal year 2007 by the Japanese Dermatological Association, recruited 76 university hospitals, 55 district-based pivotal hospitals and 59 private clinics (190 clinics in total). We analyzed data for 67 448 patients that were collected seasonally from 170 clinics. This nationwide study is first of its kind in Japan, and its nature appears to eliminate, at least in part, some of the above-mentioned biases of hospital-based prevalence study.

In fiscal year 2007, eczematous and fungal diseases were commonly reported in dermatological clinics in Japan. The 20 most common categories of skin disorder were diagnosed in more than 85% of patients presenting dermatological complaints. A

Table 4. Top five skin disorders in each age group

0–5 years old (n = 4192)		26–30 years old (n = 3516)	
Miscellaneous eczema	1229; 29.32%	Atopic dermatits	826; 23.49%
Atopic dermatitis	1078; 25.72%	Miscellaneous eczema	451; 12.83%
Molluscum contagiosum	425; 10.14%	Acne	365; 10.38%
Impetigo contagiosum	291; 6.94%	Urticaria/angioedema	230; 6.54%
Miscellaneous benign skin tumors	226; 5.39%	Viral wart	215; 6.11%
6–10 years old (n = 2099)		31–35 years old (n = 4050)	
Atopic dermatits	505; 24.06%	Atopic dermatits	824; 20.35%
Viral wart	483; 23.01%	Miscellaneous eczema	551; 13.6%
Miscellaneous eczema	355; 16.91%	Acne	305; 7.53%
Molluscum contagiosum	144; 6.86%	Urticaria/angioedema	251; 6.2%
Impetigo contagiosum	110; 5.24%	Viral wart	228; 5.63%
11–15 years old (n = 1711)		36–40 years old (n = 3807)	
Atopic dermatits	396; 23.14%	Atopic dermatits	582; 15.29%
Viral wart	294; 17.18%	Miscellaneous eczema	503; 13.21%
Acne	224; 13.09%	Urticaria/angioedema	270; 7.09%
Miscellaneous eczema	214; 12.51%	Psoriasis	215; 5.65%
Urticaria/angioedema	85; 4.97%	Viral wart	203; 5.33%
16–20 years old (n = 2270)		41–45 years old (n = 3298)	
Atopic dermatits	624; 27.49%	Miscellaneous eczema	454; 13.77%
Acne	501; 22.07%	Atopic dermatits	374; 11.34%
Miscellaneous eczema	269; 11.85%	Urticaria/angioedema	248; 7.52%
Viral wart	150; 6.61%	Tinea pedis	190; 5.76%
Urticaria/angioedema	123; 5.42%	Viral wart	175; 5.31%
21–25 years old (n = 3219)		46–50 years old (n = 3201)	
Atopic dermatits	843; 26.19%	Miscellaneous eczema	453; 14.15%
Acne	452; 14.04%	Tinea pedis	236; 7.37%
Miscellaneous eczema	407; 12.64%	Psoriasis	220; 6.87%
Urticaria/angioedema	206; 6.4%	Atopic dermatits	215; 6.72%
Viral wart	179; 5.56%	Urticaria angioedema	209; 6.53%

Table 5. Top five skin disorders in each age group

51–55 years old (n = 4062)		76–80 years old (n = 4778)	
Miscellaneous eczema	676; 16.64%	Miscellaneous eczema	1304; 27.29%
Tinea pedis	366; 9.01%	Tinea pedis	463; 9.69%
Psoriasis	239; 5.88%	Tinea unguium	401; 8.39%
Urticaria/angioedema	239; 5.88%	Seborrheic dermatitis	231; 4.83%
Tinea unguium	226; 5.56%	Psoriasis	207; 4.33%
56–60 years old (n = 5540)		81–85 years old (n = 2636)	
Miscellaneous eczema	910; 16.43%	Miscellaneous eczema	725; 27.5%
Tinea pedis	534; 9.64%	Tinea unguium	233; 8.84%
Psoriasis	409; 7.38%	Tinea pedis	230; 8.73%
Tinea unguium	331; 5.97%	Herpes zoster/zoster-associated pain	115; 4.36%
Urticaria/angioedema	281; 5.07%	Seborrheic dermatitis	93; 3.53%
61–65 years old (n = 5415)		86–90 years old (n = 1099)	
Miscellaneous eczema	1016; 18.76%	Miscellaneous eczema	307; 27.93%
Tinea pedis	519; 9.58%	Tinea unguium	86; 7.83%
Tinea unguium	393; 7.26%	Tinea pedis	79; 7.19%
Psoriasis	374; 6.91%	Pressure ulcer	65; 5.91%
Urticaria/angioedema	260; 4.8%	Skin ulcer (nondiabetic)	63; 5.73%
66–70 years old (n = 5628)		91–100 years old (n = 427)	
Miscellaneous eczema	1141; 20.27%	Miscellaneous eczema	110; 25.76%
Tinea pedis	539; 9.58%	Pressure ulcer	43; 10.07%
Tinea unguium	463; 8.23%	Squamous cell carcinoma/Bowen's disease	35; 8.2%
Psoriasis	336; 5.97%	Skin ulcer (non-diabetic)	28; 6.56%
Urticaria/angioedema	250; 4.44%	Bullous pemphigoid	22; 5.15%
71–75 years old (n = 6157)			
Miscellaneous eczema	1457; 23.66%		
Tinea pedis	596; 9.68%		
Tinea unguium	566; 9.19%		
Psoriasis	327; 5.31%		
Seborrheic dermatitis	285; 4.63%		

