Induction of the 72-kD Heat Shock Protein in Organ-Cultured Normal Human Skin

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To study the induction of heat shock protein (HSP) of normal human skin, the indirect immunofluorescence method, using monoclonal antibody directed against 72-kD HSP, was applied in organ-cultured normal human skin that was treated with heat, UV, or chemicals. The present study provided new evidence that HSP 72 was induced not only by heat and chemical agents, such as L-azetidine 2-carboxylic acid, and sodium arsenite, but also by ultraviolet (UV B and C). The result suggests that normal human skin has an induced protective function against numerous environmental stresses. *J Invest Dermatol 98:786–790, 1992*

When the cells are exposed to environmental stresses such as heat, ethanol, heavy metals, amino acid analogues, and certain metabolic poisons they respond by synthesizing a characteristic group of proteins called heat shock proteins or stress proteins [1,2]. Although the biologic role of HSP in cells is still being defined, one of the proposed functions of HSP is stabilization of stress-labile proteins. When mammalian cells are exposed to thermal stress, they acquire transient resistance to a subsequent non-lethal heat treatment. This phenomenon is termed thermotolerance. Induction of HSP has been considered to be closely related to the development of thermotolerance [3,4]. It has been reported that molecular weights of the major proteins of HSP are observed at approximately 110, 100, 90, 80, 75, 73, 72, 60, 47, 32, 28, and 8 kD [5]. The HSP that are referred to as the HSP 70 family are the most conserved and the best characterized [6–8]. The HSP 70 family is required for survival of cells during and after thermal stress [9]. In mammalian cells, there are two prominent forms of the HSP 70 family, an abundant constitution member, HSP 73, and a highly stress-induced member, HSP 72 [10,11].

It is well known that skin is the largest human organ and provides an extensive physical barrier against a variety of environmental stresses [12]. There have been a few reports about the heat shock response of cultured normal human skin keratinocytes and fibroblasts [13,14]. However, to the best of our knowledge, the HSP of human skin in organ level have not been investigated.

In order to know the stress response of normal human skin to the environmental stresses, we have examined the induction of HSP 72 in organ-cultured normal human skin by the immunofluorescence method using a monoclonal antibody specific for the induced 72-kD protein.

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Abbreviations:
AzC: L-azetidine 2-carboxylic acid
HSP: heat shock protein
mRNA: messenger RNA
PBS: phosphate-buffered saline
UVA: ultraviolet A

MATERIALS AND METHODS

Skin Explants and Culture Organ-cultured normal human skin obtained from cosmetic surgery was used with the informed consent of the patient. The surgical procedure was performed under local anesthesia with 1% lidocaine hydrochloride. After washing twice with phosphate-buffered saline (PBS, pH 7.4), the skin was sliced to a thickness of about 0.5 mm and cut into approximately 2 × 2 mm pieces. Each skin sample was placed dermis side down on a millipore filter (pore size, 0.45 μm; diameter, 13 mm) and floated on the surface of the culture medium in a 35-mm Falcon plastic culture dish. These skin explants were cultured in a humidified incubator containing 5% CO_2/95% air. The culture medium was Eagle's minimum essential medium (Nacalai Tesque, Kyoto, Japan) supplemented with 15% newborn calf serum (Nacalai Tesque). The skin explants were preliminarily incubated at 37°C for 24 h prior to the treatments of various stresses.

Heat Treatment The skin explants were transferred to a culture dish that contained culture medium previously kept at 45°C and cultured at 45°C for 1 h in an incubator containing 100% air. After heat-shock treatment, the skin explants were transferred to a culture dish that contained culture medium previously kept at 37°C and incubated at 37°C for 1 to 6 h in a 5% CO_2 incubator. The corresponding control skin explants were maintained at 37°C for 1 h in an incubator containing 100% air, and then transferred to a CO_2 incubator and further incubated at 37°C for 1 to 10 h. The temperature was controlled to within ±1°C.