Table 6. Sex differences in skin diseases

	Total; Male; Female		Total; Male; Female
Burn	892, 1.33%; 414, 1.34%; 478, 1.32%	Miscellaneous viral disorders	349, 0.52%; 171, 0.55%; 178, 0.49%
Trauma	406, 0.61%; 196, 0.63%; 210, 0.58%	Syphilis	24, 0.04%; 16, 0.05%; 8, 0.02%
Skin ulcer (nondiabetic)	1318, 1.97%; 605, 1.96%; 713; 1.97%	Miscellaneous sexually transmitted diseases	40, 0.06%; 26, 0.08%, 14, 0.04%
Pressure ulcer	606, 0.9%; 313, 1.01%; 293, 0.81%		
Miscellaneous physico-chemical skin damage	675, 1.01%; 303, 0.98%; 372, 1.03%	Bullous pemphigid	509, 0.76%; 208, 0.67%; 301, 0.83%
		Pemphigus	416, 0.62%; 180, 0.58%; 236, 0.65%
Diabetic dermatoses	432, 0.64%; 300, 0.97%; 132, 0.37%	Miscellaneous bullous diseases	139, 0.21%; 67, 0.22%; 72, 0.2%
Atopic dermatitis	6707, 10.01%; 3486, 11.28%; 3221, 8.92%	Systemic sclerosis	609, 0.91%; 94, 0.3%; 515, 1.43%
Hand eczema	2009, 3%; 532, 1.72%; 1477, 4.09%	Systemic lupus erythematosus	520, 0.78%; 72, 0.23%; 448, 1.24%
Contact dermatitis	2629, 3.92%; 902, 2.92%; 1727, 4.78%	Dermatomyositis	300, 0.45%; 76, 0.25%; 224, 0.62%
Seborrheic dermatitis	2201, 3.28%; 1295, 4.19%; 906, 2.51%	Miscellaneous collagen diseases	911, 1.36%; 209, 0.68%; 702, 1.94%
Miscellaneous eczema	12523, 18.68%; 6289, 20.35%, 6234, 17.26%	Anaphylactoid purpura	169, 0.25%; 72, 0.23%; 97, 0.27%
Urticaria/angioedema	3355, 5.01%; 1251, 4.05%; 2104, 5.82%	Reticular/racemous livedo	80, 0.12%; 21, 0.07%; 59, 0.16%
Prurigo	1216, 1.81%; 755, 2.44%; 461, 1.28%	Miscellaneous vasculitis/purpura/ circulatory disturbance	625, 0.93%; 239, 0.77%; 386, 1.07%
Drug eruption/toxicoderma	1012, 1.51%; 436, 1.41%; 576, 1.59%		
Psoriasis	2967, 4.43%; 2138, 6.92%; 829, 2.29%	Mycosis fungoides	418, 0.62%; 244, 0.79%; 174, 0.48%
Palmoplantar pustulosis	828, 1.24%; 284, 0.92%; 544, 1.51%	Miscellaneous lymphomas	283, 0.42%; 149, 0.48%; 134, 0.37%
Miscellaneous pustulosis	170, 0.255%; 67, 0.22%; 103, 0.29%	Pigmented nevus	703, 1.05%; 206, 0.67%; 497, 1.38%
Lichen planus	200, 0.3%; 80, 0.26%; 120, 0.33%	Seborrheic keratosis	1090, 1.63%; 537, 1.74%; 553, 1.53%
Miscellaneous inflammatory keratotic disorders	241, 0.36%; 95, 0.31%; 146, 0.4%	Soft fibroma/achrochordon	228, 0.34%; 78, 0.25%; 150, 0.42%
		Epidermal cyst	1183, 1.77%; 713, 2.31%; 470, 1.3%
Tylosis/clavus	911, 1.36%; 292, 0.95%; 619, 1.71%	Lipoma	171, 0.26%; 92, 0.3%; 79, 0.22%
Ichthyosis	61, 0.09%, 31, 0.1%; 30, 0.08%	Dermatofibroma	110, 0.16%; 44, 0.14%, 66, 0.18%
Miscellaneous keratinization disorders	502, 0.75%; 192, 0.62%; 310, 0.86%	Miscellaneous benign skin tumors	1651, 2.46%; 673, 2.18%; 978, 2.71%
		Actinic keratosis	256, 0.38%; 129, 0.42%; 127, 0.35%
Ingrown nail	594, 0.89%; 197, 0.64%; 397, 1.1%	Basal cell carcinoma	324, 0.48%; 166, 0.54%; 158, 0.44%
Miscellaneous nail disorder	396, 0.59%; 123, 0.4%; 273, 0.76%	Squamous cell carcinoma/Bowen's disease	447, 0.67%; 272, 0.88%; 175, 0.48%
Alopecia areata	1644, 2.45%; 557, 1.8%; 1087, 3.01%		
Androgenic alopecia	208, 0.31%; 198, 0.64%; 10, 0.03%	Paget's disease	221, 0.33%; 136, 0.44%; 85, 0.24%
Miscellaneous skin appendage disorders	266, 0.4%, 77, 0.25%; 189, 0.52%	Malignant melanoma	802, 1.2%; 395, 1.28%; 407, 1.13%
		Miscellaneous malignant skin tumors	531, 0.79%; 291, 0.94%; 240, 0.66%
Scabies	96, 0.14%; 50, 0.16%; 46, 0.13%	Vitiligo vulgaris	1123, 1.68%; 473, 1.53%; 650, 1.8%
Insect bite	762, 1.14%; 285, 0.92%; 477, 1.32%	Chloasma/senile freckle	334, 0.5%; 18, 0.06%; 316, 0.87%
Tinea pedis	4363, 6.51%; 2225, 7.2%; 2138, 5.92%	Miscellaneous pigmented disorders	154, 0.23%; 30, 0.1%; 124, 0.34%
Tinea unguium	3216, 4.8%; 1581, 5.12%; 1635, 4.53%	Erythema multiforme	194, 0.29%; 89, 0.29%; 105, 0.29%
Miscellaneous tinea	607, 0.91%; 404, 1.31%; 203, 0.56%	Erythema nodosum	111, 0.17%; 12, 0.04%; 99, 0.27%
Candidiasis	406, 0.61%; 176, 0.57%; 230, 0.64%	Miscellaneous disorders with erythematous plaques	130, 0.19%; 40, 0.13%; 90, 0.25%
Miscellaneous mycosis	209, 0.31%; 117, 0.38%; 92, 0.25%		
Acne	2423, 3.62%; 757, 2.45%; 1666, 4.61%	Nevus/phacomatosis (other than pigmented nevus)	266, 0.4%; 89, 0.29%; 177, 0.49%
Impetigo contagiosum	505, 0.75%; 283, 0.92%; 222, 0.61%		
Folliculitis	749, 1.12%; 432, 1.4%; 317, 0.88%	Rosacea/rosacea-like dermatitis	148, 0.22%; 36, 0.12%; 112, 0.31%
Erysipelas	81, 0.12%; 35, 0.11%; 46, 0.13%	Granulomatous diseases	192, 0.29%; 65, 0.21%; 127, 0.35%
Cellulitis	589, 0.88%; 304, 0.98%; 285, 0.79%	Keloid/hypertrophic scar	184, 0.27%; 73, 0.24%; 111, 0.31%
Miscellaneous bacterial infection	909, 1.36%; 497, 1.61%; 412, 1.14%	Cheilitis/angular cheilitis/mucous membrane diseases	94, 0.14%; 38, 0.12%; 56, 0.16%
Molluscum contagiosum	602, 0.9%; 327, 1.06%; 275, 0.76%		
Herpes simplex	688, 1.03%; 266, 0.86%; 422, 1.17%	Erythroderma	62, 0.09%; 44, 0.14%; 18, 0.05%
Herpes zoster/zoster-associated pain	1599, 2.39%; 694, 2.25%; 905, 2.51%	Other diseases	662, 0.99%; 315, 1.02%; 347, 0.96%
		Total	67 024, 100%; 30 899, 100%; 36 125, 100%
Viral wart	3016, 4.5%; 1388, 4.49%; 1628, 4.51%		

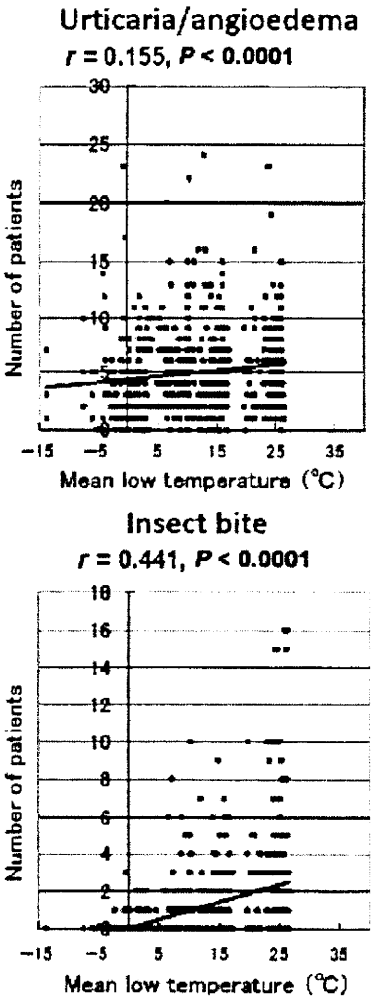

Figure 5. Correlation between patient numbers and mean low temperature in urticaria/angioedema and insect bite.