UV Irradiation The sources of UVB and UVC used here were a health lamp (280–320 nm, main peak at 315 nm, FL 20SE, Toshiba, Tokyo, Japan) and a germicidal lamp (254 nm, GL-10, Toshiba), respectively. In the case of UVB irradiation, a Kodacel filter was used for cutting off wavelengths shorter than 275 nm [15]. The intensities of the health lamp and germicidal lamp were determined by a digital radiometer (UV.103, Macam Photometrics Ltd., Livingston, Scotland) and by a UV dosimetry (UVR1, Tokyo Optical Co. Ltd., Tokyo, Japan), respectively. The fluence rate of the health lamp with Kodacel filter was 1.07 J/m²/second and the fluence rate of the germicidal lamp was 0.05 J/m²/second. In the present experiment, the skin explants were irradiated by exposure to UVB (200 J/m²) or UVC (10 J/m²) and then incubated at 37°C for 10 h, because nuclear immunofluorescence of HSP 72 in normal human fibroblasts was detected in 10 h after UVB (200 J/m²) or UVC (20 J/m²) irradiation (in preparation).

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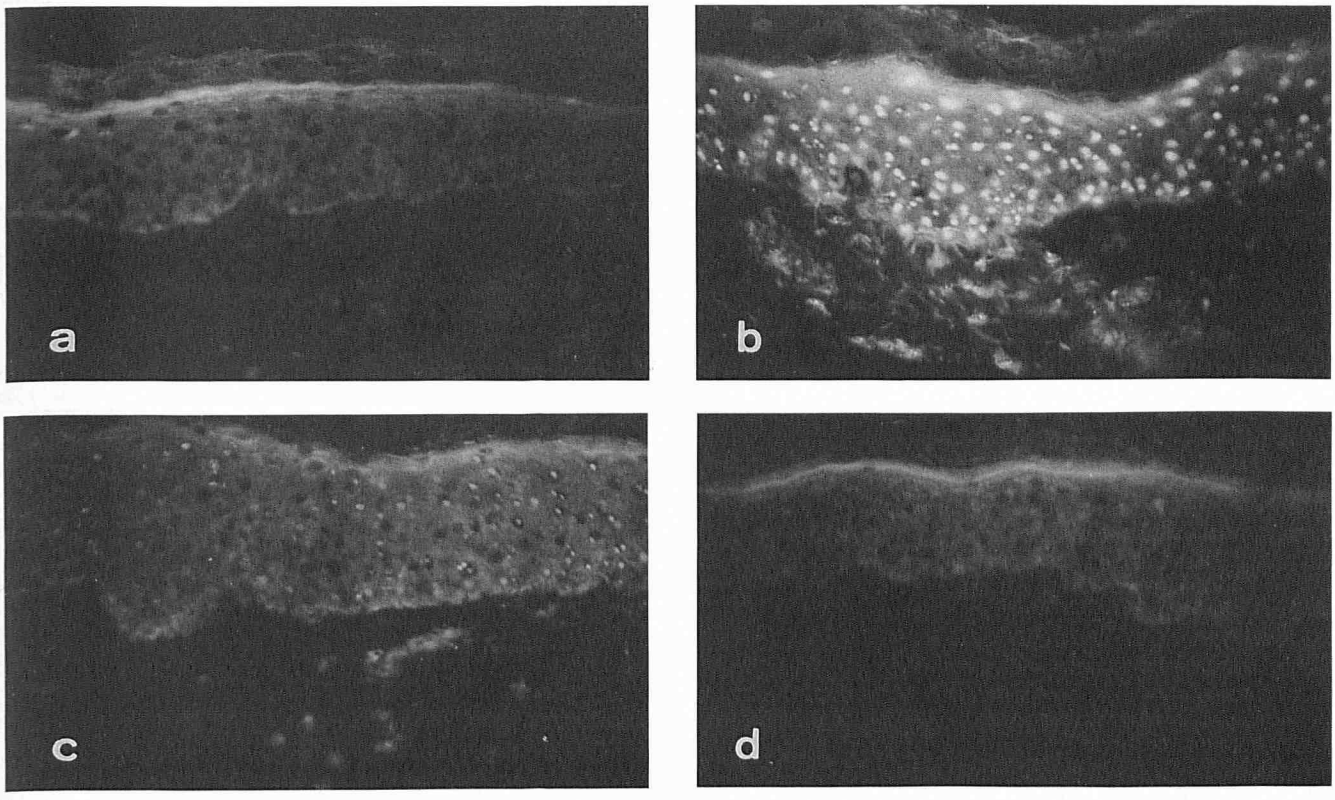

Figure 1. Distribution of the 72-kD heat shock protein in heat-treated skin explants and in skin explants recovering from heat shock treatment as determined by indirect immunofluorescence using an HSP 72 monoclonal antibody. *a)* Incubation at 37°C (control); *b)* heat shock treatment at 45°C for 1 h; *c)* heat shock treatment at 45°C for 1 h and incubation at 37°C for 1 h; *d)* heat shock treatment at 45°C for 1 h and incubation at 37°C for 3 h (magnification ×100).

Treatments with Chemicals The chemical agents used were sodium arsenite, L-azetidine 2-carboxylic acid (AzC), cadmium chloride, and zinc chloride (Nacalai Tesque). These chemicals were dissolved in a culture medium. After the preliminary incubation at 37°C for 24 h, the skin explants were treated at 37°C for 1, 4, or 6 h with the chemical agents, 10 mM AzC or 100 μM sodium arsenite (final concentration). In other cases, skin explants were treated at 37°C for 1, 3, 7, 14, or 24 h with 50 μM cadmium chloride or 200 μM zinc chloride. Control skin explants were also incubated at 37°C for up to 24 h in culture medium without chemical stressors.

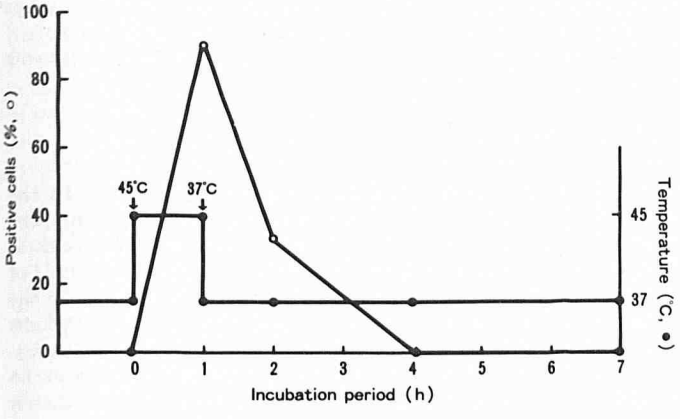

Figure 2. Time course of the induction and disappearance of HSP 72 in normal human epidermal cells. The starting time of heat treatment is shown at 0 h. The skin explants were heat treated at 45°C for 1 h. Then they were returned to 37°C and incubated for 1, 3, or 6 h. The number of epidermal cells with nuclear immunofluorescence was counted as positive. Each *point* indicates at least 300 cells. *Open* and *closed circles,* percent of positive cells and temperature of incubation, respectively.

Immunofluorescence Method Analysis of the intracellular distribution of the 72-kD protein was performed by indirect immunofluorescence using a mouse monoclonal antibody (Amersham International PLC, Amersham, UK) specific for the 72-kD heat shock protein. Skin explants were embedded in O.C.T. compound (Lab-Tek Products, IL), snap-frozen in liquid nitrogen, and kept at −70°C until used. Four-micrometer thick sections were prepared on a cryostat. These cryostat sections were air dried and then incubated with mouse monoclonal anti-72-kD antibody (diluted in 1:70 in PBS) at 37°C for 30 min, following 15-min wash with PBS, incubated with fluorescein-isothiocyanate-conjugated horse anti-mouse IgG antibody (Vector Laboratories, Inc. Burlingam, CA, USA) (diluted in 1:10 in PBS) at 37°C for 30 min. After a final wash with PBS, the sections were mounted in buffered-glycerol and examined using an Olympus fluorescence microscope. The number of cells with nuclear immunofluorescence were counted as positive. At least 300 cells, the total of two independent experiments, were examined in six to eight randomly selected microscopic fields.