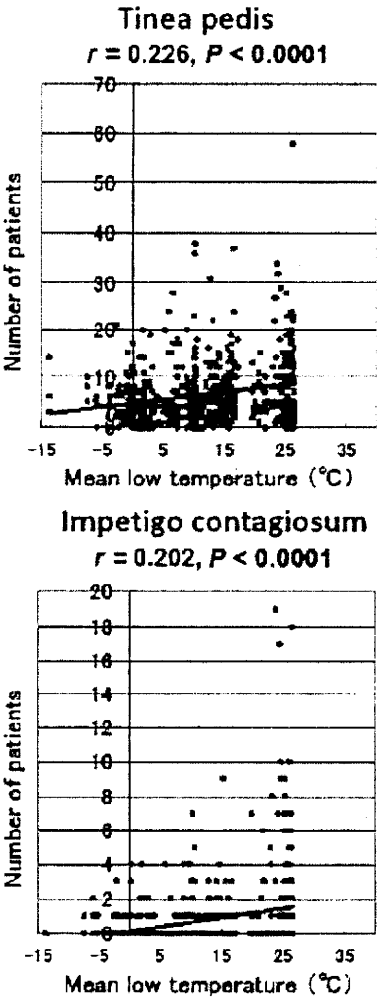

Figure 6. Correlation between patient numbers and mean low temperature in tinea pedis and impetigo contagiosum.

previous hospital-based study in Turkey[3] reported that the five most common disorders were atopic dermatitis, diaper dermatitis, impetigo, seborrheic dermatitis and miliaria in children aged 0–2 years; atopic dermatitis, impetigo, warts, contact dermatitis and insect bites in children aged 3–5 years; contact dermatitis, warts, atopic dermatitis, pruritus and impetigo in children aged 6–11 years; and acne, contact dermatitis, warts, seborrheic dermatitis and pruritus in children aged 12–16 years. For Dutch children aged 0–17 years old in 2001, the incidence rates per person-year of skin disorders were, in descending order, warts 34.3, dermatophytosis 25.4, contact dermatitis/other eczema 22.9, impetigo 20.5, laceration/cuts 20.3, atopic

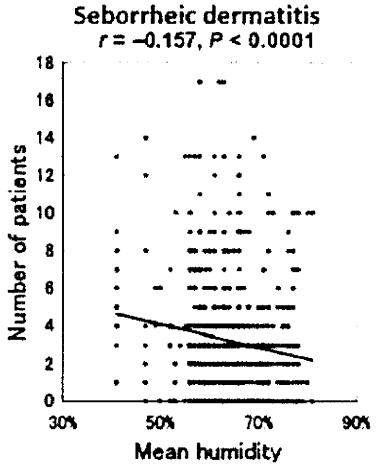

Figure 7. Negative correlation between patient numbers and mean humidity in seborrheic dermatitis.

dermatitis 16.5, moniliasis/candidasis 9.8 and molluscum contagiosum 9.5.[2] Although the order of each disease differed from country to country, atopic dermatitis, miscellaneous eczematous diseases, impetigo and warts appear to share their top rankings in pediatric dermatology, and this was also the case in Japan. Similar observations were also made in 1105 pediatric outpatients aged 0–15 years who visited the hospital of Aarau in Switzerland between 1998 and 2001.[7]

In Turkey, Yalçin *et al.*[8] examined records for 4099 geriatric patients over 65 years old who were admitted to the Ankara Numune Educational and Research Hospital from 1999 through 2003. The five most frequently diagnosed diseases were as follows: in the group aged 65–74 years, eczematous dermatitis, fungal infections, pruritus and bacterial and viral infections; in the group aged 75–84 years, eczematous dermatitis, pruritus, and fungal, viral and bacterial infections; and in the group aged over 85 years, pruritus, eczematous dermatitis, precancerous lesions and skin carcinomas, and viral and fungal infections.[8] In the present study, the Japanese geriatric population was also found to suffer very frequently from miscellaneous eczema and tinea pedis/unguium. In addition, there was a high incidence of psoriasis in elderly Japanese patients. As expected, we found conspicuous differences in the incidence of collagen diseases between the two sexes. A preponderance of collagen diseases in females was also evident in Yalçin's study.[8]

It should be emphasized again that this study was simply a measure of skin disorders in patients attending ordinary dermatology clinics in Japan. The study holds various limitations and biases, but it appears to highlight the current situation regarding patients presenting dermatological problems in Japan.

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Allergic contact cheilitis caused by ditrimethylolpropane triethylhexanoate in a lipstick

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Lipsticks are composed of dyes, flavouring agents, sunscreens, vehicle components, and preservatives. Recently, several branched fatty acid esters have been used in lipsticks as pigment solvents in place of castor oil, because

the latter is a known contact allergen (1). Ditrimethylolpropane triethylhexanoate is a new branched fatty acid ester used in lipsticks. Here, we report a case of allergic contact cheilitis caused by ditrimethylolpropane triethylhexanoate in a lipstick.

Case Report

A 38-year-old female with a history of atopic dermatitis and urticaria presented with cheilitis of several years' duration. She had been treated with topical steroids, and changed her lipstick a year ago, but her cheilitis had not improved. Patch tests were performed with the Japanese standard allergens as well as samples of her cosmetics, applied on her back with Finn Chambers® on Scanpor® tape (Epitest Ltd, Tuusula, Finland). The tests were occluded for 2 days, and read according to the International Contact Dermatitis Research Group Scoring Scale (2) on D2, D3, and D7. There was a positive reaction to her current lipstick 'as is'. The patient was subsequently patch tested according to the list of 18 ingredients in the lipstick obtained from the manufacturer. The ingredients were prepared in pet., and the patient showed a positive reaction to ditrimethylolpropane triethylhexanoate 'as is' (Table1). Ditrimethylolpropane triethylhexanoate accounted for 15% of the lipstick. A total of four consecutive controls were negative to ditrimethylolpropane triethylhexanoate 'as is'. From these observations, we diagnosed the patient as having allergic contact cheilitis caused by ditrimethylolpropane triethylhexanoate in the lipstick.

Table 1. Patch test results

Materials	Concentration	D2	D3	D7
Lipstick	As is	?+	+	+
Ditrimethylolpropane triethylhexanoate	As is	−	+	+
Other ingredients of lipstick	—	—	—	—

Discussion

There are reports of allergic contact dermatitis caused by lipsticks (1, 3–6). Cases of allergic contact cheilitis caused by isopalmityl diglyceryl sebacate (3, 6), glyceryl isostearate, and diisostearyl malate (7) – chemicals used in place of castor oil in lipsticks – have been reported. Ditrimethylolpropane triethylhexanoate was first introduced onto the market in 2003. Ditrimethylolpropane triethylhexanoate is a new pigment solvent that has superior pigment dispersibility to conventional solvents such as diisostearyl malete (8). It is a white or light yellow liquid oil, and is a triester consisting of ditrimethylolpropane and 2-ethylhexanoate (8) (Fig. 1). When ditrimethylolpropane triethylhexanoate is used together with wax, the mixture is hard, and this enables the lipstick to have a smoother texture because of the reduction in the volume of wax in the lipstick (8). Ditrimethylolpropane triethylhexanoate is stable against heat, and mixes well with other oils; it may be used for various products, such as skin care products (8). Our case demonstrates that ditrimethylolpropane triethylhexanoate has sensitizing properties. This is the first case of allergic contact dermatitis caused by ditrimethylolpropane triethylhexanoate reported in the literature.

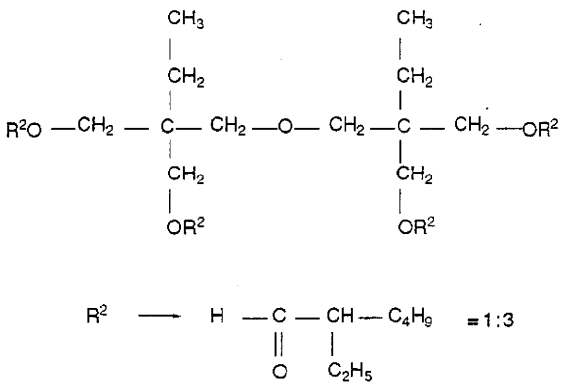

Fig. 1. Chemical structure of ditrimethylolpropane triethylhexanoate.

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Original Article

Changes of cell-surface thiols and intracellular signaling in human monocytic cell line THP-1 treated with diphenylcyclopropenone

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ABSTRACT — Changes of cell-surface thiols induced by chemical treatment may affect the conformations of membrane proteins and intracellular signaling mechanisms. In our previous study, we found that a non-toxic dose of diphenylcyclopropene (DPCP), which is a potent skin sensitizer, induced an increase of cell-surface thiols in cells of a human monocytic cell line, THP-1. Here, we examined the influence of DPCP on intracellular signaling. First, we confirmed that DPCP induced an increase of cell-surface thiols not only in THP-1 cells, but also in primary monocytes. The intracellular reduced-form glutathione/oxidized-form glutathione ratio (GSH/GSSG ratio) was not affected by DPCP treatment. By means of labeling with a membrane-impermeable thiol-reactive compound, Alexa Fluor 488 C5 maleimide (AFM), followed by two-dimensional gel electrophoresis and analysis by liquid chromatography coupled with electrospray tandem mass spectrometry (LC/MS/MS), we identified several proteins whose thiol contents were modified in response to DPCP. These proteins included cell membrane components, such as actin and β-tubulin, molecular chaperones, such as heat shock protein 27A and 70, and endoplasmic reticulum (ER) stress-inducible proteins. Next, we confirmed the expression in DPCP-treated cells of spliced XBP1, a known marker of ER stress. We also detected the phosphorylation of SAPK/JNK and p38 MAPK, which are downstream signaling molecules in the IRE1α-ASK1 pathway, which is activated by ER stress. These data suggested that increase of cell-surface thiols might be associated with activation of ER stress-mediated signaling.

Key words: Cell-surface thiols, SH test, ER stress, THP-1, Diphenylcyclopropenone, 2DE

INTRODUCTION

Skin sensitization is a cell-mediated, delayed-type hypersensitivity immune response induced by low-molecular-weight compounds called haptens. Dendritic cells, including Langerhans cells, are potent antigen-presenting cells and play an important role in the induction of skin sensitization by simple chemicals (Aiba and Tagami, 1998). Activation by haptens has been observed in cultured human monocyte-derived dendritic cells (MoDCs); these cells respond *in vitro* to haptens such as nickel chloride ($NiCl_2$) and 2,4-dinitrochlorobenzene (DNCB) by significantly augmenting their expression of CD86, CD54, HLA-DR and CCR7 (Aiba *et al.*, 1997; Coutant *et al.*, 1999; Boislève *et al.*, 2004). Furthermore, mitogen-activated protein kinases (MAPKs), such as p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinases (ERK) and c-jun N-terminal kinase (JNK), play crucial rules in the augmentation of CD86 and CCR7 expression on dendritic cells treated with haptens (Aiba *et al.*, 2003; Boislève *et al.*, 2004).

On the other hand, the reduction-oxidation (redox) state of cells reflects the balance between the levels of oxidizing and reducing equivalents. The redox balance is important for cell activation. It was demonstrat-

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ed that intracellular redox imbalance is a signaling mediator up-stream of p38 MAPK in hapten-treated MoDCs and THP-1 cells (Mizuashi *et al.*, 2005). Filomeni *et al.* (2003) reported that oxidation of exofacial membrane thiol groups by exogenous non-permeable GSSG triggered a decrease of intracellular glutathione (GSH) content, phosphorylation of p38 MAPK and apoptosis in U-937 cell lines. Becker *et al.* (2003) reported that coupling of sensitizers to thiol groups is a key event for the activation of monocytes and MoDCs. Furthermore, we demonstrated that a decrease of cell-surface thiols is a trigger of activation of intracellular signal transduction in hapten-treated MoDCs and THP-1 cells (Hirota *et al.*, 2009; Kagatani *et al.*, 2010), and that the level of cell-surface thiols on THP-1 cells decreases in response to treatment with many kinds of hapten (Suzuki *et al.*, 2009). On the other hand, increase of cell-surface thiols on cells has been observed in response to N-acetyl-L-cysteine (NAC), a thiol antioxidant, and mitogens such as concanavalin A (Con A) and phytohemagglutinin (PHA-L) (Laragione *et al.*, 2003; Lawrence *et al.*, 1996). However, the effect of cell-surface thiol-increasing chemicals on intracellular signal transduction in cells is unknown.

From such a viewpoint, we focused on the increase in cell-surface thiols in THP-1 cell lines treated with diphenylcyclopropenone (DPCP). DPCP is used as a drug to treat alopecia areata, and is a potent sensitizer (Holzer *et al.*, 2006; Basketter *et al.*, 2002). Furthermore, DPCP was reported to have peptide-binding activity in *in vitro* assay (Gerberick *et al.*, 2004). In general, skin sensitizers have protein (peptide)-binding activity, and free-thiols in cell-surface proteins were thought to decrease by the treatment with hapten (Hirota *et al.*, 2009). So, increase of cell-surface thiols by DPCP, which has peptide-binding activity, is very interesting. In this study, we examined the effect of DPCP on intracellular redox state, identified several cell membrane proteins whose thiol level is increased by DPCP, and evaluated the effects of DPCP on some intracellular signal transduction pathways.

MATERIALS AND METHODS

Chemicals

DNCB, DPCP, dithiothreitol (DTT) and NAC were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and 3-[3-cholamidopropyl] dimethylammonio-1-propanesulphonate (CHAPS) were purchased from Kanto Chemical (Tokyo, Japan). Impermeable, thiol-reactive Alexa Fluor 488 C5 maleimide (AFM) and BSA Alexa Fluor 488 conjugate (AFM-BSA) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA).

Cells and culture

THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were maintained in RPMI 1640 medium (Invitrogen Life Technologies) with 1% (v/v) antibiotic-antimycotic (Invitrogen Life Technologies), 10% fetal bovine serum (v/v) (FBS, JRH Biosciences, Lenexa, KS, USA) and 0.05 mM 2-mercaptoethanol (2-ME) (Invitrogen Life Technologies) at 37°C in a 5% CO_2 incubator. Cells were passaged by the addition of fresh medium twice a week and cell density was maintained between 0.1 and 0.5 x 10^6 cells/ml.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized fresh leukocyte-enriched buffy coats from different donors using Lymphoprep (Nycomed Pharma As, Oslo, Norway). After several washes with phosphate-buffered saline (PBS), 1 x 10^8 PBMC were treated with 150 µl of CD14 microbeads in 600 µl of PBS supplemented with 1% bovine serum albumin (BSA) and 5 mM EDTA (MACS buffer) at 4°C for 30 min. The cells coated with CD14 microbeads were washed with MACS buffer, then separated by a magnetic cell separator, MACS (Miltenyi Biotech), according to the manufacturer's protocol. Before culturing, we examined the percentage of $CD14^+$ cells in these preparations by flow cytometry (FACScalibur using CellQuest software (Becton Dickinson, San Jose, CA, USA)). Only cell specimens containing more than 90% $CD14^+$ cells were used in the experiments. This study was approved by the ethics committee of Shiseido Research Center, Yokohama, Japan and the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan, and adhered to the guidelines set forth by the Helsinki protocol. All the subjects gave informed consent before the examinations.