RESULTS

Table I shows the immunofluorescence patterns of heat-shocked, UV-irradiated, or chemical-exposed organ-cultured normal human skin. As controls, skin explants that have been maintained at 37°C for up to 24 h showed no positive immunofluorescence (Fig 1*a*). When the skin explant was heated at 45°C for 1 h, a distinct bright immunofluorescence was observed in the nuclei of about 90% of epidermal cells (Fig 1*b*). When the skin explant was transferred from 45°C to 37°C, by 1 h after heat treatment nuclear staining was

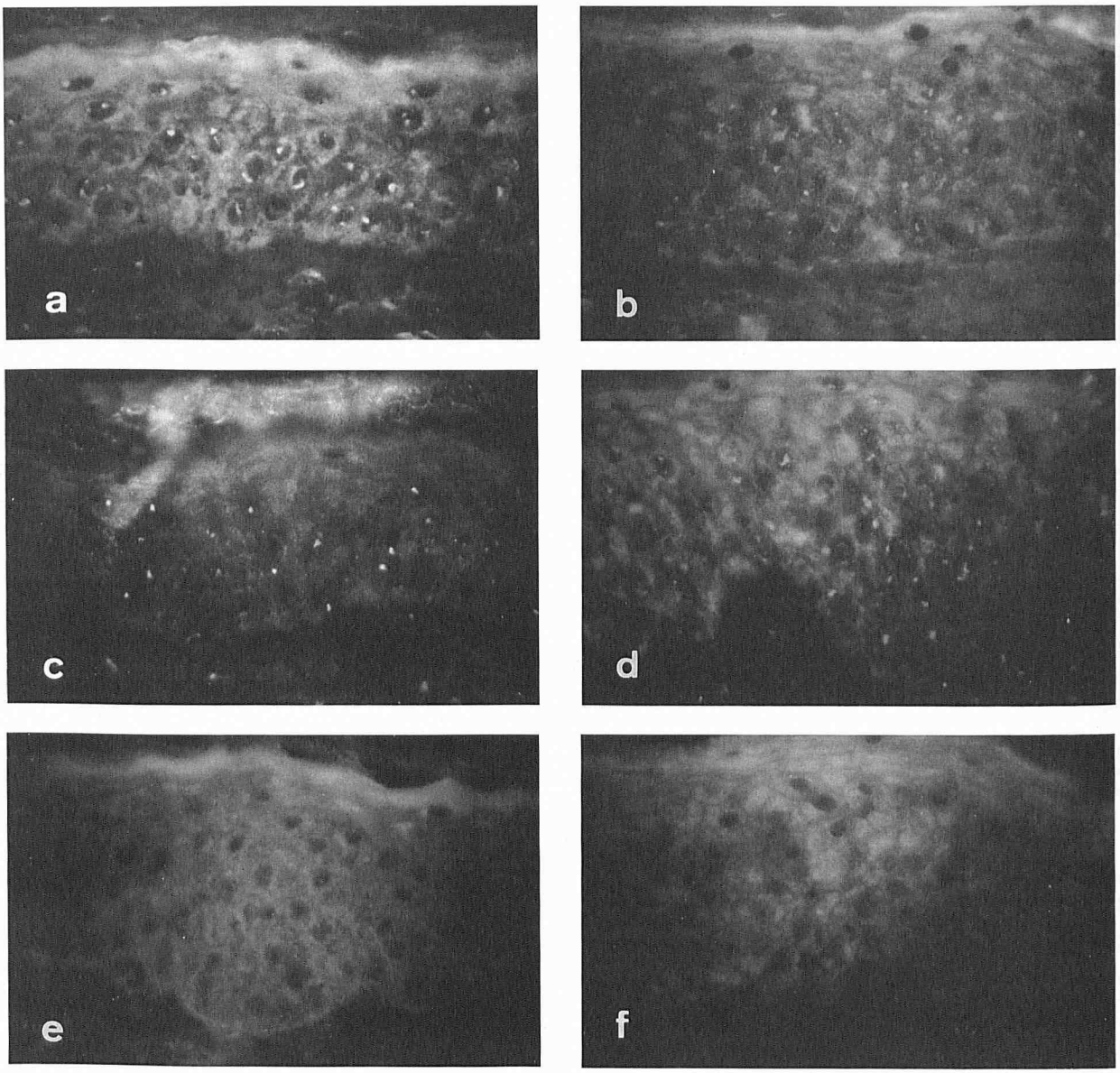

Figure 3. Distribution of the 72-kD stress protein in skin explants treated with various stresses as determined by indirect immunofluorescence using an HSP 72 monoclonal antibody. *a)* Exposure to UVB (200 J/m²) and incubation at 37°C for 10 h; *b)* exposure to UVC (10 J/m²) and incubation at 37°C for 10 h; *c)* treatment with 10 mM AzC for 6 h; *d)* treatment with 100 μM sodium arsenite for 6 h; *e)* treatment with 50 μM cadmium chloride for 7 h; *f)* treatment with 200 μM zinc chloride for 7 h (magnification ×200).

found to markedly diminish to about 30% of epidermal cells (Fig 1*c*). Furthermore, after incubation at 37°C for 3 h, nuclear staining was no longer observed (Fig 1*d*). Time course of the induction and disappearance of HSP 72 by heat treatment was shown in Fig 2.

In the UVB-irradiated skin at a dose of 200 J/m², nuclear fluorescence was observed in about 20% of the epidermal cells (Fig 3*a*). On the other hand, when the organ-cultured skin was irradiated by UVC at a dose of 10 J/m², skin explants showed weakly positive nuclear immunofluorescence (Fig 3*b*). At this dose, about 11% of epidermal cells were positive.