Analysis of cell-surface thiols by flow cytometry

THP-1 cells and monocytes were seeded at 1 x 10^6 cells/ml. After treatment with test chemicals, THP-1 cells were recovered and washed with PBS twice, then incubated with 100 µl of AFM (10 µM) PBS solution for 30 min at 37°C. After having been washed again with PBS, the cells were analyzed by flow cytometry. Flow-cytometric analyses were performed with an EPICS XL-MCL System II (Beckman Coulter, Fullerton, CA, USA) or FACScalibur (Becton Dickinson). Dead cells were gated out by staining with propidium iodide (PI, 0.625 µg/ml). A total of 10,000 living cells was analyzed. RFI was calculated by use of the following formula: RFI (% of control) = (MFI of chemical-treated cells/ MFI of vehicle control

cells) x 100.

Quantification of intracellular GSH and GSSG

After treatment with chemicals, 0.5 x 10^6 THP-1 cells were collected by pipetting and washed twice with cold PBS. The cells were immediately lysed with 100 μl of lysis buffer (0.1% Triton X-100, 0.1 M sodium phosphate buffer, and 5 mM EDTA, pH 7.5). The mixture was allowed to stand at room temperature (RT) for 5 min to lyse the cells. Thereafter, 5 μl of 0.1 N HCl and 5 μl sulfosalicylic acid (Wako Pure Chemicals, Osaka, Japan) were added. The total cellular GSH concentration was determined using a Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Gaithersburg, MD, USA). The GSSG concentration was determined according to the method of Sacchetta *et al.* (1986). Briefly, 1 μl of 2-vinylpyridine (Wako Pure Chemicals) was added to 50 ml of the cell lysate supernatant, and mixed at RT for 1 min, then the pH was adjusted to 7.5. Subsequently, the reaction mixture was allowed to stand at RT for 60 min. The levels of total GSH and GSSG were calculated by using a standard curve obtained with GSH and GSSG (Wako Pure Chemicals), and the content of GSH was obtained by subtracting the amount of GSSG from total GSH content.

Preparation of AFM-labeled cell membrane proteins derived from DPCP-treated THP-1

THP-1 cells were seeded at 1 x 10^6 cells/ml. After 2 hr treatment with DPCP, THP-1 cells were recovered, washed with PBS twice, and incubated with 500 μl of AFM (30 μM) PBS solution for 30 min at 37°C. The cells were washed again with PBS, and cell membrane proteins were extracted according to the protocol of 2-D Sample Prep for Membrane Proteins (Pierce, Milwaukee, WI, USA). The change of cell-surface thiols was checked by flow-cytometric analyses using the EPICS XL-MCL System II (Beckman Coulter) before the preparation of cell membrane proteins.

Analysis of AFM-labeled cell membrane proteins by 2DE

The membrane protein preparation (1 mg) was mixed in 2DE sample buffer (7 M urea, 2 M thiourea, 4% (v/v) CAPS, 60 mM DTT, 0.5% IPG buffer (pH 3-10) (GE Healthcare, Buckinghamshire, UK) and 0.002% BPB) and applied to the acidic end of immobilized pH gradient gels (Immobiline DryStrip, pH 3-10, 24 cm, GE Healthcare) in a strip holder. Protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Isoelectric focusing (IEF) was performed with stepwise voltage increment from 500 V to 8,000 V up to a total of 41 kV-h, using an Ettan IPGphor II (GE Healthcare). Before carrying out the second-dimensional SDS-PAGE, strip gels were subjected to a two-step equilibration in equilibration buffer including 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS and 1% DTT and 2.5% iodoacetamide (IAA). After equilibration, gel strips were transferred onto 12.5% SDS-polyacrylamide gels (20 cm x 26 cm) and the second-dimensional SDS-PAGE was performed using Ettan DALTsix Large Electrophoresis Systems (GE Healthcare). AFM-BSA was used for fluorescence correction among gels. Images of gels were digitally scanned with a FluorImager 595 (GE Healthcare) to visualize the AFM-labeled protein spots on gels. After scanning, the gels were stained using Deep Purple Total Protein stain (GE Healthcare) to visualize protein spots and scanned again. Profiles of AFM-labeled proteins and Deep Purple-stained proteins were assessed using ImageMaster 2D Platinum (GE Healthcare).

In-gel tryptic-digestion of protein spots

Protein spots of interest were manually excised from gels with a clean scalpel. The gel pieces were rinsed in deionized water, dehydrated in acetonitrile and then dried for 10 min at RT. Following reduction in 10 mM DTT and alkylation in 25 mM IAA in 25 mM ammonium bicarbonate, the gel pieces were dehydrated in acetonitrile and dried again. They were then rehydrated in 20 ng/ml of TPCK-modified trypsin (Promega, Madison, WI, USA) at 4°C. In-gel tryptic digestion was performed at 37°C overnight and digested peptides were recovered into extraction solution (2% acetonitrile, 5% formic acid). The extract was vacuum-dried and the residue was dissolved in 2% acetonitrile, 5% formic acid.

Identification of proteins using liquid chromatography coupled with electrospray tandem mass spectrometry (LC/MS/MS) analysis

Digested peptides were subjected to LC/MS/MS-based protein identification analysis as described previously (Motoyama *et al.*, 2007). Briefly, peptide extracts were loaded by an autosampler (SI-2 semi-micro HPLC system, Shiseido Co., Ltd., Tokyo, Japan) onto a fused-silica trapping column (100 μm i.d. x 1 cm, Aqua C18, Phenomenex, Torrance, CA, USA). The trapping column was desalted with a gradient starting buffer (0.1% formic acid, 5% acetonitrile/purified water) for approximately 30 min, then it was directly connected to a fused-silica analytical capillary column (100 μm i.d. x 12 cm, Aqua C18, Phenomenex) by changing the position of a two-way switching valve. The peptides were separated with a 40-min organic gradi-

ent (5-75% acetonitrile). The column flow rate was set to 300~400 nl/min by adjusting the length of a split resistant capillary (50 μm i.d. x 50~200 mm). Peptides eluted from the column were directly electrosprayed into the mass spectrometer (Deca XP, Thermo Fisher Scientific, Waltham, MA, USA), and MS/MS spectra were automatically acquired under the control of the Xcalibur datasystem (Thermo Fisher Scientific). Collected MS/MS spectra were searched to identify peptides/proteins with the SEQUEST algorithm running on Bioworks software (Thermo Fisher Scientific). A non-redundant human protein database (NCBI, downloaded in 2007) was used for protein identification. Stringent search criteria were used to minimize false-discovery rates (Sf score > 0.85, Peptide probability > 0.001, Number of top matches: > 1).

Real-time polymerase chain reaction (real-time PCR) analysis of spliced XBP1 mRNA

Following 2-h DPCP treatment, real-time PCR analysis of spliced XBP1 mRNA was performed as described previously (Hirota *et al.*, 2006). In brief, cDNA synthesis and measurement of spliced XBP1 mRNA were performed using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA) and Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen Life Technologies). The double-stranded cDNA was synthesized from single-stranded cDNA and digested with *PstI* for 1 hr. The gene-specific PCR products were measured continuously with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The PCR conditions were 95°C for 5 min, then 40 cycles of 95°C 30 sec -55°C 30 sec -72°C 30 sec. The quantity of specific mRNA was normalized as a ratio to the amount of GAPDH mRNA. Specific primer sequences for real-time PCR were as follows: XBP1 mRNA, <sense primer> 5'-CCTTGTAGTTGAGAACCAGG-3', <anti-sense primer> 5'-GGGCTTGGTATATATGTGG-3'; GAPDH mRNA, <sense primer> 5'-GAAGGTGAAGGTCGGAGTC-3' <anti-sense primer> 5'-GAAGATGGTGATGGGATTTC-3'. The values of fold increase over the control were calculated by use of the following formula: spliced XBP1 fold increase (% of control) = (normalized spliced XBP1 expression of chemical-treated cells/ normalized spliced XBP1 expression of vehicle control cells) x 100.

SDS-PAGE and analysis of phospho-p38 MAPK and SAPK/JNK by Western blot analysis

Following 2-hr DPCP treatment, phosphorylation of JNK and p38 MAPK was determined by Western blot analysis, as described previously (Hirota *et al.*, 2009). Immunoblotting of phosphorylated p38 MAPK and phosphorylated JNK was performed using a p38 MAPK immunoblotting kit and SAPK/JNK immunoblotting kit (Cell Signaling Technology, Beverly, MA, USA), respectively. Total cell lysate from chemical-treated cells was prepared using lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 137 μM NaCl, 10% glycerol, 2 mM ethylenediaminetetraacetic acid, 1% protease inhibitor cocktail (Sigma-Aldrich), and 1 mM sodium orthovanadate), and suspended in 2 x SDS sample buffer (313 mM Tris-HCl (pH 6.8), 10% SDS, 2-ME, 50% glycerol, and 0.01% bromophenol blue (BPB)). The protein samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 60 min at RT, and then with rabbit polyclonal antibodies to anti-phosphorylated p38 MAPK, anti-p38 MAPK, anti-phosphorylated SAPK1/JNK and anti-SAPK/JNK antibody for 60 min at RT. They were washed three times with Wash buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20), then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 60 min at RT, and washed again three times with Wash buffer. Immunoreactive bands were detected by chemiluminescence measurement.

Statistical analysis

The statistical significance of differences in the RFI of cell surface thiols and in spliced XBP1 expression between non-treated THP-1 and chemical-treated THP-1 cells was examined using the paired Student's t test (Snedecor and Cochran, 1989). The Bonferroni correction was applied for multiple comparisons (Wallenstein *et al.*, 1980).

RESULTS AND DISCUSSION

In our previous report, we showed that modification of cell-surface thiols induces CD86 expression in cells of the human monocytic cell line THP-1, and we confirmed that 32 of 36 skin sensitizers induced a decrease of cell-surface thiols in THP-1 cells (Hirota *et al.*, 2009; Suzuki *et al.*, 2009). However, a few haptens, such as DPCP, $MnCl_2$ and pyridine, caused an increase (Suzuki *et al.*, 2009). In this study, we focused on the increase of cell-surface thiols in DPCP-treated THP-1 cells. First, we measured cell-surface thiols in DNCB-, DPCP-, DTT- or NAC-treated THP-1 cells. Thiol antioxidants, including DTT and NAC, were reported to induce increase of cell-surface thiols (Laragione *et al.*, 2003). As shown in Fig. 1 (A), cell-sur-

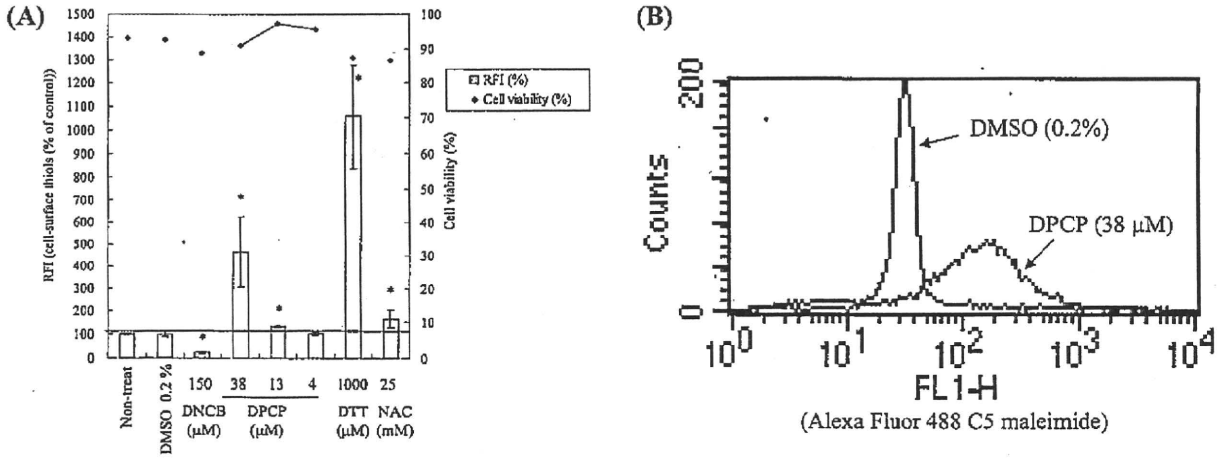


Fig. 1. Effect of chemicals on cell-surface thiols. THP-1 cells and primary monocytes (1 x 10^6 cells/ml) were exposed to the indicated chemicals for 2 hr. Cell-surface thiols was measured by flow cytometry as described in Materials and Methods. (A) Changes of cell-surface thiols on THP-1 cells treated with the indicated concentrations of DPCP, DNCB, DTT or NAC. Each value of RFI is the mean ± S.D. of at three independent experiments. Asterisks indicate a significant ($p < 0.05$) difference between chemical-treated cells and vehicle-treated cells. (B) Changes of cell-surface thiols on primary monocytes following treatment with DPCP. Representative results from three independent sets of experiments are shown.

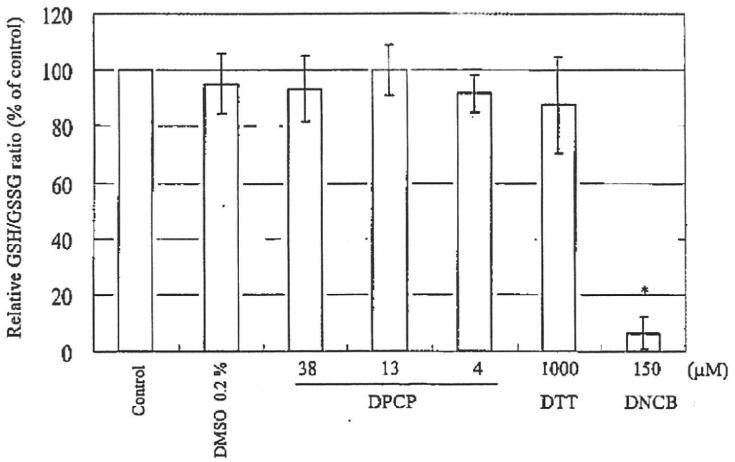
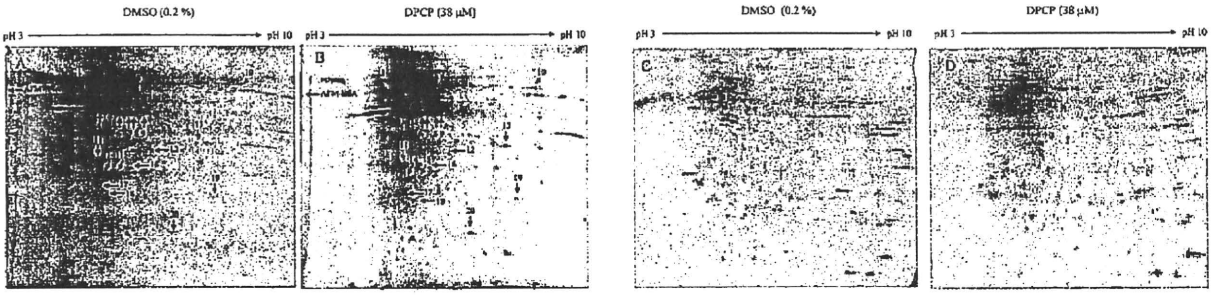