In the skin exposed to 10 mM AzC for 1 h, there was no positive immunofluorescence. However, after exposure to 10 mM AzC for 4 or 6 h, positive nuclear immunofluorescence was observed at 9 or 13% of the epidermal cells, respectively (Fig 3*c*). Similarly in the skin exposed to 100 μM sodium arsenite for 1 h, there was no positive immunofluorescence. After exposure to 100 μM sodium arsenite for 4 or 6 h, positive nuclear immunofluorescence was observed at 7 or 17% of the epidermal cells, respectively (Fig 3*d*). In the UVB-, UVC-, AzC-, or sodium arsenite–treated skin explants, the intensity of nuclear immunofluorescence was relatively weak as compared with that of the heat-treated skin explants. The grade of the intensity of immunofluorescence is indicated in Table I. In the cadmium chloride-treated or zinc chloride–treated skin explants that incubated at 37°C for 1 up to 24 h, no nuclear immunofluorescence was observed (Fig 3*e,f*). In all experiments, there was no immunofluorescence with the control pre-immune serum (data not shown).

DISCUSSION

In the present study, we have demonstrated that in normal human skin, HSP 72 was induced not only by heat treatment and chemical stressors such as AzC and sodium arsenite, but also by UVB or UVC irradiation. Heat treatment induced HSP 72 more rapidly and in-

Table I. Effects of Heat, UV, and Chemical Stressors upon the Induction of HSP 72 in Normal Human Skin[a]

Treatments	Percent of Positive Cells[b]	Nuclear Immunofluorescence[c]
37°C 1 h* → 37°C (0, 1, 3, 6, 10 h)	0	−
45°C 1 h*	90	++
45°C 1 h* → 37°C 1 h	33	+
45°C 1 h* → 37°C 3 h	0	−
45°C 1 h* → 37°C 6 h	0	−
UVB (200 J/m²) → 37°C 10 h[d]	20	+
UVC (10 J/m²) → 37°C 10 h[d]	11	±
37°C (1, 3, 4, 6, 7, 14, 24 h)	0	−
AzC (10 mM) 1 h	0	−
AzC (10 mM) 4 h	9	±
AzC (10 mM) 6 h	13	+
$NaAsO_2$ (100 μM) 1 h	0	−
$NaAsO_2$ (100 μM) 4 h	7	±
$NaAsO_2$ (100 μM) 6 h	17	+

[a] Organ-cultured normal human skin explants were treated with heat, UVB, UVC, AzC, and $NaAsO_2$ and the induction of HPS 72 was examined by indirect immunofluorescence using an anti-HPS 72 monoclonal antibody.

[b] The percent of cells with nuclear fluorescence is presented.