Fig. 2. Effect of chemicals on intracellular GSH/GSSG ratio in THP-1 cells. THP-1 cells (1 x 10^6 cells/ml) were exposed to DPCP, DNCB or DTT for 2 hr. Intracellular GSH and GSSG were measured by using colorimetric assays employing the GSH reductase-DTNB recycling procedure. The relative GSH/GSSG ratio was calculated as described in Materials and Methods. Each value of relative GSH/GSSG ratio is the mean ± S.D. of at three independent experiments. Asterisks indicate a significant ($p < 0.05$) difference between chemical-treated cells and vehicle-treated cells.

face thiols were increased by DPCP, DTT and NAC, but decreased by DNCB. The increase of cell-surface thiols by DPCP was confirmed not only in THP-1 cells, but also in primary monocytes (Fig. 1 (B)). Next, we investigated whether the intracellular GSH/GSSG ratio was affected by DPCP in THP-1 cells. It was reported that the intracellular GSH/GSSG ratio in THP-1 cells was decreased by haptens such as DNCB and $NiCl_2$, which induce decrease



E

Spot No.	Protein Name	Accession No.	Fold difference (DMSO vs DPCP)
1	β-tublin	gi|2119276	1.8 ± 0.19
2	β-tublin	gi|2119276	2.3 ± 0.63
3	heat shock protein 70 kDa protein 9B	gi|24234688	2.0 ± 0.55
4	actin	gi|7245526	2.7 ± 0.97
5	actin	gi|7245526	2.2 ± 0.52
6	actin	gi|7245526	3.9 ± 0.42
7	actin	gi|7245526	2.3 ± 0.59
8	actin	gi|7245526	1.7 ± 0.04
9	actin	gi|7245526	2.3 ± 0.61
10	adenylyl cyclase-associated protein (CAP)	gi|5453595	2.1 ± 0.67
11	p64 CLCP (GST_N family, Chloride Intracellular Channel (CLIC))	gi|895845	1.8 ± 0.15
12	Annexin IV	gi|4502105	2.1 ± 0.43
13	palmitoyl-protein thioesterase 1 (PPT1)	gi|4506031	2.7 ± 0.68
14	heat shock protein 27 kDa 1	gi|4504517	2.3 ± 0.51
15	heat shock protein 27 kDa 1	gi|4504517	2.0 ± 0.41
16	prohibitin	gi|4505773	2.6 ± 0.19
17	glyoxalase I	gi|5729842	2.3 ± 0.69
18	RAB1B, member RAS oncogene family	gi|13569962	2.1 ± 0.19
19	stromal-cell derived factor2-like 1 (SDF2L1) precursor	gi|56243533	2.3 ± 0.74
20	cofilin 1	gi|5031635	2.9 ± 0.88

Fig. 3. AFM labeling pattern of membrane proteins from THP-1 cells treated with DMSO (0.2%, solvent control) (A) or DPCP (38 μM) (B) and Deep Purple-stained gel from solvent control (0.2% DMSO) cells (C) and DPCP (38 μM) treated cells (D). Proteins were separated by 2DE based on pI (3-10, left to right) and molecular weight (SDS 12.5% PAGE, top to bottom). Thiol expression patterns were compared using ImageMaster 2D Platinum software. The average fold differences ± S.D. ($p < 0.05$) were calculated for four different gels (E).

of cell-surface thiols (Mizuashi *et al.*, 2005; Hirota *et al.*, 2009). As shown in Fig. 2, the GSH/GSSG ratio did not change in response to treatment with DPCP or DTT. On the other hand, DNCB induced a significant decrease of the GSH/GSSG ratio. These data indicated that increase of cell-surface thiols might not to be related to intracellular redox imbalance. So, we attempted to identify cell membrane proteins whose thiols were increased by means of a redox proteomics approach. For 2DE, we used AFM, which is a membrane-impermeable thiol-reactive reagent, together with flow cytometric analysis.

Fig. 3 shows the SH group content and total protein expression of cell membrane proteins derived from cells exposed to 0.2% DMSO (solvent control, SH groups; Fig. 3A, total proteins; Fig. 3C) and 38 μM DPCP (SH groups; Fig. 3B, total proteins; Fig. 3D).

We identified twenty protein spots whose thiols were significantly augmented by DPCP treatment (Fig. 3E). At a > 2 arbitrary fold difference cut-off, thiols of twelve proteins were augmented by DPCP treatment. Among them, actin and β-tubulin are cytoskeletal components. It was reported that the thiol content of β-actin was increased by

NAC treatment in human PBMC (Laragione *et al.*, 2003). Adenylyl cyclase-associated protein (CAP) was reported to bind actin (Hubberstey *et al.*, 1996). Cofilin and Hsp 27 were reported to be important in the control of the actin cytoskeletal network (de Graauw *et al.*, 2005). Furthermore, cofilin is reported to translocate to plasma membranes, and to be involved in actin depolymerization, cell motility, and apoptosis in human prostate cancer cells (Suzuki *et al.*, 1995; Zhu *et al.*, 2006). Prohibitin is located in plasma membrane and mitochondria, and was reported to be responsive to apoptosis signaling by TGF-β (Mielenz *et al.*, 2005; Zhu *et al.*, 2006). Annexin IV was also reported to be located in plasma membrane, and to interact with activated protein kinase C alpha (Diakonova *et al.*, 1997; Schmitz-Peiffer *et al.*, 1998). These data supported the idea that conformational changes of cell membrane proteins, such as actin and β-tubulin, may result in apoptotic signaling.

Rab1B, belonging to the Ras superfamily of GTPases, is required for protein transport from ER to Golgi (Alvarez *et al.*, 2003). Glyoxalase I is related to the detoxification of α-oxoaldehyde and its expression is augmented in Parkinson's disease, possibly via endoplasmic reticulum (ER) stress (de Hemptinne *et al.*, 2007; Ryu *et al.*, 2002; Werner *et al.*, 2008).

From the 2DE data, we identified molecular chaperones (Hsp 27 and Hsp 70) and ER stress-related proteins (palmitoyl-protein thioesterase 1 (PPT1), stromal-cell derived factor 2-like 1 (SDF2L1)). Hsp 27, which was identified from two spots with the same molecular weight and different PI, might have been phosphorylated. PPT is related to the post-translational modification of proteins and PPT gene mutation was reported to cause ER stress (Kim *et al.*, 2006). SDF2L1 was also reported to be induced by ER stress (Fukuda *et al.*, 2001). These data may indicate that increase of cell-surface thiols is associated with ER stress.