[c] The grade of the intensity of nuclear immunofluorescence was judged by two independent observers, ++, clearly positive; +, positive; ±, weakly positive; and −, negative.

[d] Skin explants were irradiated by exposure to UV, and then followed by incubation at 37°C for 10 h. *, incubation under 100% air condition. Otherwise mentioned, skin explants were cultured under 5% CO_2/95% air condition.

tensely than UV irradiation or chemical exposure. After heat treatment at 45°C for 1 h, the nuclear immunofluorescence of HSP 72 markedly increased, and by 1 h after heat treatment the nuclear staining rapidly decreased. By 3 h after heat treatment, the HSP 72 was not detected in the nuclei of epidermal cells.

On the other hand, Welch and Feramisco [10] have shown that in cultured mammalian cells after 4 h incubation at 42°C, intense nuclear staining with prominent staining of the nucleoli was observed. In this cell-culture system, returning the 4-h heat shock–treated cells to 37C for 30 min resulted in little or no change in the distribution of the 72-kD protein. In addition, after 2 or 4 h incubation at 37°C, nuclear staining was no longer apparent [10]. In organ cultured skin presented here, although the nucleolar staining was not so clearly identified, we detected apparent nuclear staining by heat treatment. The time course of the induction of HSP 72 in organ-cultured cells was almost the same as that in the cultured cells [10,11]. Under normal conditions, the immunofluorescent antibody staining of the 72-kD protein was reported to be weakly positive in the nucleus and cytoplasm in the majority of the cultured cells [10]. This background staining may be caused by another form of stress such as an exposure to serum in cell-culture system [16]. In our organ-culture system, however, such background staining was not detectable. Therefore, it is suggested that the skin explants used in this study may display more clearly the characteristics of normal human skin in vivo as compared with cell-culture systems. Because the cells with nuclear immunofluorescence were observed throughout the epidermis, it is suggested that the induction of HSP 72 is not associated with the differentiation of epidermal cells, namely, the degree of keratinization.

Concerning the UV-induced HSP, it has been reported that solar near UV (UVA) induced a 32-kD stress protein in cultured normal skin fibroblasts [17], and the 32-kD stress protein was identified as heme oxygenase [18,19]. In addition, in mouse epidermis the relative amount of mRNA hybridizing to the HSP 70 DNA probe increased with the UVB fluences [20]. The present study has shown that UVB irradiation induced HSP 72 in normal human skin and this finding implies that the induced HSP 72 might play a protective role against UVB-induced cell damage of the skin. Although UVC irradiation induced the synthesis of several proteins in human epidermal keratinocytes [21], there have been no reports about UVC-induced HSP 72. The present study verified that HSP 72 was induced not only by UVB irradiation but also by UVC irradiation in normal human skin.

Similar to the results obtained from cultured fibroblasts [22], HSP 72 was also induced by treatment with AzC or sodium arsenite in organ-cultured human epidermal cells. In contrast to AzC and sodium arsenite, cadmium chloride and zinc chloride had no effect on the induction of HSP 72 in the organ-cultured epidermal cells. Previously, Ohtsuka et al[11] reported that in HeLa cells nuclear staining was not detected by treatment with cadmium chloride. In contrast, another report showed that in cultured human keratinocytes, cadmium sulfate is one of the inducing agents of HSP 72 [14]. The discrepancy of these results might be due to the different sensitivity of these two cell lines to the cadmium compounds or to the different solubility or permeability of the cadmium compounds.

The biologic role of the HSP 70 family has been studied primarily in association with thermotolerance [3,13,23]. In the field of dermatology, therapeutic hyperthermia as well as UV irradiation have been used for the treatment of various cutaneous diseases such as psoriasis [24,25]. Considering these facts, the understanding of the biologic effects of HSP on the skin may be of use in the treatment of a number of skin dermatoses [26], and organ-cultured skin might be a useful experimental model for the study of HSP in human skin diseases.

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The changed meeting format will include an invited lecture from an eminent international dermatologist and dedicated sessions with expert introductions on immune diseases and biology of the pilosebaceous unit, for which abstracts are particularly invited. Non-members are welcome to attend.