In general, heat shock proteins, such as Hsp 27 and Hsp 70 act as molecular chaperones, promoting the folding of proteins. Protein folding is performed in the ER, and the aggregation of unfolded or misfolded proteins leads to apoptosis via ER stress (Rao *et al.*, 2004). Thiols in proteins are known to play a crucial role in protein folding. Cell-surface proteins include thiol-containing ion channels and membrane receptors that are redox-sensitive (Garant *et al.*, 1999; Lipton *et al.*, 2002; Zeng *et al.*, 2003). Based on our data and previous reports, we speculated that chemically induced increase of thiols may result in alterations of protein structure that lead to ER stress. Therefore, we examined the increase of spliced XBP1 mRNA expression, a biomarker of ER stress, and the phosphor-

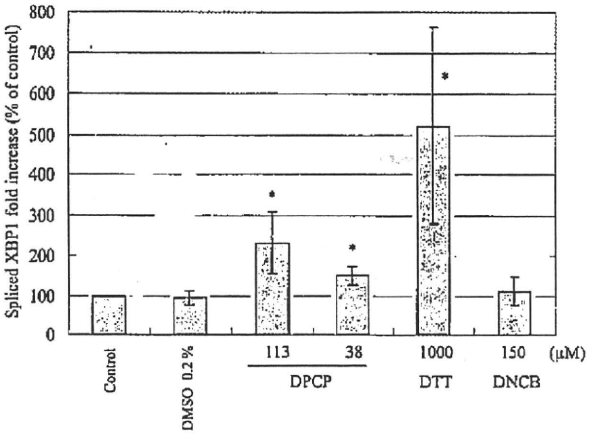


Fig. 4. Effect of chemicals on spliced XBP1 in THP-1 cells. THP-1 cells (1 x 10^6 cells/ml) were exposed to DPCP, DNCB or DTT for 2 hr. RNA was extracted, reverse-transcribed, and analyzed with the real-time PCR system after synthesis of double-stranded cDNA followed by *PstI* treatment as described in Materials and Methods. The increases (fold) of mRNA levels were determined and normalized (Materials and Methods). Each value is the mean ± S.D. of at least three independent experiments. Asterisks indicate a significant ($p < 0.05$) difference between chemical-treated cells and vehicle-treated cells.

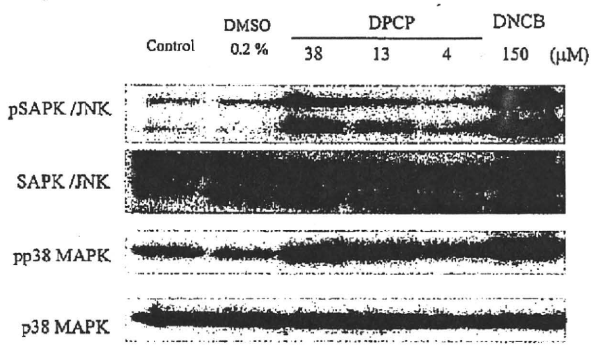


Fig. 5. Effect of chemicals on phosphorylation of intracellular SAPK/JNK and p38 MAPK in THP-1 cells. THP-1 cells were exposed to test chemicals for 2 hr and cell lysate was prepared as described in Materials and Methods. Western blot analysis of phosphorylated SAPK/JNK and p38 MAPK was performed using p38 MAPK immunoblotting kit and SAPK/JNK immunoblotting kit, respectively. Representative results from three independent sets of experiments are shown.

ylation of p38 MAPK and JNK/SAPK (Figs. 4 and 5). In Fig. 4, we used 113 μM DPCP as well as 38 μM DPCP in order to examine the dose-dependence of the expression of spliced XBP1 mRNA. XBP1 mRNA was reported to be spliced by activated IRE1α, a sensor protein of ER stress (Yoshida *et al.*, 2001). IRE1α was reported to activate apoptosis signal-regulating kinase 1 (ASK1) and its downstream signal transduction molecules, such as p38 MAPK and c-Jun NH_2-terminal kinases (JNK, including JNK1, JNK2 and JNK3 isoforms) (Nagai *et al.*, 2007). These data are consistent with the activation of MAPK pathways via IRE1α by DPCP. Among cell-surface thiol-increasing reagents, DTT provoked ER stress, including JNK activation (Urano *et al.*, 2000), while $MnCl_2$ induced ER stress, including augmentation of BiP and activation of caspase-12 (Chun *et al.*, 2001).

Based on the above results, we hypothesized that conformational change of cytoskeletal proteins might be induced by way of intracellular signaling molecules such as HSP27 and cofilin in DPCP-treated THP-1 cells. Furthermore, increase of thiols in cell-surface proteins might lead to activation of signal transduction in the MAPK pathway via IRE1α, resulting in ER stress.

However, our results did not establish the mechanism of the increase of cell-surface thiols. For example, protein disulfide isomerase (PDI) was not hit in the redox proteomics analysis, though it was reported to control membrane thiols, and might be a target of DPCP (Jiang *et al.*, 1999). Further work is needed to investigate the effects of chemicals on key molecules which control the redox state of cell membrane proteins.

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Further Verification of an *In Vitro* Tier System for the Identification of Cosmetic Ingredients that are Not Ocular Irritants

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Summary — A tier evaluation system for the identification of cosmetic ingredients that are not ocular irritants was applied to 59 cosmetic ingredients, for which *in vivo* data were available. The tier system employs monolayer cultures of SIRC cells, an established cell line originally derived from rabbit cornea, and a three-dimensional living dermal model (LDM; MATREX™), which consists of human dermal fibroblasts in a contracted collagen lattice. The effects of ingredients on monolayer cultures of SIRC cells were determined by Crystal Violet staining (in the SIRC-CVS assay), and the effects on the LDM were measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (in the LDM-MTT assay). The classifications of eye irritancy predicted by the *in vitro* system were compared with previously reported data obtained with the *in vivo* Draize rabbit eye test. The *in vivo* classification was based on appearance of corneal damage, or a maximal average score (MAS) of 15 as the cut-off point. The SIRC-CVS assay was effective in the prediction of compounds that would be non-irritants at a concentration of 10%, while the subsequent LDM-MTT assay could predict non-irritancy at various lower and higher concentrations, including 10%. The tier system gave very few false-negative predictions, though false positives were unavoidable. Performing the LDM-MTT assay with an additional 73 ingredients gave similar results in the prediction of non-irritancy at various concentrations. Our findings indicate that the tier system may be suitable for the safety assessment of eye irritancy of ingredients intended to be used in cosmetics and medicated cosmetics in Japan.

Key words: *alternative method, cytotoxicity, Draize eye test, eye irritation, MATREX™, SIRC cells, three-dimensional dermal model, tier evaluation system.*

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Introduction

The determination of eye irritation potential is required for the hazard assessment of chemicals. The Draize eye test, which was developed by Draize *et al.* (1), has become the international standard assay for acute ocular toxicity (as in OECD Test Guideline 405; 2). However, the method has been criticised for scientific and animal welfare reasons. On 11 March 2009, *in vivo* eye irritation testing, together with other animal-based testing, of cosmetic ingredients was banned in the European Union (EU; 3). This legislation also prohibited the marketing in the EU of cosmetic products containing ingredients tested on animals. Great efforts to develop substitute *in vitro* eye irritation tests have been made in academia, and by individual companies, industry trade associations and public institutions (4), especially the European Centre for the Validation of Alternative Methods (ECVAM), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the Japanese Centre for the Validation of Alternative Methods (JaCVAM). Two alternative tests, in which tissues collected from slaughterhouses are used, i.e. the bovine corneal opacity and permeability (BCOP) test and the isolated chicken eye (ICE) test, were endorsed as alternative methods for the identification of ocular corrosives and severe irritants, by ICCVAM in 2006, by ECVAM in 2007, and by the OECD in 2009. These were important steps toward elimination of the use of animals in eye irritation testing. However, the Draize eye test has also been used to identify non-irritating ingredients in chemical safety assessments, especially for ingredients for use in cosmetics and medicated cosmetics in Japan. Therefore, to further reduce the use of animals in testing, an *in vitro* test method to identify non-irritating ingredients needs to be developed, validated and endorsed as soon as possible. In the EU, ECVAM validation studies on reconstructed human tissue models (EpiOcular™ and SkinEthic HCE™ assays) are in progress. Though the cytosensor microphysiometer test was endorsed as an *in vitro* eye irritation test for the identification of non-irritants by the ECVAM Scientific Advisory Committee in 2009, its scope is very restricted, in that it is applicable only to water-soluble surfactants and water-soluble surfactant-containing mixtures.